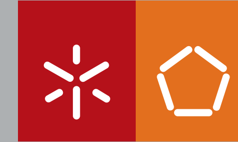


Universidade do Minho
Escola de Engenharia

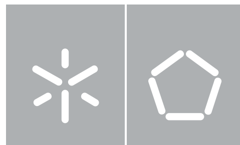
Lígia Raquel Marona Rodrigues

Biosurfactants Production by Probiotic Bacteria and Inhibition of Voice Prostheses Microbial Colonization

Setembro de 2005

Lígia Raquel Marona Rodrigues **Biosurfactants Production by Probiotic Bacteria and Inhibition of Voice Prostheses Microbial Colonization**

UM | 2005



Universidade do Minho
Escola de Engenharia

Lígia Raquel Marona Rodrigues

Biosurfactants Production by Probiotic Bacteria and Inhibition of Voice Prostheses Microbial Colonization

Tese de Doutoramento
Doutoramento em Engenharia Química e Biológica

Trabalho efectuado sob a orientação dos
Professor Doutor José António Couto Teixeira
Professora Doutora Domingas do Rosário Veríssimo Jacinto Tavares de Oliveira

Setembro de 2005

Autora: Lígia Raquel Marona Rodrigues

email: lrmr@deb.uminho.pt

Tel:+ 351 253604400

BI: 10267744

Título tese: *Biosurfactants production by probiotic bacteria and inhibition of voice prostheses microbial colonization*

Orientadores:

Professor Doutor José António Couto Teixeira

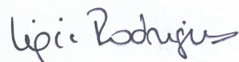
Professora Doutora Domingas do Rosário Veríssimo Jacinto Tavares de Oliveira

Ano de conclusão: 2005

Doutoramento em Engenharia Química e Biológica

É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTA TESE APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE

Universidade do Minho, 20 de Setembro de 2005



AGRADECIMENTOS

Ao terminar esta tese, não posso deixar de expressar o meu sincero agradecimento a todas as pessoas que me acompanharam e apoiaram no decorrer destes últimos anos. Em primeiro lugar, gostaria de agradecer aos meus orientadores científicos, Professor José Teixeira e Professora Rosário Oliveira, pelo apoio concedido ao longo destes anos. Indiscutivelmente as suas excepcionais qualidades científicas e humanas, disponibilidade, optimismo e constantes desafios foram determinantes para a realização deste trabalho. Ao director do Laboratório de Tecnologia Enzimática e Bio-Separações (LTEB), Professor Miguel Gama, pelas facilidades na cedência de alguns equipamentos. Ao Professor Manuel Mota, na qualidade de director do Centro de Engenharia Biológica, pela oportunidade e acolhimento como investigadora deste centro.

To Prof. Dr. Henny van der Mei I would like to express my deep thank for the possibility to integrate her research team. The nice work and excellent time I spent in Groningen were only possible due to her extraordinary qualities both as a scientist and a person. I also would like to thank all the good friends I left in Groningen that were crucial for such a nice stay. Dank u wel! To Joop de Vries a special thanks for running the XPS analysis. To Prof. Dr. Henk Busscher I would like to thank for the opportunity to develop a part of my work in the Biomedical Engineering Department, University of Groningen (The Netherlands).

To Prof. Dr. Ibrahim Banat I would like to thank for the cooperation and great help regarding the final part of the work developed. His wise advices and constant challenges were decisive for the progress of the work. To Eddie O’Kane and Stephen McClean (University of Ulster, UK) I would like to thank for performing LC-MS experiments

I also acknowledge CACTI (Spain) for running the FTIR experiments.

À Ana Moldes gostaria de agradecer a ajuda e colaboração no desenvolvimento de uma parte do trabalho, quer no que diz respeito à aplicação das técnicas desenvolvidas às suas estirpes, bem como no tratamento dos resultados.

Ao Fernando Dourado gostaria de agradecer a ajuda e disponibilidade na execução dos ensaios de cromatografia gasosa.

Aos colegas do laboratório (LTEB) são devidos também os agradecimentos pelo bom ambiente, companheirismo e paciência nos momentos mais cinzentos.

É devido também o agradecimento pelo apoio concedido para a realização deste trabalho à FCT pela bolsa SFRH/BD/4700/2001.

Gostaria ainda de agradecer à minha família e aos meus amigos todo o apoio, incentivo e ajuda prestados ao longo destes anos. Não posso deixar de destacar o meu reconhecimento à Isabel Rocha, Cândida Silva e Rosa Silva pela amizade incondicional e pela disponibilidade sempre demonstradas.

Aos meus pais gostaria de agradecer todo o apoio que sempre me deram, incentivo, ajuda, meios que me proporcionaram, carinho e inesgotável paciência.

Finalmente, gostaria de dedicar este trabalho aos meus Pais.

Lígia

ABSTRACT

BIOSURFACTANTS PRODUCTION BY PROBIOTIC BACTERIA AND INHIBITION OF VOICE PROSTHESES MICROBIAL COLONIZATION

The main purposes of this thesis were the optimization of fermentation conditions for the production of biological antifouling agents, namely biosurfactants from probiotic bacteria, in order to develop new strategies for the prevention of microbial colonization of silicone rubber voice prostheses. Probiotic bacteria *Lactococcus lactis* 53 and *Streptococcus thermophilus* A were found to be biosurfactant-producing strains. The improvement of the standard culture medium for biosurfactant production by response surface methodology, using a compilation of mathematical and statistical techniques, was used and an effective increase in the production yields was achieved.

Economical alternatives were pursed using non conventional low cost raw materials as molasses or cheese whey instead of synthetic medium. An improvement of biosurfactants production yields with 60 to 80% medium preparation costs reduction was achieved.

The ability of the biosurfactants obtained from probiotic bacteria to inhibit adhesion of microbial strains isolated from explanted voice prostheses to silicone rubber surfaces with and without an adsorbed biosurfactant layer was studied in a parallel plate flow chamber. Biosurfactants produced by the *L. lactis* 53 and *S. thermophilus* A reduced about 90% both deposition rates and number of adhering microorganisms after 4 hours. The biosurfactant obtained from *S. thermophilus* A proved to be much more efficient against *Rothia dentocariosa* GBJ 52/2B that is the most frequently isolated bacteria in the group of patients whose prostheses fail after a short time of use forcing replacement.

An artificial throat model was used to assess the influence of biosurfactants from probiotic bacteria on the formation of voice prosthetic biofilms. Both biosurfactants were found to be antimicrobial agents and greatly reduced microbial numbers on prostheses and also induced a decrease in the airflow resistance of voice prostheses after biofilm formation.

The key components of the crude biosurfactant mixtures produced by *L. lactis* 53 and *S. thermophilus* A, including their molecular composition (by Fourier transform infrared spectroscopy), elemental composition (by X-ray photoelectron spectroscopy), molecular mass (by mass spectrometry) and monosaccharide composition (by gas-liquid chromatography) were studied. Moreover, partial functional characterization was established using the following techniques: blood agar test, oil spreading test, critical micelle concentration determination, antimicrobial activity and anti-adhesion test. Finally, desorption of biosurfactants

from silicone rubber and their stability at several pH were evaluated. The most surface-active fractions isolated from the crude biosurfactant mixtures were found to be rich in glycoproteins and glycolipids for *L. lactis* 53 and *S. thermophilus* A, respectively. In addition, these fractions showed antimicrobial and anti-adhesive activities against microbial strains isolated from explanted voice prostheses. Furthermore, the most surface-active fractions stay adsorbed onto silicone rubber surfaces up to 2 months at effective concentrations against microbial colonization. Therefore an increase in voice prostheses lifespan is achievable, as well as the consequent reduction of the health costs associated with prostheses replacement.

Keywords: voice prostheses, biosurfactants, *Lactococcus lactis* 53, *Streptococcus thermophilus* A, microbial adhesion, biofilm formation, low cost fermentative medium, optimization of fermentative medium, physicochemical and functional characterization.

SUMÁRIO

PRODUÇÃO DE BIOSURFACTANTES POR BACTÉRIAS PROBIÓTICAS E INIBIÇÃO DA COLONIZAÇÃO MICROBIANA DAS PRÓTESES DA FALA

A presente tese teve como principais objectivos a optimização das condições fermentativas para a produção de compostos inibidores da adesão microbiana, nomeadamente biosurfactantes, por bactérias probióticas; de forma a desenvolver novas estratégias de prevenção da colonização microbiana das próteses da fala. Demonstrou-se que as bactérias probióticas *Lactococcus lactis* 53 e *Streptococcus thermophilus* A são estirpes produtoras de biosurfactantes. O melhoramento dos meios de cultura para a produção de biosurfactantes foi efectuado usando a metodologia de optimização factorial, fazendo recurso a uma série de ferramentas matemáticas e estatísticas, tendo-se obtido um efectivo aumento dos rendimentos de produção.

Alternativas mais económicas foram desenvolvidas usando matérias-primas não convencionais de baixo custo, como os melaços e o soro de queijo. Atingiu-se uma melhoria razoável dos rendimentos de produção de biosurfactantes tendo sido estimado um decréscimo de 60-80% dos custos associados.

A capacidade dos biosurfactantes de inibir a adesão de microorganismos isolados de próteses da fala (removidas de doentes) a superfícies de borracha de silicone com e sem uma camada de biosurfactante adsorvida, foi estudada usando para o efeito uma célula de fluxo laminar. Ambos os biosurfactantes promoveram uma redução de cerca de 90% das taxas de deposição iniciais de microorganismos, bem como do número total de microorganismos aderidos ao final de 4 horas. O biosurfactante obtido a partir de *S. thermophilus* A provou ser muito mais eficiente na inibição da adesão de *Rothia dentocariosa* GBJ 52/2B que é a bactéria mais frequentemente isolada em doentes cujas próteses falham após um curto período de uso, forçando a sua substituição.

Um modelo de garganta artificial com próteses da fala foi utilizado para estudar o efeito dos biosurfactantes na formação de biofilmes. Ambos os biosurfactantes apresentaram actividade antimicrobiana e reduziram significativamente o número de microorganismos presentes nas próteses, bem como promoveram um decréscimo da resistência à passagem de ar através da válvula da prótese.

Os biosurfactantes produzidos por *L. lactis* 53 e *S. thermophilus* A foram parcialmente purificados numa coluna de interacção hidrofóbica. As estruturas físico-químicas e características funcionais das fracções isoladas foram estudadas. A composição molecular (por FTIR), a composição elementar (por XPS), a massa molecular (por espectrometria de massa) e a composição em açúcares simples (por cromatografia gasosa) foram estudadas. A caracterização funcional foi estabelecida

com recurso a técnicas como: teste do agar de sangue para avaliar a actividade hemolítica; teste para avaliar a dispersão de óleos; determinação da concentração micelar critica e a determinação das actividades antimicrobianas e anti-adesivas. Adicionalmente foi efectuado um estudo da desorção das fracções activas ligadas a superfícies de borracha de silicone, bem como da sua estabilidade a vários valores de pH. Concluiu-se que as fracções com maior actividade de superfície obtidas a partir de *L. lactis* 53 e *S. thermophilus* A são ricas em glicoproteínas e glicolípidos, respectivamente. Estas fracções demonstraram possuir actividade antimicrobiana, bem como anti-adesiva contra os microorganismos isolados de próteses de doentes. Finalmente, verificou-se que as fracções com maior actividade de superfície permanecem adsorvidas à borracha de silicone até cerca de 2 meses em concentrações inibitórias da colonização microbiana, o que poderá permitir um aumento do tempo de vida das próteses da fala e consequentemente reduzir os custos de saúde associados à sua frequente substituição.

Palavras chave: próteses da fala, biosurfactantes, *Lactococcus lactis* 53, *Streptococcus thermophilus* A, adesão microbiana, biofilmes, optimização de meios de fermentação alternativos, caracterização físico-química e funcional.

LIST OF PUBLICATIONS

This thesis is based on the work contained in the following papers:

Rodrigues, L.R., Teixeira, J.A. and Oliveira, R. (2005) Low cost fermentative medium for biosurfactant production by probiotic bacteria. *Biochemical Engineering Journal*. (*submitted*)

Rodrigues, L.R., Banat, I.M., Teixeira, J.A. and Oliveira, R. (2005) Biosurfactants: Potential applications in medicine. *Journal of Antimicrobial Chemotherapy*. (*submitted*)

Rodrigues, L.R., Banat, I.M., Van der Mei, H.C., Teixeira, J.A. and Oliveira, R. (2005) Interference in adhesion of bacteria and yeasts isolated from explanted voice prostheses to silicone rubber by rhamnolipid biosurfactant. *Journal of Applied Microbiology*. (*in press*)

Rodrigues, L.R., Moldes, A., Teixeira, J.A. and Oliveira, R. (2005) Kinetic study of fermentative biosurfactant production by *Lactobacillus* strains. *Biochemical Engineering Journal*. (*in press*)

Rodrigues, L.R., Van der Mei, H.C., Banat, I.M., Teixeira, J.A. and Oliveira, R. (2005) Inhibition of microbial adhesion to silicone rubber treated with biosurfactant from *Streptococcus thermophilus* A. *FEMS Immunology and Medical Microbiology*. (*in press*)

Rodrigues, L.R., Van der Mei, H.C., Teixeira, J.A. and Oliveira, R. (2004) Biosurfactant from *Lactococcus lactis* 53 inhibit microbial adhesion on silicone rubber. *Applied Microbiology and Biotechnology*. 66 (3): 306-311

Rodrigues, L.R., Van der Mei, H.C., Teixeira, J.A. and Oliveira, R. (2004) Influence of biosurfactants from probiotic bacteria on formation of biofilms on voice prostheses. *Applied and Environmental Microbiology* 70 (7): 4408-4410

Rodrigues, L.R., Teixeira, J.A., Oliveira, R. and Van der Mei, H.C. (2005) Response surface optimization of the medium components for the production of biosurfactants by probiotic bacteria. *Process Biochemistry*. (*in press*)

TABLE OF CONTENTS, LIST OF FIGURES, LIST OF TABLES AND LIST OF SYMBOLS

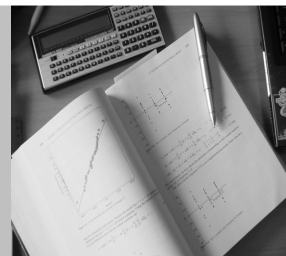


TABLE OF CONTENTS

	GENERAL INTRODUCTION	**1**
	CONTEXT AND MOTIVATION	2
	RESEARCH AIMS	4
	OUTLINE OF THE THESIS	4
	REFERENCES	7
	CHAPTER 1: STRATEGIES FOR THE PREVENTION OF BIOFILM FORMATION ON SILICONE RUBBER VOICE PROSTHESES	**9**
1.1	INTRODUCTION	10
1.2	BIOFILM FORMATION ON VOICE PROSTHESES	12
1.2.1	Microbial Adhesion	12
1.2.2	Microbial Biofilms	13
1.2.3	Characteristics of Biofilms on Silicone Rubber Voice Prostheses	15
1.3	MODIFICATIONS OF SILICONE RUBBER SURFACES	17
1.3.1	Metal Coating	18
1.3.2	Plasma Treatment	21
1.3.3	Perfluoro-alkylsiloxane Treatment	21
1.3.4	Covalently Coupled Quaternary Ammonium Silane Coatings	22
1.3.5	Bulk Surface Photografting	23
1.3.6	Biosurfactants	23

TABLE OF CONTENTS (CONT.)

1.4	PROPHYLACTIC TREATMENT ON SILICONE RUBBER VOICE PROSTHESES	26
1.4.1	Probiotics	26
1.4.2	Dairy Products	28
1.4.3	Caffeinated Soft Drinks	29
1.4.4	Antifungal Agents	29
1.4.5	Synthetic Salivary Peptides	31
1.5	CONCLUSIONS	32
1.6	REFERENCES	33
	CHAPTER 2: BIOSURFACTANTS: POTENTIAL APPLICATIONS IN MEDICINE	**43**
2.1	INTRODUCTION	44
2.2	BIOSURFACTANTS: MECHANISMS OF INTERACTION	47
2.2.1	Synthetic Surfactants and Bacteria	48
2.2.2	Biosurfactants and Bacteria	48
2.2.3	Surface Active Approach to Bacterial Adhesion/Detachment	49
2.3	BIOLOGICAL ACTIVITY OF BIOSURFACTANTS	54
2.3.1	Lipopeptides	54
2.3.2	Glycolipids	56
2.3.3	Other Biosurfactants with Surface Activity	58
2.4	ANTIMICROBIAL ACTIVITY OF BIOSURFACTANTS	58
2.5	ANTI-ADHESIVE ACTIVITY OF BIOSURFACTANTS	61
2.6	BIOMEDICAL AND THERAPEUTICAL APPLICATIONS OF BIOSURFACTANTS	62
2.7	CONCLUSIONS	66
2.8	REFERENCES	66

TABLE OF CONTENTS (CONT.)

CHAPTER 3: RESPONSE SURFACE OPTIMIZATION OF THE MEDIUM COMPONENTS FOR THE PRODUCTION OF BIOSURFACTANTS BY PROBIOTIC BACTERIA **77**

3.1 INTRODUCTION 78

3.2 MATERIALS AND METHODS 79

3.2.1 Strains and Culture Conditions 79

3.2.2 Cell Growth and Biosurfactant Production 80

3.2.3 Biosurfactants Surface-activity Determination 80

3.2.4 Mass of Produced Biosurfactants 81

3.2.5 Experimental Designs 81

3.3 RESULTS 82

3.3.1 Biosurfactant Production by Growing Cells 82

3.3.2 Effects of Different MRS or M17 Medium Components in Cell Growth 85

3.3.3 The Path of Steepest Ascent 91

3.3.4 Central Composite Design (*CCD*) 91

3.3.5 Mass Recovery and Surface-activity 96

3.4 DISCUSSION 96

3.5 CONCLUSIONS 98

3.6 REFERENCES 98

CHAPTER 4: FERMENTATIVE BIOSURFACTANT PRODUCTION BY *LACTOBACILLUS* STRAINS – A KINETIC STUDY **101**

4.1 NOMENCLATURE 102

4.2 INTRODUCTION 103

4.3 MATERIALS AND METHODS 104

4.3.1 Strains and Culture Conditions 104

TABLE OF CONTENTS (CONT.)

4.3.2	Growth Curves	104
4.3.3	Blood Agar Screening	105
4.3.4	Biosurfactant Production	105
4.3.5	Analytical Methods	106
4.3.6	Surface-activity Assay	106
4.3.7	Glucose consumption and Biosurfactant Production – Fitting of Data	107
4.4	RESULTS	108
4.4.1	Blood Agar Screening Method	108
4.4.2	Biosurfactant Production	110
4.4.3	Biosurfactant Extraction with PBS	112
4.4.4	Fermentation in MRS Broth	113
4.4.5	*Lactobacillus pentosus* Fermentation in Whey	113
4.5	DISCUSSION	115
4.6	CONCLUSIONS	118
4.7	REFERENCES	118
	CHAPTER 5: BIOSURFACTANT FROM *LACTOCOCCUS LACTIS* 53 INHIBIT MICROBIAL ADHESION ON SILICONE RUBBER	**121**
5.1	INTRODUCTION	122
5.2	MATERIALS AND METHODS	123
5.2.1	Biosurfactant Production	123
5.2.2	Microbial Strains and Growth Conditions	123
5.2.3	Contact Angle Measurements and Surface Free Energy Calculation	124
5.2.4	Parallel Plate Flow Chamber and Image Analysis	125

TABLE OF CONTENTS (CONT.)

5.3	RESULTS	127
5.3.1	Microbial Cell Surface and Silicone Rubber Characterization	127
5.3.2	Microbial Adhesion	128
5.4	DISCUSSION	130
5.5	CONCLUSIONS	134
5.6	REFERENCES	134
	CHAPTER 6: INHIBITION OF MICROBIAL ADHESION TO SILICONE RUBBER TREATED WITH BIOSURFACTANT FROM *STREPTOCOCCUS THERMOPHILUS* A	**139**
6.1	INTRODUCTION	140
6.2	MATERIALS AND METHODS	141
6.2.1	Biosurfactant Production	141
6.2.2	Biosurfactant Surface-activity Determination	141
6.2.3	Microbial Strains and Culture Conditions	142
6.2.4	Contact Angle Measurements	142
6.2.5	Adhesion Experiments	142
6.2.6	Statistical Analysis	143
6.3	RESULTS	143
6.4	DISCUSSION	147
6.5	CONCLUSIONS	149
6.6	REFERENCES	149

TABLE OF CONTENTS (CONT.)

CHAPTER 7: INTERFERENCE IN ADHESION OF BACTERIA AND YEASTS ISOLATED FROM EXPLANTED VOICE PROSTHESES TO SILICONE RUBBER BY RHAMNOLIPIDS **153**

7.1	INTRODUCTION	154
7.2	MATERIALS AND METHODS	155
7.2.1	Biosurfactant Production and Extraction	155
7.2.2	Derivatization and HPLC Analysis of Rhamnolipid	156
7.2.3	Surface-activity Determination	156
7.2.4	Contact Angle Measurements	156
7.2.5	Microbial Strains and Growth Conditions	157
7.2.6	Adhesion Assay in 96 Wells Plate	157
7.2.7	Adhesion Experiments in the Parallel Plate Flow Chamber	158
7.2.8	Detachment Protocol	159
7.2.9	Statistical Analysis	159
7.3	RESULTS	159
7.3.1	Surface-activity and Biosurfactant Concentration	159
7.3.2	Anti-adhesive Activity of the Rhamnolipid	160
7.3.3	Microbial Adhesion in the Parallel Plate Flow Chamber	161
7.3.4	Detachment Protocol	165
7.4	DISCUSSION	167
7.5	CONCLUSIONS	169
7.6	REFERENCES	170

TABLE OF CONTENTS (CONT.)

	CHAPTER 8: THE INFLUENCE OF BIOSURFACTANTS FROM PROBIOTIC BACTERIA ON FORMATION OF BIOFILMS ON VOICE PROSTHESES	**175**
8.1	INTRODUCTION	176
8.2	MATERIALS AND METHODS	179
8.2.1	Biosurfactant Production	179
8.2.2	Microbial Growth Inhibition Test	179
8.2.3	Voice Prostheses	180
8.2.4	Biofilm Formation	180
8.2.5	Measurement of Airflow Resistance	181
8.2.6	Evaluation of Biofilms	182
8.3	RESULTS	182
8.3.1	Biosurfactant Antimicrobial Activity	182
8.3.2	Biofilms Evaluation and Airflow Resistance	183
8.4	DISCUSSION	186
8.5	CONCLUSIONS	188
8.6	REFERENCES	188
	CHAPTER 9: LOW COST FERMENTATIVE MEDIUM FOR BIOSURFACTANT PRODUCTION BY PROBIOTIC BACTERIA	**193**
9.1	NOMENCLATURE	194
9.2	INTRODUCTION	195
9.3	MATERIALS AND METHODS	196
9.3.1	Microorganisms and Inoculums	196
9.3.2	Fermentation Experiments	196
9.3.3	Cheese Whey Preparation	197

TABLE OF CONTENTS (CONT.)

9.3.4	Molasses Preparation	197
9.3.5	Bacterial Growth Determination	198
9.3.6	Sugar Analysis	198
9.3.7	Surface-activity Determination	198
9.3.8	Sugar Consumption, Biosurfactant Production and Biomass Growth	199
9.4	RESULTS	200
9.4.1	Biosurfactant Production Using Conventional Synthetic Medium	200
9.4.2	Biosurfactant Production Using Cheese Whey	203
9.4.3	Biosurfactant Production Using Molasses	203
9.5	DISCUSSION	206
9.6	CONCLUSIONS	209
9.7	REFERENCES	209
	CHAPTER 10: ISOLATION AND PARTIAL PHYSICOCHEMICAL AND FUNCTIONAL CHARACTERIZATION OF BIOSURFACTANTS PRODUCED BY PROBIOTIC BACTERIA	**213**
10.1	INTRODUCTION	214
10.2	MATERIALS AND METHODS	216
10.2.1	Strains and Culture Conditions	216
10.2.2	Biosurfactant Production and Isolation	216
10.2.3	Hydrophobic Interaction Chromatography	216
10.2.4	Blood Agar Test	217
10.2.5	Oil Spreading Test	217
10.2.6	Critical Micelle Concentration	218
10.2.7	Anti-adhesion Assay in 96 Wells Plate	218
10.2.8	Antimicrobial Assay	219

TABLE OF CONTENTS (CONT.)

10.2.9	Biosurfactant Desorption Assay	219
10.2.10	Biosurfactant Stability	219
10.2.11	Fourier Transform Infrared Spectroscopy	220
10.2.12	X-ray Photoelectron Spectroscopy	220
10.2.13	Mass Spectrometry	221
10.2.14	Quantitative Determination of Monosaccharides	221
10.3	RESULTS	221
10.3.1	Partial Purification of the Biosurfactants	221
10.3.2	Haemolytic Activity	223
10.3.3	Oil Spreading Capacity	223
10.3.4	Critical Micelle Concentration	224
10.3.5	Anti-adhesive Activity	225
10.3.6	Antimicrobial Activity	227
10.3.7	Desorption Assay	229
10.3.8	Biosurfactant Stability	231
10.3.9	Forrier Transform Infrared Spectroscopy	231
10.3.10	X-ray Photoelectron Spectroscopy	233
10.3.11	Mass Spectrometry	235
10.3.12	Quantitative Determination of Monosaccharides	238
10.4	DISCUSSION	238
10.5	CONCLUSIONS	243
10.6	REFERENCES	243

TABLE OF CONTENTS (CONT.)

	CHAPTER 11: GENERAL CONCLUSIONS AND RECOMMENDATIONS	**249**
11.1	CONCLUSIONS	250
11.2	RECOMMENDATIONS	252

LIST OF FIGURES

CHAPTER 1

Figure 1.1 Schematic, sequential presentation of the steps in biofilm formation. 14

Figure 1.2 Micrographs of *Staphylococcus aureus* GB 2/1 (bar is 40 mm) adhering after 2h on silicone rubber with and without an adsorbed biosurfactant layer obtained from *Lactococcus lactis* 53. 26

CHAPTER 2

Figure 2.1 Orientation of microbial biosurfactants at the microbial cell surface. The binding of the biosurfactants to the microbial cell surface may be mediated by hydrophobic interactions (top illustration) or ionic interactions (bottom illustration). The hydrophobic part of the biosurfactant is indicated by a straight line (hydrophobic tail) and the hydrophilic by a circle (polar head). The possible adhesion of microorganisms to interfaces with hydrophilic (hatched) or hydrophobic (dotted) properties is indicated. Adapted from Neu, 1996. 50

Figure 2.2 Adhesion of hydrophilic and hydrophobic microorganisms to a hydrophilic (hatched) and hydrophobic (dotted) interface. The interface is covered with a microbial conditioning film of biosurfactants. Depending on the surface energy of the interface, the conditioning film of biosurfactants will have a different orientation. The biosurfactants may be bound to the interface by means of hydrophobic interactions or ionic interactions. The hydrophobic part of the biosurfactant is indicated by a straight line (hydrophobic tail). Adapted from Neu, 1996. 52

Figure 2.3 Detachment of microorganisms from hydrophilic (hatched) and hydrophobic (dotted) interfaces by excretion or release of biosurfactants. The surface active compounds will result in a microbial produced footprint or conditioning film consisting of biosurfactants. The orientation of the biosurfactants which form the footprint or conditioning film is determined by the surface energy of the interface. The microbial footprint or conditioning film may later influence the interaction of other bacteria with this interface. The hydrophobic part of the biosurfactant is indicated by a straight line (hydrophobic tail). Adapted from Neu, 1996. 53

CHAPTER 3

Figure 3.1 Fermentation evolution for *L. lactis* 53: variation of biomass concentration (g l^{-1}) (■) and surface tension (mN m^{-1}) (▲), in time. The biomass concentration is a measure of the cell growth, while surface tension is a measure of the biosurfactant activity. A) *L. lactis* 53 grown in MRS medium before experimental design optimization of the media composition. B) *L. lactis* 53 grown in MRS optimized by experimental design. 83

LIST OF FIGURES (CONT.)

Figure 3.2 Fermentation evolution for *S. thermophilus* A: variation of biomass concentration (g l^{-1}) (■) and surface tension (mN m^{-1}) (▲), in time. The biomass concentration is a measure of the cell growth, while surface tension is a measure of the biosurfactant activity. A) *S. thermophilus* A grown in M17 medium before experimental design optimization of media composition. B) *S. thermophilus* A grown in M17 optimized by experimental design. 84

Figure 3.3 Plots of Observed *versus* expected biomass concentration (g l^{-1}) values for *L. lactis* 53 (A) and *S. thermophilus* A (B). The biomass concentration is the response variable of interest. The expected biomass concentration values are determined by the model equations determined for fractional factorial design (*FFD*). 90

Figure 3.4 Response surface contour plots of biomass concentration (g l^{-1}) for *L. lactis* 53 and *S. thermophilus* A. The biomass concentration is the response variable of interest. The contour plots represent the effect of the significant variables and their interaction in the response variable. All the other variables non significant are held at zero level of the central composite design (*CCD*). A) The effect of peptone, lactose and their mutual interaction on biomass concentration for *L. lactis* 53. B)The effect of lactose, sodium glycerophosphate and their mutual interaction on biomass concentration for *S. thermophilus* A. 94

Figure 3.5 Plots of observed *versus* expected biomass concentration (g l^{-1}) values for *L. lactis* 53 (A) and *S. thermophilus* A (B). The biomass concentration is the response variable of interest. The expected biomass concentration values are determined by the model equations determined for central composite design (*CCD*). 95

CHAPTER 4

Figure 4.1 Blood agar screening method results for *L. casei* CECT-5275 (up-left spot), *L. rhamnosus* CECT-288 (up-right spot), *L. pentosus* CECT-4023 (down-left spot) and *L. coryniformis* subsp. *torquens* CECT-25600 (down-right spot). 109

Figure 4.2 Representation of the surface tension variation (--*--), experimental data and calculated time courses of biomass (◆, -----), glucose (▲, — - —) and biosurfactant concentrations (■,——) during fermentations carried out with MRS broth using (A) *L. casei* CECT-5275, (B) *L. rhamnosus* CECT-288, (C) *L. pentosus* CECT-4023, (D) *L. coryniformis* subsp. *torquens* CECT-25600. Results represent the average of three independent experiments. 110

Figure 4.3 Representation of the surface tension variation (--*--), experimental data and calculated time courses of biomass (◆, -----), glucose (▲, — - —) and biosurfactant concentrations (■,——) during fermentations carried out with whey broth using *L. pentosus* CECT-4023. Results represent the average of three independent experiments. 115

LIST OF FIGURES (CONT.)

CHAPTER 5

Figure 5.1 Surface tension of crude biosurfactant concentrations obtained from *L. lactis* 53 in PBS (pH 7.0) after 2 h as measured by ADSA-P. Results are averages of triplicate experiments varying within 2-7% (ANOVA) and the standard deviation represented by error bars. 126

Figure 5.2 The initial deposition rates (j_0) of the bacterial strains (*Staphylococcus epidermidis* GB 9/6, *Streptococcus salivarius* GB 24/9, *Staphylococcus aureus* GB 2/1,and *Rothia dentocariosa* GBJ 52/2B) and yeast (*Candida albicans* GBJ 13/4A and *Candida tropicalis* GB 9/9) isolated from explanted voice prostheses on silicone rubber with and without an adsorbed biosurfactant layer. Results are averages of triplicate experiments varying within 10-15% (ANOVA) and the standard deviation represented by error bars. 129

Figure 5.3 The number of microorganisms adhering after 4 h (n_{4h}) on silicone rubber with and without an adsorbed biosurfactant layer. The codification of the microorganisms is presented in Fig. 5.2. Results are averages of triplicate experiments varying within 10-15% (ANOVA) and the standard deviation represented by error bars. 130

CHAPTER 6

Figure 6.1 Surface tension of crude biosurfactant concentrations obtained from *S. thermophilus* A in PBS (pH 7.0) after 2 h as measured by ADSA-P. Results are averages of triplicate experiments varying within 3-8% (ANOVA) and the standard deviation represented by error bars. 144

Figure 6.2 The initial deposition rates (j0) of the bacterial strains (*Staphylococcus epidermidis* GB 9/6, *Streptococcus salivarius* GB 24/9, *Staphylococcus aureus* GB 2/1 and *Rothia dentocariosa* GBJ 52/2B) and yeast (*Candida albicans* GBJ 13/4A and *Candida tropicalis* GB 9/9) isolated from explanted voice prostheses on silicone rubber with and without an adsorbed biosurfactant layer. Results are averages of triplicate experiments varying within 10-15% (ANOVA) and the standard deviation represented by error bars. 145

Figure 6.3 The number of microorganisms adhering after 4 h (n_{4h}) on silicone rubber with and without an adsorbed biosurfactant layer. The microorganisms used are as described in Fig. 6.2. Results are averages of triplicate experiments varying within 10-15% (ANOVA) and the standard deviation represented by the error bars. 146

CHAPTER 7

Figure 7.1 Surface tension of several rhamnolipid dilutions in PBS (pH 7.0) as measured by the Ring method. Results are averages of triplicate experiments varying within 2-7% (ANOVA) and the standard deviation represented by error bars. 160

LIST OF FIGURES (CONT.)

Figure 7.2 The initial deposition rates (j_0) of the bacterial (*S. epidermidis* GB 9/6, *S. salivarius* GB 24/9, *S. aureus* GB 2/1 and *R. dentocariosa* GBJ 52/2B) and yeast (*C. albicans* GBJ 13/4A and *C. tropicalis* GB 9/9) strains isolated from explanted voice prostheses on silicone rubber with and without an adsorbed rhamnolipid layer. The undiluted rhamnolipid-containing solution used had a 4 g l^{-1} rhamnolipid concentration. Black column – without rhamnolipid; grey column – with rhamnolipid dilution 1:1000 and white column – with undiluted rhamnolipid. Results are averages of triplicate experiments varying within 10-15% (ANOVA) and the standard deviation represented by the error bars. 162

Figure 7.3 The number of microorganisms adhering after 4 h (n_{4h}) on silicone rubber with and without an adsorbed rhamnolipid layer (1:1000 dilution and undiluted rhamnolipid). The codification of the microorganisms is presented in 163

Figure 7.4 Deposition kinetics observed for the microbial strains isolated from explanted voice prostheses adhering to silicone rubber, and the subsequent effects of perfusing a rhamnolipid solution (dilution 1:15) through the chamber followed by the passage of a liquid-air interface. R, denotes the perfusion of the flow chamber with the rhamnolipid solution. D, denotes the passage of a liquid-air interface. Results are averages of duplicates experiments varying within 10-15% (ANOVA). 166

CHAPTER 8

Figure 8.1 (Adapted from Neu et al. 1994) A) Anatomy of the esophageal region before laryngectomy (arrows indicate parts to be removed). B) Anatomy after laryngectomy showing the separation of the airway and the digestive tract (arrow indicates tracheostoma). C) Schematic drawing of the Groningen button silicone rubber voice prosthesis. D) Groningen button inserted into the tracheo-esophageal shunt (arrow). 176

Figure 8.2 Schematic presentation of the artificial throat, equipped with three Groningen button voice prostheses. 180

Figure 8.3 Examples of pressure-flow diagrams of "Low-Resistance" Groningen button voice prostheses prior and after 7-days of biofilm formation in the artificial throat: (A) control voice prosthesis; (B) voice prosthesis pre-conditioned with biosurfactant 1; (C) voice prosthesis pre-conditioned with biosurfactant 2. 185

CHAPTER 9

Figure 9.1 Representation of the surface tension variation (--*--), experimental data and calculated time courses of biomass (◆, -----), lactose (▲, — - —) and biosurfactant concentrations (■, ——) during fermentations carried out with medium D (whey (50 g l^{-1} lactose content) + 5.8 g l^{-1} yeast extract + 44.8 g l^{-1} peptone) or medium J (whey (50 g l^{-1} lactose content) + 22 g l^{-1} yeast extract + 43.8 g l^{-1} peptone + 231.6 g l^{-1} sodium glycerophosphate) for (A) *L. lactis* 53 or (B) *S. thermophilus* A, respectively. Results represent the average of three independent experiments. 204

LIST OF FIGURES (CONT.)

Figure 9.2 Representation of the surface tension variation (--*--), experimental data and calculated time courses of biomass (◆, -----), lactose (▲, — - —) and biosurfactant concentrations (■, ——) during fermentations carried out with medium F (molasses (20 g l^{-1} sucrose content) + 2.3 g l^{-1} yeast extract + 18 g l^{-1} peptone) or medium M (molasses (20 g l^{-1} sucrose content) + 8.8 g l^{-1} yeast extract + 17.5 g l^{-1} peptone + 92.6 g l^{-1} sodium glycerophosphate) for (A) *L. lactis* 53 or (B) *S. thermophilus* A, respectively. Results represent the average of three independent experiments. 205

CHAPTER 10

Figure 10.1 Elution profile of the crude biosurfactant obtained from *L. lactis* 53 on Octyl Sepharose 4 FF Prep column. Fractions were eluted with a linear gradient from 1 to 0 M $(NH_4)_2SO_4$ in PBS buffer. Fractions were collected and monitored by absorbance at 280 nm record, total sugars (phenol-sulfuric method), total protein (Bradford method) and surface tension (Ring method). Results represent the average of three independent experiments. 222

Figure 10.2 Elution profile of the crude biosurfactant obtained from *S. thermophilus* A on Octyl Sepharose 4 FF Prep column. Fractions were eluted with a linear gradient from 1 to 0 M $(NH_4)_2SO_4$ in PBS buffer. Fractions were collected and monitored by absorbance at 280 nm record, total sugars (phenol-sulfuric method), total protein (Bradford method) and surface tension (Ring method). Results represent the average of three independent experiments. 223

Figure 10.3 Surface tension *versus* concentrations of the crude biosurfactants (1 and 2) and isolated fractions obtained from (A) *L. lactis* 53 or (B) *S. thermophilus* A, respectively. Results represent the average of three independent experiments. 225

Figure 10.4 Fourier transform infrared absorption spectra of crude biosurfactants (1 and 2) and respective isolated fractions (A and F) obtained from (A) *L. lactis* 53 or (B) *S. thermophilus* A. The absorption bands used for quantification are indicated. 233

Figure 10.5 Mass spectrum of crude biosurfactant obtained from *L. lactis* 53 (A) and respective isolated fraction (B). 236

Figure 10.6 Mass spectrum of crude biosurfactant obtained from *S. thermophilus* A (A) and respective isolated fraction (B). 237

LIST OF TABLES

CHAPTER 1

Table 1.1 Comparison between the most commonly used silicone rubber voice prostheses. 11

Table 1.2 Bacterial and yeast strains most frequently isolated from silicone rubber voice prostheses removed from laryngectomized patients. 16

Table 1.3 Modifications of silicone rubber surfaces. 19

Table 1.4 Percentages of bacteria and yeasts isolated from Groningen button and Provox®2 voice prostheses after biofilm formation in artificial throat and perfusion with various probiotic suspensions. As control, set at 100%, perfusion with phosphate-buffered saline solution was used. Adapted from Free *et al.* 2001. 27

Table 1.5 Percentages of bacteria and yeasts isolated from Groningen button and Provox®2 voice prostheses after biofilm formation in artificial throat and perfusion with various dairy products. As control, set at 100%, perfusion with phosphate-buffered saline solution was used. Adapted from Free *et al.* 2000b. 29

CHAPTER 2

Table 2.1 Examples of biosurfactants application in the medical field. 45

Table 2.2 Antimicrobial activity of biosurfactants at different concentrations against several bacterial and yeast strains isolated from explanted voice prostheses. The experiments were scored as positive (+) when growth inhibition was observed (no colonies formed); a (±) sign indicated some colonies formed within the zones; and no growth inhibition was marked as negative (-). For details see Chapter 8 and 2004b.Rodrigues *et al.* 2004b. 59

Table 2.3 Desorption percentages of microorganisms isolated from explanted voice prostheses adhered to silicone rubber as result of rhamnolipid perfusion through the parallel plate flow chamber with and without a following passage of a liquid-air interface. Results are averages of duplicate experiments varying within 10-15%. For details see Chapter 7 and Rodrigues *et al.* 2005c. 62

CHAPTER 3

Table 3.1 Experimental range and levels of the independent variables (X_i and Z_i, i = 1, 2, 3, 4, 5, 6) used in the Fractional Factorial Design (*FFD*). 85

Table 3.2 Experimental design and results of the Fractional Factorial Design (*FFD*). 86

Table 3.3 Analysis of Variance (ANOVA) for the first order models determined from the Fractional Factorial Design (*FFD*). 89

Table 3.4 Experimental design and results of the central composite design (*CCD*). 92

LIST OF TABLES (CONT.)

CHAPTER 4

Table 4.1 Surface tension values (mN m^{-1}) obtained for the culture broth supernatants during the 72 h fermentation (the control surface tension (MRS) was 50 mN m^{-1}) and from the biosurfactant extraction with PBS during 24 h (the control surface tension (PBS) was 72 mN m^{-1}). Results are expressed as means ± standard deviations of values from triplicate experiments. 109

Table 4.2 Results obtained by regression of biosurfactant, biomass and glucose concentration data in MRS broth fermentations. 114

Table 4.3 Results obtained by regression of biosurfactant, biomass and lactose concentration data for *L. pentosus* in whey broth fermentation 114

CHAPTER 5

Table 5.1 Contact angles (degrees) measured at 25ºC on silicone rubber with and without an adsorbed biosurfactant layer, and on microorganisms isolated from explanted voice prostheses, as determined by the sessile drop technique. The surface free energy (mN m^{-1}) component γ_{sv}^{LW}, electron-accepting γ_{sv}^{+} and electron-donating γ_{sv}^{-} parameters are presented. Standard deviations (±) were determined over three separate measurements. 128

Table 5.2 Interfacial free energies of adhesion between the several microorganisms isolated from explanted voice prostheses and silicone rubber with an adsorbed biosurfactant layer. The total free energy (ΔG) as well as the Lifshitz-Van der Waals (ΔG^{LW}) and the acid-base (ΔG^{AB}) components are presented. Experiments were performed in triplicate and correspond within 15%. 133

CHAPTER 7

Table 7.1 Microbial inhibition percentages obtained from the microtiter-plate anti-adhesion assay with several rhamnolipid concentrations. The dilution factor is a direct measure of the rhamnolipid concentration. The undiluted rhamnolipid-containing solution used had a 4 g l^{-1} rhamnolipid concentration. PBS was used as control and set at 0% as no microbial inhibition occurs. Experiments were carried out in triplicate and correspond within 15%. 161

Table 7.2 Contact angles (degrees) measured at 25ºC on silicone rubber with and without an adsorbed rhamnolipid layer as determined by the sessile drop technique. Standard deviations (±) were determined over three separate measurements. 164

LIST OF TABLES (CONT.)

CHAPTER 8

Table 8.1 Antimicrobial activity of biosurfactants with different concentrations against several bacterial and yeast strains isolated from explanted voice prostheses. Biosurfactant 1 was obtained from *L. lactis* 53 and biosurfactant 2 from *S. thermophilus* A. The experiments were scored as positive (+) when growth inhibition was observed; a (±) sign indicated some colonies formed within the zones; and no growth inhibition was marked as negative (-). 183

Table 8.2 The percentage of viable bacteria and yeasts isolated from the voice prostheses, with and without adsorbed biosurfactants, after biofilm formation in the artificial throat. Both for bacteria and yeasts, the number of organisms found after using PBS as a control was set at 100%. Also included are the decreases in airflow resistance caused by biofilms influenced by biosurfactants, compared with the effects of PBS as a control. The relative decrease in airflow resistance caused by the control was set as 0 cm H2O.s l^{-1}. Biosurfactant 1 was obtained from *L. lactis* 53 and biosurfactant 2 from *S. thermophilus* A. All experiments were carried out in triplicate with separately cultured strains. 184

CHAPTER 9

Table 9.1 Medium composition used in the fermentation experiments for both tested strains. 197

Table 9.2 Results obtained by regression of glucose, lactose or sucrose, biomass and biosurfactant concentration data in several fermentation medium for *Lactococcus lactis* 53. 201

Table 9.3 Results obtained by regression of glucose, lactose or sucrose, biomass and biosurfactant concentration data in several fermentation medium for *Streptococcus thermophilus* A. 202

CHAPTER 10

Table 10.1 Diameters (mm) of the clearing zones on the oil surface obtained from oil spreading assay with several crude biosurfactants (1 and 2) and isolated fractions concentrations. PBS was used as control with a diameter of 0.0 mm as no clearing zone occurs. Standard deviations (±) were determined over three separate measurements. 224

Table 10.2 Microbial inhibition percentages obtained from the microtiter-plate anti-adhesion assay with several crude biosurfactant 1 and correspondent isolated fractions (A, B and C) concentrations (g l^{-1}). PBS was used as control and set at 0% as no microbial inhibition occurs. Experiments were carried out in triplicate and correspond within 20%. 226

Table 10.3 Microbial inhibition percentages obtained from the microtiter-plate anti-adhesion assay with several crude biosurfactant 2 and correspondent isolated fractions (D, E and F) concentrations (g l^{-1}). PBS was used as control and set at 0% as no microbial inhibition occurs. Experiments were carried out in triplicate and correspond within 15%. 227

LIST OF TABLES (CONT.)

Table 10.4 Antimicrobial activities of fractions isolated from crude biosurfactant 1 with different concentrations against several bacterial and yeast strains isolated from explanted voice prostheses. The experiments were scored as positive (+) when growth inhibition was observed (no colonies formed); a (±) sign indicated some colonies formed within the zones; and no growth inhibition was marked as negative (-). 228

Table 10.5 Antimicrobial activities of fractions isolated from crude biosurfactant 2 with different concentrations against several bacterial and yeast strains isolated from explanted voice prostheses. The experiments were scored as positive (+) when growth inhibition was observed (no colonies formed); a (±) sign indicated some colonies formed within the zones; and no growth inhibition was marked as negative (-). 229

Table 10.6 Contact angles (degrees) measured over a period of 3 months at 25ºC on silicone rubber with adsorbed crude biosurfactants (1 and 2) or isolated fractions layers as determined by sessile drop technique. Bare silicone rubber was used as control with a contact angle of 109 ± 2 degrees. Standard deviations (±) were determined over three separate measurements. 230

Table 10.7 Surface tension measurements of crude biosurfactants (1 and 2) or isolated fractions with a concentration of 40 mg/ml at several pH values, as determined by the Ring method. Experiments were carried out in triplicate and correspond within 18%. 231

Table 10.8 Chemical composition data of crude biosurfactants (1 and 2) and isolated fractions (A and F) expressed as infrared absorption band ratios by Fourier transform infrared spectroscopy. 232

Table 10.9 Chemical composition data by XPS of crude biosurfactants (1 and 2), isolated fractions (A and F) and reference compounds (for comparison). 234

Table 10.10 Monosaccharide composition of the crude biosurfactants (1 and 2) and isolated fractions (A and F) as determined by gas-liquid chromatography. 238

LIST OF SYMBOLS

Uppercase Latin Letters

F value	*F*-test statistical parameter	
P	Biosurfactant concentration	(ML^{-3})
P value	Statistical parameter	
P_0	Initial biosurfactant concentration	(ML^{-3})
P_{max}	Maximum biosurfactant concentration	(ML^{-3})
P_r	Ratio between initial volumetric rate of biosurfactant formation (rp) and initial biosurfactant concentration (Po)	(t^{-1})
S	Substrate (glucose, lactose or sucrose) concentration	(ML^{-3})
S_0	Initial substrate (glucose, lactose or sucrose) concentration	(ML^{-3})
X	Biomass concentration	(ML^{-3})
X_0	Initial biomass concentration	(ML^{-3})
X_{max}	Maximum biomass concentration	(ML^{-3})
$Y_{P/S}$	Yield of biourfactant production per substrate consumption	(MM^{-1})
$Y_{P/X}$	Yield of biosurfactant production per biomass growth	(MM^{-1})
$Y_{X/S}$	Yield of biomass growth per substrate consumption	(MM^{-1})

Lower Latin Letters

cmc	Critical micelle concentration	(ML^{-3})
j_o	Initial deposition rate (number of microorganisms adhering per area per time)	$(L^{-2}\ t^{-1})$
n_{4h}	Number of adhering microorganisms after 4 h	(L^{-2})
r^2	Correlation coefficient	
r_p	Initial volumetric rate of biosurfactant production	$(ML^{-3}t^{-1})$

Greek Letters

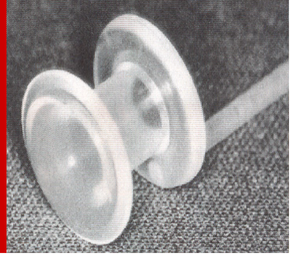
μ_{max}	Maximum specific growth rate	(t^{-1})
θ	Contact angle (degrees)	
γ	Surface free energy	$((MLt^{-2})L^{-1})$
γ^{LW}	Surface free energy: Lifshitz-Van der Waals component	$((MLt^{-2})L^{-1})$
γ^{AB}	Surface free energy: Acid-base component	$((MLt^{-2})L^{-1})$
γ^{-}	Electron-donating surface free energy parameter	$((MLt^{-2})L^{-1})$
γ^{+}	Electron-accepting surface free energy parameter	$((MLt^{-2})L^{-1})$
ΔG	Interfacial free energy	$((MLt^{-2})L^{-1})$

Abbreviations

ADSA-P	Axisymmetric drop shape analysis by profile
ANOVA	Analysis of variance
ATP	Adenosine 5'-triphosphate
BOD	Biochemical oxygen demand
cAMP	Cyclic adenosine monophosphate
CCD	Central composite design
CFU	Colony forming unit
DLVO	Theory advanced independently by Derjaguin & Landau and by Vervey & Overbeek
DNA	Deoxyribonucleic acid
EPS	Exopolysaccharide
ESI	Electrospray ionization
FEP	Fluoroethylene-propylene
FFD	Fractional factorial design
FPLC	Fast protein liquid chromatography

FTIR	Fourier transform infrared spectroscopy
HL60	Human promyelocytic leukaemia cell line
IPN	Interpenetrating polymer network
LC-MS	Liquid chromatography mass spectrometry
LPS	Lipopolysaccharide
M17 broth	Medium for improved growth of lactic streptococci and their bacteriophages and the selective enumeration of *Streptococcus thermophilus* from yoghurt
MEL	Mannosylerythritol lipid
MRS broth	Medium introduced by De Man, Rogosa and Sharpe for cultivation of *Lactobacillus* species
NGF	Nerve growth factor
PAS	Perfluoro-alkylsiloxane
PBS	Phosphate-buffered saline (PBS: 10 mM KH_2PO_4/K_2HPO_4 and 150 mM NaCl, pH 7.0)
PC12	Cell line derived from a rat pheochromocytoma
PDMS	Polydimethylsiloxane
PHEMA	Poly(2-hydroxylethyl methacrylate)
PLA2	Cytosolic phospholipase A2
PMMA	Poly(methyl methacrylate)
PVC	Poly(vinyl chloride)
QAC	Quaternary ammonium compounds
QAS	Quaternary ammonium silane
RTF	Reduced transport fluid
STL	Succinoyl trehalose lipid
TLC	Thin layer chromatography
XPS	X-ray photoelectron spectroscopy

GENERAL INTRODUCTION



"Whatever you do will be insignificant, but it is very important that you do it".

Mahatma Gandhi.

This thesis addresses the optimization of fermentation conditions for the production of biological antifouling agents, namely biosurfactants from probiotic bacteria, in order to develop new strategies for the prevention of microbial colonization of silicone rubber voice prostheses. The use of biosurfactants as coating agents for biomaterials is not yet a common issue as often the physiological role of biosurfactant production by microorganisms is unknown and the quantities released are too small for detailed research. Thus, fermentation must be cost competitive with chemical synthesis and many of the potential applications that have been considered for biosurfactants depend on whether they can be produced economically. Nevertheless, the interest in biosurfactants has increased considerably in recent years, as they have several advantages over chemical surfactants, therefore they are potential candidates for many applications in the biomedical field.

CONTEXT AND MOTIVATION

RESEARCH AIMS

OUTLINE OF THE THESIS

REFERENCES

CONTEXT AND MOTIVATION

Total laryngectomy remains the procedure of choice for advanced laryngeal carcinoma, either as a primary procedure or as a salvage following irradiation alone or concurrent chemoradiation therapy (Brown *et al.* 2003). The laryngeal cancers are by far the most frequently occurring cancers in the upper airway and digestive tract with reported incidences in Europe that account for 31.400 newly diagnosed cases in 2000 and a number of deaths estimated in 14.900 persons. Portugal is one of the leaders in the incidence and mortality from larynx cancer together with Spain, France, Belgium and Italy which is related with the smoking habits. Between 5-20% of the larynx cancers will require treatment with total laryngectomy leaning on tumour stage and differing treatment protocols. The inability to speak is the most disabling consequence of total laryngectomy and different methods of rehabilitating the lost voice of laryngectomized patients have been developed. The insertion of silicone rubber voice prosthesis in a surgically created tracheoesophageal shunt or fistula was a major step forward in the speech rehabilitation of laryngectomized patients.

Voice prostheses are not permanent implants and although silicone rubber is an ideal material for their manufacturing because of its ease of moulding and excellent mechanical properties; in laryngectomized patients, the hydrophobic surface becomes colonized rapidly with a thick biofilm, consisting of a variety of bacterial and yeast strains (Neu *et al.* 1993). Voice prostheses are replaced on average every 3-4 months when, due to biofilm formation, patients complain about leakage of food and liquid or increased air flow resistance (Busccher *et al.* 1997). Different strategies have been developed to prolong the lifetime of voice prostheses (Mahieu *et al.* 1986, Van Weissenbruch *et al.* 1997, Everaert *et al.* 1999, Elving *et al.* 2001, Gottenbos *et al.* 2002). Antifouling improvement of the silicone rubber material is desirable and modification of the silicone rubber surface to discourage biofilm formation is an obvious strategy to prolong the lifetime of voice prostheses. Although voice prostheses will become covered by a conditioning film of adsorbed salivary components prior to adhesion of bacteria or yeasts, experiments in the human oral cavity have demonstrated that the properties of this conditioning film are determined by the material itself (Busscher *et al.* 1997). In this context, biofilm formation can be influenced by adjusting the properties of the voice prosthesis material or by surface modification.

Within patient support groups in The Netherlands, laryngectomized patients have suggested that the consumption of buttermilk, containing antimycotic releasing *Lactococcus lactis*, positively influence the lifetime of their prostheses (Van der Mei *et al.* 1999). Similarly, active bioyogurt containing active *Streptococcus thermophilus* has been suggested to have such beneficial effects. The mechanism by which this occurs has not been investigated, but it is hypothesized that the presence of *S. thermophilus* and *Lactobacillus bulgaricus*, two well-known probiotic bacterial strains, in active bioyogurt may interfere with the adhesion of yeast to the silicone rubber possibly due to the release of biosurfactants (Van der Mei *et al.* 1999, 2000). Evaluations in the artificial throat model have furthermore indicated that the development of an oropharyngeal biofilm on silicone rubber voice prostheses can be delayed by exposure to caffeinated soft drinks (Free *et al.* 2000) or suspensions of active probiotics, such as *Lactococcus lactis* 53 and *S. thermophilus* B.

Little research has been done on biosurfactant production by probiotic bacteria and their interference in microbial adhesion, and only a few studies suggest that such a mechanism is applicable in the interference of uropathogenic enterococcal adhesion by lactobacilli and streptococci (Millsap *et al.* 1994, Velraeds *et al.* 1996, 1998, Reid *et al.* 1999, Gan *et al.* 2002). Biosurfactants are biological surface-active compounds produced by some microorganisms and can have some influence on interfaces. They include a wide variety of chemical structures such as glycolipids, lipopeptides, polysaccharide-protein complexes, phospholipids, fatty acids, and neutral lipids (Desai and Banat 1997). With regard to an anti-adhesive effect of biosurfactants, hypotheses have been forwarded in which adsorption of biosurfactants to a substratum surface alters the hydrophobicity of the surface and causes interference in microbial adhesion and desorption processes (Desai and Banat 1997). Biosurfactants have also been reported to have various degrees of antimicrobial activity (Banat *et al.* 2000). Some biosurfactants have strong antibacterial, antifungal and antiviral activity (Singh and Cameotra 2004).

Several problems and complications are associated with the use of voice prostheses and despite the significant improvement in the rehabilitation of laryngectomized patients over the last 30 years; both development of microbial-resistant biomaterials and optimization of life quality of patients will remain an interesting area of research for years to come.

RESEARCH AIMS

The main purposes of this thesis were the optimization of fermentation conditions for the production of biological antifouling agents, namely biosurfactants from probiotic bacteria, in order to develop new strategies for the prevention of microbial colonization of silicone rubber voice prostheses. The mechanisms by which these probiotic bacteria act are not yet clear and possibly involve biosurfactant release. Thus, the aims of this thesis were:

1- to establish whether probiotic bacteria *L. lactis* 53 and *S. thermophilus* A produce biosurfactants and to provide a physicochemical and biochemical characterization of these biosurfactants

2- to determine a possible role of the biosurfactants produced by these probiotic bacteria as anti-adhesive and antimicrobial coatings on silicone rubber voice prostheses

3- to increase the production yields of biosurfactants from probiotic bacteria by using non conventional low cost raw materials

The produced and characterized biosurfactants described in this thesis are intended to be applicable not only to the studied silicone rubber voice prostheses, but also to a vast class of biomaterials.

OUTLINE OF THE THESIS

This thesis is organized in eleven chapters that cover the research aims stated above. The thesis subjects are introduced in this chapter, while Chapter 11 respects to the main conclusions and recommendations extracted from the current work. In the other chapters, the research fields are covered as follows:

• In Chapter 1, an overview on the different approaches available and future perspectives to solve the frequent replacements of voice prostheses in laryngectomized patients is given. The main advantages and disadvantages of several techniques that have been employed in the recent years to improve antifouling properties of silicone rubber voice prostheses are discussed.

• In Chapter 2, medicinal and therapeutic perspectives of biosurfactants applications are reviewed. The biosurfactants antibacterial, antifungal and antiviral activities, making them relevant molecules for applications in treating many diseases and as therapeutic agents, are described. Also, their role as anti-adhesive agents against several pathogens indicating their utility as suitable anti-adhesive coatings for medical insertional materials is discussed.

• In Chapter 3, the improvement of the standard culture medium for biosurfactant production by response surface methodology, using a compilation of mathematical and statistical techniques, is described. The yields of biosurfactant production determined before and after optimization procedure are presented.

• The screening of a number of *Lactobacillus* strains by blood agar method and surface tension determination is described in Chapter 4. The relation between cellular growth and surface-activity of the biosurfactant during the fermentation is determined for all the strains and adequate models of the biosurfactant production as well as the time courses of glucose consumption and biomass growth are presented.

• In Chapter 5, the ability of the biosurfactant obtained from the probiotic bacterium, *L. lactis* 53 to inhibit adhesion of four bacterial and two yeast strains isolated from explanted voice prostheses to silicone rubber with and without an adsorbed biosurfactant layer in a parallel plate flow chamber is discussed.

• Microbial adhesion of four bacterial and two yeast strains isolated from explanted voice prostheses to silicone rubber before and after conditioning with a biosurfactant obtained from the probiotic bacterium *S. thermophilus* A in a parallel plate flow chamber is described in Chapter 6.

• In Chapter 7, the effects and extent of adhesion of four different bacterial and two yeast strains isolated from explanted voice prostheses to silicone rubber with and without an adsorbed rhamnolipid biosurfactant layer obtained from *Pseudomonas aeruginosa* DS10-129 is presented. The ability of rhamnolipid biosurfactant to inhibit adhesion of microorganisms to silicone rubber was investigated in a parallel plate flow chamber.

• The influence of biosurfactants from probiotic bacteria on the formation of biofilms on voice prostheses in an artificial throat model is evaluated in Chapter 8. The two tested biosurfactants are obtained from the probiotic bacteria *L. lactis* 53 and *S. thermophilus* A. A microbial growth inhibition test is first performed in order to estimate the concentration of biosurfactant to use in the artificial throat experiments. To this end, biofilms are grown on voice prostheses in the artificial throat model. In addition to biofilm evaluation, the effects of biosurfactant adsorption to voice prostheses on airflow resistances are determined.

• In Chapter 9, the development of a low-cost alternative medium for biosurfactant production by *L. lactis* 53 and *S. thermophilus* A is described. Molasses and cheese whey are evaluated as alternative media and compared with the conventional synthetic media. The yields of biosurfactant production for both strains are determined for all tested media. Additionally, the time courses of biosurfactant production, glucose, sucrose or lactose consumption and biomass growth are modelled.

• In Chapter 10, the key components of the crude biosurfactant mixtures produced by *L. lactis* 53 and *S. thermophilus* A, including their molecular composition (by Fourier transform infrared spectroscopy), elemental composition (by X-ray photoelectron spectroscopy), molecular mass (by mass spectrometry) and monosaccharide composition (by gas-liquid chromatography) are described. Moreover, partial functional characterization is established using the following techniques: blood agar test, oil spreading test, critical micelle concentration determination, antimicrobial activity and anti-adhesion test. Finally, desorption of biosurfactants from silicone rubber and their stability at several pH is evaluated.

REFERENCES

Banat, I.M., Makkar, S. and Cameotra, S. (2000) Potential commercial application of microbial surfactants - A review article. *Applied Microbiology and Biotechnology*. 53: 495-508.

Brown, D., Hilgers, F., Irish, J. and Balm, A. (2003) Postlaryngectomy voice rehabilitation: state of the art at the millenium. *World Journal of Surgery*. 27: 824-831.

Busscher, H.J., Van Hoogmoed, C.G., Geertsema-Doornbusch, G.I., Van der Kuijl-Booij, M. and Van der Mei, H.C. (1997) *Streptococcus thermophilus* and its biosurfactants inhibit adhesion by *Candida* spp. on silicone rubber. *Applied and Environmental Microbiology.* 63: 3810-3817.

Desai, J. and Banat, I.M. (1997) Microbial production of surfactants and their commercial potential. *Microbiology and Molecular Biology Reviews*. 61:47-64.

Elving, G., Van der Mei, H.C., Busscher, H.J., Van Weissenbruch, R. and Albers, F.W.J. (2001(a)) Air-flow resistences of silicone rubber voice prosthesis after formation of bacterial and fungal biofilms. *Journal Biomedical Materials Research*. 58: 421-426.

Everaert, E., Mahieu, H., Van de Belt-Gritter, B., Peeters, J., Verkerke, G., Van der Mei ,H.C. and Busscher,H. (1999) Biofilm formation *in vivo* on perfluoro-alkylsiloxane-modified voice prostheses. *Archives of Otolaryngology Head Neck Surgery.* 125: 1329-1332.

Free, R.H., Elving, G., Van der Mei, H.C., Van Weissenbruch, R., Albers, F.W.J. and Busscher, H.J. (2000) Caffeinated soft drinks reduce bacterial prevalence in voice prosthetic biofilms. *Biofouling.* 16: 69-76.

Gan, B., Kim, J., Reid, G., Cadieux, P. and Howard, J. (2002) *Lactobacillus fermentum* RC-14 inhibits *Staphylococcus aureus* infection of surgical implants in rats. *The Journal of Infectious Diseases*. 185: 1369-1372.

Gottenbos, B., Van der Mei, H.C., Klatter, F., Nieuwenhuis, P. and Busscher, H.J. (2002) *In vitro* and *in vivo* antimicrobial activity of covalently coupled quaternary ammonium silane coatings on silicone rubber. *Biomaterials.* 23: 1417-1423.

Mahieu, H.F., Van Saene, J.J., Van den Besten, J. and Van Saene, H.K. (1986) Oropharynx decontamination preventing *Candida* vegetation on voice prostheses. *Archives of Otolaryngology: Head and Neck Surgery*. 112: 1090-1092.

Millsap, K., Reid, G., Van der Mei, H.C. and Busscher, H.J. (1994) Displacement of *Enterococcus faecalis* from hydrophobic and hydrophilic substrata by *Lactobacillus* and *Streptococcus* spp.

as studied in a parallel plate flow chamber. *Applied and Environmental Microbiology*. 60: 1867-1874.

Neu, T.R., Van der Mei, H.C., Busscher, H.J., Dijk, F. and Verkerke, G.J. (1993) Biodeterioration of medical-grade silicone rubber used for voice prostheses: a SEM study. *Biomaterials*. 14: 459-464.

Reid, G., Heinemann, C., Velraeds, M., Van der Mei, H.C. and Busscher, H.J. (1999) Biosurfactants produced by *Lactobacillus*. *Methods in Enzymology*. 310: 426-433.

Singh, P. and Cameotra, S. (2004) Potential applications of microbial surfactants in biomedical sciences. *Trends in Biotechnology*. 22, 142-146.

Van der Mei, H.C., Free, R.H., Elving, G.J., Van Weissenbruch, R., Albers, F.W.J. and Busscher, H.J. (2000) Effect of probiotic bacteria on prevalence of yeasts in oropharyngeal biofilms on silicone rubber voice prostheses *in vitro*. *Journal of Medical Microbiology*. 49: 713-718.

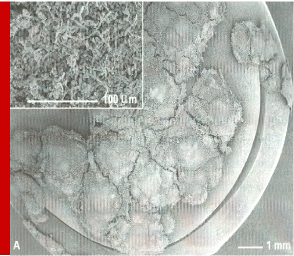
Van der Mei, H.C., Van de Belt-Gritter, B., Van Weissenbruch, R., Dijk, F., Albers, F.W. and Busscher, H.J. (1999) Effect of consumption of dairy products with probiotic bacteria on biofilm formation on silicone rubber implant surfaces in an artificial throat. *Trans IChemE*. 77: 156-158.

Van Weissenbruch, R., Bouckaert, S., Remon, J., Nelis, H.J., Aerts, R. and Albers, F.W. (1997) Chemoptophylaxis of fungal deterioration of the Provox prostheses. *Annals of Otology, Rhinology and Laryngology.* 106: 329-337.

Velraeds, M., Van de Belt-Gritter, B., Van der Mei, H.C., Reid, G. and Busscher, H.J. (1998) Interference in initial adhesion of uropathogenic bacteria and yeasts to silicone rubber by a *Lactobacillus acidophilus* biosurfactant. *Journal of Medical Microbiology.* 47: 1081-1085.

Velraeds, M., Van der Mei, H.C., Reid, G. and Busscher, H.J. (1996) Inhibition of initial adhesion of uropathogenic *Enterococcus faecalis* by biosurfactants from *Lactobacillus* isolates. *Applied and Environmental Microbiology*. 62: 1958-1963.

CHAPTER 1



STRATEGIES FOR THE PREVENTION OF BIOFILM FORMATION ON SILICONE RUBBER VOICE PROSTHESES

"Try to learn something about everything and everything about something"

Thomas H. Huxley.

In this Chapter an overview is given on the different approaches available and future perspectives to solve the frequent replacements of voice prostheses in laryngectomized patients due to microbial colonization.

1.1 INTRODUCTION

1.2 BIOFILM FORMATION ON VOICE PROSTHESES

1.2.1 Microbial Adhesion

1.2.2 Microbial Biofilms

1.2.3 Characteristics of Biofilms on Silicone Rubber Voice Prostheses

1.3 MODIFICATIONS OF SILICONE RUBBER SURFACES

1.3.1 Metal Coating

1.3.2 Plasma Treatment

1.3.3 Perfluoro-alkylsiloxane Treatment

1.3.4 Covalently Coupled Quaternary Ammonium Silane Coatings

1.3.5 Bulk Surface Photografting

1.3.6 Biosurfactants

1.4 PROPHYLACTIC TREATMENT ON SILICONE RUBBER VOICE PROSTHESES

1.4.1 Probiotics

1.4.2 Dairy Products

1.4.3 Caffeinated Soft Drinks

1.4.4 Antifungal Agents

1.4.5 Synthetic Salivary Peptides

1.5 CONCLUSIONS

1.6 REFERENCES

1.1 INTRODUCTION

Different methods of rehabilitating the lost voice of laryngectomized patients have been developed as the inability to speak is the most disabling consequence of total laryngectomy. The major step forward in the speech rehabilitation of patients was the insertion of a silicone rubber voice prosthesis in a surgically created tracheoesophageal fistula and since the introduction of the first reliable voice prosthesis by Singer and Blom in 1980 the success rate of vocal rehabilitation after total laryngectomy has improved considerably (Blom 2000, Singer and Blom 1980). There are different types of voice prostheses mentioned, nonindwelling (removable) devices, which have to be removed regularly for cleaning purposes, such as the Blom-Singer and Panje (Singer and Blom 1980, Panje 1981); and the indwelling devices, which remain in the stand for a longer period of time, such as the Groningen button (Nijdam *et al.* 1982), Traissac (Traissac *et al.* 1987), Nijdam (Nijdam *et al.* 1990), Provox® (Hilgers and Schouwenburg 1990), and Staffieri (Staffieri and Staffieri 1988). Indwelling voice prostheses are generally preferred by laryngectomees as many patients are inept due to lack of manual dexterity, fear or incomprehension to accurately remove and replace prostheses. Therefore, nonindwelling voice prostheses are especially allocated to motivated patients willing to be autonomous.

The self-retaining low resistance Provox® voice prosthesis, developed in the Netherlands Cancer Institute in 1988 (Hilgers and Schouwenburg 1990, Hilgers *et al.* 1993) together with the Groningen button voice prostheses are presently one of the more widely used devices in Europe. Table 1.1 summarizes a comparison of the features, advantages and disadvantages for the most common Dutch voice prostheses.

However, all indwelling silicone rubber voice prostheses will suffer from biofilm formation in time, leading to dysfunction and, eventually, replacement. In the literature it has been reported that the causes for often replacement of voice prostheses are mainly related with salivary leakage through the prosthesis valve, salivary leakage around the prosthesis, deterioration of the prosthesis, and increased airflow resistance due to valve mechanism blocking (Mahieu *et al.* 1986, Van den Hoogen *et al.* 1996, Laccourreye *et al.* 1997).

Table 1.1 Comparison between the most commonly used silicone rubber voice prostheses.

Voice Prostheses	Description	Advantages	Disadvantages	Reference
Groningen button	• medical grade silicone biflanged voice button • single-valve • esophageal side constructed as a one-way valve • valve outlet designed to allow passage of sufficient quantities of air from the trachea to the esophagus • tracheal side open • flange provided with small silicone string used during insertion • low pressure, indwelling and interchangeable (with Nijdam and Provox) voice prostheses. • average lifetime estimated = 15.8 weeks	• insertion simple and one-stage procedure • successfully restored speech in over 80% patients treated in primary procedure • secure, self-retaining flanges, no adhesives necessary • rapid rehabilitation • simple patient instruction • cheaper than Provox® voice prostheses • use of antimicrobial agents prolongs device lifetime	• local anaesthesia for insertion procedure • increased airflow resistance (45.4%) • leakage (58.8%) • silicone material susceptible to *Candida* colonization	Van den Hoogen *et al.* 1996 Van Lith-Bijl *et al.* 1992 Laccourreye *et al*. 1997 Leunisse *et al*. 2001
Provox®	• medical grade silicone rubber voice button • available in four shaft lengths (distance between the esophageal and tracheal flange) and supplied with a guidewire for retrograde insertion. • one-way valve • low-pressure, self-retaining, indwelling device • interchangeable with Nijdam and Groningen button. • average lifetime estimated = 13 weeks	• easy to manage • diminished airflow resistance (22.7%) • 80% of the patients report fair to good intelligibility both in face-to-face conversations and in the speaking in the telephone. • only maintenance required from the patient is the daily brush-cleaning of the device • replacement method safe and reliable (ensures correct position of the esophageal flange)	• more expensive • leakage (80.2%) • silicone material susceptible to *Candida* colonization and ingrowth • replacement method uncomfortable for the patient • local anaesthesia for insertion procedure	Van den Hoogen *et al.* 1996 Ackerstaff *et al.* 1999 Laccourreye *et al*. 1997 Leunisse *et al*. 2001

1.2 BIOFILM FORMATION ON VOICE PROSTHESES

Several strategies seem useful to prevent biofilm formation on voice prostheses. In general, the main goal is to reduce the attractive force between bacteria and biomaterial surface by optimizing the physicochemical surface properties of the biomaterial. For example, bacterial adhesion is low on extremely hydrophobic surfaces (Everaert *et al*. 1999), while also more negatively charged biomaterials attract fewer bacteria (Hoght *et al.* 1986). Keogh and Eaton (1994) have shown that albumin and heparin coatings decrease the adhesiveness of biomaterials. However, microorganisms always seem to be able to adhere to some extent to solid materials. Moreover, when proteins are present they can cover an anti-adhesive biomaterial and be the anchors for the adhesion of microorganisms.

Another approach to prevent biofilm formation is to avoid the growth of adhering microorganisms. This can be achieved by application of antimicrobial agents near the biomaterial surface and one way to do this is designing antibiotic releasing biomaterials. A disadvantage of these applications is that they usually only work for a few days to weeks, as the amount of antibiotic that is actually released is extremely limited and does not exceed 15% of the total amount incorporated (Van de Belt *et al.* 2000). However, there is a serious problem with antibiotic releasing materials and the low dose actually released, which is the development of antibiotic resistant microbial strains (Douglas 2003). Other approach would be to couple an antimicrobial agent covalently onto the biomaterial surface, while maintaining its activity. As in this approach the antimicrobial agent can only reach the outside of the microbial cells, it can only be employed with antibiotics working at the level of the cell wall or membrane. Polymers with incorporated quaternary ammonium groups have shown such antimicrobial activity *in vitro* (Flemming *et al.* 2000, Gottenbos *et al.* 2002b), thus these compounds might have the required properties (Gottenbos *et al.* 2002a).

1.2.1 Microbial Adhesion

When microorganisms have reached the biomaterial surface, initial microbial adhesion can occur. Microbial adhesion is mediated by specific interactions between cell surface structures and specific molecular groups on the substratum surface (Christensen *et al.* 1989), or by non-specific interaction forces, including Lifshitz-Van der Waals forces, electrostatic forces, acid-base interactions and Brownian motion forces (Van Oss 1991). The specific interactions are forces acting on highly localized regions of the interacting surfaces over distances smaller

than 5 nm, while non-specific interaction forces have a long-range character and originate from the entire body of the interacting surfaces (Gottenbos *et al*. 2002a). Upon approach of a surface, organisms will be attracted or repelled by the surface (DLVO theory), depending on the resultant of the different non-specific interaction forces (Reid 1999, Oliveira 1992).

Thus, the physicochemical surface properties of the biomaterial, with or without conditioning film of epithelial cells, and microorganisms play a major role in this process. The conditioning film on the biomaterial surface (and on the bacterial cell surface) plays an important role, as it changes the physicochemical properties of the interacting surfaces (Reid 1999). Albumin is a strong adhesion inhibitor, for unknown reasons, although changes in hydrophobicity and sterical hindrance are proposed mechanisms (Christensen *et al.* 1989). Fibronectin and fibrinogen have been shown to promote the adhesion of *Staphylococcus aureus* and certain *Staphylococcus epidermidis* strains, which is mediated by specific adhesive cell structures directed to these proteins (An and Friedman 1988).

After adhesion to biomaterials most microorganisms start secreting slime and embed themselves in a slime layer that provides protection against humoral and excreted cellular immune components. However, to do real damage the adhering microorganisms first have to grow.

The adhesion of bacteria to a surface is one of the first stages in the development of a biofilm and is believed to be influenced by a number of factors. As the substrate is essential in the development of a biofilm, an understanding of how substrate properties affect adherence of bacterial cells may assist in designing or modifying substrates inhibitory to bacterial adhesion.

1.2.2 Microbial Biofilms

The formation of an infectious biofilm on biomaterials appears to involve several sequential steps as can be seen in Figure 1.1, and includes adsorption of host conditioning film onto the device and adhesion of the infectious microorganisms anchoring by exopolymer production, growth and spread of the organisms in large clumps separated by water channels (Reid 1999).

Immediately after exposure of a device to body fluids, such as blood, saliva, or urine, macromolecular components adsorb to form a conditioning film (Gristina 1987, Reid *et al.* 1992). Many of the molecules are proteinaceous, such as serum albumin, fibrogen, collagen, and fibronectin, and some have been shown to affect subsequent bacterial adhesion (Keogh and Eaton, 1994, Christensen *et al.* 1989, An and Friedman 1988).

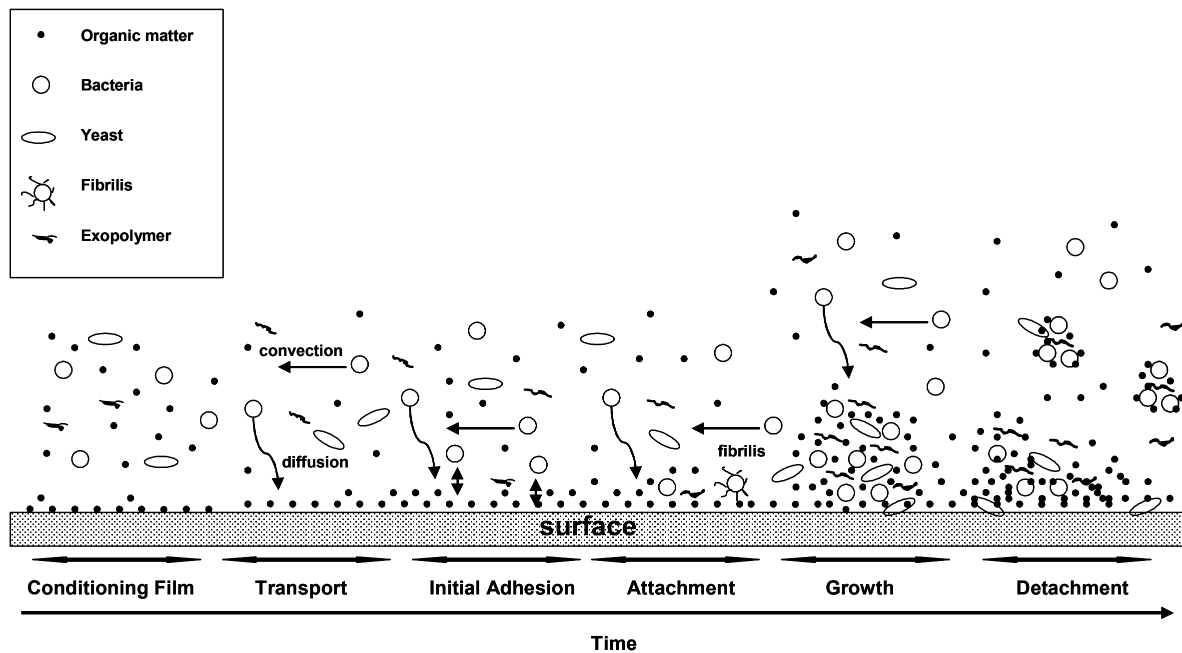


Figure 1.1 Schematic, sequential presentation of the steps in biofilm formation.

The film deposition consists in the first link in the chain of events leading to the formation of a mature biofilm and is the anchor from which further events take place. If it was possible to weaken or break this link, for example under the influence of fluctuating shear forces or using surfactants (Velraeds *et al.* 1996, Rodrigues *et al*. 2004a,b), it could be feasible for the entire biofilm to detach.

Detachment of parts of a biofilm can occur by failure inside the bulk of the biofilm, or by failure in the linking film, involving either the detachment of the initially adhering organisms, cohesive failure in conditioning film or interfacial rupture. Furthermore, as the number of biofilm organisms increases, growth rates will decrease due to nutrient and oxygen limitations and accumulation of organic acids, eventually leading to a stationary biofilm thickness, where adhesion and growth counterbalance detachment. Additionally, the quorum sensing phenomenon can occur, whereby the accumulation of a low-molecular mass signalling molecule enables individual cells to sense when the minimal population unit or “quorum” of bacteria has been achieved for a concerted action to be initiated.

1.2.3 Characteristics of Biofilms on Silicone Rubber Voice Prostheses

It has been well-documented that microorganisms can colonize surfaces of synthetic biomedical devices *in vivo*, resulting in disruption of prosthetic devices and sometimes in infection, although such an infection is rarely seen in the use of voice prostheses. Voice prostheses are non-implanted devices that contact with open air and consequently are not in a sterile environment.

All voice prostheses are mainly made of medical grade silicone rubber because of its excellent mechanical and moulding properties. However, silicone rubber materials have the tendency to become quickly colonized by microorganisms (Neu *et al.* 1992, 1993, Press *et al.* 1992), most notably *Candida* species (Mahieu *et al.* 1986, Palmer *et al.* 1993, Neu *et al.* 1994a,b, Everaert *et al.* 1997) resulting in frequent replacement of indwelling voice prostheses (Van den Hoogen *et al.* 1996, Mahieu *et al.* 1986, Neu *et al.* 1994a, Van Weissenbruch *et al.* 1997a). Table 1.2 summarizes the bacterial and yeast strains most frequently isolated from silicone rubber voice prostheses removed from laryngectomized patients.

Palmer and co-workers (1993) studied the microbial colonization of Blom-Singer valves obtained from laryngectomized patients both by standard microbial culture methods and scanning electron microscopy (SEM) and found that *S. aureus* was the significant colonizing organism. Two main types of microbial colonization forms with mixed biofilms of mainly cocci and yeast were distinguished on Groningen button voice prostheses (Neu *et al.* 1992). Moreover, Neu and co-workers (1993) studied the biodeterioration by yeasts of medical-grade silicone used for voice prostheses. The bacteria in the mixed biofilm found on the prostheses do not seem to be directly responsible for the material defects, since they were only associated with the surface parts of the yeast colonies and were found directly on the silicone surface. Additionally, the same authors (Neu *et al.* 1994a) investigated the taxonomy of the microflora on explanted silicone rubber voice prostheses and found that most of the bacteria were Gram-positive/Catalase-positive cocci and the yeasts were mainly *Candida* species.

Van der Mei *et al.* (1996) studied, by electron microscopy, the ingrowth features as seen *in vivo* for *Candida* strains using a modified Robbins device. The onset of the ingrowth features observed *in vivo* were shown by all strains: sometimes in the form of a small group of yeasts growing into a hole-like defect or, at other times in the form of clearly visible imprints in the silicone rubber, left after detachment of adhering yeasts during preparation of the samples for electron microscopy.

Although *Candida* species are mainly held responsible for microbial overgrowth of prostheses, the role of bacteria has also been emphasized (Ell *et al*. 1996, Van Weissenbruch *et al.* 1997a, Eerenstein *et al.* 1999, Free *et al.* 2000a, Millsap *et al.* 2001).

Table 1.2 Bacterial and yeast strains most frequently isolated from silicone rubber voice prostheses removed from laryngectomized patients.

Identification	Prosthesis type	Nº*	Reference
Candida species; *Staphylococcus aureus*	Groningen button	10	Mahieu *et al.* 1986
Bacteria: cocci, rods and filamentous forms; Yeasts	Groningen button	19	Neu *et al.* 1992
C. albicans; S. aureus	Blom-Singer	44	Palmer *et al.* 1993
C. albicans	Provox	3	Natarajan *et al*. 1994
C. albicans, C. tropicalis; Unidentified *cocci*	Eska-Herrmann	7	Neu *et al.* 1994(b)
C. albicans, C. tropicalis, *C. glabrata*, *C.krusei; Streptococcus mitis, S. salivarius; S. epidermidis, S. aureus*	Groningen button	26	Neu *et al.* 1994(a)
Candida species; staphylococci; streptococci; enterococci	Groningen button	55	Ell *et al*. 1996
C. albicans, C. glabrata, *C. krusei; S. mitis, S.salivarius; S.aureus, S. epidermidis*	Groningen button	7	Everaert *et al.* 1997
C. albicans, C. tropicalis, *C. krusei, C. glabrata, C. guillermondi; S.aureus*	Provox	55	Van Weissenbruch *et al.* 1997(b)
C. albicans, C. glabrata, C. krusei, C. tropicalis, C. parapsilosis, C. maris, C. lusitaniae, C. famata, C. guillermondi, C. kefyr	Provox Blom-Singer	170	Bauters *et al.* 2002
C. albicans I, C. humicola, C. glabrata, C. tropicalis, C. parapsilosis, C. krusei; Saccharomyces cerevisiae; Trichosporon cutaneum; Rothia dentocariosa; Streptococcus vestibularis, S. cricetus, S. suis, S. salivarius, S. mitis, S. pyogenes; S. epidermidis, S. aureus, S. lugdumensis, S. warneni, S. cohnii, S. intermedius; Escherichia coli ; Stomatococcus mucilaginous	Groningen button	38	Elving *et al*. 2002

* Number of voice prosthesis tested

Ell and co-workers (1996) studied the microflora of 55 failed Groningen buttons. In case of valve failure due to leakage (n=25) there was a positive correlation between biofouling in the lumen of the valve and the number of streptococci cultured. In valves failing due to increased air flow resistance, enterococci were particularly found on the esophageal surface. In another study, Van Weissenbruch *et al.* (1997a) identified 14 different yeast species in association with other commensals of the oral flora. The yeast strains were the most distinctive colonizers of the prostheses (72.9%), being *Candida albicans* and *Candida glabrata* the most often

isolated. *S. aureus* was found to be another dominant microorganism in all cultures and it was often isolated in association with *Candida* strains (Van Weissenbruch *et al.* 1997a, Free *et al.* 2000a). In addition, *Rothia dentocariosa* have been suggested as causative organisms for prosthesis failure by Elving and co-workers (2001). Only *R. dentocariosa* and *S. aureus* appear to positively influence adhesion to silicone rubber of yeast species from saliva, especially of *C. albicans* (Millsap *et al.* 2001). Interestingly, this observation coincides with clinical findings that malfunctioning of silicone rubber voice prostheses occurs more rapidly when either *R. dentocariosa* or *S. aureus* are present in the voice prosthetic biofilm in combination with *C. albicans*. This suggests that these bacterial strains enhance the prevalence of *C. albicans* on voice prostheses and therewith contribute to valve failure.

Although silicone rubber has long been considered as an inert biomaterial, this notion has been questioned (Press *et al.* 1992) due to the problems arising with silicone-filled breast implants. Also in dental materials science, it has been frequently observed that silicone rubber denture liners are apt to colonization and biodegradation by yeasts (Gettleman *et al.* 1983) similar to silicone rubber voice prostheses (Mahieu *et al.* 1986, Neu *et al.* 1993).

1.3 MODIFICATIONS OF SILICONE RUBBER SURFACES

Silicone rubber is used for a wide variety of biomedical applications due to its good mechanical properties, combined with a hydrophobic surface. For instance, silicone rubber has been used for voice prostheses (Neu *et al.* 1993), urinary catheters (Farber and Wolff 1993) and contact lens materials (Holly and Owen 1983).

Patients using voice prostheses are continuously exposed to saliva, food and drinks that together with the oropharyngeal microflora contribute to valve failure and frequent exchange of the implant (Mahieu *et al.* 1986). In this context, antifouling improvement of the silicone rubber material is desirable. In case of laryngectomized patients with voice prostheses lifetimes less than two months, there is need for employing “antibiofilm” therapy from the time of insertion of the voice prostheses, preferably without using antimycotics or antibiotics because of the risk of inducing resistant strains (Foley and Gilbert 1996, Mahieu *et al.* 1986, Van Weissenbruch *et al.* 1997b). Therefore, research into the development of new methods for preventing or retarding biofilm formation on voice prostheses is worthwhile. Different approaches have been undertaken to modify the silicone rubber surface as an obvious strategy to discourage biofilm formation and consequently to prolong the lifetime of voice prostheses. Although voice prostheses will become covered by a conditioning film of

adsorbed salivary components prior to adhesion of bacteria or yeasts, experiments in the human oral cavity have demonstrated that the properties of this conditioning film are determined by the material itself (Busscher *et al.* 1992, 1997a). By consequence, biofilm formation can be influenced by adjusting the properties of the voice prosthesis material or by surface modification.

A brief description on the several methods for silicone rubber surface modification is provided in Table 1.3.

1.3.1 Metal Coating

In order to create surfaces resistant to bacterial adhesion and colonization, several methods of silicone rubber surface modification have been described. Thicker coatings, such as those produced by solutions or sprays, scale off, and electrochemical treatment are not possible because silicone itself acts as an insuflator (Arweiler-Harbeck *et al*. 2001). Arweiler-Harbeck and co-workers (2001) aimed to create a *Candida*-resistant surface by either gold or titanium coating of silicone voice prostheses using a new method of surface modification by anodic vacuum arc coating. Although no functional change in the properties of the prosthesis and no difference in the quality of speech were reported by the patients as a result of metal coating, further studies are needed to show that metal coating with gold or titanium lead to a retardation of *Candida* growth.

Silver impregnation of poly(vinyl chloride) (PVC) completely inhibited *Pseudomonas aeruginosa* adhesion and efficiently prevented colonization over a longer period (Balazs *et al.* 2004). However and despite the high antimicrobial activity of silver coatings, because of two major disadvantages silver is not frequently considered as a suitable coating for silicone voice prostheses. Firstly, silver is toxic if ingested, which is an obvious drawback as a voice prosthesis is nothing more than a tracheoesophageal shunt. Secondly, and more importantly, silver produces a rough, high-energy surface. Rough surfaces promote plaque formation and maturation and high-energy surfaces are known to collect more plaque, to bind the plaque more strongly and to select specific bacteria (Quirynen and Bollen, 1995).

Table 1.3 Modifications of silicone rubber surfaces.

Method	Description	Advantages	Disadvantages	Reference
Gold and Titanium coating	• anodic arc plasma treatment • anode heated by means of particle bombardment from the cathode and pure gold is ionized • ionized anodic gold expands into the ambient vaccum forming an anodic arc which deposits onto the silicone surface	• suitable for coating medical silicone products • titanium produces a homogeneous coating • no functional change in the properties of the prosthesis was found • no difference in quality of speech observed • no visible irritation of mucosa	• time consuming • expensive method • no homogeneous coating on the entire prosthesis by gold	Arweiler-Harbeck *et al*. 2001
Silver treatment	• medical grade poly(vinyl chloride) PVC surface • oxygen glow discharge treatment followed by a two-step wet-treatment in sodium hydroxide and silver nitrate solutions	• high antimicrobial activity against *Staphylococcus aureus*, *Candida albicans*, *Pseudomonas aeruginosa* and others • reproducibility • reduces biofilm formation over a prolonged period of time	• expensive method • silver is toxic if ingested • produces a rough and high-energy surface prone to plaque formation • no improved yield of silver on the surface	Balazs *et al.* 2004
Palladium/tin salt mixture treatment	• voice prostheses immersed in palladium/tin salt solution • rinse and immersion in a colloidal palladium/tin solution	• simple procedure • biofilm formation was significantly less • this treatment does not negatively affect the airflow resistance of the voice prostheses • this treatment does not induce cytotoxicity • commercially available solutions	• some ingrowing microcolonies were observed • little known about the mechanism of microbial adhesion to metals	Dijk *et al.* 2000
Plasma treatment	• in a low-pressure, high-frequency discharge, the heavy particles (gas molecules and ions) are at ambient temperature while the electrons have enough kinetic energy to break covalent bonds and cause further ionization • the chemically reactive species can react with a solid surface in contact with the plasma	• conducted at near-ambient temperature • suited for processing thermally sensitive materials (semiconductors and polymers) • surface cleaning by removal of organic contaminants • *in vitro* microbial adhesion and growth reduced	• time consuming • *in vivo* biofilm formation oppositely enhanced by hydrophilizing the silicone rubber surface	Everaert *et al.* 1998a, 1999

Table 1.3 Modifications of silicone rubber surfaces. *Continuation*

Method	Description	Advantages	Disadvantages	Reference
Perfluoro-alkylsiloxane (PAS) treatment	• silicone rubber surface oxidized once in an argon plasma (Ar-SR) • PAS were chemisorbed onto Ar-SR surfaces • silane compounds were diluted in perfluoroheptane and subsequently the Ar-SR surfaces were put into these solutions for a period of time	• suitable for coating medical silicone products • adhesion of bacteria/yeasts significantly reduced • treatment increased the percentage detachment of bacteria/yeasts to almost 100% • low surface free energy and mobility of the adsorbed chain	• multi-step procedure • number of adhering organisms was not zero and growth may, albeit delayed, ultimately lead to the formation of a biofilm on the modified silicone rubber	Everaert *et al.* 1998b, 1999
Covalently coupled quaternary ammonium silane coatings	• silicone rubber surfaces were oxidized once in an argon plasma (Ar-SR) • oxidized silicone rubber was covered with quaternary ammonium silane (QAS) and allowed to react and dry	• suitable for coating medical silicone products • it was found antimicrobial activity *in vitro* against *Staphylococcus aureus*, *Streptococcus epidermidis*, *Escherichia coli* and *Pseudomonas aeruginosa*	• time consuming • multi-step procedure • *in vivo* the antimicrobial activity was detected just for *S. aureus*	Angelova and Hunkeler 1999 Gottenbos *et al.* 2002b
Bulk surface photografting	• laser-induced surface grafting of poly(2-hydroxyethyl methacrylate) (PHEMA) and a sequential method for preparation of interpenetrating polymer networks (IPNs) of PDMS/PHEMA were used for surface and bulk modifications, respectively	• suitable for coating medical silicone products • polymers anchored very strongly to the SiO_2 surfaces and survive even drastic extraction conditions without loss of chains • simple to implement and reagents readily available • platelet adhesion greatly reduced	• time consuming • multi-step procedure	Abbasi *et al.* 2002 Ramakrishnan *et al.* 2002
Biosurfactants	• produced by probiotic bacteria *Lactococcus lactis* 53 and *Streptococcus thermophilus* A strains • biosurfactant recovered after 2h extraction in phosphate saline buffer from cells in stationary growth phase • adsorption of biosurfactant onto surface	• suitable for coating medical silicone products • simple and reliable procedure • adhesion of bacteria/yeasts significantly reduced (over 90%) • promising strategy to prevent the microbial colonization of silicone rubber voice prostheses	• little known about the mechanism of microbial adhesion inhibition • amounts of produced biosurfactant are very low	Busscher *et al.* 1997b, 1999a Rodrigues *et al.* 2004a,b

Dijk and co-workers (2000) treated Groningen button voice prostheses with a colloidal palladium/tin solution to form a thin metal coat intended to discourage biofilm formation. Results showed that biofilm formation was significantly less on the heavily treated palladium/tin prostheses than it was on the untreated prostheses although some ingrowing microcolonies also were observed on the treated prostheses. Nevertheless, the spread of the biofilms was smaller on the treated prostheses than on the untreated ones. Therefore, palladium/tin-treated silicone rubber may have potential for extending the lifetime of indwelling voice prostheses.

1.3.2 Plasma Treatment

Polymeric surfaces can be efficiently modified by a glow-discharge plasma treatment, in which a non-polymer forming plasma (i.e. plasma of argon, oxygen or nitrogen) is used. Plasma treatments deals with overall effects of very complex reactions and essentially modify composition and structure of a few molecular layers at or near the surface of the material without affecting the bulk properties (Abbasi *et al*. 2002).

Everaert and co-workers (1998a) reported a study on the effects of repeated argon plasma treatment of medical grade hydrophobic silicone rubber on *in vitro* adhesion and growth of bacteria and yeasts isolated from voice prostheses, as well as *in vivo* biofilm formation. Briefly, this study demonstrated that *in vitro* microbial adhesion and growth on silicone rubber can be reduced by plasma treatment, but *in vivo* biofilm formation on silicone rubber voice prostheses is oppositely enhanced by hydrophilizing the silicone rubber surface. Several reasons can explain why *in vitro* and *in vivo* evaluation of the fouling properties of biomaterials surfaces might give contradictory results. Firstly, the number of strains and species occurring *in vivo* and their variability in cell surface properties is much larger than can be evaluated *in vitro*. Moreover, co-adhesion phenomena between bacteria or yeasts, as well as between yeasts and bacteria, occur *in vivo* but make *in vitro* evaluation even more difficult. Finally, the conditions in the oropharyngeal cavity (*in vivo*) are highly dynamic with regard to nutrient availability, temperature, humidity and shear.

1.3.3 Perfluoro-alkylsiloxane Treatment

As demonstrated by Everaert *et al* (1998a) biofilm formation on voice prostheses surfaces *in vivo* is governed by substratum hydrophobicity. For an improved antifouling performance of voice prostheses, increasing the hydrophobicity of the silicone rubber, like, for example, by adsorption of fluorocarbons, could be a possibility. Fluorocarbon surfaces (i.e. Teflon) are

slightly more hydrophobic than silicone rubber and hardly attracted any dental plaque during 9 days of exposure to dynamic conditions of the human oral cavity (Quirynen *et al.* 1989).

Everaert *et al.* (1998b) prepared reactive silicone rubber surfaces by argon plasma glow discharge prior to anchoring fluoro-alkyltrichlorosilanes. As a result of the increased hydrophobicity and mobility of the silicone rubber surface after fluoro-alkylsilane chemisorption, the adhesion of bacteria and yeasts was significantly reduced. However, despite a significant reduction in microbial adhesion, the number of adhering organisms was not zero and growth may, albeit delayed, ultimately lead to the formation of a biofilm on the modified silicone rubber when used, e.g. as a voice prostheses. More important than adhesion is the ability of the initially adhering microorganisms to withstand occasionally high detachment forces. A very promising aspect of chemisorbed long chain fluoro-alkylsiloxanes to silicone rubber is that they not only reduce microbial adhesion but also increase the percentage detachment by the passage of an air-bubble to almost 100%. This promising feature of chemisorbed long chain fluoro-alkylsilanes is probably caused by a combination of the low surface free energy of the surface created in combination with the mobility of the adsorbed chain. In another study, the same authors (Everaert *et al.* 1999) evaluated the potential of perfluoro-alkylsiloxane (PA) chemisorption on silicone rubber voice prostheses as a mean to reduce biofilm formation and thereby prolong the prostheses lifetime. It was demonstrated that biofilm formation on silicone rubber Groningen button voice prostheses over an evaluation period of approximately 2 to 8 weeks can be reduced by chemisorption of long (8 fluorocarbon units) PA polymer chains owing to the high hydrophobicity and mobility of these chemisorbed chains.

1.3.4 Covalently Coupled Quaternary Ammonium Silane Coatings

Another possible advance to prevent voice prostheses colonization is to render the silicone rubber surface antimicrobial properties by functionalization with quaternary ammonium groups, which are widely known as disinfectants. In this approach no antimicrobial agents are leaching from the surface, providing long term protection against bacterial colonization, and reducing the risk of developing antimicrobial resistant microbial strains, as the concentration of antimicrobial groups is constantly above the minimal inhibitory concentration. As quaternary ammonium functionalized surfaces have a high positive surface charge, they exert a strong adhesive force on negatively charged bacteria, which has been proposed to physically inhibit surface growth of rod-shaped bacteria. Poly(methacrylates) with methyl or ethyl quaternary ammonium chloride side groups showed antimicrobial activity (Gottenbos *et*

al. 2001, Kenawy *et al.* 1998, Angelova and Hunkeler 1999) toward Gram-negative strains, although Gram-positive *staphylococci* were little affected by these polymers.

Gottenbos and co-workers (2002b) determined the antimicrobial activity of 3-(trimethoxysilyl)-propyldimethyloctadecylammonium chloride (QAS) coating on silicone rubber. Antimicrobial activity of QAS coated silicone rubber was demonstrated both *in vitro* and *in vivo*. The application of positively charged biomaterial surfaces to prevent infection is a 180 degrees turn in thinking, as current research has been directed toward designing non-adhesive surfaces. Positively charged surfaces are strongly adhesive with regard to negatively charged bacteria, but the positive charge inhibited biofilm formation to proceed from the stage of initial adhesion toward growth since immobilized QAS molecules still interact with the cell membranes of adhering bacteria, presumably causing membrane leakage and cell death.

1.3.5 Bulk Surface Photografting

Bulk modification methods can be divided into blending, copolymerization, interpenetrating polymer networks and functionalization. Among the mentioned bulk modification techniques, laser-induced surface grafting and sequential method for interpenetrating polymer network preparation have the potential for local modification of a specific section of a polymeric sample.

In a recent study, Abbasi *et al.* (2002) attempted to modify the surface and bulk properties of polydimethylsiloxane (PDMS) polymers to improve their properties for biomedical applications. Data from *in vitro* results showed that platelet adhesion is greatly reduced by the surface graft polymerization of hydroxyethyl metacrylate (HEMA). HEMA-grafted PDMS surfaces showed better inhibition platelet adhesion results.

A simple procedure for synthesizing dense and homogeneous poly(methyl methacrylate) (PMMA) brushes on silicon substrates by a room temperature atom-transfer radical polymerization process starting from self-assemble monolayers of covanlently anchored initiators was described by Ramakrishnan *et al.* (2002). An advantage of the system described in comparison to similar systems on gold surfaces is that the polymers are anchored very strongly to the SiO_2 surfaces and survive even drastic extraction conditions without loss of chains.

1.3.6 Biosurfactants

Biosurfactants are microbial amphiphilic polymers and polyphilic polymers that tend to interact with the phase boundary between two phases in a heterogeneous system, defined as

interface. For all interfacial systems, it is known that organic molecules from the aqueous phase tend to immobilize at the solid interface. There they eventually form a film known as conditioning film, which will change the properties (wettability and surface energy) of the original surface (Neu 1996). In an analogy to organic conditioning films, biosurfactants may interact with the interfaces and will affect the adhesion and detachment of bacteria. Additionally, the substratum surface properties will determine the composition and orientation of the molecules conditioning the surface during the first hour of exposure. After about 4 hours, a certain degree of uniformity is reached and the composition of the adsorbed material becomes substratum independent (Neu 1996). Microorganisms excrete fatty acids, lipids, and biosurfactants into the surrounding media. A microbial cell able to excrete biosurfactants into the aqueous phase may be responsible for a microbially created conditioning film at an interface. On a hydrophobic interface, this conditioning film will change the interface from hydrophobic to hydrophilic. Biosurfactants may be oriented in different ways at the microbial cell surface. However, regardless of their orientation, if they are released from the cell surface or excreted into the area between the cell surface and interface, they will probably lead to detachment of bacteria from the interface.

Biosurfactants have become an important product of biotechnology for industrial and medical applications. The reason for their popularity, as high value microbial products, is primarily in their specific action, low toxicity, relative ease of preparation and widespread applicability. They can be used as emulsifiers, de-emulsifiers, wetting agents, spreading agents, foaming agents, functional food ingredients and detergents in various industrial sectors. Several studies concerning the applications, production methods and characterization of biosurfactants are described in the literature (Kosaric 1992, Lin 1996, Fiechter 1992).

Research into the use of biosurfactants as new methods for preventing or retarding biofilm formation on medical devices has been reported in the literature. A role for biosurfactants as defense weapons in postadhesion competition with other strains or species has been suggested for biosurfactants released by *Streptococcus mitis* strains against *Streptococcus mutans* adhesion (Pratt-Terpstra *et al*. 1989, Van Hoogmoed *et al.* 2000) and for biosurfactants released by lactobacilli against adhesion of uropathogens (Velraeds *et al.* 1996). Studies have shown than certain strains of bacteria, such as *Lactobacillus* strains, commonly found in healthy urogenital microflora, can protect the host against infection by invading uropathogens (Reid *et al.* 1998, 1999). The mechanism by which lactobacilli exert this protection is not yet known; however, it is speculated that biosurfactant production (Reid *et al.* 1984, Velraeds *et al.* 1996) and adhesion to the surface are prerequisites. Millsap and co-workers (1996) demonstrated that the adhesion of lactobacilli to silicone rubber differs with

strain hydrophobicity and charge, and that urinary components can affect the ability of hydrophilic *Lactobacillus* strains to adhere to substrata. Velraeds *et al.* (1996, 1998) determined the role of some *Lactobacillus* biosurfactants as anti-adhesive, non-antibiotic coatings on catheter surfaces. Since only one pathogen (*E. faecalis*) was studied and several *Lactobacillus* strains investigated did not inhibit the uropathogen equally well, it should not be expected that biosurfactants of different *Lactobacillus* strains will produce equivalent results for any given pathogen.

Other microorganisms were reported in the literature as biosurfactant producers, namely *Streptococcus thermophilus* strains isolated from heat exchanger plates in the downstream side of the regenerator section of pasteurizers in the dairy industry (Busscher *et al.* 1994). Busscher and co-workers (1997b) determined whether biosurfactant release by *S. thermophilus* might constitute a mechanism by which the lifetime of indwelling silicone rubber voice prostheses could become prolonged. To this end, the adhesion of different yeast strains, isolated from Groningen button voice prostheses, to silicone rubber in the absence and presence of biosurfactant-releasing *S. thermophilus* B cells was studied. The results obtained provide evidence in support of rumours circulating among laryngectomized patients and certain groups to ear-nose-throat clinicians that the consumption of dairy products with active bacteria may prolong the lifetime of indwelling silicone rubber voice prostheses. Later on, the same authors (Busscher *et al.* 1999a) extended this study into growth phase of biofilm formation. Also at the growth phase, distinct effects of the presence of biosurfactant-releasing streptococci on biofilm were observed.

Moreover, the ability of a biosurfactant obtained from the probiotic bacterium, *Lactococcus lactis* 53 to inhibit adhesion of microbial strains isolated from explanted voice prostheses to silicone rubber with and without an adsorbed biosurfactant layer was investigated in a parallel plate flow chamber, as described in Chapter 5 (Rodrigues *et al.* 2004a).

Figure 1.2 shows an example of the inhibitory effect of the biosurfactant obtained from *L. lactis* 53 on *S. aureus* GB 2/1 adhesion onto silicone rubber after 2 hours. The results obtained showed that the biosurfactant was effective in decreasing the initial deposition rates of *S. epidermidis* GB 9/6, *Streptococcus salivarius* GB 24/9 and *S. aureus* GB 2/1, allowing for a 90% reduction of the deposition rates. The deposition rates of *R. dentocariosa* GBJ 52/2B, *C. albicans* GBJ 13/4A and *C. tropicalis* GB 9/9 were far less reduced in the presence of the biosurfactant as compared with the other strains. Further work was developed (Rodrigues *et al.* 2004b) to assess the influence of the biosurfactants obtained from *L. lactis* 53 and *S. thermophilus* A in the growth phase of a prosthetic biofilm formation, as can be seen in detail on Chapter 8. Both biosurfactants greatly reduced microbial numbers on

prostheses and also induced a decrease in the airflow resistance of voice prostheses after biofilm formation. These results represent a promising strategy to prevent the microbial colonization of silicone rubber voice prostheses with the change of the surface properties of silicone rubber by adsorbing biosurfactants obtained from probiotic bacteria, thus prolonging the lifetime of voice prostheses.

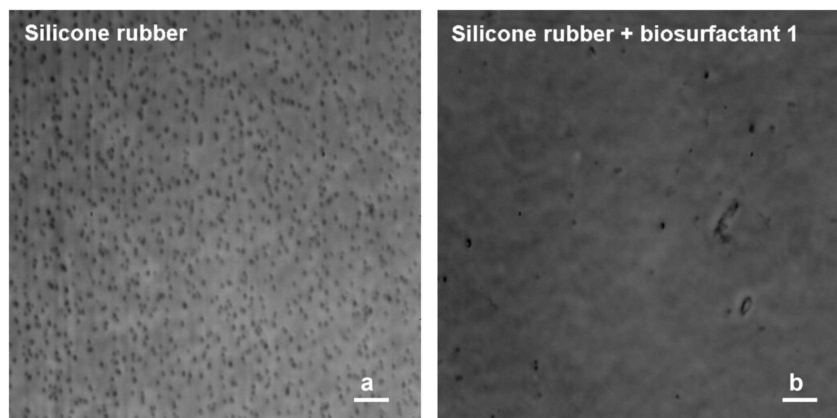


Figure 1.2 Micrographs of *Staphylococcus aureus* GB 2/1 (bar is 40 mm) adhering after 2h on silicone rubber with and without an adsorbed biosurfactant layer obtained from *Lactococcus lactis* 53.

1.4 PROPHYLATIC TREATMENT ON SILICONE RUBBER VOICE PROSTHESES

It is well-known that the biofilms are resistant to a range of antifungal agents currently in clinical use, including amphotericin B and fluconazole, and there appear to be multiple resistance mechanisms, thus alternative prophylactic treatments are being explored to prolong the lifetime of voice prostheses.

1.4.1 Probiotics

As nowadays antimicrobial resistance is a growing source of concern in modern medicine and health-improving functional foods are gaining in popularity, the development of alternative prophylactic and therapeutic agents, including probiotics, has been revitalized (Free *et al*. 2001). *Lactobacilli* are one of the most well-known probiotic bacterial genera and play an important role in the maintenance of a healthy intestinal and urogenital tract (Reid *et al.* 1984). Other bacterial species known to have probiotic effects are lactococci, enterococci,

Bifidobacterium and streptococci. The mechanisms by which probiotic bacterial exert their beneficial effects are not yet entirely clear. Some strains are able to release biosurfactants, while others are known to have antimycotic effects and produce lactic acid or hydrogen peroxide. Also, competitive adhesion (Batish *et al.* 1990, Busscher *et al.* 1997b), activation of the immune system (Perdigon *et al*. 1986), and nutrient competition (Free *et al*. 2001) have been suggested as mechanisms by which probiotic bacteria exert their beneficial effects.

In a study published by Wagner and co-workers (1997), it was demonstrated that probiotic bacteria have biotherapeutic potential for prophylaxis and therapy against candidiasis.

Free and co-workers (2001) determined the influence of probiotic bacteria (*L. lactis* 53 and *S. thermophilus* B) on biofilm formation on Groningen and Provox®2 voice prostheses in an artificial throat. Briefly, biofilms were grown on both types of voice prostheses and exposed 3 times daily to a probiotic bacterial suspension. It was found that yeast prevalence in voice prosthetic biofilms can be affected by introduction of probiotic bacteria in the ecosystem through the consumption of *L. lactis* 53 when the foreign body is the Groningen prosthesis, whereas introduction of *S. thermophilus* B has the greatest beneficial influence on yeast prevalence in biofilms on Provox®2 prostheses. Table 1.4 provides the percentages of bacteria and yeasts isolated from both Groningen button and Provox®2 voice prostheses after biofilm formation in artificial throat and perfusion with various probiotic suspensions.

Table 1.4 Percentages of bacteria and yeasts isolated from Groningen button and Provox®2 voice prostheses after biofilm formation in artificial throat and perfusion with various probiotic suspensions. As control, set at 100%, perfusion with phosphate-buffered saline solution was used. Adapted from Free *et al.* 2001.

	Groningen button		**Provox®2**	
Probiotic strain	Bacteria	Yeasts	Bacteria	Yeasts
Control	100	100	100	100
Streptococcus thermophilus B	52	33	15	0
Lactococcus lactis 53	17	22	20	48
Lactococcus rhamnosus 744	45	50	70	120
Lactobacillus fermentum B54	210	60	148	98
Enterococcus faecium 603	260	80	20	123
Lactobacillus casei Shirota	82	39	80	180
Bifidobacterium infantis 420	61	74	45	60
Lactococcus lactis cremoris SK11	50	62	10	270

Increased prostheses lifetimes can be expected from carefully designed food supplements containing these bacteria. The bacteria can be administered in different forms, such as freeze-dried in sachets or in a dairy product.

1.4.2 Dairy Products

Several studies to assess the beneficial effect of different dairy products on prolonging the lifetime of voice prostheses are reported in the literature (Busscher *et al.* 1998, 1999b, 2000, Van der Mei 1999, 2000, Free *et al.* 2000b). Within patient support groups in The Netherlands, laryngectomees have suggested that the consumption of buttermilk not only prolongs the clinical usefulness of indwelling silicone rubber voice prostheses but can also resolve early leakage of dysfunctioning valves. Scientific evidence for a potential beneficial effect of buttermilk consumption is lacking and probably impossible to obtain from clinical studies due to the necessary duration of such studies and also factors related to the patient environment. Busscher and co-workers (1998) simulated the consumption of buttermilk in an artificial throat and found that it almost fully prevented the formation of a biofilm during the experimental period. The mechanism by which the consumption of buttermilk interferes with biofilm formation on voice prostheses can only be speculated upon. Buttermilk is a mildly acidic dairy product with a pH of 4.5 due to the fermentation of sugars into lactic acid by *L. lactis* and *Streptococcus cremoris* and contains a number of enzymes in addition to high calcium content (110-120 mg per 100 g). *L. lactis* strains are known to release antimycotic substances, while the proteins present in buttermilk include casein, lactoglobulin and immunoglobulins and may have detergent properties. Clearly, the combined effect of all properties of buttermilk contributes to the control of biofilm formation on indwelling silicone rubber voice prostheses.

The influence of various dairy products on biofilm formation on Groningen and Provox®2 voice prostheses was studied (Free *et al.* 2000b), using the artificial throat model. In such experiments, the artificial throats were inoculated with the total microflora isolated from an explanted Groningen voice prosthesis. It was demonstrated that it is feasible to formulate a dairy product based on probiotics that will strongly prevent, if not fully eliminate, biofilm formation on voice prostheses. Differences were obtained between the two types of voice prostheses tested, being the effects less pronounced for Provox®2. Percentages of bacteria and yeasts isolated from both types of voice prostheses after biofilm formation in artificial throat and perfusion with various dairy products are shown in Table 1.5.

Table 1.5 Percentages of bacteria and yeasts isolated from Groningen button and Provox®2 voice prostheses after biofilm formation in artificial throat and perfusion with various dairy products. As control, set at 100%, perfusion with phosphate-buffered saline solution was used.
Adapted from Free *et al.* 2000b.

	Groningen button		**Provox®2**	
Probiotic strain	Bacteria	Yeasts	Bacteria	Yeasts
Control	100	100	100	100
Buttermilk	3	15	76	65
Pasteurized buttermilk	601	522	> 400	186
Yakult	12	74	91	> 400
Buttermilk/Yakult	33	217	19	488
Mona mild yoghurt	86	100	111	41
Mona vifit yoghurt	51	125	178	200
Semi-skimmed milk	62	200	74	88
Low-fat yoghurt	47	117	144	> 400

The Groningen voice prostheses contain fewer retention sites for colonizing organisms to find a niche than Provox®2 voice prostheses, due to the differences in design.

1.4.3 Caffeinated Soft Drinks

The influence of caffeinated soft drinks on biofilm formation on silicone rubber voice prostheses was investigated (Free *et al.* 2000a) in a modified Robbins device. The caffeinated soft drinks studied reduced bacterial prevalence in the biofilms to 1-5% of the control, while yeasts thrived in voice prosthetic biofilms exposed to caffeinated soft drinks. Free and co-workers (2000a) suggested that caffeine, or a combination of a low pH and high sugar content, might be essential to the inhibitory effects measured. The relevance of the results achieved for laryngectomized patients has to be established in a clinical trial, which might be difficult because of the multiple factors influencing biofilm formation on voice prostheses *in vivo*. However, this study points to rather simple alternative to antibiotics and antimycotics to influence biofilm formation on voice prostheses and prolong their lifetime through the incorporation of caffeinated soft drinks in a carefully designed diet for laryngectomees.

1.4.4 Antifungal Agents

A strategy frequently applied by otolaryngologists to solve the rapid colonization of voice prostheses is oropharyngeal yeast decontamination by using antifungal agents, despite the

fact that there is no compelling evidence that prescription of antifungal agents will prolong the lifetime of voice prostheses. Moreover, the prophylactic use of antifungal agents contributes to the development of resistant strains. Many efforts have been made to develop new antifungal drugs, as well as to clarify their effects on the lifetime of voice prostheses (Graybill 2000, Mahieu *et al.* 1986, Van Weissenbruch *et al.* 1997b, Bodey 1988, Georgopapadakou and Walsh 1996, Sheehan *et al.* 1999, Andriole 1999, Ackerstaff *et al.* 1999, Bauters *et al.* 2002). Oropharyngeal yeast decontamination by using amphotericin B lozenges and buccal adhesive slow-release tablets containing miconazole nitrate has been applied by otolaryngologists (Mahieu *et al.* 1986, Van Weissenbruch *et al.* 1997b) to increase the lifetime of voice prostheses. In studies (Mahieu *et al.* 1986) with Groningen button voice prostheses, the successful decontamination of the oropharynx with amphotericin B lozenges (10 mg) four times daily was also associated with a prolonged device life and lower intratracheal phonatory pressures. One of the drawbacks encountered in the use of this agent is certainly the frequent daily applications, leading to poor compliance by the patients. In 1988, Bodey (1988) reviewed the available antifungal drugs and described new imidazoles, such as itraconazole and fluconazole that at the time were undergoing evaluation. Also, liposomal preparations of amphotericin B were described as substantially less toxic and more effective, but still clinical trials had to be performed. Weissenbruch and co-workers (1997) conducted a double-blind randomized trial among 36 laryngectomees to assess the influence of a buccal bioadhesive slow-release tablet (10 mg) containing miconazole nitrate on the lifetime of the Provox® voice prosthesis. All patients colonized with *Candida* strains and treated with miconazole showed a significant decrease of colonization at the end of the study. The intratracheal phonatory pressures were remarkably higher after 2 months of follow-up in the placebo group. No local or systemic adverse reactions to miconazole were observed during this study. Patient compliance was acceptable according to regular miconazole determination in saliva samples. The device lifetime was significantly higher in patients treated with miconazole even after 1 year of follow-up. The use of a buccal bioadhesive slow-release tablet containing an antimycotic agent proves to be an adequate method of preventing fungal colonization and deterioration of silicone voice prosthesis. Also, inclusion of antimycotics in the devices during manufacturing and application of specific coating may be considered as possible options.

Bauters *et al.* (2002) assessed the colonization of tracheoesophageal voice prostheses by albicans and non-albicans *Candida* species and determined their susceptibility for three antimycotics that are frequently used for prophylaxis or treatment of oral candidiasis (i.e. miconazole, fluconazole, and nystatin). In this study, *C. albicans* was the predominant yeast

isolated, but its occurrence was superseded by that of the non-*albicans Candida* species as a group. It was found in the susceptibility data that *C. albicans* is more susceptible to azoles than non-*albicans* species. All isolates of *C. glabrata* proved sensitive to miconazole, and a large majority of them to fluconazole also. In contrast, 17.2% and 65.5% of isolates of *C. albicans* were resistant to miconazole and fluconazole, respectively. Moreover, it was found that 13.1% of all *Candida* species and 17.2% of *C. albicans* isolates were resistant to miconazole.

1.4.5 Synthetic Salivary Peptides

Salivary dysfunction as a result of surgical therapy, radiation therapy, aging or medication is frequently a problem for many laryngectomees. The low salivary secretion reduces the amount of histatins in saliva, yielding better chances for opportunistic microorganisms such as *C. albicans*, because histatins are the most significant source of fungicidal activity in saliva (Tenovuo 1998). Ruissen and co-workers (1999) developed a formulation which will prolong the retention time of antimicrobial agents at the site of application. Artificial salivary substitutes, commonly used by xerostomic patients and sometimes by laryngectomy patients, now mainly contain carboxymethylcellulose, animal mucins, or xanthan, but these substances present an excellent vehicle for novel antifungal agents. Promising antifungal agents are synthetic salivary peptides, which can possess bactericidal and fungicidal activities (Helmerhorst *et al.*, 1999). Moreover, these salivary peptides so far have not been associated with the development of microbial resistance. Helmerhorst and co-workers (1999) showed that a number of basic antifungal peptides, including human salivary histatin 5, a designed histatin analog designated dhvar4, and a peptide from frog skin, PGLa, are active against amphotericin B- resistant *C. albicans*, *C. krusei*, and *Aspergillus fumigatus* strains and against a fluconazole-resistant *C. glabrata* isolate. In addition, Elving *et al.* (2000) studied the antimicrobial activity of different synthetic salivary peptides derived from histatin against a variety of oropharyngeal microorganisms from explanted voice prostheses. Dhvar4 and dhvar5 were the only synthetic peptides with an antimicrobial spectrum broad enough to cover the variety of oropharyngeal microorganisms found on voice prostheses.

Recently, Oosterhof *et al.* (2003) conducted experiments in an artificial throat to determine the effectiveness of dhvar 4 and dhvar5 against the formation of the oropharyngeal biofilm. Biofilms consisting of bacteria and yeasts offered full protection against dhvar4, while dhvar5 was effective in reducing the number of bacteria and yeasts in mixed species biofilms. Unfortunately, this reduction was not accompanied by a reduction in airflow resistance, suggesting that the integrity of the biofilm was not affected. This could be due to

exopolysaccharide (EPS) and connecting slime threads still being present in the biofilm. The integrity of a biofilm is determined by the EPS production rather than by the number of organisms in the biofilm. This was confirmed by the observation that both ascorbic acid and N-acetylcysteine induced almost the same reduction in the number of bacteria and yeasts, probably due to their antioxidant natures, but with opposite effects on airflow resistances. Treatment with ascorbic acid did not result in a decrease in airflow resistance, whereas treatment with the mucolytic N-acetylcysteine did. Perez-Giraldo *et al.* (1997) studied the influence of various concentrations of N-acetylcysteine on the formation of biofilms of different strains of *S. epidermidis* and found a dose-related decrease in biofilm and slime formation. N-acetylcysteine, therefore, is a promising chemical to disrupt the integrity of voice prosthetic biofilms, especially since it can be swallowed and used over a long period without adverse effects.

1.5 Conclusions

In recent years there have been major new developments of methods for restoring the lost voice of laryngectomized patients. The main step forward in the speech rehabilitation was the insertion of silicone rubber voice prostheses in a surgically created tracheoesophageal shunt and is now generally considered to be superior to any other form of substitute voice production. Voice prostheses are not permanent implants, but need to be replaced when patients complain about leakage through or around the prosthesis, or increased efforts to produce tracheoesophageal speech. A continuous exposure to saliva, food, drinks and oropharyngeal microflora contribute to rapid colonization of the voice prostheses by mixed biofilm of bacteria and yeasts, leading to valve failure and frequent exchange of the implant. In this context, antifouling improvement of the silicone rubber material is desirable whether by the development of new biomaterials or new antimicrobial agents. This review describes the different approaches available and future perspectives on solving the voice prostheses drawbacks. When designing new biomaterials, inhibition of surface growth should be achieved by changing the physicochemical properties of the biomaterial surface or by binding antimicrobial agents covalently to the biomaterial surface. Detailed information on the methods to modify silicone rubber surfaces, and the several prophylactic treatments adequate for silicone rubber voice prostheses, has been provided. Moreover, as nowadays antimicrobial resistance is a growing source of concern in modern medicine the development of alternative prophylactic and therapeutic agents, including probiotics and biosurfactants, has been revitalized.

In conclusion, the combination of the proposals discussed in this review will improve the antifouling properties of silicone rubber voice prostheses. As a consequence, the lifetime of voice prostheses may be lengthened which would directly benefit laryngectomized patients. Nevertheless, laryngectomees should keep in mind that the use of medical devices do not cure diseases, rather they correct the functional consequences of disease.

1.6 REFERENCES

Abbasi, F., Mirzadeh, H. and Katbab, A. (2002) Bulk and surface modification of silicone rubber for biomedical applications. *Polymer International*. 51: 882-888.

Ackerstaff, A., Hilgers, F., Meeuwis, C., Van der Velden, L., Van den Hoogen, F., Marres, H., Vreeburg,G. and Manni, J. (1999) Multi-institutional assessment of the Provox 2 voice prosthesis. *Archives Otolaryngology Head Neck Surgery*. 125: 167-173.

An, Y.H. and Friedman, R.J. (1988) Concise review of mechanisms of bacterial adhesion to biomaterial surfaces. *Journal of Biomedical Materials Research*. 43: 338-348.

Andriole, V. (1999) Current and future antifungal therapy: new targets for antifungal agents. *Journal of Antimicrobial Chemotherapy*. 44: 151-162.

Angelova, N. and Hunkeler, D. (1999) Rationalizing the design of polymeric biomaterials. *TIBETECH.* 17: 409-421.

Arweiler-Harbeck, D., Sanders, A., Held, M., Jerman, M., Ehrich, H. and Jahnke, K. (2001) Does metal coating improve the durability of silicone rubber voice prosthesis? *Acta Otolaryngologica*. 121: 643-646.

Balazs, D., Triandafillu, K., Wood, P., Chevolot, Y., Van Delden, C., Harms, H., Hollenstein, C. and Mathieu, H. (2004) Inhibition of bacterial adhesion on PVC endotracheal tubes by RF-oxygen glow discharge, sodium hydroxide and silver nitrate treatments. *Biomaterials.* 25: 2139-2151.

Batish, V., Lal, R. and Chander, H. (1990) Effect of nutritional factors on the production of antifungal sustance by *Lactococcus lactis* subsp. *lactis biovar diacetylactis*. *Australian Journal of Dairy Technology.* 45: 74-76.

Bauters, T., Moerman, M., Vermeersch, H. and Nelis, H. (2002) Colonization of voice prostheses by *Albicans* and non-*albicans Candida* species. *The Laryngoscope*. 112: 708-712.

Blom, E. (2000) Tracheoesophageal voice restoration: origin - evolution - state-of-the-art. *Folia Phoniatrica et Logopaedica*. 52: 1-3.

Bodey, G. (1988) Topical and systemic antifungal agents *Medical Clinics of North America*. 72: 637-659.

Busscher, H.J., Bruinsma, G., Van Weissenbruch, R., Leunisse, C., Van der Mei, H.C., Dijk, F. and Albers, F.W.J. (1998) The effect of buttermilk consumption on biofilm formation on silicone rubber voice prostheses in an artificial throat. *European Archives Otorhinolaryngology.* 255: 410-413.

Busscher, H.J., Cowan, M.M. and Van der Mei, H.C. (1992) On the relative importance of specific and non-specific approaches to oral microbial adhesion. *FEMS Microbiology Letters.* 88: 199-210.

Busscher, H.J., Free, R.H., Van Weissenbruch, R., Albers, F.W.J. and Van der Mei, H.C. (2000) Preliminary observations on influence of dairy products on biofilm removal from silicone rubber voice orostheses *in vitro*. *Journal of Dairy Science.* 83: 641-647.

Busscher, H.J., Geertsema-Doornbusch, G.I. and Van der Mei, H.C. (1997a) Adhesion to silicone rubber of yeasts and bacteria isolated from voice prostheses: influence of salivary conditioning films. *Journal of Biomedical Materials Research*. 34: 201-210.

Busscher, H.J., Mulder, A. and Van der Mei, H.C. (1999b) *In vitro* adhesion to enamel and *in vivo* colonization of tooth surfaces by *lactobacilli* from a bio-yoghurt. *Caries Research*. 33: 403-404.

Busscher, H.J., Neu, T. and Van der Mei, H.C. (1994) Biosurfactant production by *thermophilic* dairy streptococci. *Applied Microbiology and Biotechnology.* 41: 4-7.

Busscher, H.J., Van de Belt-Gritter, B., Westerhof, M., Van Weissenbruch, R., Albers, F.W.J. and Van der Mei, H.C (1999a) Microbial interference in the colonization of silicone rubber implant surfaces in the oropharynx: *Streptococcus thermophilus* against a mixed fungal/bacterial biofilm. In: *Microbial Ecology and Infectious Disease.* Ed. Rosenberg, E. Washington D.C.: American Society for Microbiology. Pp. 66-74.

Busscher, H.J., Van Hoogmoed, C.G., Geertsema-Doornbusch, G.I., Van der Kuijl-Booij, M. and Van der Mei, H.C. (1997b) *Streptococcus thermophilus* and its biosurfactants inhibit adhesion by candida spp on silicone rubber. *Applied and Environmental Microbiology.* 63: 3810-3817.

Christensen, G.D., Baddour, L.M., Hasty, D.L., Lowrance, J.H. and Simpson, W.A. (1989) Microbial and foreign body factors in the pathogenesis of medical device infections. In *Infections associated with indwelling medical devices*. Ed. Bisno, A.L. and Waldvogel, F.A. Washington DC: American Society of Microbiology. Pp. 27-59.

Dijk, F., Westerhof, M., Busscher, H.J., Van Luyn, M. and Van der Mei, H.C. (2000) *In vitro* formation of oropharyngeal biofilms on silicone rubber treated with a palladium/tin salt mixture. *Journal of Biomedical Materials Research*. 51: 408-412.

Douglas, L. (2003) *Candida* biofilms and their role in infection. *TRENDS in Microbiology*. 11: 30-36.

Eerenstein, S., Grolman, W. and Schouwenburg, P. (1999) Microbial colonization of silicone rubber voice prostheses used in laryngectomized patients. *Clinical Otolaryngology*. 24: 398-403.

Ell, S.R., Davies, C.M., Clegg, R.T. and Parker, A.J. (1996) Do bacteria play a role in silastic speaking valve failure? In: *Surgery and Prosthetic voice restoration after total and subtotal laryngectomy*. Ed. Algaba, J. Amesterdam: Elsevier Science. Pp. 363-365.

Elving, G., Van der Mei, H.C., Busscher, H.J., Amerongen, A. and Veerman, E. (2000) Antimicrobial activity of synthetic salivary peptides against voice prosthetic microrganisms. *The Laryngoscope*. 110: 321-24.

Elving, G., Van der Mei, H.C., Busscher, H.J., Van Weissenbruch, R. and Albers, F.W.J. (2001) Air-flow resistences of silicone rubber voice prosthesis after formation of bacterial and fungal biofilms. *Journal Biomedical Materials Research*. 58, 421-26.

Elving, G.J., Van der Mei, H.C., Busscher, H.J., Van Weissenbruch, R. and Albers, F.W. (2002) Comparison of the microbial composition of voice prosthesis biofilms from patients requiring frequent *versus* infrequent replacement. *Annals of Otology, Rhinology & Laryngology.* 111: 200-203.

Everaert, E., Mahieu, H., Chung, R., Verkerke, G., Van der Mei, H.C. and Busscher,H.J. (1997) A new method for *in vivo* evaluation of biofilms on surface-modified silicone rubber voice prostheses. *European Archives Otorhinolaryngology*. 254: 261-263.

Everaert, E., Mahieu, H., Reitsma, A., Van de Belt-Gritter, B., Van der Mei, H.C., Verkerke, G. and Busscher, H.J. (1998a) *In vitro* and *in vivo* microbial adhesion and growth on argon plasma treated silicone rubber voice prostheses. *Journal of Materials Science: Materials in Medicine*. 9: 147-157.

Everaert, E., Mahieu, H., Van de Belt-Gritter, B., Peeters, J., Verkerke, G., Van der Mei ,H.C. and Busscher,H.J (1999) Biofilm formation *in vivo* on perfluoro-alkylsiloxane-modified voice prostheses. *Archives of Otolaryngology Head Neck Surgery.* 125: 1329-1332.

Everaert, E., Van der Mei , H.C. and Busscher,H.J. (1998b) Adhesion of yeasts and bacteria to fluoro-alkylsiloxane layers chemisorbed on silicone rubber. *Colloids and Surface B: Biointerfaces.* 10: 179-190.

Farber, B.F. and Wolff, A.G. (1993) Salicylic acid prevents the adherence of bacteria and yeast to silastic catheters. *Journal Biomedical Materials Research*. 27: 599-602.

Fiechter, A. (1992) Biosurfactants: moving towards industrial application. *Trends in Biotechnology*. 10: 208-218.

Flemming, R.G., Capelli, C.C., Cooper, S.L. and Proctor, R.A. (2000) Bacterial colonization of functionalized polyurethanes. *Biomaterials*. 21: 273-281.

Foley, I. and Gilbert, P. (1996) Antibiotic resistance of biofilms. *Biofouling*. 10: 331-346.

Free, R.H., Elving, G., Van der Mei, H.C., Van Weissenbruch, R., Albers, F.W.J. and Busscher, H.J. (2000a) Caffeinated soft drinks reduce bacterial prevalence in voice prosthetic biofilms. *Biofouling* 16: 69-76.

Free, R.H., Elving, G., Van Weissenbruch, R., Busscher, H.J., Van der Mei, H.C. and Albers, F.W.J. (2000b) Biofilm formation on voice prostheses: influence of dairy products *in vitro*. *Acta Otolaryngologica.* 120: 92-99.

Free, R.H., Van der Mei, H.C., Dijk, F., Van Weissenbruch, R., Busscher, H.J. and Albers, F.W.J. (2001) Biofilm formation on voice prostheses: *in vitro* influence of probiotics. *Annals of Otology, Rhinology & Laryngology.* 110: 946-951.

Georgopapadakou, N. and Walsh, T. (1996) Antifungal agents: chemotherapeutic targets and immunologic strategies. *Antimicrobial Agents and Chemotherapy*. 40: 279-291.

Gettleman, L., Fisher, D.J. and Farris, C. (1983) Self-sanitizing soft denture liners: paradoxical results. *Journal Biomedical Materials Research*. 17: 731-734.

Gottenbos, B., Busscher, H.J. and Van der Mei, H.C. (2002a) Pathogenesis and prevention of biomaterial centered infections. *Journal of Materials Science: Materials in Medicine*. 13: 717-722.

Gottenbos, B., Grijpma, D., Van der Mei, H.C., Feijen, J. and Busscher, H.J. (2001) Antimicrobial effects of positively charged surfaces on adhering Gram-positive and Gram-negative bacteria. *Journal of Antimicrobial Chemotherapy*. 48: 7-13.

Gottenbos, B., Van der Mei, H.C., Klatter, F., Nieuwenhuis, P. and Busscher, H.J. (2002b) *In vitro* and *in vivo* antimicrobial activity of covalently coupled quaternary ammonium silane coatings on silicone rubber. *Biomaterials*. 23: 1417-1423.

Graybill, J. (2000) Systemic antifungal drugs. *Revista Iberoamericana de Micologia.* 168: 174.

Gristina, A.G. (1987) Biomaterial centered infection: microbial adhesion *versus* tissue integration. *Science*. 237: 1588-1595.

Helmerhorst, E., Reijnders, I., Van't Hof, W., Simoons-Smit, I., Veerman, E. and Amerongen, A. (1999) Amphotericin B- and Fluconazole-resistant *Candida* spp, *Aspergillus fumigatus*, and other newly emerging pathogenic fungi are susceptible to basic antifungal peptides. *Antimicrobial Agents and Chemotherapy*. 43: 702-704.

Hilgers, F.J.M. and Schouwenburg, P.F. (1990) A new low-resistance, self-retaining prosthesis (Provox®) for voice rehabilitation after total laryngectomy. *Laryngoscope*. 100: 1202-1207.

Hilgers, F.J.M., Cornelissen, M.W. and Balm, A.J.M. (1993) Aerodynamic characteristics of the low-resistance, indwelling Provox® voice prosthesis. *European Archives Otorhinolaryngology*. 250: 375-380.

Hoght, A.H., Dankert, J. and Feijen, J. (1986) Adhesion of coagulase-negative staphylococci to methacrylate polymers and copolymers. *Journal of Biomedical Materials Research*. 20: 533-545.

Holly, F.J. and Owen, M.J. (1983) In: *Physicochemical Aspects of Polymer Surfaces.* Ed. Mittal, K.L. New York: Plenum Press.

Kenawy, E., Abdel-Hay, F.I., El-Shanshoury, A.R. and El-Newehy, M.H. (1998) Biologically active polymers: synthesis and antimicrobial activity of modified glycidyl methacrylate polymers having a quaternary ammonium and phosphonium groups. *Journal of Controlled Release*. 50: 145-152.

Keogh, J.R. and Eaton, J.W. (1994) Albumin binding surfaces for biomaterials. *Journal of Laboratory and Clinical Medicine*. 124: 537-545.

Kosaric, N. (1992) Biosurfactants in industry. *Journal of American Oil Chemists Society*. 64: 1731-1737.

Laccourreye, O., Menard, M., Crevier-Buchman, L., Couloigner, V. and Brasnu, D. (1997) *In situ* lifetime, causes for replacement, and complications of the Provox voice prosthesis. *The Laryngoscope*. 107: 527-530.

Leunisse, C., Van Weissenbruch, R., Busscher, H.J., Van der Mei, H.C., Dijk, F. and Albers, F.W.J. (2001) Biofilm formation and design features of indwelling silicone rubber tracheoesophageal voice prostheses - an electron microscopical study. *Journal of Biomedical Materials Research*. 58: 556-563.

Lin, S. (1996) Biosurfactants: recent advances. *Journal of Chemical Technology and Biotechnology*. 66: 109-120.

Mahieu, H., Van Saene, H., Rosingh, H. and Schutte, H. (1986) *Candida* vegetations on silicone voice prostheses. *Archives of Otolaryngology Head Neck Surgery*. 112: 321-325.

Millsap, K., Bos, R., Van der Mei, H.C. and Busscher, H.J. (2001) Adhesive interactions between voice prosthetic yeast and bacteria on silicone rubber in the absence and presence of saliva. *Antonie van Leeuwenhoek*. 79: 337-343.

Millsap, K., Reid, G., Van der Mei, H.C. and Busscher, H.J. (1996) Adhesion of *Lactobacillus* species in urine and phosphate buffer to silicone rubber and glass under flow. *Biomaterials*. 18: 87-91.

Natarajan, B., Richardson, M.D., Path, M.R.C., Irvine, B.W.H. and Thomas, M. (1994) The ProvoxTM voice prosthesis and *Candida albicans* growth: a preliminary report of clinical, mycological and scanning electron microscopc assessment. *Journal of Laryngology and Otology.* 108: 666-668.

Neu, T. (1996) Significance of bacterial surface active compounds in interaction of bacteria with interfaces. *Microbiological Reviews.* 60: 151-166.

Neu, T., De Boer, C., Verkerke, G., Schutte, H., Rakhorst, G., Van der Mei, H.C. and Busscher, H.J. (1994b) Biofilm development in time on a silicone voice prosthesis - a case study. *Microbial Ecology in Health and Disease*. 7: 27-33.

Neu, T., Dijk, F., Verkerke, G., Van der Mei, H.C. and Busscher, H.J. (1992) Scanning electron microscopy study of biofilms on silicone voice prostheses. *Cells and Materials.* 2: 261-269.

Neu, T., Van der Mei, H.C., Busscher, H.J., Dijk, F. and Verkerke, G. (1993) Biodeterioration of medical-grade silicone rubber used for voice prostheses: a SEM study. *Biomaterials.* 14: 459-464.

Neu, T., Verkerke, G., Herrmann, I., Schutte, H., Van der Mei, H.C. and Busscher, H.J. (1994a) Microflora on explanted silicone rubber voice prostheses: taxonomy, hydrophobicity and electrophorectic mobility. *Journal of Applied Bacteriology*. 76: 521-528.

Nijdam, H.F., Annyas, A.A., Schutte, H.K. and Leever, H. (1982) A new prosthesis for voice rehabilitation after laryngectomy. *Archives of Otorhinolaryngology.* 237: 27-29.

Nijdam, H.F., Manni, J.J. and Nicolasen, M. (1990) A new valveless low-pressure voice prostheses. *Clinical Otolaryngology.* 15: 561.

Oliveira, D.R. (1992) Physico-chemical aspects of adhesion. In: *Biofilms – Science and Technology*. Ed. Melo, L.F., Bott, T.R., Fletcher, M. and Capdeville, B. Dordrecht: Kluwer Academic Publishers. Pp. 45-58.

Oosterhof, J., Elving, G., Stokroos, I., Amerongen, A., Van der Mei, H.C., Busscher, H.J., Van Weissenbruch, R. and Albers, F.W.J. (2003) The influence of antimicrobial peptides and mucolytics on the integrity og biofilms consisting of bacteria and yeasts as affecting voice prosthesis air flow resistances. *Biofouling*. 19: 347-353.

Palmer, M., Johnson, A. and Elliott, T. (1993) Microbial colonization of Blom-Singer prostheses in postlaryngectomy patients. *Laryngoscope*. 103: 910-914.

Panje, W.R. (1981) Prosthetic vocal rehabilitation following laryngectomy. *Annals of Otology, Rhinology & Laryngology*. 90: 116-120.

Perdigon, G., De Marcias, M.E., Alvaraz, S., Oliver, G. and De Ruiz-Holgado, A.A. (1986) Effect of perorally administrated lactobacilli on macrophage activation in mice *Infectious Immunology*. 53: 404-410.

Perez-Giraldo, C., Rodriguez-Benito, A., Moran, F.J., Hurtado, C., Blanco, M.T. and Gomez-Garcia, A.C. (1997) Influence of N-acetylcysteine on the formation of biofilm by *Staphylococcus epidermidis*. *Journal of Antimicrobial Chemotherapy*. 39: 643-646.

Pratt-Terpstra, I.H., Weerkamp, A.H. and Busscher, H.J. (1989) Microbial factors in a thermodynamic approach of oral streptococcal adhesion to solid substrata. *Journal Colloid Interface Science*. 129: 568-574.

Press, R.I., Peebles, C.L., Kumagai, Y., Ochs, R.L. and Tan, E.M. (1992) Antinuclear auto-antibodies in woman with silicone breast implants. *The Lancet*. 340: 1304-1307.

Quirynen, M. and Bollen, C.M. (1995) The influence of surface roughness and surface-free energy on supra- and subgingival plaque formation in man. A review of the literature. *Journal Clinical Periodontology*. 22: 1-14.

Quirynen, M., Marechal, M., Busscher, H.J., Weerkamp, A., Arends, J., Darius, P. and Van Steenberghe, D. (1989) The influence of surface free-energy on planimetric plaque growth in man. *Journal of Dental Research*. 68: 796-799.

Ramakrishnan, A., Dhamodharan, R. and Ruhe, J. (2002) Controlled growth of PMMA brushes on silicon surfaces at room temperature. *Macromolecules Rapid Communications*. 23: 612-616.

Reid, G. (1999) Biofilms in infectious disease and on medical devices. *International Journal of Antimicrobial Agents*. 11: 223-226.

Reid, G., Bruce, A. and Smeianov, V. (1998) The role of *Lactobacilli* in preventing urogenital and intestinal infections. *International Dairy Journal*. 8: 555-562.

Reid, G., Heinemann, C., Velraeds, M., Van der Mei, H.C. and Busscher, H.J. (1999) Biosurfactants produced by *Lactobacillus*. *Methods in Enzymology*. 310: 426-433.

Reid, G., Tieszer, C., Foerch, R., Busscher, H.J., Khoury, A.E. and Van der Mei, H.C. (1992) The binding of urinary components and uropathogens to a silicone latex urethral catheter. *Cells Materials.* 2: 253-260.

Reid, G., Zalai, C. and Gardiner, G. (1984) Urogenital *Lactobacilli* probiotics, reliability, and regulatory issues. *Journal of Dairy Science*. 84E: 164-169.

Rodrigues, L.R., Van der Mei, H.C., Teixeira, J. and Oliveira, R. (2004a) Biosurfactant from *Lactococcus lactis* 53 inhibit microbial adhesion on silicone rubber. *Applied Microbiology and Biotechnology*. 66: 306-311.

Rodrigues, L.R., Van der Mei, H.C., Teixeira, J. and Oliveira, R. (2004b) Influence of biosurfactants from probiotic bacteria on formation of biofilms on voice prostheses. *Applied and Environmental Microbiology*. 70: 4408-4410.

Ruissen, A., Van der Reijden, W., Van't Hof, W., Veerman, E. and Amerongen, A. (1999) Evaluation of the use of xanthan as vehicle for cationic anfifungal peptides *Journal of Controlled Release*. 60: 49-56.

Sheehan, D., Hitchcock, C. and Sibley, C. (1999) Current and emerging azole antifungal agents. *Clinical Microbiology Reviews*. 12: 40-79.

Singer, M.I.M. and Blom E. (1980) An endoscopic technique for restoration of voice after laryngectomy. *Annals of Otology, Rhinology & Laryngology.* 89: 529-531.

Staffieri, M. and Staffieri, A. (1988) A new voice button for post-total laryngectomy speech rehabilitation. *Laryngoscope*. 98: 1027-1029.

Tenovuo, J. (1998) Antimicrobial function of human saliva - how important is it for oral health? *Acta Odontologica Scandinavica.* 56: 250-256.

Traissac, L., Devars, F., Gioux, M., Petit, J., Benjebria, A., Henry, C.H. and Ucelay, I. (1987) La réhabilitation vocale du laryngectomisé par implant phonatoire. *Revue de Laryngologie*. 108: 157-159.

Van de Belt, H., Neut, D., Uges, D.R.A., Schenk, W., Van Horn, J.R., Van der Mei, H.C. and Busscher, H.J. (2000) Surface roughness, porosity and wettability of gentamicin-loaded bone cements and their antibiotic release. *Biomaterials*. 21: 1981-1987.

Van den Hoogen, F., Oudes, M., Hombergen, G., Nijdam, H. and Manni, J. (1996) The Groningen, Nijdam and Provox Voice prostheses: a prospective clinical comparison based on 845 replacements. *Acta Otolaryngologica*. 116: 119-124.

Van der Mei, H.C., Van de Belt-Gritter, B., Dijk, F. and Busscher,H.J. (1996) Initial deterioration of silicone rubber by *Candida albicans* and *Candida tropicalis* strains isolated from voice prostheses. *Cells and Materials.* 6: 157-163.

Van der Mei, H.C., Van de Belt-Gritter, B., Van Weissenbruch, R., Dijk, F., Albers, F.W.J. and Busscher, H.J. (1999) Effect of the consumption of dairy products with probiotic bacteria on biofilm formation on silicone rubber implant surfaces in an artificial throat. *Trans IChemE*. 77C: 156-158.

Van Hoogmoed, C.G., Van der Kuijl-Booij, M., Van der Mei, H.C. and Busscher, H.J. (2000) Inhibition of *Streptococcus mutans* NS adhesion to glass with and without a salivary conditioning film by biosurfactant-releasing *Streptococcus mitis* strain. *Applied and Environmental Microbiology.* 66: 659-663.

Van Lith-Bijl, J., Mahieu, H., Patel, P. and Zijlstra, R. (1992) Clinical experience with the low-resistance Groningen button. *European Archives Otorhinolaryngology*. 249: 354-357.

Van Oss, C.J. (1991) The forces involved in bioadhesion to flat surfaces and particles – their determination and relative roles. *Biofouling*. 4: 25-35.

Van Weissenbruch, R., Albers, F.W.J., Bouckaert, S., Nelis, H., Criel, G., Remon, J. and Sulter, A. (1997a) Deterioration of the Provox silicone tracheoesophageal voice prosthesis: microbial aspects and structural changes. *Acta Otolaryngologica (Stockholm)*. 117: 452-458.

Van Weissenbruch, R., Bouckaert, S. and Remon, J. (1997b) Chemoprophylaxis of fungal deterioration of the Provox silicone tracheoesophageal prosthesis in postlaryngectomy patients. *Annals of Otology, Rhinology & Laryngology*. 106: 329-337.

Velraeds, M., Van de Belt-Gritter, B., Van der Mei, H.C., Reid, G. and Busscher, H.J. (1998) Interference in initial adhesion of uropathogenic bacteria and yeasts to silicone rubber by a *Lactobacillus acidophilus* biosurfactant. *Journal of Medical Microbiology.* 47: 1081-1085.

Velraeds, M., Van der Mei, H.C., Reid, G. and Busscher, H.J. (1996) Inhibition of initial adhesion of uropathogenic *Enterococcus faecalis* by biosurfactants from *Lactobacillus* isolates. *Applied and Environmental Microbiology*. 62: 1958-1963.

Wagner, R., Pierson, C., Warner, T., Dohnalek, M., Farmer, J., Roberts, L., Hilty, M. and Balish, E. (1997) Biotherapeutic effects of probiotic bacteria on candidiasis in immunodeficient mice. *Infection and Immunity*. 65: 4165-4172.

CHAPTER 2

BIOSURFACTANTS: POTENTIAL APPLICATIONS IN MEDICINE

"The more knowledge you have more you will appreciate it".

Leonardo da Vinci.

The use and potential commercial application of biosurfactants in the medical field has increased during the past decade. Their antibacterial, antifungal and antiviral activities make them relevant molecules for applications in combating many diseases and as therapeutic agents. In addition their role as anti-adhesive agents against several pathogens indicates their utility as suitable anti-adhesive coating agents for medical insertional materials leading to a reduction of a large number of hospital infections without the use of synthetic drugs and chemicals. Medicinal and therapeutic perspectives of biosurfactants applications are reviewed in this Chapter.

2.1 INTRODUCTION

2.2 BIOSURFACTANTS: MECHANISMS OF INTERACTION

2.2.1 Synthetic Surfactants and Bacteria

2.2.2 Biosurfactants and Bacteria

2.2.3 Surface Active Approach to Bacterial Adhesion/Detachment

2.3 BIOLOGICAL ACTIVITY OF BIOSURFACTANTS

2.3.1 Lipopeptides

2.3.2 Glycolipids

2.3.3 Other Biosurfactants with Biological Activity

2.4 ANTIMICROBIAL ACTIVITY OF BIOSURFACTANTS

2.5 ANTI-ADHESIVE ACTIVITY OF BIOSURFACTANTS

2.6 BIOMEDICAL AND THERAPEUTICAL APPLICATIONS OF BIOSURFACTANTS

2.7 CONCLUSIONS

2.8 REFERENCES

2.1 INTRODUCTION

Microbial compounds which exhibit pronounced surface and emulsifying activities are classified as biosurfactants. Biosurfactants constitute a wide variety of chemical structures, such as glycolipids, lipopeptides, polysaccharide-protein complexes, phospholipids, fatty acids and neutral lipids (Morikawa *et al.* 1993, Lin 1996, Desai and Banat 1997, Angelova and Shmauder 1999, Ahimou *et al.* 2000). For instance, Cooper and Goldenberg (1987) described different bioemulsifiers produced by two *Bacillu*s species in water-soluble substrates with distinct emulsifying and surface activities. It is, therefore, reasonable to expect diverse properties and physiological functions for different groups of biosurfactants. Moreover, these molecules can be tailor-made to suit different applications by changing the growth substrate or growth conditions (Fiechter 1992). Although most biosurfactants are considered as secondary metabolites, some may play essential roles for the survival of the producing-microorganisms either through facilitating nutrient transport, microbe-host interactions or as biocide agents. Biosurfactant roles include increasing the surface area and bioavailability of hydrophobic water-insoluble substrates, heavy metal binding, bacterial pathogenesis, quorum sensing and biofilm formation (Singh and Cameotra 2004). Biosurfactants are amphipatic molecules with both hydrophilic and hydrophobic moieties that partition preferentially at the interface between fluid phases that have different degrees of polarity and hydrogen bonding, such as oil and water, or air and water interfaces; which explains their broad use in environmental applications (Banat 1995a, b, Mulligan 2005). Most work on biosurfactant applications has been limited to their use in environmental applications due to their diversity, environmentally friendly nature, possibility of large-scale production and selectivity (Banat *et al.* 2000). Despite their potentials and biological origin only few studies were carried out on biomedical applications (Benincasa *et al.* 2004, Flasz *et al.* 1998, Makkar and Cameotra 2002). Some biosurfactants are suitable alternatives to synthetic medicines and antimicrobial agents, and may be used as safe and effective therapeutic agents (Table 2.1).

Table 2.1 Examples of biosurfactants application in the medical field.

Microorganism	Biosurfactant type	Activity/Application	Reference
Pseudomonas aeruginosa	Rhamnolipid	• antimicrobial activity against *Mycobacterium tuberculosis* • anti-adhesive activity against several bacterial and yeast strains isolated from voice prostheses	Gerard *et al.* 1997; Lang *et al.* 1999; Maier *et al.* 2000; Rodrigues *et al.* 2005c
Bacillus subtilis	Surfactin	• antimicrobial and antifungal activities • inhibition of fibrin clot formation • hemolysis and formation of ion channels in lipid membranes • antitumor activity against Ehrlich's ascite carcinoma cells • antiviral activity against human immunodeficiency virus 1 (HIV-1)	Bernheimer *et al.* 1970; Kameda *et al.* 1974; Sheppard *et al.* 1991 ; Itokawa *et al.* 1994 ; Vollenbroich *et al.* 1997a,b
Bacillus pumilus	Pumilacidin (surfactin analog)	• antiviral activity against herpes simplex virus 1 (HSV-1) • inhibitory activity against H^+, K^+-ATPase and protection against gastric ulcers *in vivo*.	Naruse *et al.* 1990
Bacillus subtilis	Iturin	• antimicrobial activity and antifungal activity against profound mycosis • effect on the morphology and membrane structure of yeast cells • increase in the electrical conductance of biomolecular lipid membranes • nontoxic and nonpyrogenic immunological adjuvant	Besson *et al.* 1976; Thimon *et al.* 1995 ; Tanaka *et al.* 1997 ; Mittenbuhler *et al.* 1997 ; Ahimou *et al.* 2000
Bacillus licheniformis	Lichenysin	• antibacterial activity • chelating properties that might explain the membrane disrupting effect of lipopeptides	Jenny *et al.* 1991; Lin *et al.* 1994 ; Yakimov *et al.* 1995; Grangemard *et al.* 2001

Table 2.1 Examples of biosurfactants application in the medical field. *Continuation*

Microorganism	Biosurfactant type	Activity/Application	Reference
Candida antartica	Mannosylerythritol lipids	• antimicrobial, immunological and neurological properties • induction of cell differentiation in the human promyelocytic leukemia cell line HL60	Kitamoto *et al.* 1993; Isoda *et al.* 1997, 1999; Zhao *et al.* 1999, 2000 ; Shibahara *et al.* 2000; Wakamatsu *et al.* 2001
Rodococcus erythropolis	Treahalose lipid	• antiviral activity against HSV and influenza virus	Uchida *et al.* 1989a,b
Streptococcus thermophilus	Glycolipid	• anti-adhesive activity against several bacterial and yeast strains isolated from voice prostheses	Busscher *et al.* 1994, 1997, 1999; Rodrigues *et al*. 2004b, 2005b
Streptococcus mitis	Not identified	• anti-adhesive activity against *Streptococcus mutans*	Pratt-Terpstra *et al.* 1989; Van Hoogmoed *et al.* 2000
Lactobacillus	Surlactin	• anti-adhesive activity against several pathogens including enteric bacteria	Reid *et al.* 1984, 1999; Velraeds *et al.* 1996a,b, 1997
Lactococcus lactis	Not identified	• anti-adhesive activity against several bacterial and yeast strains isolated from voice prostheses	Rodrigues *et al.* 2004a,b

Microbial surfactants have several advantages over chemical surfactants including lower toxicity and higher biodegradability, and effectiveness at extreme temperatures or pH values (Kosaric 1992, Cameotra and Makkar 1998). Many of the potential applications that have been considered for biosurfactants depend on whether they can be produced economically, however much effort in process optimization and at the engineering and biological levels has been carried out. Biosurfactant production from inexpensive waste substrates, thereby decreasing their production cost (Makkar and Cameotra 2002, Otto *et al.* 1999) has been reported. Additionally, legal aspects such as stricter regulations concerning the environmental pollution by industrial activities, as well as health regulations, will also strongly influence the chances of biodegradable biosurfactants replacing their chemical counterparts (Fiechter 1992).

This review aims to cover the applications of various biosurfactants in the medical field and also to provide an overview of biosurfactant activities and mechanisms of interaction that could be exploited further in developing alternative drugs, lines of therapy or biomaterials.

2.2 BIOSURFACTANTS: MECHANISMS OF INTERACTION

Biosurfactants are microbial amphiphilic polymers and polyphilic polymers that tend to interact with the phase boundary between two phases in a heterogeneous system, defined as interface. For all interfacial systems, it is known that organic molecules from the aqueous phase tend to immobilize at the solid interface. There they eventually form a film known as conditioning film, which will change the properties (wettability and surface energy) of the original surface (Neu 1996). In an analogy to organic conditioning films, biosurfactants may interact with the interfaces and will affect the adhesion and detachment of bacteria. Additionally, the substratum surface properties will determine the composition and orientation of the molecules conditioning the surface during the first hour of exposure. After about 4 hours, a certain degree of uniformity is reached and the composition of the adsorbed material becomes substratum independent (Neu and Marshall 1990).

2.2.1 Synthetic Surfactants and Bacteria

A wide variety of experiments investigating the microbial cell surface and the interaction of microorganisms with interfaces using synthetic surfactants have been reported.

The use of surfaces conditioned with synthetic surfactants aiming the development of nonfouling coatings and materials has been studied. The success of these nonfouling coatings would have an enormous economic significance for technical and medical applications in which interfacial processes are critical. Several studies employed cationic surfactants such as quaternary ammonium compounds (QACs). QACs bind by chemisorption to the cell surface of bacteria because the microbial cell surface at physiological pH is negatively charged. Thus, the QACs do influence the zeta potential of bacteria. QACs have been also reported to enhance the biological inactivation of adhering *Listeria monocytogenes* by listeriaphages (Roy *et al.* 1993).

Also promising in the development of antifouling coatings are the block copolymer surfactants. These compounds are polymeric surfactants, which adsorb via their hydrophobic part to hydrophobic surfaces while the hydrophilic segments extend freely into the water. It was suggested that the protruding hydrophilic chains form a static barrier, which prevents protein, bacterial or eukaryotic fouling of the coated surfaces. It was generally observed that surfactants with either polyethylene glycol or propylene glycol chains could inhibit bacterial adhesion to hydrophobic surfaces (Humphries *et al.*1987, Vladkova 2004).

Microbial adhesion and its prevention in the medical field are a key factor for successfully applying biomaterials. Therefore, several groups evaluated the effect of block copolymer surfactants in preventing the adhesion of bacteria to a variety of biomaterials and usually reported approximately 95% reduction of adhesion (Neu 1996).

2.2.2 Biosurfactants and Bacteria

The biocidal activity of biosurfactants is closely related to the lipid moiety of the molecules and the consequences of the interaction of these compounds with eukaryotic cells are well known and include pyrogenicity, lethal toxicity, immunogenicity, mitogenicity and other molecular effects (Wicken and Knox 1980). The lytic activity of biosurfactants produced on media without hydrocarbons was also described as a selection criterion for microorganisms producing biosurfactants (Mulligan *et al.* 1984).

2.2.3 Surface-active Approach to Bacterial Adhesion/Detachment

Due to the amphiphilic nature of the biosurfactants, not only hydrophobic but a range of interactions is involved in the possible adsorption of charged biosurfactants to interfaces. Most natural interfaces have an overall negative or, rarely, positive charge. Thus, the ionic conditions and the pH are important parameters if interactions of ionic biosurfactants with interfaces are to be investigated (Craig *et al.* 1993). Gottenbos *et al.* (2001) demonstrated that positively charged biomaterial surfaces exert an antimicrobial effect on adhering gram-negative bacteria, but not on gram-positive ones. In addition, the molecular structure of a surfactant will influence its behaviour at interfaces. In describing the surface-active approach, an effort is made to elaborate on the possible theoretical locations and orientations of the biosurfactants. Nevertheless, it must be kept in mind that the situation in natural systems is far more complex and requires the consideration of many additional parameters.

2.2.3.1 Biosurfactants and Adhesion to Interfaces

Two different situations can be observed concerning adhesion to interfaces in the presence of biosurfactants, namely if they are cell-bound or if they are excreted into the environment.

2.2.3.1.1 Cell Bound Biosurfactants

A biosurfactant may be bounded by the hydrophobic part in the outer layers of the cell surface. In this case, the cell can interact with a hydrophilic interface but not with a hydrophobic interface (Figure 2.1). An example of this case is provided by the lipids in the outer layer of the outer membrane of gram-negative cells which, depending on the cell surface structure, may be involved to a certain degree in the interaction with interfaces. The reduction of cell surface hydrophobicity by the presence of a serratamolide (an amphipathic aminolipid present on the surface of *Serratia marcescens*) was suggested (Bar-Ness *et al.* 1988). Lipid-modified polypeptides which are tightly associated with the cell membrane, may be involved in the expression of cell surface properties associated with the colonization of the human oral cavity by *Streptococcus gordonii* (Jenkinson 1992).

The biosurfactant may also be oriented the other way around, thereby exposing the hydrophobic part to the outside and in this case the cell can only interact with a hydrophobic interface. Several examples were reported for *Pseudomonas aeruginosa* strains producing a rhamnolipid surfactant. It was found that the rhamnolipid increased cell hydrophobicity of slow octadecane degraders, which in turn was related to the rate of octadecane degradation. These findings imply a binding of the rhamnolipid in the outer cell layers by the hydrophilic

part while the hydrophobic moiety is directed into the environment (Zhang and Miller 1994, Zhang et al. 1997). Al-Tahhan *et al.* (2000) reported that rhamnolipid biosurfactant causes the cell surface of *Pseudomonas* spp. to become hydrophobic through the release of lipopolysaccharides (LPS). In their study, two *P. aeruginosa* strains were grown on glucose and hexadecane to investigate the chemical and structural changes that occur in the presence of a rhamnolipid biosurfactant, and it was concluded that rhamnolipid-induced LPS release was the probable mechanism of enhanced cell surface hydrophobicity.

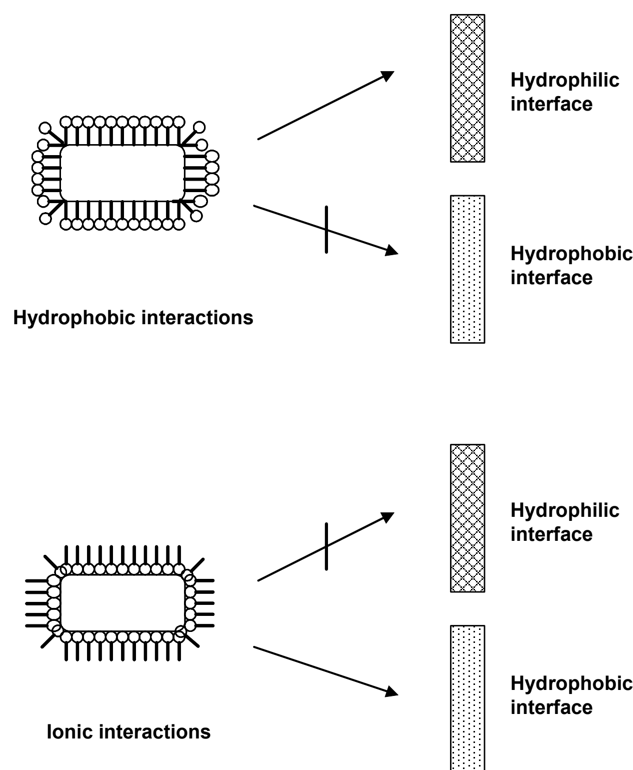


Figure 2.1 Orientation of microbial biosurfactants at the microbial cell surface. The binding of the biosurfactants to the microbial cell surface may be mediated by hydrophobic interactions (top illustration) or ionic interactions (bottom illustration). The hydrophobic part of the biosurfactant is indicated by a straight line (hydrophobic tail) and the hydrophilic by a circle (polar head). The possible adhesion of microorganisms to interfaces with hydrophilic (hatched) or hydrophobic (dotted) properties is indicated. Adapted from Neu, 1996.

2.2.3.1.2 Excreted Biosurfactants

When a microorganism excretes a biosurfactant into the aqueous phase it may be responsible for a microbially created conditioning film at an interface. On a hydrophobic interface, this conditioning film will change the interface from hydrophobic to hydrophilic, which means that hydrophilic but not hydrophobic cells may now interact with the interface (Figure 2.2). Biosurfactant-producing microorganisms that have been isolated from habitats with hydrophobic interfaces represent an illustrative example. It was reported that most phytopathogenic *Corynebacteria* isolated from the hydrophobic cuticle of plants produce surface-active compounds (Akit *et al.*1981). Similar findings were reported for *Pseudomonas* species by Bunster and co-workers (1989). One such isolated bacterium was a *Pseudomonas* strain producing viscosin, a peptidolipid antibiotic biosurfactant that lowers the surface tension of water to the lowest theoretical possible value of 27 mN/m (Neu *et al.* 1990).

On a hydrophilic interface, the excreted biosurfactants may change the properties of the interface from hydrophilic to hydrophobic. Only hydrophobic cells are able to interact with this hydrophobic conditioning film (Figure 2.2). An example of this is the surface active exolipid of *S. marcescens*. This lipopeptide was shown to promote the flagellum-independent spreading of the bacteria on a hydrophilic surface (Pratt-Terpstra *et al.* 1989, Matsuyama *et al.* 1992).

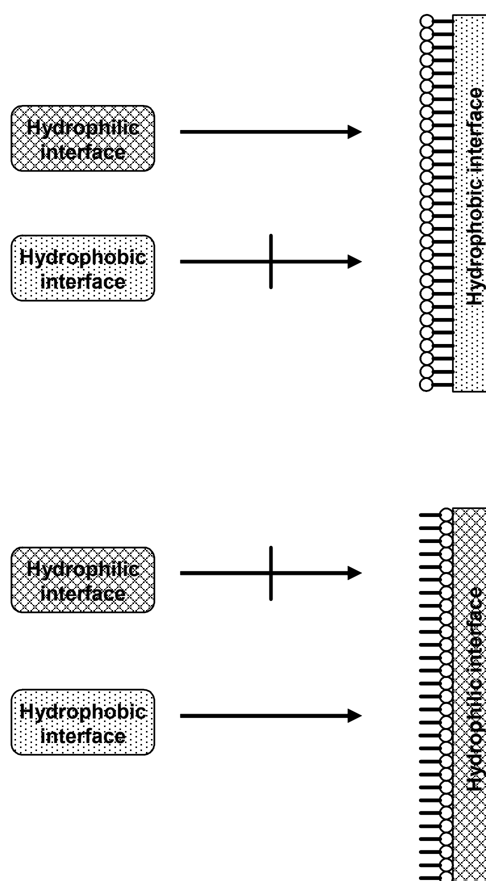


Figure 2.2 Adhesion of hydrophilic and hydrophobic microorganisms to a hydrophilic (hatched) and hydrophobic (dotted) interface. The interface is covered with a microbial conditioning film of biosurfactants. Depending on the surface energy of the interface, the conditioning film of biosurfactants will have a different orientation. The biosurfactants may be bound to the interface by means of hydrophobic interactions or ionic interactions. The hydrophobic part of the biosurfactant is indicated by a straight line (hydrophobic tail). Adapted from Neu, 1996.

2.2.3.2 Biosurfactants and Detachment from Interfaces

Regardless of the biosurfactants orientation at the microbial cell surface, if they are excreted into the area between the cell surface and interface, they will probably lead to detachment of the bacterium from the interface. Depending on the hydrophilic or hydrophobic properties of the interface, the bacteria will leave a microbial conditioning film with hydrophilic or hydrophobic properties (Figure 2.3). Several examples have been described for streptococci (Cowan, and Busscher 1993, Van Hoogmoed *et al.* 2000, Busscher *et al.* 1994). When biosurfactants modified the substratum this influenced the adhesion properties of *Streptococcus mitis* and *Streptococcus mutans* cells (Van Hoogmoed *et al.* 2000). Similar

observations were made in other experiments with *Streptococcus thermophilus*, (Busscher *et al.* 1994). It was generally believed that during detachment, the initially deposited cells leave biosurfactants on the substratum, thereby conditioning it with an anti-adhesive layer.

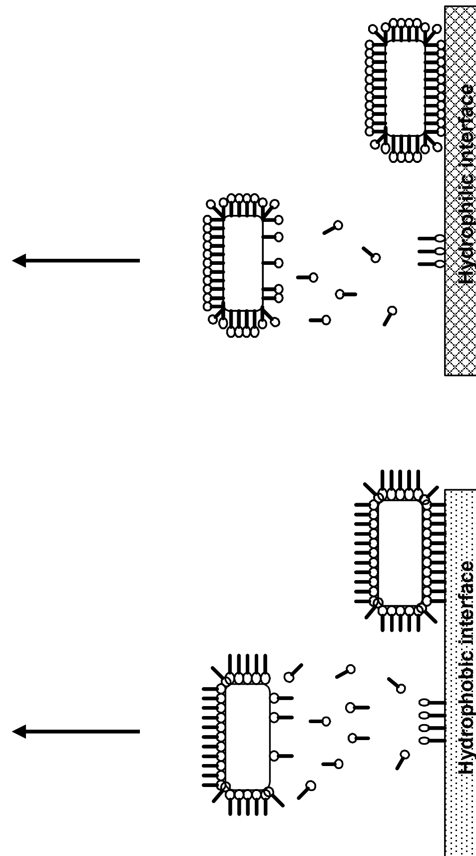


Figure 2.3 Detachment of microorganisms from hydrophilic (hatched) and hydrophobic (dotted) interfaces by excretion or release of biosurfactants. The surface active compounds will result in a microbial produced footprint or conditioning film consisting of biosurfactants. The orientation of the biosurfactants which form the footprint or conditioning film is determined by the surface energy of the interface. The microbial footprint or conditioning film may later influence the interaction of other bacteria with this interface. The hydrophobic part of the biosurfactant is indicated by a straight line (hydrophobic tail). Adapted from Neu, 1996.

2.3 BIOLOGICAL ACTIVITY OF BIOSURFACTANTS

As described above a broad range of chemical structures, such as glycolipids, lipopeptides, polysaccharide-protein complexes, phospholipids, fatty acids and neutral lipids, have been attributed to biosurfactants (Lin 1996, Ahimou *et al.* 2000, Angelova and Shmauder 1999, Morikawa *et al.* 1993). Some of these biosurfactants were described for their potential as biological active compounds and applicability in the medical field.

2.3.1 Lipopeptides

Among the many classes of biosurfactants, lipopeptides are particularly interesting because of their high surface activities and antibiotic potential. Lipopeptides can act as antibiotics, antiviral and antitumor agents, immunomodulators or specific toxins and enzyme inhibitors. Ahimou *et al.* (2000) reported that lipopeptide profile and bacterial hydrophobicity vary greatly with the strains, iturin A being the only lipopeptide type produced by all *Bacillus subtilis* strains. Lipopeptides enhance or decrease the bacterial surface hydrophobicity following that the surface is less or more hydrophobic. Surfactin was found to be more efficient than iturin A in modifying the *B. subtilis* surface hydrophobic character. This aspect appears essential in association with the antifungal properties of lipopeptides involved in the biological control of plant diseases. Morikawa *et al.* (1993) identified and characterized a biosurfactant, arthrofactin, produced by *Arthrobacter* species that was found to be seven times more effective than surfactin.

2.3.1.1 Iturin Biosurfactants

Produced by strains of *B. subtilis*, iturin A is a potent antifungal lipopeptide of which antimicrobial activity was the first reported (Besson *et al.* 1996, Ahimou *et al.* 2000). Iturin A mechanism of action is related to the disruption of the plasma membrane by the formation of small vesicles and the aggregation of intramembranous particles, in yeast cells. Moreover, it also significantly increases the electrical conductance of biomolecular lipid membranes (Thimon *et al.* 1995). Iturin A has been proposed as an effective antifungal agent for profound mycosis (Tanaka *et al.* 1997). Other members of the iturin group, including bacillomycin D and bacillomycin Lc were also found to have antimicrobial activity against *Aspergillus flavus*, but the different lipid chain length apparently affected the activity of the lipopeptide against other fungi (Moyne *et al.* 2001). Thus, the members of the iturin-like biosurfactant group have the potential to be used as alternative potent antifungal agents.

2.3.1.2 Surfactin Biosurfactants

Surfactin, a cyclic lipopeptide, is also produced by *B. subtilis* strains and has well-known antimicrobial properties (Ahimou *et al.* 2000). It has been reported to interact with artificial and biomembrane systems, for example bacterial protoplasts or enveloped viruses (Vollenbroich *et al.* 1997a). There are three different types of surfactins, A, B and C, which are classified according to the differences in their amino acid sequences.

In addition to antifungal and antibacterial properties, surfactin has also been related to several biological activities, namely the inhibition of fibrin clot formation, the induction of ion channels formation in lipid bilayer membranes, the inhibition of cyclic adenosine monophosphate (cAMP), the inhibition of platelet and spleen cytosolic phospholipase A2 (PLA2) and antiviral and antitumor activities (Kim *et al.* 1998). Kim and co-workers (1998) demonstrated that surfactin is a selective inhibitor for cytosolic PLA2 and a putative anti-inflammatory agent through the inhibitory effect produced by direct interaction with cytosolic PLA2, and that inhibition of cytosolic PLA2 activity may suppress inflammatory responses. Vollenbroich *et al.* (1997a) showed that surfactin treatment improved proliferation rates and lead to changes in the morphology of mammalian cells that had been contaminated with mycoplasma. In addition, the low cytotoxicity of surfactin to mammalian cells permitted specific inactivation of mycoplasmas without significant damaging effects on cell metabolism and the proliferation rate of cells in culture. In another study, the same authors (Vollenbroich *et al.* 1997b) showed that surfactin is active against several viruses, including Semliki Forest virus, herpes simplex virus, suid herpes virus, vesicular stomatitis virus, simian immunodeficiency virus, feline calicivirus and murine encephalomyocarditis virus. The inactivation of enveloped viruses, especially herpesviruses and retroviruses, was significantly more efficient than that of non-enveloped viruses, suggesting that the antiviral action of surfactin is primarily due to a physicochemical interaction between the membrane active surfactant and the outer part of the virus lipid membrane bilayer, which causes permeability changes and at higher concentrations leading finally to the disintegration of the mycoplasma membrane system by a detergent effect.

Surfactin C was found to enhance the activation of prourokinase (plasminogen activator) and the conformational change in plasminogen, leading to increased fibrinolysis *in vitro* and *in vivo* (Kikuchi and Hasumi 2002). The plasminogen-plasmin system is involved in blood clot dissolution as well as in a variety of physiological and pathological processes requiring localized proteolysis. In a rat pulmonary embolism model, surfactin C increases plasma clot lyses when injected in combination with prourokinase (Kikuchi and Hasumi 2003). These

results point to the potential use of surfactin in thrombolytic therapy related to pulmonary, myocardial and cerebral disorders.

Various nosocomial infections such as those related to the use of central venous catheters, urinary catheters, prosthetic heart valves, voice prostheses and orthopaedic devices are clearly associated with biofilms that adhere to the biomaterial surface (Stewart and Costeron, 2001). These infections share common characteristics even though the microbial causes and host sites vary greatly. The most important of these characteristics is that bacteria in biofilms evade host defences and withstand antimicrobial chemotherapy. As antimicrobial resistance is nowadays a growing source of concern in modern medicine, genetic engineering of the known biosurfactant molecules is a key factor for the development of alternative prophylactic and therapeutic agents. Symmank *et al.* (2002) produced a novel lipohexapeptide with altered antimicrobial activities by genetic engineering of the surfactin biosynthesis mechanism. Reduced detectable haemolytic activity concomitant with an increase in growth inhibition of bacterial cells, including *Bacillus licheniformis* was observed. Thus, similar surfactin derivatives might exhibit reduced toxicity against eukaryotic cells, which could improve their therapeutic applications.

2.3.2 Glycolipids

Glycolipids are the most common class of biosurfactants of which the most effective from the point of view of surface active properties are the trehalose lipids of *Mycobacterium* and related bacteria, the rhamnolipids of *Pseudomonas* sp. and the sophorolipids of yeasts. Otto and co-workers (1999) described the production of sophorose lipids from deproteinized whey concentrate by a two-stage process. Several antimicrobial, immunological and neurological properties have been attributed to mannosylerythritol lipid (MEL), a yeast glycolipid biosurfactant, produced from vegetable oils by *Candida* strains. Kitamoto *et al.* (1993) shown that MEL exhibits antimicrobial activity particularly against gram-positive bacteria. Isoda *et al.* (1997) investigated the biological activities of seven extracellular microbial glycolipids including MEL-A, MEL-B, polyol lipid, rhamnolipid, sophorose lipid, and succinoyl trehalose lipid STL-1 and STL-3. Except for rhamnolipid, all the other tested glycolipids were found to induce cell differentiation instead of cell proliferation in the human promyelocytic leukaemia cell line HL60. STL (succinoyl trehalose lipid) and MEL differentiation-inducing activity was attributed to a specific interaction with the plasma membrane instead of a simple detergent-like effect.

In addition the effects of several kinds of microbial extracellular glycolipids on neutrite initiation in PC12 cells were investigated (Isoda *et al.* 1999). The PC12 cell line derived from

a rat pheochromocytoma, provides a relatively simple, and homogeneous system for studying various aspects of neuronal differentiation, because PC12 cells can survive and proliferate without requiring the presence of neutrotrophic factors. A significant neutrite outgrowth was observed as consequence of the addition of MEL-A, MEL-B and sophorose lipid (SL) to PC12 cells. MEL-A increased acetylcholinesterase activity to an extent similar to nerve growth factor (NGF). MEL-A induced neutrite outgrowth after treatment of PC12 cells with an anti-NGF receptor antibody that obstructed NGF action. It was shown that MEL-A and NGF induce differentitation of PC12 cells through different mechanisms. Moreover, MEL was found to induce the outgrowth of neutrites, enhance the activity of acetylcholinesterase and increase the levels of galactosylceramide from PC12 pheochromocytoma cells (Shibahara *et al.* 2000).

Glycolipids have also been implicated with growth arrest, apoptosis and the differentiation of mouse malignant melanoma cells (Zhao *et al.* 1999, 2000). Exposure of B16 cells to MEL resulted in the condensation of the chromatin, DNA fragmentation and sub-G1 arrest (the sequence of events of apoptosis). Additionally, MEL was also reported to markedly inhibit the growth of mouse melanoma B16 cells in a dose-dependent manner. Moreover, MEL exposure stimulated the expression of differentiation markers of melanoma cells, such as tyrosinase activity and the enhanced production of melanin, which is an indication that MEL triggered both apoptotic and cell differentiation programs. In addition, exposure of PC12 cells to MEL enhanced the activity of acetylcholinesterase and interrupted the cell cycle at the G1 phase, with resulting outgrowth of neutrites and partial cellular differentiation (Wakamatsu *et al.* 2001). MEL has been implicated in the induction of neuronal differentiation in PC12 cells and therefore provides the basis for the use of glycolipids as therapeutical agents for cancer cells treatment. Nevertheless, further studies of the molecular basis of the signalling cascade that follows exposure of PC12 cells to MEL may ultimately lead to a better understanding of the processes that result in the outgrowth of neutrites and the commitment to differentiation of PC12 cells.

In other studies four analogs of STL-3 at their critical micelle concentration were evaluated for their ability to inhibit growth and induce differentiation of HL60 human promyelocytic leukaemia cells (Sudo *et al.* 2000). It was found that the effect of STL-3 and its analogs on HL60 cells was dependent on the hydrophobic moiety of STL-3. Furthermore, a high binding-affinity of MEL towards human immunoglobulin G (HIgG) was shown by Im *et al.* (2001). It was suggested by their report the possibility of using MEL-A as an alternative ligand for immunoglobulins. In further studies they evaluated the potential of MEL (-A, -B and –C) attached to PHEMA beads (poly(2-hydroxyethyl methacrylate)), for binding and affinity to

HIgG (Im *et al.* 2003). Of these three composite compounds, those bearing MEL-A exhibited the highest binding capacity for HIgG. More significantly, the bound HIgG was efficiently recovered (approximately 90%) under significantly mild elution conditions, with phosphate buffer at pH 7, indicating a great potential of the glycolipids as an affinity ligand material. Inoh *et al.* (2001, 2004) reported that MEL-A significantly increased the efficiency of gene transfection mediated by cationic liposomes with a cationic cholesterol derivative. Among the cationic liposomes tested, the liposome bearing cholesteryl-3β-carboxyamindoethylene-*N*-hydroxyethylamine and MEL-A showed the best efficiency for delivery pf plasmids encoding luciferase (ρGL3) into the target cells (NIH3T3, COS-7 and HeLa). The properties, production and applications of MEL were widely studied by Kitamoto and co-workers (2002), particularly the excellent interfacial properties and differentiation-inducing activities of MEL. They also focused on the excellent biological and self-assembling actions of MEL and examined the effect of MEL-A on the gene transfection using cationic liposomes.

2.3.3 Other Biosurfactants with Biological Activity

Nielsen and co-workers (1999) reported viscosinamide, a cyclic depsipeptide, as a new antifungal surface active agent produced by *Pseudomonas fluorescens*, and with different properties to the biosurfactant viscosin, known to be produced from the same species and to have antibiotic activity (Neu *et al.* 1990). Massetolides A-H, also cyclic depsipeptides, were isolated from *Pseudomonas* species, derived from a marine habitat, and found to exhibit *in vitro* antimicrobial activity against *Mycobacterium tuberculosis* and *Mycobacterium avium-intracellulare* (Gerard *et al.* 1997).

Precursors and degeneration products of sphingolipids biosurfactants were found to inhibit the interaction of *S. mitis* with buccal epithelial cells and of *Staphylococcus aureus* with nasal mucosal cells (Bidel *et al*. 1992). Gram-positive *Bacillus pumilis* cells were found to produce pumilacidin A, B, C, D, E, F and G which exhibited antiviral activity against HSV-1, inhibitory activity against H^+, K^+-ATPase, and was found to be protective against gastric ulcers (Naruse *et al.* 1990) probably through inhibiting microbial activity contributing to these ulcers.

2.4 ANTIMICOBRIAL ACTIVITY OF BIOSURFACTANTS

The antimicrobial activity of several biosurfactants has been reported in the literature for many different applications (Cameotra and Makkar 2004). For instance, the antimicrobial activity of two biosurfactants obtained from probiotic bacteria, *Lactococcus lactis* 53 and *S.*

thermophilus A, against a variety of bacterial and yeast strains isolated from explanted voice prostheses was evaluated as can be seen in Table 2.2 and in detail on Chapter 8 (Rodrigues *et al.* 2004b).

Table 2.2 Antimicrobial activity of biosurfactants at different concentrations against several bacterial and yeast strains isolated from explanted voice prostheses. The experiments were scored as positive (+) when growth inhibition was observed (no colonies formed); a (±) sign indicated some colonies formed within the zones; and no growth inhibition was marked as negative (-). For details see Chapter 8 and Rodrigues *et al.* 2004b.

Biosurfactant 1 obtained from *L. lactis* 53					
Microorganism	**5 g l^{-1}**	**10 g l^{-1}**	**25 g l^{-1}**	**50 g l^{-1}**	**100 g l^{-1}**
S. epidermidis GB 9/6	±	±	+	+	+
S. salivarius GB 24/9	-	-	±	±	+
S. aureus GB 2/1	±	±	±	+	+
R. dentocariosa GBJ 52/2B	-	-	±	±	±
C. albicans GBJ 13/4A	-	±	±	+	+
C. tropicalis GB 9/9	+	+	+	+	+
Biosurfactant 2 obtained from *S. thermophilus* A					
Microorganism	**3 g l^{-1}**	**5 g l^{-1}**	**10 g l^{-1}**	**50 g l^{-1}**	**100 g l^{-1}**
S. epidermidis GB 9/6	±	±	±	+	+
S. salivarius GB 24/9	-	-	±	±	+
S. aureus GB 2/1	-	-	±	±	+
R. dentocariosa GBJ 52/2B	-	-	±	±	±
C. albicans GBJ 13/4A	-	-	±	+	+
C. tropicalis GB 9/9	+	+	+	+	+

It was found that both biosurfactants have a high antimicrobial activity even at low concentrations against *C. tropicalis* GB 9/9, one of the strains held responsible for prostheses failure. At the highest concentration tested both biosurfactants were active against all bacterial and yeast strains studied. In another study, Reid (1998, 2001) emphasized a possible probiotic role for the biosurfactant-producing lactobacilli in the restoration and maintenance of healthy urogenital and intestinal tracts, conferring protection against pathogens, and suggested a reliable alternative treatment and preventive regimen to antibiotics in the future. The first clinical evidence that probiotic lactobacilli can be delivered to the vagina following oral intake was provided (Reid *et al.* 2001) and although only a limited

set of strains have any proven clinical effect or scientific basis, there are sufficient data to suggest that this approach could provide a valuable alternative to antibiotic prophylaxis and treatment of infection. By the use of a rat model of surgical implant infection, Gan *et al.* (2002) determined that the probiotic strain, *Lactobacillus fermentum* RC-14, and its secreted biosurfactant reduced infections associated with surgical implants, which are mainly caused by *S. aureus* through inhibition of growth and reduction of adherence to surgical implants. A recent *in vitro* study of *Lactobacillus plantarum* 299v and *Lactobacillus rhamnosus* GG showed that these probiotic strains could inhibit the adhesion of *Escherichia coli* to intestinal epithelial cells by stimulating epithelial expression of mucins (Mack *et al.* 1999). However, these strains were also found to be biosurfactant producers as can be seen in Chapter 4 (Rodrigues *et al.* 2005a). These observations generally indicated that biosurfactants might also contain signalling factors that interact with host and/or bacterial cells leading to the inhibition of infections. Moreover, they support the assertion of a possible role in preventing microbial adhesion (Millsap *et al.* 1996, Velraeds *et al.* 1997) and their potential in developing anti-adhesion biological coatings for implant materials (Chapter 7, Rodrigues *et al.* 2005c).

Bechard *et al.* (1998) isolated and partially characterized the chemical structure of a peptide antibiotic (fatty acid-containing peptides) produced by *B. subtilis* found in apple fruit. This biosurfactant was reported to have a broad spectrum of activity against Gram-negative bacteria, little activity against Gram-positive organisms and it is active against *Botrytis cinera*.

Antibacterial and antiphytoviral effects of various rhamnolipids have been described in the literature (Bai *et al.* 1997, Benincasa *et al.* 2004). Seven different rhamnolipids were identified in cultures of *P. aeruginosa* AT10 from soybean oil refinery wastes and showed excellent antifungal properties against various *fungi* (Abalos *et al.* 2001). Golubev *et al.* (2001) reported the production of an extracellular, low molecular weight, protease-resistant thermostable glycolipids fungicide from the yeast *Pseudomonas fusiformata* (*Ustilaginales*). This fungicide was active against more than 80% of the 280 yeast and yeast-like species tested under acidic conditions (pH 4.0) at 20-30ºC (Kulakovskaya *et al.* 2003). The purified glycolipids enhanced non-specific permeability of the cytoplasmatic membrane in sensitive cells, which resulted in ATP leakage.

Borrelia burgdorferi glycolipids were suggested as promising candidates for diagnosis of Lyme disease and possible vaccination (Hossain *et al.* 2001). Immunoassays with sera from patients with Lyme disease showed antibody reactivity to the glycolipids, which is present in all stages of the disease.

2.5 ANTI-ADHESIVE ACTIVITY OF BIOSURFACTANTS

Biosurfactants have been found to inhibit the adhesion of pathogenic organisms to solid surfaces or to infection sites, thus prior adhesion of biosurfactants to solid surfaces might constitute a new and effective means of combating colonization by pathogenic microorganisms (Singh and Cameotra 2004). Precoating vinyl urethral catheters by running a surfactin solution through them before inoculation with media resulted in a decrease of the amount of biofilm formed by *Salmonella typhimurium*, *Salmonella enterica*, *Escherichia coli* and *Proteus mirabilis* (Mireles *et al.* 2001). Given the importance of opportunistic infections with *Salmonella* species, including urinary tract infections of AIDS patients, these results have great potential for practical applications. In addition, the use of lactobacilli as a probiotic for the prevention of urogenital infections has been widely studied. The role of *Lactobacillus* species in the female urogenital tract as a barrier to infection is of considerable interest (Boris and Barbés 2000). These organisms are believed to contribute to the control of vaginal microbiota by competing with other microorganisms for adherence to epithelial cells and by producing biosurfactants. There are reports of inhibition of biofilm formed by uropathogens and yeast on silicone rubber by biosurfactants produced by *Lactobacillus acidophilus* (Velraeds *et al.* 1998, Reid 2000). Heinemann and co-works showed that *L. fermentum* RC-14 releases surface-active components that can inhibit adhesion of uropathogenic bacteria, including *Enterococcus faecalis* (Heinemann *et al.* 2000). Efforts in the development of strategies to prevent the microbial colonization of silicone rubber voice prostheses have been reported in Chapters 5 and 6 (Rodrigues *et al.* 2004a, 2005b). The ability of biosurfactants obtained from the probiotic strains, *L. lactis* 53 and *S. thermophilus* A, to inhibit adhesion of four bacterial and two yeast strains isolated from explanted voice prostheses to precoated silicone rubber was evaluated. The results obtained showed that the biosurfactants were effective in decreasing the initial deposition rates, as well as the number of bacterial cells adhering after 4 h, for all microorganisms tested. Over 90% reductions in the initial deposition rates were achieved for most of the bacterial strains tested. The biosurfactant obtained from *S. thermophilus* A was more effective against *Rothia dentocariosa* GBJ 52/2B, which is one of the strains responsible for valve prosthesis failure. The yeast strains initial deposition rates were far less reduced in the presence of the biosurfactant as compared to the other tested strains. Recently the authors also demonstrated that when rinsing flow chambers, designed to monitor microbial adhesion, with a rhamnolipid biosurfactant containing solution the rate of deposition and adhesion was significantly reduced for a variety of bacterial and yeast strains

isolated from explanted voice prostheses to silicone rubber as can be seen in Table 2.3 and in detail on Chapter 7 (Rodrigues *et al.* 2005c).

Table 2.3 Desorption percentages of microorganisms isolated from explanted voice prostheses adhered to silicone rubber as result of rhamnolipid perfusion through the parallel plate flow chamber with and without a following passage of a liquid-air interface. Results are averages of duplicate experiments varying within 10-15%. For details see Chapter 7 and Rodrigues *et al.* 2005c.

Microorganism	Desorption Percentages (%)	
	Rinsing with rhamnolipid solution	**Passage air-liquid interface**
Staphylococcus epidermidis GB 9/6	80.2	89.5
Streptococcus salivarius GB 24/9	87.3	98.7
Staphylococcus aureus GB 2/1	21.0	67.4
Rothia dentocariosa GBJ 52/2B	63.3	98.9
Candida albicans GBJ 13/4A	81.8	95.5
Candida tropicalis GB 9/9	74.2	95.5

Therefore, this may be useful for use as a biodetergent solution for prostheses cleaning, prolonging their lifetime and directly benefiting laryngectomized patients.

The role for surfactants in the defence against infection and inflammation in the human body is a well-known phenomenon. The pulmonary surfactant is a lipoprotein complex synthesized and secreted by the epithelial lung cells into the extracellular space, where it lowers the surface tension at the air-liquid interface of the lung and represents a key factor against infections and inflammatory lung diseases (Wright 2003).

2.6 BIOMEDICAL AND THERAPEUTICAL APPLICATIONS OF BIOSURFACTANTS

Some biosurfactants are a suitable alternative to synthetic medicines and antimicrobial agents, and may be used as safe and effective therapeutic agents (Sing and Cameotra 2004, Banat *et al.* 2000). There has been an increasing interest in the effect of biosurfactants on human and animal cells and cell lines.

Mannosylerythritol lipids from *Candida antartica* (Kitamoto *et al.* 1993), rhamnolipids produced by *P. aeruginosa* (Lang and Wullbrandt 1999, Maier and Soberon-Chavez 2000)

and lipopeptides produced by *B. subtilis* (Vollenbroich *et al.* 1997a) and *B. licheniformis* (Jenny *et al.* 1991, Fiechter 1992, Lin et al. 1994, Yakimov *et al.* 1995) have been shown to have antimicrobial activities. Jenny and co-workers (1991) performed the structural analysis and characterized surface activities of biosurfactants produced by *B. licheniformis*, while Lin *et al.* (1994) described their continuous production (1994). Yakimov and co-workers (1995) demonstrated the antibacterial activity of lichenysin A, a biosurfactant produced by *B. licheniformis* that favourably compared to others surfactants. More recently Grangemard *et al.* (2001) reported the chelating properties of lichenysin, which might explain the membrane disrupting effect of lipopeptides. In another study, Carrillo *et al.* (2003) noted a molecular mechanism of membrane permeabilization by surfactin, which may explain surfactin induced pore formation underlying the antibiotic and haemolytic action of these lipopeptides. This study also suggested that the membrane barrier properties are likely to be damaged in the areas where surfactin oligomers interact with the phospholipids, at concentrations much below the onset for solubilization. Such properties can cause structural fluctuations that may well be the primary mode of the antibiotic action of this lipopeptide. Surfactin type peptides that can rapidly act on membrane integrity rather than other vital cellular processes may perhaps constitute the next generation of antibiotics. Lipopeptide surfactin was also reported to have an antitumor activity against Ehrlich's ascite carcinoma cells (Kameda *et al.* 1974) and having antifungal properties, as well as various pharmacological applications such as inhibiting fibrin clot formation and haemolysis (Bernheimer and Avigad 1970) and formation of membrane ion channels (Sheppard *et al.* 1991). In addition, surfactin and surfactin analogs have been reported as antiviral agents, namely it was demonstrated a significant inhibitory effect of pumilacidin on herpes simplex virus 1 (HSV-1) (Naruse *et al.* 1990) and an inhibitory activity against H^+, K^+-ATPase and protection against gastric ulcers *in vivo*. The potential of surfactin against human immunodeficiency virus 1 (HIV-1) was reported by Itokawa *et al.* (1994). The antiviral action of surfactin was suggested to be due to physicochemical interaction between the membrane-active surfactant and the virus lipid membrane (Vollenbroich *et al.* 1997b).

Another lipopeptide, iturin, produced by *B. subtilis* was reported to have antifungal properties (Thimon *et al.* 1995), which affects the morphology and membrane structure of yeast cells. The iturin was shown to pass through the cell wall and disrupt the plasma membrane with the formation of small vesicles and the aggregation of intramembranous particles. Iturin also passes through the plasma membrane and interacts with the nuclear membrane and probably with membranes of other cytoplasmic organelles.

Possible applications of biosurfactants as emulsifying aids for drug transport to the infection site, for supplementing pulmonary surfactant and as adjuvants for vaccines were suggested by Kosaric (1996).

Mittenbuhler *et al.* (1997) showed that bacterial lipopeptides constitute potent non-toxic and nonpyrogenic immunological adjuvants when mixed with conventional antigens. A marked enhancement of the humoral immune response was obtained with the low molecular mass antigens iturin AL, herbicolin A and microcystin (MLR) coupled to poly-L-lysine (MLR-PLL) in rabbits and in chickens. Conjugates of lipopeptide –Th-cell epitopes also constituted effective adjuvants for the *in vitro* immunization of either human mononuclear cells or mouse B cells with MLR-PLL and result in a significantly increased yield of antibody-secreting hybridomas.

Mannosylerythritol lipids biological activities from *C. antartica* were investigated by Isoda *et al.* (1997), who reported an induction of cell differentiation in the human promyelocytic leukaemia cell line HL60. These glycolipids induced the human myelogenous leukaemia cell line K562 and the human basophilic leukaemia cell line Ku812 to differentiate into monocytes, granulocytes and megakaryocytes. The succinoyl trehalose lipid produced by *Rhodococcus erythropolis* has also been reported to inhibit HSV and influenza virus (Uchida *et al.* 1989a,b). The deficiency of pulmonary surfactant described earlier and which is responsible for respiration failure in premature infants (Wright 2003) may be corrected through the isolation of genes for protein molecules of this surfactant and cloning in bacteria for possible fermentative production for use in medical application (Lang and Wullbrandt 1999). Sano *et al.* (1999) demonstrated the different actions of pulmonary surfactant protein A upon distinct serotypes of LPS which is major constituent of the outer membrane of gram-negative bacteria.

Although there is an increasing potential for the application of biosurfactants in the biomedical field, some of these molecules may constitute risks for humans. For instance, *P. aeruginosa* is a bacterium responsible for severe nosocomial infections, life-threatning infections in immunocompromised persons, and chronic infections in cystic fibrosis patients; thus rhamnolipids therefore have to be well investigated prior to such uses. *P. aeruginosa* strains virulence depends on a large number of cell-associated and extracellular factors (Van Delden and Iglewski 1998, Ramisse *et al.* 2000). Cell-to-cell signalling systems control the expression and allow a coordinated cell-density-dependent production of many extracellular virulence factors. The possible role of cell-to-cell signalling in the pathogenesis of *P. aeruginosa* infections and a rationale for targeting cell-to-cell signalling systems in the development of new therapeutic approaches was discussed by Van Delden and Iglewski (1998). Synthesis of rhamnolipids is regulated by a very complex genetic regulatory system

that also controls different *P. aeruginosa* virulence-associated traits (Maier and Soberon-Chavez 2000). The cosmetic and health care industries use large amounts of surfactants for a wide variety of products including insect repellents, acne pads, contact lens solutions, hair colour and care products, deodorants, nail care products, lipstick, eye shadow, mascara, toothpaste, denture cleaners, lubricated condoms, baby products, foot care products, antiseptics, shaving and depilatory products (Kosaric 1992). Biosurfactants are known to have advantages over synthetic surfactants such as low irritancy or anti-irritating effects and compatibility with skin. Rhamnolipids in particular are being used as cosmetic additives and have been patented to make some liposomes and emulsions (Maier and Soberon-Chavez 2000, Ishigami and Suzuki 1997), both of which are important in the cosmetic industry.

Another approach on the use of biosurfactants in biomedical applications is the development of suitable anti-adhesion biological coatings for implant materials. Dairy *S. thermophilus* strains produced a biosurfactant which caused its own desorption from glass, leaving a completely non-adhesive coating (Busscher *et al.* 1994). Busscher *et al.* (1997, 1999) also showed that biosurfactant release by *S. thermophilus* inhibited adhesion onto silicone rubber and growth of several bacterial and yeast strains isolated from explanted voice prostheses. On Chapter 8, by using an artificial throat model it is showed that biosurfactants obtained from probiotic strains greatly reduce microbial numbers on voice prostheses and also induce a decrease in the airflow resistance of voice prostheses after biofilm formation which may constitute a mechanism by which the lifetime of indwelling silicone rubber voice prostheses can be prolonged (Rodrigues *et al.* 2004a). A role for biosurfactants as defence weapons in post adhesion competition with other strains or species has to date been suggested only for biosurfactants released by *S. mitis* strains against *S. mutans* adhesion (Pratt-Terpstra *et al.* 1989, Van Hoogmoed *et al.* 2000) and for biosurfactants released by lactobacilli against adhesion of uropathogens (Reid *et al.* 1984, 1999). The biosurfactant surlactin (Velraeds *et al.* 1996a), produced by several *Lactobacillus* isolates, was suggested as a suitable anti-adhesive coating for catheter materials. Velraeds *et al.* (1996b) also reported on the inhibition of adhesion of pathogenic enteric bacteria by biosurfactant produced by a *Lactobacillus* strain and later showed that the biosurfactant caused an important, dose-related inhibition of the initial deposition rate of *E. coli* and other bacteria adherent on both hydrophobic and hydrophilic substrata (Velraeds *et al.* 1997).

2.7 CONCLUSIONS

A host of interesting features of biosurfactants have lead to a wide range of potential applications in the medical field. They are not only useful as antibacterial, antifungal and antiviral agents, but also have potential for use as major immunomodulatory molecules, adhesive agents and even in vaccines and gene therapy. Biosurfactants have been used for gene transfection, as ligands for binding immunoglobulins, as adjuvants for antigens and also as inhibitors for fibrin clot formation and activators of fibrin clot lyses. Promising alternatives to produce potent biosurfactants with altered antimicrobial profiles and decreased toxicity against mammalian cells may be exploited by genetic alteration of biosurfactants. Furthermore, biosurfactants have the potential to be used as anti-adhesive biological coatings for medical insertional materials, thus reducing hospital infections and use of synthetic drugs and chemicals. They may also be incorporated into probiotic preparations to combat urogenital tract infections and in pulmonary immunotherapy.

In spite of the immense potential in this field, the use of biosurfactants still remains limited, possibly due to their production and extraction high costs and lack of information on their toxicity towards human systems. Further investigations on human cells and natural microbiota are needed to validate the use of biosurfactants in several biomedical and health related areas. Nevertheless, there appears to be great potential for their use in the medical science arena waiting to be fully exploited.

2.8 REFERENCES

Abalos, A., Pinazo, A., Infante, M.R., Casals, M., Garcia, F. and Manresa, A. (2001) Physicochemical and antimicrobial properties of new rhamnolipids produced by *Pseudomonas aeruginosa* AT10 from soybean oil refinery wastes. *Langmuir.* 17: 1367-1371.

Ahimou, F., Jacques, P. and Deleu, M. (2001) Surfactin and iturin A effects on *Bacillus subtilis* surface hydrophobicity. *Enzyme and Microbial Technology*. 27: 749-754.

Akit, J., Cooper, D.G., Manninen, K.I. and Zajic, J.E. (1981) Investigation of potential biosurfactant production among phytopathogenic corynebacteria and related soil microbes. *Current Microbiology*. 6: 145-150.

Al-Tahhan, R., Sandrin, T., Bodour, A. and Maier, R. (2000) Rhamnolipid-induced removal of lipopolysaccharide from *Pseudomonas aeruginosa*: effect on cell surface properties and

interaction with hydrophobic substrates. *Applied and Environmental Microbiology*. 66: 3262-3268.

Angelova, B. and Schmauder, H.-P. (1999) Lipophilic compounds in biotechnology - interactions with cells and technological problems. *Journal of Biotechnology*. 67: 13-32.

Bai, G., Brusseau, M.L. and Miller, R.M. (1997) Influence of a rhamnolipid biosurfactant on the transport of bacteria through a sandy soil. *Applied and Environmental Microbiology*. 63: 1866-1873.

Banat, I.M. (1995a) Biosurfactants production and use in microbial enhanced oil recovery and pollution remediation: A Review. *Bioresource Technology*. 51: 1-12.

Banat, I.M. (1995b) Biosurfactants characterization and use in pollution removal: State of the art. A review. *Acta Biotechnologica*. 15: 251-267.

Banat, I.M., Makkar, R. and Cameotra, S. (2000) Potential commercial applications of microbial surfactants. *Applied Microbiology and Biotechnology*. 53: 495-508.

Bar-Ness, R., Avrahamy, N., Matsuyama, T. and Rosenberg, M. (1988) Increased cell surface hydrophobicity of a *Serratia marcescens* NS38 mutant lacking wetting activity. *Journal of Bacteriology.* 170: 4361-4364.

Bechard, J., Eastwell, K., Sholberg, P., Mazza, G. and Skura, B. (1998) Isolation and partial chemical characterization of an antimicrobial peptide produced by a strain of *Bacillus subtilis*. *Journal of Agriculture and Food Chemistry*. 46: 5355-5361.

Benincasa, M., Abalos, A., Oliveira, I. and Manresa, A. (2004) Chemical structure, surface properties and biological activities of the biosurfactant produced by *Pseudomonas aeruginosa* LB1 from soapstock. *Antonie van Leeuwenhoek*. 85: 1-8.

Bernheimer, A. and Avigad, L. (1970) Nature and properties of a cytolytic agent produced by *Bacillus subtilis*. *Journal of General Microbiology*. 61: 361-369.

Besson, F., Peypoux, F., Michel, G. and Delcambe, L. (1976) Characterization of iturin A in antibiotics from various strains of *Bacillus subtilis*. *Journal of Antibiotics.* 29: 1043-1049.

Bidel, D.J., Aly, R. and Shinefield, H.R. (1992) Inhibition of microbial adherence by sphinganine. *Canadian Journal of Microbiology.* 38: 983-985.

Boris, S. and Barbés, C. (2000) Role played by *lactobacilli* in controlling the population of vaginal pathogens. *Microbes and Infection*. 2: 543-546.

Bunster, L., Fokkema, N.J. and Shippers, B. (1989) Effect of surface-active *Pseudomonas* spp. of leaf wettability. *Applied and Environmental Microbiology.* 55: 1340-1345.

Busscher, H. J., Van Hoogmoed, C.G., Geertsema-Doornbusch, G.I., Van der Kuijl-Booij, M. and Van der Mei, H.C. (1997) *Streptococcus thermophilus* and its biosurfactants inhibit adhesion by *candida* spp on silicone rubber. *Applied and Environmental Microbiology.* 63: 3810-3817.

Busscher, H.J., Neu, T. and Van der Mei, H.C. (1994) Biosurfactant production by thermophilic dairy *streptococci*. *Applied Microbiology and Biotechnology*. 41: 4-7.

Busscher, H.J., Van de Belt-Gritter, B., Westerhof, M., Van Weissenbruch, R., Albers, F.W.J. and Van der Mei, H.C. (1999) Microbial interference in the colonization of silicone rubber implant surfaces in the oropharynx: *Streptococcus thermophilus* against a mixed fungal/bacterial biofilm. In: *Microbial Ecology and Infectious Disease*. Ed. Rosenberg, E. Washington D.C.: American Society for Microbiology. Pp. 66-74.

Cameotra, S. and Makkar, R. (1998) Synthesis of biosurfactants in extreme conditions. *Applied Microbiology and Biotechnology*. 50: 520-529.

Cameotra, S. and Makkar, R. (2004) Recent applications of biosurfactants as biological and immunological molecules. *Current Opinion in Microbiology*. 7: 262-266.

Carrillo, C., Teruel, J., Aranda, F. and Ortiz, A. (2003) Molecular mechanism of membrane permeabilization by the peptide antibiotic surfactin. *Biochimica et Biophysica Acta*. 1611: 91-97.

Cooper, D. and Goldenberg, B. (1987) Surface-active agents from two *Bacillus* species. *Applied and Environmental Microbiology.* 53: 224-229.

Cowan, M. and Busscher, H.J. (1993) Flow chamber study of the adhesion of *Prevotella intermedia* to glass after preconditioning with mutants streptococcal species: kinetics and spatial arrangement. *Microbios.* 73: 135-144.

Craig, V.S.J., Ninham, B.W. and Pashley, R.M. (1993) Effect of electrolytes on bubble coalescence. *Nature (London).* 364: 317-319.

Desai, J.D. and Banat (1997) Microbial production of surfactants and their commercial potential. *Microbiology and Molecular Biology Reviews.* 61: 47-64.

Fiechter, A. (1992) Biosurfactants: moving towards industrial application. *Trends in Biotechnology*. 10: 208-218.

Flasz, A., Rocha, C.A., Mosquera, B. and Sajo, C. (1998) A comparative study of the toxicity of a synthetic surfactant and one produced by *Pseudomonas aeruginosa* ATCC 55925. *Medical Science Research.* 6: 181-185.

Gan, B., Kim, J., Reid, G., Cadieux, P. and Howard, J. (2002) *Lactobacillus fermentum* RC-14 inhibits *Staphylococcus aureus* infection of surgical implants in rats. *The Journal of Infectious Diseases*. 185: 1369-1372.

Gerard, J., Lloyd, R., Barsby, T., Haden, P., Kelly, M.T. and Andersen, R.J. (1997) Massetolides A-H, antimycobacterial cyclic depsipeptides produced by two pseudomonads isolated from marine habitats. *Journal of Natural Products.* 60: 223-229.

Golubev, W.I., Kulakovskaya, T.V. and Golubeva, E.W. (2001) The yeast *Pseudozyma fusiformata* VKM Y-2821 producing an antifungal glycolipid. *Microbiology.* 70: 553-556.

Gottenbos, B., Grijpma, D., Van der Mei, H.C., Feijen, J. and Busscher, H.J. (2001) Antimicrobial effects of positively charged surfaces on adhering Gram-positive and Gram-negative bacteria. *Journal of Antimicrobial Chemotherapy*. 48: 7-13.

Grangemard, I., Wallach, J., Maget-Dana, R. and Peypoux, F. (2001) Lichenysin: A more efficient cation chelator than surfactin. *Applied Biochemistry and Biotechnology.* 90: 199-210.

Heinemann, C., Van Hylckama, V., Janssen, D., Busscher, H.J., Van der Mei, H.C. and Reid, G. (2000) Purification and characterization of a surface-binding protein from *Lactobacillus fermentum* RC-14 that inhibits adhesion of *Enterococcus faecalis* 1131. *FEMS Microbiology Letters*. 190: 177-180.

Hossain, H., Wellensiek, H-J., Geyer, R. and Lochnit, G. (2001) Structural analysis of glycolipids from *Borrelia burgdorferi*. *Biochemie.* 83: 683-692.

Humphries, M., Jaworzyn, J.F., Cantwell, J.B. and Eakin, A. (1987) The use of non-ionic ethoxylated and propoxylated surfactants to prevent the adhesion of bacteria to solid surfaces. *FEMS Microbiology Letters.* 42: 97-101.

Im, J., Nakane, T., Yanagishita, H., Ikegami, T. and Kitamoto, D. (2001) Mannosylerythritol lipid, a yeast extracellular glycolip, shows high binding affinity towards human immunoglobulin G. *BMC Biotechnology*. 1-5.

Im, J.H., Yanagishita, H., Ikegami, T., Takeyama, Y., Idemoto, Y., Koura, N. and Kitamoto, D. (2003) Mannosylerythritol lipids, yeast glycolipid biosurfactants, are potential affinity ligand materials for human immunoglobulin G. *Journal Biomedical Materials Research.* 65: 379-385.

Inoh, Y., Kitamoto, D., Hirashima, N. and Nakanishi, M. (2001) Biosurfactants of MEL-A increase gene transfection mediated by cationic liposomes. *Biochemical and Biophysical Research Communications.* 289: 57-61.

Inoh, Y., Kitamoto, D., Hirashima, N. and Nakanishi, M. (2004) Biosurfactant MEL-A dramatically increases gene transfection via membrane fusion. *Journal of Controlled Release*. 94: 423-431.

Ishigami, Y. and Suzuki, S. (1997) Development of biochemicals - functionalization of biosurfactants and natural dyes. *Progress in Organic Coatings*. 31: 51-61.

Isoda, H., Kitamoto, D., Shinmoto, H., Matsumoto, M. and Nakahara, T. (1997) Microbial extracellular glycolipid induction of differentiation and inhibition of protein kinase C activity of human promyelocytic leukaemia cell line HL60. *Bioscience, Biotechnology and Biochemistry.* 61: 609-614.

Isoda, H., Shinmoto, H., Matsumura, M. and Nakahara,T. (1999) The neurite-initiating effect of microbial extracellular glycolipids in PC12 cells. *Cytotechnology*. 31: 163-170.

Itokawa, H., Miyashita, T., Morita, H., Takeya, K., Hirano, T., Homma, M. and Kitaro, O. (1994) Structural and conformational studies of [Ile7] and [Leu7] surfactins from *Bacillus subtilis*. *Chemical Pharmacological Bulletin.* 42: 604-607.

Jenkinson, H.F. (1992) Adherence, coaggregation and hydrophobicity of *Streptococcus gordonii* associated with expression of cell surface lipoproteins. *Infectious Immunology*. 60: 1225-1228.

Jenny, K., Kappeli, O. and Fietcher, A. (1991) Biosurfactants from *Bacillus licheniformis*: structural analysis and characterization. *Applied Microbiology and Biotechnology*. 36: 5-13.

Kameda, Y., Ouchira, S., Matsui Kkanatomo, S., Hase, T. and Atsusaka, T. (1974) Antitumor activity of *Bacillus natto* V. Isolation and characterization of surfactin in the culture medium of *Bacillus natto* KMD 2311. *Chemical Pharmacological Bulletin.* 22: 938-944.

Kikuchi, T. and Hasumi, K. (2002) Enhancement of plasminogen activation by surfactin C: augmentation of fibrinolysis *in vitro* and *in vivo*. *Biochimica Biophysica Acta.* 1596: 234-245.

Kikuchi, T. and Hasumi, K. (2003) Enhancement of reciprocal activation of prourokinase plasminogen by the bacterial lipopeptide surfactins and iturins. *Journal of Antibiotics.* 56: 34-37.

Kim, K., Jung, S.Y., Lee, D.K., Jung, J.K., Park, J.K., Kim, D.K. and Lee, C.H. (1998) Suppression of inflammatory responses by surfactin, a selective inhibitor of platelet cytosolic phospholipase A2. *Biochemical Pharmacology.* 55: 975-985.

Kitamoto, D, Isoda, H. and Nakahara, T. (2002) Functions and potential applications of glycolipid biosurfactants - from energy-saving materials to gene delivery carriers. *Journal of Bioscience and Bioengineering*. 94: 187-201.

Kitamoto, D, Yanagishita, H., Shinbo, T., Nakane, T., Kamisawa, C. and Nakahara, T. (1993) Surface active properties and antimicrobial activities of mannosylerythritol lipids as biosurfactants produced by *Candida antarctica*. *Journal of Biotechnology*. 29: 91-96.

Kosaric, N. (1992) Biosurfactants in industry. *Journal of American Oil Chemists Society*. 64: 1731-1737.

Kosaric, N. (1996) Biosurfactants. In: *Biotechnology.* Ed. Rehm, H.J., Reed, G., Puhler, A. and Stadler, P. Weinheim: VCH. Pp. 659-717.

Kulakovskaya, T., Kulakovskaya, E. and Golubev, W. (2003) ATP leakage from yeast cells treated by extracellular glycolipids of *Pseudozyma fusiformata*. *FEMS Yeast Research*. 3: 401-404.

Lang, S. and Wullbrandt, D. (1999) Rhamnose lipids - biosynthesis, microbial production and application potential. *Applied Microbiology and Biotechnology*. 51: 22-32.

Lin, S. (1996) Biosurfactants: recent advances. *Journal of Chemical Technology and Biotechnology*. 66: 109-120.

Lin, S., Carswell, K. and Sharma, M. (1994) Continuous production of the lipopeptide biosurfactant of *Bacillus licheniformis* JF-2. *Applied Microbiology and Biotechnology*. 41: 281-285.

Mack, D.R., Michail, S., Wei, S., McDougall, L. and Hollingsworth, M.A. (1999) Probiotics inhibit enteropathogenic *E. coli* adherence in vitro by inducing intestinal mucin gene expression. *American Journal of Physiology.* 276: 941-950.

Maier, R. and Soberon-Chavez, G. (2000) *Pseudomonas aeruginosa* rhamnolipids: biosynthesis and potential applications. *Applied Microbiology and Biotechnology*. 54: 625-633.

Makkar, R. and Cameotra, S. (2002) An update on the use of unconventional substrates for biosurfactant production and their new applications. *Applied Microbiology and Biotechnology*. 58: 428-434.

Matsuyama, T., Kaneda, K., Nakagawa, Y., Isa, K., Hara-Hotta, H. and Yano, I. (1992) A novel extracellular cyclic lipopeptide which promotes flagellum-dependent and –independent spreading growth of *Serratia marcescens*. *Journal of Bacteriology.* 174: 1769-1776.

Millsap, K., Reid, G., Van der Mei, H.C. and Busscher, H.J. (1996) Adhesion of *Lactobacillus* species in urine and phosphate buffer to silicone rubber and glass under flow. *Biomaterials*. 18: 87-91.

Mireles, J.R., Toguchi, A. and Harshey, R.M. (2001) *Salmonella enterica serovar typhimurium* swarming mutants with altered biofilm formation abilities: surfactin inhibits biofilm formation. *Journal of Bacteriology*. 183: 5848-5854.

Mittenbuhler, K., Loleit, M., Baier, W., Fischer, B., Sedelmeier, E., Jung, G., Winkelmann, G., Jacobi, C., Weckesser, J., Erhard, M.H., Hofmann, A., Bessler, W. and Hoffmann, P. (1997) Drug specific antibodies: T-cell epitope-lipopeptide conjugates are potent adjuvants for small antigens *in vivo* and *in vitro*. *International Journal of Immunopharmacology.* 19: 277-287.

Morikawa, M., Daido, H., Takao, T., Murata, S., Shimonishi, Y. and Imanaka, T. (1993) A new lipopeptide biosurfactant produced by *Arthrobacter* sp. strain MIS38. *Journal of Bacteriology*. 175: 6459-6466.

Moyne, A.L, Shelby, R., Cleveland, T.E. and Tuzun, S. (2001) Bacillomycin D: an iturin with antifungal activity against *Aspergillus flavus*. *Journal of Applied Microbiology.* 90: 622-629.

Mulligan, C.N. (2005) Environmental application for biosurfactants. *Environmental Pollution.* 133: 183-198.

Mulligan, C.N., Cooper, D.G. and Neufeld, R.J. (1984) Selection of microbes producing biosurfactants in media without hydrocarbons. *Journal of Fermentation Technology.* 62: 311-314.

Naruse, N., Tenmyo, O., Kobaru, S., Kamei, H., Miyaki, T., Konishi, M. and Oki, T. (1990) Pumilacidin, a complex of new antiviral antibiotics: production, isolation, chemical properties, structure and biological activity. *Journal of Antibiotics.* 43: 267-280.

Neu, T. (1996) Significance of bacterial surface active compounds in interaction of bacteria with interfaces. *Microbiological Reviews*. 60: 151-166.

Neu, T. and Marshall, K.C. (1990) Bacterial polymers: physicochemical aspects of their interactions at interfaces. *Journal of Biomaterials Applications.* 5: 107-133.

Neu, T., Hartner, T. and Poralla, K. (1990) Surface active properties of viscosin: a peptidolipid antibiotic. *Applied Microbiology and Biotechnology*. 32: 518-520.

Nielsen, T., Christophersen, C., Anthoni, U. and Sorensen, J. (1999) Viscosinamide, a new cyclic depsipeptide with surfactant and antifungal properties produced by *Pseudomonas fluorescens* DR54. *Journal of Applied Microbiology*. 86: 80-90.

Otto, R.T., Daniel, H.-J., Pekin, G. and Muller-Decker, K. (1999) Production of sophorolipids from whey II. - Product composition, surface active properties, cytotoxicity and stability against hydrolases by enzimatic treatment. *Applied Microbiology and Biotechnology*. 52: 495-501.

Pratt-Terpstra, I.H., Weerkamp, A.H. and Busscher, H.J. (1989) Microbial factors in a thermodynamic approach of oral streptococcal adhesion to solid substrata. *Journal Colloid Interface Science.* 129: 568-574.

Ramisse, F., Delden, C., Gidenne, S., Cavallo, J.- and Hernandez, E. (2000) Decreased virulence of a strain of *Pseudomonas aeruginosa* O12 overexpressing a chromosomal type 1 b-lactamase could be due to reduced expression of cell-to-cell signalling dependent virulence factors." *FEMS Immunology and Medical Microbiology*. 28: 241-245.

Reid, G. (2000) *In vitro* testing of *Lactobacillus acidophilus* NCFM as a possible probiotic for the urogenital tract. *International Dairy Journal*. 10: 415-419.

Reid, G., Bruce, A. and Smeianov, V. (1998) The role of *Lactobacilli* in preventing urogenital and intestinal infections. *International Dairy Journal.* 8: 555-562.

Reid, G., Bruce, A., Fraser, N., Heinemann, C., Owen, J. and Henning, B. (2001) Oral probiotics can resolve urogenital infections. *FEMS Immunology and Medical Microbiology*. 30: 49-52.

Reid, G., Heinemann, C., Velraeds, M., Van der Mei, H.C. and Busscher, H.J. (1999) Biosurfactants produced by *Lactobacillus*. *Methods in Enzymology*. 310: 426-433.

Reid, G., Zalai, C. and Gardiner, G. (1984) Urogenital *Lactobacilli* probiotics, reliability, and regulatory issues. *Journal of Dairy Science*. 84: 164-169.

Rodrigues, L.R., Banat, I.M., Van der Mei, H.C., Teixeira, J. and Oliveira, R. (2005c) Interference in adhesion of bacteria and yeasts isolated from explanted voice prostheses to silicone rubber by rhamnolipid biosurfactants. *Journal of Applied Microbiology*. In press.

Rodrigues, L.R., Moldes, A., Teixeira, J. and Oliveira, R. (2005a) Kinetic study of fermentative biosurfactant production by *Lactobacillus* strains. *Biochemical Engineering Journal*. In press.

Rodrigues, L.R., Van der Mei, H.C., Banat, I.M., Teixeira, J. and Oliveira, R. (2005b) Inhibition of microbial adhesion to silicone rubber treated with biosurfactant from *Streptococcus thermophilus* A. *FEMS Immunology and Medical Microbiology*. In press.

Rodrigues, L.R., Van der Mei, H.C., Teixeira, J. and Oliveira, R. (2004a) Biosurfactant from *Lactococcus lactis* 53 inhibit microbial adhesion on silicone rubber. *Applied Microbiology and Biotechnology*. 66: 306-311.

Rodrigues, L.R., Van der Mei, H.C., Teixeira, J. and Oliveira, R. (2004b) Influence of biosurfactants from probiotic bacteria on formation of biofilms on voice prostheses. *Applied and Environmental Microbiology*. 70: 4408-4410.

Roy, B., Ackermann, H.-W., Pandian, S., Picard, G. and Goulet, J. (1993) Biological inactivation of adhering *Listeria monocytogenes* by listeriaphages and a quaternary ammonium compound. *Applied and Environmental Microbiology.* 59: 2914-2917.

Sano, H., Sohma, H., Muta, T., Nomura, S.-, Voelker, D. and Kuroki, Y. (1999) Pulmonary surfactant protein A modulates the cellular response to smooth and rough lipopolysaccharides by interaction with CD14. *Journal of Immunology.* 163: 387-395.

Sheppard, J.D., Jumarie, C., Cooper, D.G. and Laprade, R. (1991) Ionic channels induced by surfactin in plannar lipid bilayer membranes. *Biochimica et Biophysica Acta.* 1064: 13-23.

Shibahara, M., Zhao, X., Wakamatsu, Y., Nomura, N., Nakahara, T., Jin, C., Nagaso, H., Murata, T. and Yokoyama, K.K. (2000) Mannosylerythritol lipid increases levels of galactoceramide in and neutrite outgrowth from PC12 pheochromocytoma cells. *Cytotechnology.* 33: 247-251.

Singh, P. and Cameotra, S. (2004) Potential applications of microbial surfactants in biomedical sciences. *Trends in Biotechnology*. 22: 142-146.

Sudo, T., Zhao, X., Wakamatsu, Y., Shibahara, M., Nomura, N., Nakahara, T., Suzuki, A., Kobayashi, Y., Jin, C., Murata, T. and Yokoyama, K.K. (2000) Induction of the differentiation of human HL-60 promyelocytic leukemia cell line by succinoyl trehalose lipids. *Cytotechnology.* 33: 259-264.

Symmank, H., Franke, P., Saenger, W. and Bernhard, F. (2002) Modification of biologically active peptides production of a novel lipohexapeptide after engineering of *Bacillus subtilis* surfactin synthetase. *Protein Engineering.* 15: 913-921.

Tanaka, Y., Takashi, T., Kazuhik, U., Jun, U. Yasushi, U. and Chiba, S.O. (1997) Method of producing iturin A and antifungal agent for profound mycosis. *Biotechnology Advances.* 15: 234-235.

Thimon, L., Peypoux, F., Wallach, J. and Michel, G. (1995) Effect of lipopeptide antibiotic, iturin A, on morphology and membrane ultrastructure of yeast cells. *FEMS Microbiology Letters.* 128: 101-106.

Uchida, Y., Misava, S., Nakahara, T. and Tabuchi, T. (1989b) Factor affecting the production of succinotrehalose lipids by *Rodococcus erythropolis* SD-74 grown on n-alkanes. *Agriculture and Biological Chemistry.* 53: 765-769.

Uchida, Y., Tsuchiya, R., Chino, M., Hirano, J. and Tabuchi, T. (1989a) Extracellular accumulation of mono and di succinyl trehalose lipids by a strain of *Rodococcus erythropolis* grown on n-alkanes. *Agriculture and Biological Chemistry.* 53: 757-763.

Van Delden, C. and Iglewski, B. (1998) Cell-to-cell signaling and *Pseudomonas aeruginosa* infections. *Emerging Infectious Diseases*. 4: 551-560.

Van Hoogmoed, C. G., Van der Kuijl-Booij, M., Van der Mei, H.C. and Busscher, H.J. (2000) Inhibition of *Streptococcus mutans* NS adhesion to glass with and without a salivary conditioning film by biosurfactant-releasing *Streptococcus mitis* strain. *Applied and Environmental Microbiology.* 66: 659-663.

Velraeds, M., Van de Belt-Gritter, B., Van der Mei, H.C., Reid, G. and Busscher, H.J. (1998) Interference in initial adhesion of uropathogenic bacteria and yeasts to silicone rubber by a *Lactobacillus acidophilus* biosurfactant. *Journal of Medical Microbiology*. 47: 1081-1085.

Velraeds, M., Van der Mei, H.C., Reid, G. and Busscher, H.J. (1996a) Physicochemical and biochemical characterization of biosurfactants released by *Lactobacillus* strains. *Colloids and Surfaces B: Biointerfaces*. 8: 51-61.

Velraeds, M., Van der Mei, H.C., Reid, G. and Busscher, H.J. (1996b) Inhibition of initial adhesion of uropathogenic *Enterococcus faecalis* by biosurfactants from *Lactobacillus* isolates. *Applied and Environmental Microbiology.* 62: 1958-1963.

Velraeds, M., Van der Mei, H.C., Reid, G. and Busscher, H.J. (1997) Inibition of initial adhesion of uropathogenic *enterococcus faecalis* to solid substrata by an adsorbed biosurfactant layer from *Lactobacillus Acidophilus*. *Urology*. 49: 790-794.

Vladkova, T. (2004) Surface modification of silicone rubber with poly(ethylene glycol) hydrogel coatings. *Journal of Applied Polymer Science*. 92: 1486-1492.

Vollenbroich, D., Ozel, M., Vater, J., Kamp, R.M. and Pauli, G. (1997b) Mechanism of inactivation of enveloped viruses by the biosurfactant surfactin from *Bacillus subtilis*. *Biologicals.* 25: 289-297.

Vollenbroich, D., Pauli, G., Ozel, M. and Vater, J. (1997a) Antimycoplasma properties and applications in cell culture of surfactin, a lipopeptide antibiotic from *Bacillus subtilis*. *Applied and Environmental Microbiology.* 63: 44-49.

Wakamatsu, Y., Zhao, X., Jin, C., Day, N., Shibahara, M., Nomura, N., Nakahara, T., Murata, T. and Yokoyama, K.K. (2001) Mannosylerythritol lipid induces characteristics of neuronal differentiation in PC12 cells through an ERK-related signal cascade. *European Journal of Biochemistry*. 268: 374-383.

Wicken, A.J. and Knox, K.W. (1980) Bacterial cell surface amphiphiles. *Biochimica Biophysica Acta.* 604: 1-26.

Wright, J.R. (2003) Pulmonary surfactant: a front line of lung host defense. *Journal of Clinical Investigation.* 111: 1453-1455.

Yakimov, M., Timmis, K., Wray, V. and Fredrickson, H. (1995) Characterization of a new lipopeptide surfactant produced by thermotolerant and halotolerant subsurface *Bacillus licheniformis* BAS50. *Applied and Environmental Microbiology.* 61: 1706-1713.

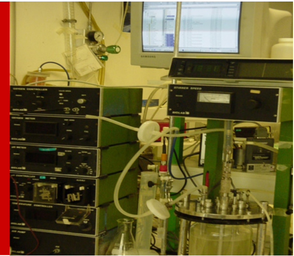
Zhang, Y. and Miller, R.M. (1994) Effect of a *Pseudomonas* rhamnolipid biosurfactant on cell hydrophobicity and biodegradation of octadecane. *Applied and Environmental Microbiology.* 60: 2101-2106.

Zhang, Y., Maier, W. and Miller, R. (1997) Effect of rhamnolipids on the dissolution, bioavailability, and biodegradation of phenanthrene. *Environmental Science Technology*. 31: 2211-2217.

Zhao, X., Geltinger, C., Kishikawa, S., Ohshima, K., Murata, T., Nomura, N., Nakahara, T. and Yokoyama, K. (2000) Tretament of mouse melanoma cells with phorbol 12-myristate 13-acetate counteracts mannosylerythritol lipid-induced growth arrest and apoptosis. *Cytotechnology*. 33: 123-130.

Zhao, X., Wakamatsu, Y., Shibahara, M., Nomura, N., Geltinger, C., Nakahara, T., Murata, T. and Yokoyama, K.K. (1999) Mannosylerythritol lipid is a potent inducer of apoptosis and differentiation of mouse melanoma cells in culture. *Cancer Research.* 59: 482-486.

CHAPTER 3



RESPONSE SURFACE OPTIMIZATION OF THE MEDIUM COMPONENTS FOR THE PRODUCTION OF BIOSURFACTANTS BY PROBIOTIC BACTERIA

"As far as the laws of mathematics refer to reality, they are not certain; and as far as they are certain, they do not refer to reality".

Albert Einstein.

The optimization of the medium for biosurfactants production by probiotic bacteria (*Lactococcus lactis* 53 and *Streptococcus. thermophilus* A) using experimental factorial design and response surface analysis is described in this Chapter. The optimization procedure allowed an increase about 2 times in the biosurfactants production yields. The response surface methodology proved to be an attractive tool for biosurfactant production medium definition.

3.1	INTRODUCTION	3.3.1	Production and Cell Growth
3.2	MATERIALS AND METHODS	3.3.2	Effect of Medium Compositions on Cell Growth
3.2.1	Strains and Culture Conditions	3.3.3	The Path of Steepest Ascent
3.2.2	Cell Growth and Biosurfactant Production	3.3.4	Central Composite Design
3.2.3	Biosurfactants Surface-activity Determination	3.3.5	Mass Recovery and Surface-activity
3.2.4	Mass of Produced Biosurfactants	3.4	DISCUSSION
3.2.5	Experimental Designs	3.5	CONCLUSIONS
3.3	RESULTS	3.6	REFERENCES

3.1 INTRODUCTION

Interest in biosurfactants has increased considerably in recent years, as they are potential candidates for many commercial applications in the petroleum, pharmaceuticals, biomedical and food processing industries (Desai and Banat 1997). Dairy *Streptococcus thermophilus* strains, for example, can produce biosurfactants that cause their own desorption (Busscher *et al.* 1991). *Lactobacillus* and *Streptococcus* species have been shown to be able to displace adhering uropathogenic *Enterococcus faecalis* from hydrophobic and hydrophilic substrata in a parallel plate flow chamber, possibly through biosurfactant production (Velraeds *et al.* 1996). Biosurfactants have special advantages over synthetic surfactants such as their biodegradability, lower toxicity and greater diversity, as they present a much broader range of surfactant types and properties than the available synthetic surfactants (Cooper and Zajic 1980). Depending upon the nature of the biosurfactant and the producing microrganisms, the following patterns of biosurfactant production by fermentation are possible: (a) growth-associated production, (b) production under growth limiting conditions, (c) production by resting/nongrowing cells, and (d) production associated with the precursor augmentation. In the case of growth-associated biosurfactant production, there exists a parallel relationship between the substrate utilization, growth and biosurfactant production (Desai and Desai 1993).

Cell growth and the accumulation of metabolic products are strongly influenced by medium composition such as carbon source, nitrogen source, growth factors, and inorganic salts. Thus, it is difficult to search for the major factors and to optimize them for biotechnological processes as several parameters are involved (Li *et al.* 2002). Environmental factors and growth conditions such as pH, temperature, agitation, and oxygen availability also affect biosurfactant production through their effects on cellular growth or activity (Desai and Banat 1997).

The classical method of medium optimization involves changing one variable at a time, keeping the others at fixed levels. Being single dimensional, this laborious and time-consuming method often does not guarantee determination of optimal conditions. On the other hand carrying out experiments with every possible factorial combination of the test variables is impractical because of the large number of experiments required (Sen 1997).

Experimental design and optimization are tools that are used to systematically examine different types of problems that arise within, e.g., research, development and production.

Response surface methodology is a collection of mathematical and statistical techniques that are useful for the modelling and analysis of problems in which a response of interest is influenced by several variables and the purpose is to optimize this response. In addition, it is a good way to graphically illustrate the relation between different experimental variables and the responses. In the first screening it is recommended to evaluate the result and estimate the main effects of the variables according to a linear model. After this evaluation, the variables that have the largest influence on the result are selected for new studies. Thus, a large number of experimental variables can be investigated without having to increase the number of experiments to the extreme (Montgomery 1997).

The aim of the present study was to improve the standard media, using lactose as carbon source instead of glucose, for growing biosurfactant-producing lactic acid bacteria. The optimization of cellular growth of the probiotic bacteria *Lactococcus lactis* 53 and *S. thermophilus* A was achieved using a 2^{6-2} fractional factorial central composite design and surface modelling method, after establishing that their biosurfactants are growth-associated. The yields of biosurfactant production for both strains were determined before and after optimization, as well as its surface-activity. The relation between cellular growth and surface-activity of the biosurfactant during the fermentation (as a measure of its production) was determined for both strains before and after the optimization procedure.

3.2 MATERIALS AND METHODS

3.2.1 Strains and Culture Conditions

The bacterial strains *L. lactis* 53 and *S. thermophilus* A were stored at –20ºC in MRS (De Man *et al.* 1960) or M17 (Terzaghi and Sandine 1975) broth, respectively. From frozen stock, bacteria were streaked on MRS or M17 agar plates and incubated at 37ºC. To prepare subcultures, the respective medium was inoculated with a colony from the plate and incubated overnight under the same conditions. In the experimental design assays optimization of the standard MRS and M17 media was performed by changing the carbon source from glucose to lactose, as well as the concentrations of the key factors as described below.

3.2.2 Cell Growth and Biosurfactants Production

Cellular growth was measured by optical density of the culture at 600 nm and biomass concentrations (g dry weight l^{-1}) were determined using a calibration curve. The calibration curve was calculated for each strain using dilutions of a biomass suspension with known optical density. A fixed volume of the dilutions was filtered (0.45 µm) and left to dry at 105ºC for 24 h. All the filters were weighed before filtration and after drying. Thus, a relationship between biomass concentration (g l^{-1}) and optical density (600 nm) can be determined.

For the bacterial strains *L. lactis* 53 and *S. thermophilus* A, 600 ml cultures in MRS and M17 broth respectively were grown overnight (18 h) with inoculums volume of 6 ml. The growth media used for the production of these biosurfactants were the standard media MRS and M17, and the optimized media obtained by experimental design for higher yields of biosurfactant production. Cells were harvested by centrifugation (10000 × g, 5 min, 10ºC), washed twice with demineralized water, and ressuspended in 100 ml of phosphate-buffered saline (PBS: 10 mM KH_2PO_4/K_2HPO_4 and 150 mM NaCl with pH adjusted to 7.0). The bacteria were left at room temperature for 2 h with gentle stirring for cell-bound biosurfactants release. Subsequently, the bacteria were removed by centrifugation and the remaining supernatant liquid was filtered through a 0.22 µm pore-size filter (Millipore). The supernatant was dialyzed against demineralized water at 4ºC in a Spectrapor membrane tube (molecular weight cut off 6000 to 8000, Spectrum Medical Industries Inc., CA) and freeze-dried.

3.2.3 Biosurfactants Surface-activity Determination

Axisymmetric drop shape analysis by profile (ADSA-P) is a technique for determining liquid surface tensions based on the shape of an axisymmetric droplet on a solid substratum. In order to measure the surface-activity of both cell-bound biosurfactants obtained in the stationary growth phase (as described previously) by ADSA-P, a 100 µl droplet of a biosurfactant solution was placed on fluoroethylene-propylene (FEP)-Teflon (Fluorplast, The Netherlands) in an enclosed chamber to prevent evaporation from the droplet. The shape of the droplet was monitored for 2 h at room temperature and the surface tension of the droplet was calculated from its shape as a function of time (Van der Vegt *et al.* 1991). Surface-activity of biosurfactant produced by bacteria in time was also measured by ADSA-P. Bacterial suspensions were prepared as follows. The *L. lactis* 53 and *S. thermophilus* A were grown in 200 ml of MRS and M17 broth respectively, inoculated with 10 ml of an overnight pre-culture. After 3, 6, 9 and 24 h, 10 ml of the culture was harvested by centrifugation (10000 × g, 5 min, 10ºC) and washed twice in fresh PBS. Bacteria were counted in a Bürker-

Türk counting chamber and diluted in PBS to a final concentration of 5×10^9 cell ml^{-1}, and used immediately as described above in the ADSA-P procedure.

3.2.4 Mass of Produced Biosurfactants

In order to compare the amount of cell-bound biosurfactants produced by the bacteria grown in standard and optimized medium, the biosurfactants were released by the stationary phase cells using the PBS extraction procedure described below. Briefly, the bacteria were left at room temperature for 2 h with gentle stirring for cell-bound biosurfactants release. Subsequently, the bacteria were removed by centrifugation and the remaining supernatant liquid was filtered through a 0.22 µm pore-size filter (Millipore). The supernatant was dialyzed against demineralized water at 4ºC in a Spectrapor membrane tube (molecular weight cut off 6000 to 8000, Spectrum Medical Industries Inc., CA) and freeze-dried. The mass of produced biosurfactant (mg per gram cell dry weight) was determined.

3.2.5 Experimental Designs

Response surface methodology is a collection of mathematical and statistical techniques that are useful for the modelling and analysis of problems in which a response of interest is influenced by several variables and the objective is to optimize this response (Montgomery 1997).

3.2.5.1 Fractional Factorial Designs (*FFD*)

In order to identify which component(s) of the medium has a significant effect on cellular growth a first optimization step was developed. In a factorial design the influences of all experimental variables, factors, and interaction effects on the response or responses are investigated (Montgomery 1997). Six major components in MRS (peptone, meat extract, yeast extract, lactose, ammonium citrate and KH_2PO4) and M17 medium (peptone, meat extract, yeast extract, lactose, soya peptone and sodium glycerophosphate) to be set as factors in the factorial designs were selected. According to factorial designs 2^6 experiments have to be performed. If the experimenter can reasonably assume that certain high-order interactions are negligible, information on the main effects and low-order interactions may be obtained by running only a fraction of the complete factorial experiment. The number of experiments can then be reduced by using only a part of the factorial designs (fractional factorial design) without loss of information about the main effects. For a moderately large number of factors, smaller fractions of the 2^k design are frequently useful (Montgomery 1997). Therefore, for a 2^{6-2} fractional factorial design with six factors at two levels, only 16

experimental runs are required. A first-order model was then fitted to the data obtained from the *FFD* experiments. Frequently, the initial estimate of the optimum operating conditions for the system will be far from the actual optimum. In such circumstances, the objective is to move rapidly to the general vicinity of the optimum. The method of steepest ascent is a procedure for moving sequentially along the path of steepest ascent, that is, in the direction of the maximum increase in the response. Further studies for the optimization involved experiments carried out along the path of steepest ascent, which means, the direction at right angles to the contour lines representing equal yield, that shows the relative amounts by which the factors have to vary in order to attain a maximum increase of responses.

3.2.5.2 Central Composite Designs (*CCD*)

The objective of this second experiment is to develop an empirical model of the process and to obtain a more precise estimate of the optimum operating conditions for the factors involved. This approach to process optimization is called response surface methodology and the second design is a central composite design, one of the most important experimental designs used in process optimization studies (Montgomery 1997). In order to describe the nature of the response surface in the optimum region, a central composite design with five coded levels was performed. For the two factors, this design was made up a full 2^2 factorial design with its four cube points, augmented with five replications of the center points and the four star points, that is, points having for one factor an axial distance to the center of $\pm \alpha$, whereas the other factor is at level 0. The axial distance α was chosen to be 1.414 to make this design rotatable. A center point is a point in which all variables are set at their mid value. Three or four center experiments should always be included in factorial designs because the risk of missing non-linear relationships in the middle of the intervals has to be minimized, and also because the repetition allows for determination of confidence intervals (Montgomery 1997). To estimate the optimal point, a third-order polynomial function was fitted to the experimental results.

3.3 RESULTS

3.3.1 Biosurfactant Production by Growing Cells

The relation between cell growth and surface-activity of the biosurfactant in time was determined for both strains before the optimization procedure (Fig. 3.1 A and 3.2 A).

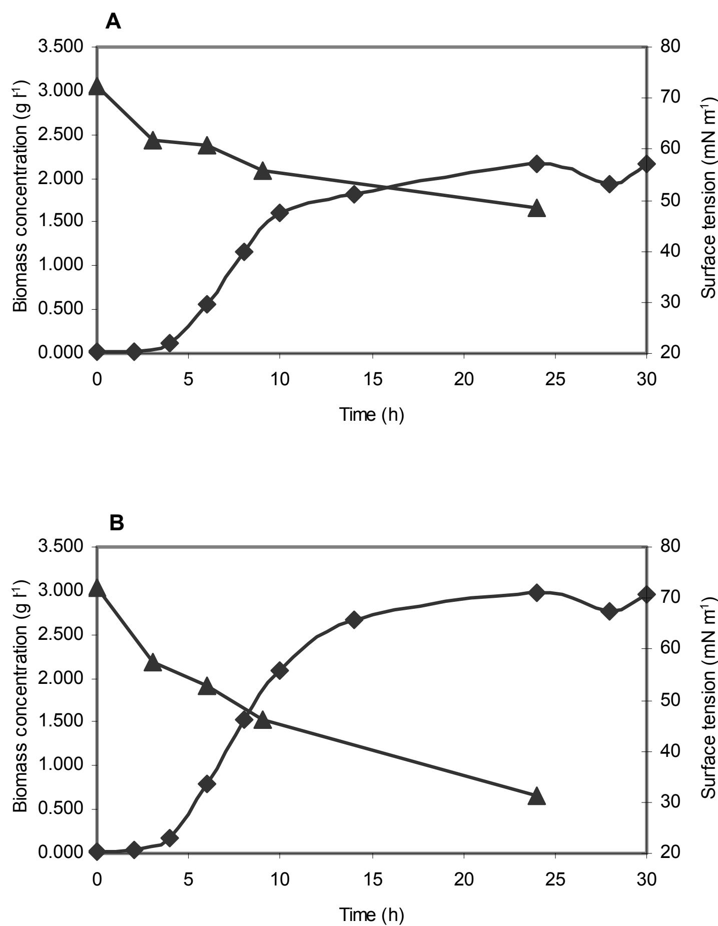


Figure 3.1 Fermentation evolution for *L. lactis* 53: variation of biomass concentration (g l^{-1}) (■) and surface tension (mN m^{-1}) (▲), in time. The biomass concentration is a measure of the cell growth, while surface tension is a measure of the biosurfactant activity. **A)** *L. lactis* 53 grown in MRS medium before experimental design optimization of the media composition. **B)** *L. lactis* 53 grown in MRS optimized by experimental design

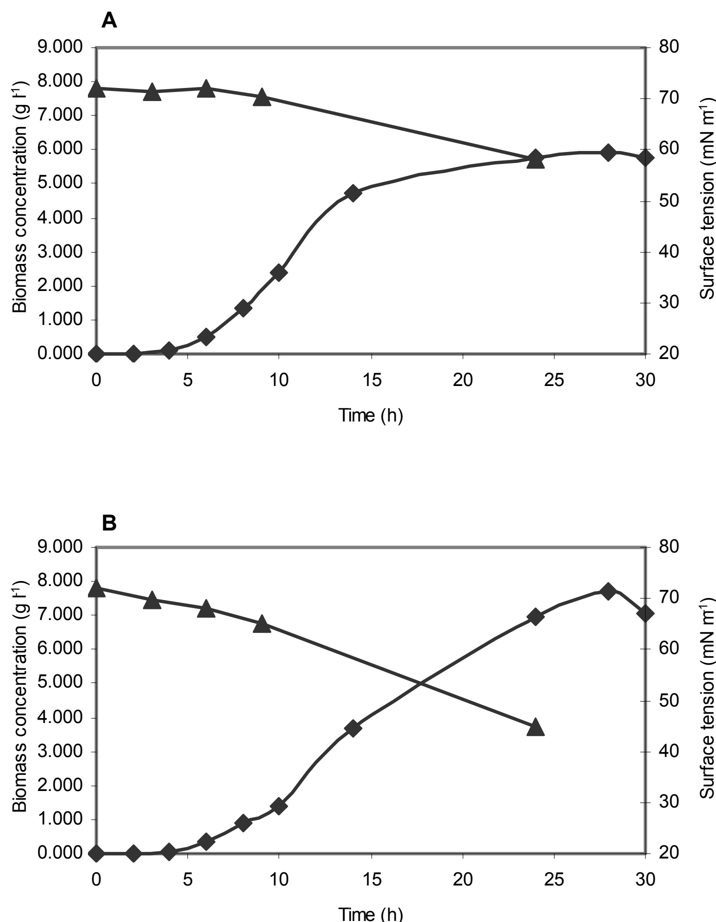


Figure 3.2 Fermentation evolution for *S. thermophilus* A: variation of biomass concentration (g l^{-1}) (■) and surface tension (mN m^{-1}) (▲), in time. The biomass concentration is a measure of the cell growth, while surface tension is a measure of the biosurfactant activity. **A)** *S. thermophilus* A grown in M17 medium before experimental design optimization of media composition. **B)** *S. thermophilus* A grown in M17 optimized by experimental design.

For both strains the biosurfactant production is associated with the cellular growth, as an increase in the biomass concentration leads to a decrease in the surface tension. In the case of a growth associated biosurfactant production there is a parallel relationship between the substrate utilization, growth and biosurfactant production (Desai and Desai 1993). The lowest values of surface tension were achieved in the stationary phase for both bacterial strains.

3.3.2 Effects of Different MRS or M17 Medium Components in Cell Growth

The factorial design enables the identification of the medium components that play a significant role on cell growth, as well as the ranges within the medium components vary. For each medium, six components were set as variables for the optimization procedure and the concentration for each component in the medium was appropriately enlarged as the ranges for the variables. The independent variables, experimental range and levels investigated in this study, for both media, are given in Table 3.1.

Table 3.1 Experimental range and levels of the independent variables (X_i and Z_i, i = 1, 2, 3, 4, 5, 6) used in the Fractional Factorial Design (*FFD*).

MRS medium optimization			
Independent variables (g l^{-1})	**Range and Levels**		
	-1	**0**	**1**
X_1 – peptone	5.0	10.0	15.0
X_2 – meat extract	5.0	10.0	15.0
X_3 – yeast extract	2.5	5.0	7.5
X_4 – lactose	10.0	20.0	30.0
X_5 – ammonium citrate	1.0	2.0	3.0
X_6 – KH_2PO_4	1.0	2.0	3.0
M17 medium optimization			
Independent variables (g l^{-1})	**Range and Levels**		
	-1	**0**	**1**
Z_1 – peptone	2.5	5.0	7.5
Z_2 – meat extract	2.5	5.0	7.5
Z_3 – yeast extract	1.25	2.5	3.75
Z_4 – lactose	5.0	10.0	15.0
Z_5 – soya peptone	2.5	5.0	7.5
Z_6 – sodium glycerophosphate	9.5	19.0	28.5

In developing the regression equation, the test variables were coded according to the equation:

$$x_i = \left(\frac{X_i - X_i^*}{\Delta X_i} \right)$$ Equation 3.1

where x_i is the coded value of the *i*th independent variable, X_i is the uncoded value for the *i*th independent variable, X_i^* is the uncoded value of the ith independent variable at the center point and ΔX_i is the step change value.

Results of the experimental design performed to achieve MRS medium optimization are shown in Table 3.2.

Table 3.2 Experimental design and results of the Fractional Factorial Design (*FFD*).

MRS medium optimization								
Run	**x1**[a]	**x2**[a]	**x3**[a]	**x4**[a]	**x5**[a]	**x6**[a]	**Biomass concentration g l^{-1}**	
							Observed[b]	**Expected**[c]
1	-1	-1	-1	-1	-1	-1	2.179	1.960
2	-1	+1	+1	-1	-1	-1	1.811	2.180
3	-1	+1	+1	+1	-1	+1	2.434	2.570
4	0	0	0	0	0	0	2.584	2.490
5	-1	-1	+1	-1	+1	+1	2.721	2.200
6	-1	+1	-1	+1	+1	-1	2.407	2.340
7	-1	-1	-1	+1	-1	+1	2.140	2.350
8	+1	-1	+1	+1	-1	-1	4.250	3.480
9	0	0	0	0	0	0	2.491	2.490
10	+1	+1	+1	-1	+1	-1	2.963	3.060
11	0	0	0	0	0	0	2.407	2.490
12	+1	-1	+1	-1	-1	+1	2.800	3.060
13	+1	+1	-1	+1	-1	-1	3.393	3.200
14	-1	-1	+1	+1	+1	-1	2.096	2.620
15	+1	+1	+1	+1	+1	+1	3.565	3.450
16	+1	-1	-1	+1	+1	+1	2.968	3.230
17	+1	+1	-1	-1	-1	+1	2.582	2.780
18	-1	+1	-1	-1	+1	+1	2.366	1.930
19	+1	-1	-1	-1	+1	-1	2.588	2.840

Table 3.2 Experimental design and results of the Fractional Factorial Design (*FFD*). *Continuation*

M17 medium optimization								
Run	$z1^a$	$z2^a$	$z3^a$	$z4^a$	$z5^a$	$z6^a$	Biomass concentration g l^{-1}	
							Observed [b]	Expected [c]
1	-1	+1	-1	-1	+1	+1	5.558	5.720
2	-1	-1	+1	-1	+1	+1	5.100	5.880
3	0	0	0	0	0	0	4.627	4.480
4	0	0	0	0	0	0	6.489	4.480
5	-1	+1	-1	+1	+1	-1	0.840	-0.053
6	-1	-1	-1	+1	-1	+1	4.912	3.680
7	0	0	0	0	0	0	3.866	4.480
8	+1	-1	+1	+1	-1	-1	1.056	1.480
9	-1	-1	+1	+1	+1	-1	1.045	0.100
10	+1	+1	-1	+1	-1	-1	0.798	1.320
11	+1	+1	+1	-1	+1	-1	3.471	3.530
12	+1	+1	-1	-1	-1	+1	7.782	7.100
13	+1	-1	+1	-1	-1	+1	8.405	7.250
14	0	0	0	0	0	0	2.945	4.480
15	-1	+1	+1	+1	-1	+1	3.068	3.840
16	-1	-1	-1	-1	-1	-1	2.489	3.320
17	+1	+1	+1	+1	+1	+1	4.334	3.880
18	+1	-1	-1	-1	+1	-1	3.889	3.370
19	+1	-1	-1	+1	+1	+1	1.901	3.720
20	-1	+1	+1	-1	-1	-1	2.960	3.480

[a] The coded variables xi and zi (i = 1, 2, 3, 4, 5, 6) are defined in Table 3.1.

[b] Observed biomass concentration stands for the experimental data.

[c] Expected biomass concentration is calculated from the first-order model approach (Equation 3.2 and 3.3).

The biomass concentration varied markedly from 1.811 to 4.250 g l^{-1} with the different levels of components in the medium. The concentration of lactose and peptone strongly affected the cell growth, with *P*-values of 0.0766 and 0.0015 respectively, whereas ammonium citrate and KH_2PO_4 did not significantly influence cell growth. Furthermore, it was found that the yeast extract is more important for the cell growth than the meat extract. The values of the regression coefficients were calculated and the response variable Yb^*_{Ll} could be written as a fit of the experimental data:

$$Yb_{Ll}^{*} = 2.70 + 0.43x_1 - 0.01x_2 + 0.13x_3 + 0.20x_4 + 0.01x_5 - 0.01x_6 \qquad \text{Equation 3.2}$$

The results for M17 medium optimization demonstrated that the biomass concentration varied markedly from 0.798 to 8.405 g l^{-1} with the different levels of components in the medium. The concentration of lactose and sodium glycerophosphate strongly affected the cell growth, with *P*-values of 0.0009 and 0.0003 respectively. All the other medium components did not significantly influence cell growth. The desired response variable (Yb^{*}_{St}) was set as biomass concentration (g l^{-1}) in the stationary phase. The values of the regression coefficients were calculated and the response variable Yb^{*}_{St} could be written as a fit of the experimental data:

$$Yb_{St}^{*} = 3.60 + 0.35z_1 + 0.01z_2 + 0.08z_3 - 1.361z_4 - 0.33z_5 + 1.531z_6 \qquad \text{Equation 3.3}$$

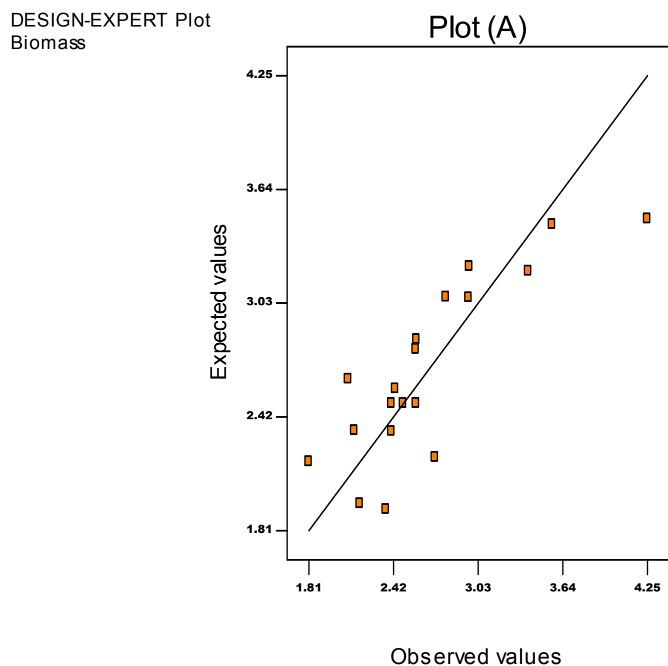
For the MRS medium optimization the regression analysis of the *FFD* showed that peptone (x_1) and lactose (x_4) were significant at the probability levels of 99 and 95%, respectively, for cell growth and proved to be the two most important components of the medium. All the other components of the medium, except for the yeast extract, were not found to be significant at the probability level of 90% for cell growth. Table 3.3 presents the coefficient of determination R^2 of the model to be 0.68, which means that the model explains 68% of the variability in the data. This ensured a satisfactory adjustment of the first order model to the experimental data. The statistical significance of the model equation was also confirmed by an *F*-test. The model *F*-value of 3.81 implies the model is significant, which means that there is only 2.64% chance that a model *F*-value this large could occur due to noise. The lack of fit *F*-value of 26.62 is significant, which means that there is only a 3.67% chance that this value could occur due to noise. A significant lack of fit is bad because we want the model to fit. The purpose of statistical analysis is to determine which experimental factors generate signals, which are large in comparison to the noise. The adequate precision value measures signal to noise ratio and a ratio greater than 4 is desirable. The adequate precision value shows an adequate signal, which means that this model can be used to navigate the design space and for further optimization. Figure 3.3 A represents the relationship between the observed biomass concentration values and the expected values determined by the model Equation 3.2 for *L. lactis* 53. It can be observed that most points are nearby the line adjustment which

means that the values determined experimentally are similar to those determined by the model. Also for *S. thermophilus* A it can be observed in Figure 3.3 B the same tendency.

Table 3.3 Analysis of Variance (ANOVA) for the first order models determined from the Fractional Factorial Design (*FFD*).

Values	MRS medium optimization	M17 medium optimization
R^2	0.68	0.80
Adjusted R^2	0.50	0.69
Predicted R^2	-0.01	0.47
Adequate precision	5.79	9.38
Model *F*-value	3.81	7.78
Lack of fit *F*-value	26.62	0.56

For the M17 medium optimization the regression analysis of the *FFD* showed that lactose (z_4) and sodium glycerophosphate (z_6) were significant at the probability level of 99% for cell growth and consequently the two most important components. All the other components of the medium were not found to be significant at the probability level of 90% for cell growth. The ANOVA summarized in Table 3.3 showed a coefficient of determination R^2 of 0.80, which means that the model explains 80% of the variability in the data. The model *F*-value of 7.78 implies that the model is significant and there is only 0.14% chance that this value could occur due to noise. The lack of fit *F*-value of 0.56 is not significant relative to true pure error, and there is a 77.9% chance that this value could occur due to noise. This model was found to be adequate to navigate the design space and further optimization.



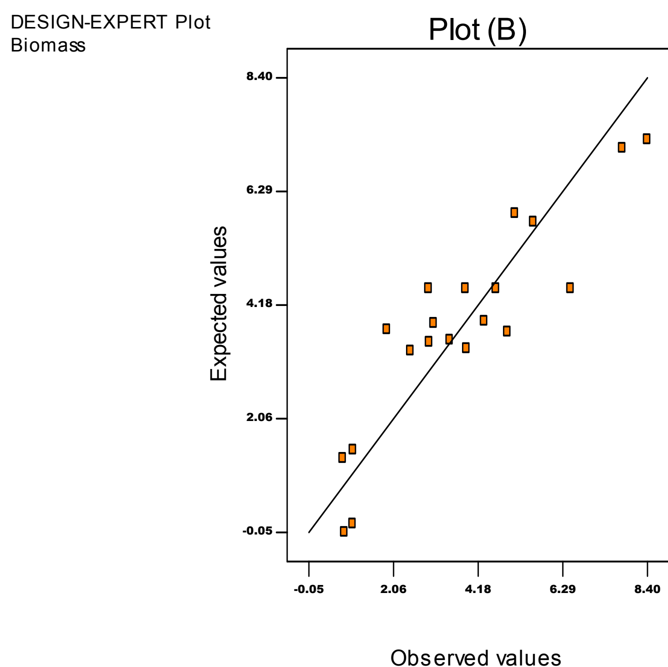


Figure 3.3 Plots of Observed *versus* expected biomass concentration (g l^{-1}) values for *L. lactis* 53 **(A)** and *S. thermophilus* A **(B)**. The biomass concentration is the response variable of interest. The expected biomass concentration values are determined by the model equations determined for fractional factorial design (*FFD*).

3.3.3 The Path of Steepest Ascent

The path of steepest ascent was determined by first-order model (Equation 3.2 and 3.3) and regression analysis for both bacterial strains. Besides the previously determined significant factors (peptone (x_1) and lactose (x_4) for MRS medium optimization; lactose (z_4) and sodium glycerophosphate (z_6) for M17 medium optimization), all the other components were fixed at the center level of the *FFD* because they were not significant at the probability level of 90% for cell growth. According to the signs of their main effects, the concentrations of the significant factors were increased or decreased, in order to achieve a positive consequence in the response variable. In MRS medium optimization, peptone and lactose were increased serially by 0.5% and 0.25% respectively, while for M17 medium optimization lactose was decreased serially by 2.0%, and sodium glycerophosphate was increased serially by 1.0%. The higher biomass concentration (2.453 g l^{-1}) has been reached with 30 g l^{-1} peptone and 38.6 g l^{-1} lactose for MRS medium optimization. For M17 medium optimization, 3.2 g l^{-1} lactose and 26.6 g l^{-1} sodium glycerophosphate allowed a 6.656 g l^{-1} biomass concentration.

3.3.4 Central Composite Design (*CCD*)

By determining the path of steepest ascent the vicinity of the optimum was reached. Thus, for MRS medium optimization, the levels of the two significant variables, peptone (x_1) and lactose (x_4) were further optimized using a central composite design. The ranges of the variables are 30-40 g l^{-1} for peptone, and 34-43 g l^{-1} for lactose. The experimental design and the results are presented in Table 3.4. The experimental results of the *CCD* were fitted with a third-order polynomial function for estimation of biomass concentration:

$$Yb_{Ll} = 2.55 + 0.46x_1 + 0.15x_4 + 0.15x_1x_4 - 0.15x_1^2 + 0.11x_4^2 - 0.26x_1^3 - 0.11x_4^3 \quad \text{Equation 3.4}$$

The model adequacy was checked and it was found to be adequate, the goodness of fit of the model was expressed by the coefficient of determination R^2, which was calculated to be 0.75, indicating that 75% of the variability in the response could be explained by the model. The *P*-value obtained for the significant variables was 0.0870. This proves that the model equation, as expressed in Equation 3.4, provides a suitable model to describe the response of the experiment pertaining to cell growth.

Table 3.4 Experimental design and results of the central composite design (*CCD*).

MRS medium optimization				
Run	**x1** [a]	**x4** [a]	**Biomass concentration (g/l)**	
			Observed [b]	**Expected** [c]
1	0	0	2.463	2.550
2	1.414	0	2.069	2.170
3	0	1.414	2.564	2.670
4	+1	+1	3.016	2.910
5	-1	+1	2.305	2.200
6	0	0	2.469	2.550
7	+1	-1	2.630	2.520
8	0	-1.414	2.778	2.880
9	-1.414	0	2.232	2.340
10	0	0	2.832	2.550
11	0	0	2.499	2.550
12	-1	-1	2.536	2.430
13	0	0	2.501	2.550
M17 medium optimization				
Run	**z4** [a]	**z6** [a]	**Biomass concentration (g/l)**	
			Observed [b]	**Expected** [c]
1	0	0	6.138	6.10
2	0	0	5.960	6.10
3	1.414	0	5.862	5.64
4	+1	+1	6.201	6.42
5	0	-1.414	6.290	6.07
6	0	0	6.184	6.10
7	0	0	6.132	6.10
8	0	1.414	6.042	5.82
9	-1	+1	5.711	5.93
10	0	0	6.100	6.10
11	-1	-1	5.613	5.84
12	+1	-1	5.305	5.53
13	-1.414	0	6.422	6.20

[a] The coded variables x_i (i = 1 or i = 4) and z_j (j = 4 or j = 6) are defined in Table 3.1.

[b] Observed biomass concentration stands for the experimental data.

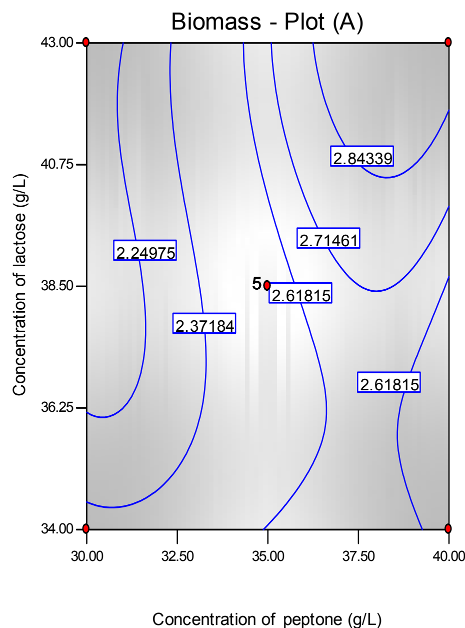
[c] Expected biomass concentration is calculated from the third-order model approach (Equation 3.4 and 3.5).

Figure 3.4 A shows the surface response plot of the model equation. From equations derived by differentiation of Equation 3.4, we can obtain the maximum point of the model, which was 38.6 g l^{-1} of peptone and 43.0 g l^{-1} lactose. The model predicted a maximum response for biomass concentration of 2.9722 g l^{-1} for this point. In order to confirm the predicted results of the model, experiments using the medium representing this maximum point were performed and a value of 3.213 g l^{-1} (triplicate experiments were carried out and correspond within 15%) was obtained. Thus, the optimum medium composition for growing *L. lactis* 53 consists of: 38.6 g l^{-1} peptone, 43.0 g l^{-1} lactose, 10 g l^{-1} meat extract, 5 g l^{-1} yeast extract, 1.08 g l^{-1} Tween®80, 2 g l^{-1} KH_2PO_4, 2 g l^{-1} CH_3COONa, 2 g l^{-1} ammonium citrate, 0.2 g l^{-1} $MgSO_4.7H_2O$ and 0.05 g l^{-1} $MnSO_4.4H_2O$.

For the M17 medium optimization the procedure adopted was similar to the one described above, thus the levels of the two significant variables, lactose (z_4) and sodium glycerophosphate (z_6) were further optimized using a central composite design. The ranges of the variables are 3.2-6.6 g l^{-1} for lactose, and 22.8-26.6 g l^{-1} for sodium glycerophosphate. The experimental design and the results are also presented in Table 3.4. Also for the M17 medium optimization a third-order polynomial function was fitted to the *CCD* data for estimation of biomass concentration:

$$Yb_{St} = 6.10 + 0.29z_4 + 0.58z_6 + 0.20z_4z_6 - 0.09z_4^2 - 0.08z_6^2 - 0.24z_4^3 - 0.34z_6^3 \quad \text{Equation 3.5}$$

The model adequacy was checked and it was found to be adequate, the goodness of the fit was expressed by the coefficient of determination R^2, which was 0.62, indicating that 62% of variability in the response was explained by the model. Figure 3.4 B shows the surface response plot of the model equation. The *P*-value obtained for the significant variables was 0.1338. Differentiation of Equation 3.5 allowed the determination of the maximum point of the model, which was 5.7 g l^{-1} of lactose and 26.4 g l^{-1} sodium glycerophosphate. The model predicted a maximum response for biomass concentration of 6.4983 g l^{-1} for this point. The validation of the model was performed using the medium representing this maximum point and a value of 6.184 g l^{-1} (triplicate experiments were carried out and correspond within 15%) was obtained. Thus, the optimum medium composition for growing *S. thermophilus* A consists of: 5.0 g l^{-1} peptone, 5.7 g l^{-1} lactose, 5.0 g l^{-1} meat extract, 2.5 g l^{-1} yeast extract, 5.0 g l^{-1} soya peptone, 26.4 g l^{-1} sodium glycerophosphate, 0.5 g l^{-1} ascorbic acid and 0.25 g l^{-1} $MgSO_4.7H_2O$.



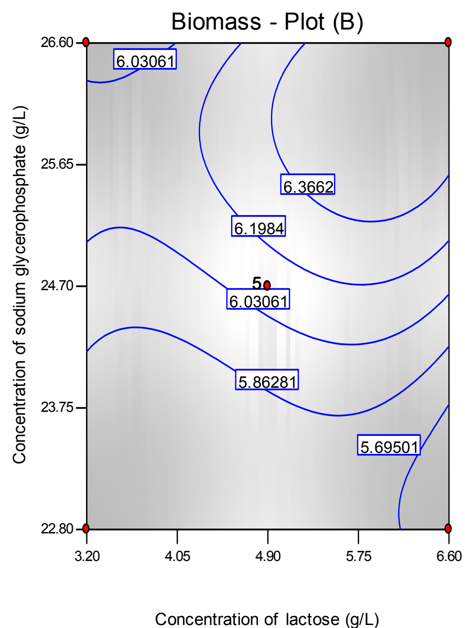
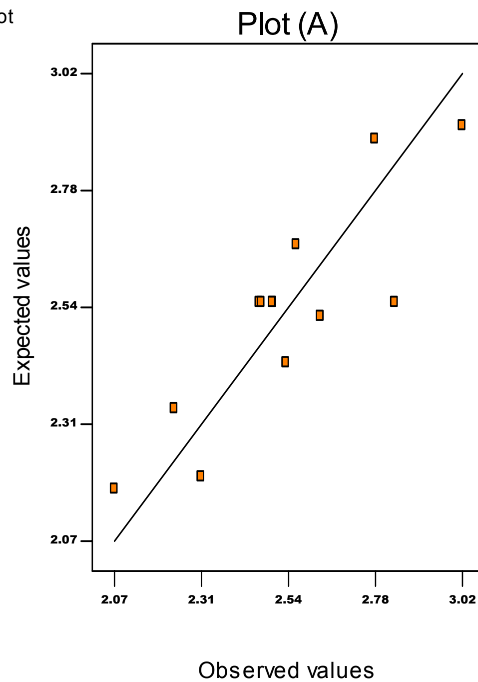


Figure 3.4 Response surface contour plots of biomass concentration (g l^{-1}) for *L. lactis* 53 and *S. thermophilus* A. The biomass concentration is the response variable of interest. The contour plots represent the effect of the significant variables and their interaction in the response variable. All the other variables non significant are held at zero level of the central composite design (*CCD*). **A)** The effect of peptone, lactose and their mutual interaction on biomass concentration for *L. lactis* 53. **B)** The effect of lactose, sodium glycerophosphate and their mutual interaction on biomass concentration for *S. thermophilus* A.



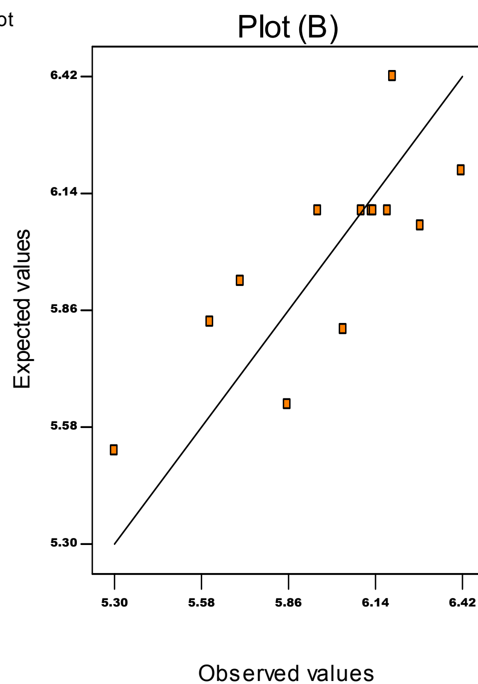


Figure 3.5 Plots of observed *versus* expected biomass concentration (g l^{-1}) values for *L. lactis* 53 **(A)** and *S. thermophilus* A **(B)**. The biomass concentration is the response variable of interest. The expected biomass concentration values are determined by the model equations determined for central composite design (*CCD*).

Figure 3.5 represents the relationship between the observed biomass concentration values and the expected values determined by the model Equation 3.4 and 3.5 for *L. lactis* 53 and *S. thermophilus* A, respectively. It can be observed that most points are nearby the line adjustment which means that the values determined experimentally are similar to those determined by the model.

3.3.5 Biosurfactants Mass Recovery and Surface-activity

After the optimization procedure the evaluation of fermentation for both probiotic strains was performed (Fig. 3.1 B and 3.2 B). Comparing results before and after the optimization procedure for *L. lactis* 53 (Fig. 3.1 A and 3.1 B), it can be observed for the same fermentation time, a higher biomass concentration and surface-activity of the biosurfactant. The optimization procedure allowed an increase of 1.6 times in the mass recovery of biosurfactant produced (mg per gram cell dry weight). Also for *S. thermophilus* A (Fig. 3.2 A and 3.2 B), with the optimization procedure a higher biomass concentration and surface-activity of the biosurfactant was achieved. The mass of biosurfactant produced (mg per gram cell dry weight) increased 2.1 times. For both bacterial strains a stronger decrease in the surface tension along the fermentation before the optimization procedure was observed.

3.4 Discussion

Biosurfactants produced by the probiotic bacteria *L. lactis* 53 and *S. thermophilus* A were found to be growth-associated, the biosurfactant yield of production was increased using a response surface optimization of medium composition for cell growth. Growth-associated biosurfactant production has been described for the production of biodispersan by *Acinetobacter calcoaceticus* (Desai and Desai 1993). In addition, biosurfactant production may occur, or be stimulated, by growing the microbial cells under growth-limiting conditions. *Pseudomonas aeruginosa* shows an overproduction of rhamnolipid when the culture reaches the stationary growth phase due to limitation of the nitrogen source (Desai and Desai 1993). Velraeds *et al.* (1996) showed that biosurfactant release by lactobacilli is maximum for cell in the stationary phase, thus a growth-associated biosurfactant production. Hence, our present observation that cell-bound biosurfactant production by *L. lactis* 53 and *S. thermophilus* A is maximal for stationary phase cells is in accordance with the general notion on this point in the literature. Moreover, a direct relation exists between biosurfactant production (shown by a decrease in the surface tension) and cell growth along the fermentation process, thus the biosurfactants are both growth-associated.

This study focused on the optimization of the medium composition for cell growth, although process parameters also play an important role and could as well be improved. Optimization through factorial design and response surface analysis is a common practice in biotechnology and various research workers have applied this technique for the optimization of culture conditions (Li *et al*. 2002, Sen 1997, Vohra and Satyanarayana 2002, Chen 1996), such as pH, temperature, aeration (Harris *et al*. 1990) and feeding rates (Bazaraa and Hassan 1996). The approach used in this study allowed the determination of the media composition that give the highest biomass concentration for *L. lactis* 53 and *S. thermophilus* A. In both cases, suitable models were found to describe the response of the experiments pertaining to cell growth, as the values obtained experimentally are in accordance with the expected values determined by the models. The models were validated by comparing the observed and predicted values in the optimum point, and a deviation of about 5% was found. The optimization procedure allowed an increase in biomass concentration and surface-activity of the biosurfactant.

The low levels of biosurfactants produced have greatly hampered research on the role of biosurfactants. However, a number of attempts have been made to increase biosurfactant productivity by manipulating physiological conditions and medium composition. Recent developments in the area of optimization of fermentation conditions have resulted in a significant increase in production yields, making them more commercially attractive. These developments include for example, the use of a fed batch technique in which the yield of sophorolipids by *Torulopsis bombicola* increased from 0.37 g per g substrate in batch culture to 0.6 g per g substrate (Lee and Kim 1993). In the present study for both bacterial strains an increase about 2 times in the mass of produced cell-bound biosurfactant (mg) per gram cell dry weight was achieved. It is not surprising the increase in the cell-bound biosurfactant mass recovery with the optimization procedure, as it is a growth-associated biosurfactant production and the cell growth was improved. However, it is interesting to notice that the change in the carbon source (from glucose to lactose) induced the cells to produce more biosurfactant. Lactic acid bacteria ferment sugars via different pathways and are also capable of forming other products, e.g. flavours such as diacetyl and acetoin, bacteriocins or biosurfactants. The different carbon sources give varying amounts of by-products (Hofvendahl and Hahn-Hägerdal 2000). Hence, it can be speculated that the use of lactose as carbon source instead of glucose induced the cells to use another metabolic pathway, and therefore the amount of mass of cell-bound biosurfactant produced mg per gram cell dry weight varied. Lactic acid bacteria have already proven to be ideal hosts for metabolic engineering. The efficacy of metabolic engineering of lactic acid bacteria for the increased

production of biosynthetic metabolites is yet to be demonstrated, but based on the results gathered in this study it seems to be an interesting approach for developing new strategies of biosurfactant production. Moreover, since both bacterial strains shown higher amounts of cell-bound biosurfactant produced with the optimized medium, this study constitutes a step in developing strategies to produce biosurfactants from cheese whey by *L. lactis* 53 and *S. thermophilus* A. Whey is a waste product from cheese production normally used as animal feed, which contains proteins, salts and lactose. For instance, sophorolipids production using whey was reported by Otto and his co-workers (1999).

3.5 Conclusions

In conclusion, using the method of experimental factorial design and response surface analysis, it was possible to determine optimal operating conditions to obtain a higher cellular growth, thus a higher cell-bound biosurfactant production yield. The validity of the model was proven by fitting the values of the variables in the model equation and by actually carrying out the experiment at those values of the variables.

3.6 References

Bazaraa, W.A. and Hassan, E.E. (1996) Response surface optimization for the continuous glucose isomerization process. *Journal of Industrial Microbiology*. 17:100-103.

Busscher, H.J., Noordmans, J., Meinders, J.M. and Van der Mei, H.C. (1991) Analysis of spatial arrangement of microorganisms adhering to solid surfaces – methods of presenting results. *Biofouling*. 4:71-80.

Chen, H.C. (1996) Optimizing the concentrations of carbon, nitrogen and phosphorous in a citric acid fermentation with response surface method. *Food Biotechnology*. 10:13-27.

Cooper, D.G. and Zajic, J.E. (1980) Surface-active compounds from microorganisms. *Advances in Applied Microbiology*. 26:229-253.

De Man, J.C., Rogosa and M., Sharpe, E. (1960) A medium for cultivation of Lactobacilli. *Journal of Applied Bacteriology*. 23:130-135.

Desai, J. and Banat, I.M. (1997) Microbial production of surfactants and their commercial potential. *Microbiology and Molecular Biology Reviews*. 61:47-64.

Desai, J. and Desai, A. (1993) Production of biosurfactants. In: *Biosurfactants: Production, Properties and Applications*. Ed. Kosaric, N. New York: Marcel Dekker. Pp 65-97.

Harris, P.L., Cuppet, S.L. and Bullerman, L.B. (1990) Optimization of lipase synthesis by *Pseudomonas fluorescens* by response surface methodology. *Journal of Food Protection*. 53:481-483.

Hofvendahl, K. and Hahn-Hägerdal, B. (2000) Factors affecting the fermentative lactic acid production from renewable resources. *Enzyme and Microbial Technology*. 26:87-107.

Lee, L.H. and Kim, J.H. (1993) Distribution of substrate carbon in sophorose lipid production by *Torulopsis bombicola*. *Biotechnology Letters*. 15:263-266.

Li, C., Bai, J., Cai, Z. and Ouyang, F. (2002) Optimization of a cultural medium for bacteriocin production by *Lactococcus lactis* using a response surface methodology. *Journal of Biotechnology*. 93:27-34.

Montgomery, D.C. (1997) Response surface methods and other approaches to process optimization. In: *Design and analysis of experiments*. Ed. Montgomery, D.C. New York: John Wiley and Sons. Pp 427-510.

Otto, R.T., Daniel, H.J., Pekin, G. and Muller-Decker, K. (1999) Production of sophorolipids from whey II. - Product composition, surface active properties, cytotoxicity and stability against hydrolases by enzymatic treatment. *Applied Microbiology and Biotechnology*. 52:495-501.

Sen, R. (1997) Response surface optimization of the critical media components for the production of surfactin. *Journal of Chemical Technology & Biotechnology*. 68:263-270.

Terzaghi, B. and Sandine, W.E. (1975) Improved medium for lactic streptococci and their bacteriophages. *Applied Microbiology*. 29:807-813.

Van der Vegt, W., Van der Mei, H.C., Noordmans, J. and Busscher, H.J. (1991) Assessment of bacterial biosurfactant production through axisymmetric drop shape analysis by profile. *Applied Microbiology and Biotechnology*. 35:766-770.

Velraeds, M.M., Van der Mei, H.C., Reid, G. and Busscher, H.J. (1996) Physico-chemical and biochemical characterization of biosurfactants released by *Lactobacillus* strains. *Colloid Surface B Biointerfaces*. 8:51-61.

Vohra, A. and Satyanarayana, T. (2002) Statistical optimization of the medium components by response surface methodology to enhance phytase production by *Pichia anomala*. *Process Biochemistry*. 37:999-1004.

CHAPTER 4

FERMENTATIVE BIOSURFACTANT PRODUCTION BY *LACTOBACILLUS* STRAINS - KINETIC STUDY

"Dissatisfaction is the first step for the progress of a man…".

Oscar Wilde.

In this section, screening of biosurfactant-producing ability of four *Lactobacillus* strains is evaluated, being shown that, for all the tested strains, biosurfactant production occurs mainly in the first 4 hours. The minimum surface tension value of the fermentation broth achieved was 39.5 mN m^{-1} for *Lactobacillus pentosus* CECT-4023. Time courses of glucose or lactose, biomass and biosurfactant were modelled according to reported models. The results obtained for *L. pentosus* CECT-4023 showed that this is a strong biosurfactant producer strain and that cheese whey can be used as an alternative medium for biosurfactant production.

4.1 NOMENCLATURE
4.2 INTRODUCTION
4.3 MATERIALS AND METHODS
4.3.1 Strains and Culture Conditions
4.3.2 Growth Curves
4.3.3 Blood Agar Screening
4.3.4 Biosurfactant Production
4.3.5 Analytical Methods
4.3.6 Surface-activity Assay
4.3.7 Glucose Consumption and Biosurfactant Production – Fitting of Data
4.4 RESULTS
4.4.1 Blood Agar Screening Method
4.4.2 Biosurfactant Production
4.4.3 Biosurfactant Extraction with PBS
4.4.4 Fermentation in MRS Broth
4.4.5 *L. pentosus* Fermentation in Whey
4.5 DISCUSSION
4.6 CONCLUSIONS
4.7 REFERENCES

4.1 NOMENCLATURE

F value	*F* test statistical parameter
MRS broth	Medium introduced by De Man, Rogosa and Sharpe for cultivation of *Lactobacillus* species, purchased from OXOID, Basingstoke, England
P	Biosurfactant concentration (g l^{-1})
P_0	Initial biosurfactant concentration (g l^{-1})
PBS	Phosphate-buffered saline (PBS: 10 mM KH_2PO_4/K_2HPO_4 and 150 mM NaCl with pH adjusted to 7.0)
P_{max}	Maximum biosurfactant concentration (g l^{-1})
P_r	Ratio between initial volumetric rate of biosurfactant formation (r_p) and initial biosurfactant concentration P_0 (h^{-1})
r^2	Correlation coefficient
r_p	Initial volumetric rate of biosurfactant production (g l^{-1} h^{-1})
S	Substrate (glucose or lactose) concentration (g l^{-1})
S_0	Initial substrate (glucose or lactose) concentration (g l^{-1})
X	Biomass concentration (g l^{-1})
X_0	Initial biomass concentration (g l^{-1})
X_{max}	Maximum biomass concentration (g l^{-1})
$Y_{P/S}$	Yield of biosurfactant production per substrate consumption (g g^{-1})
$Y_{X/S}$	Yield of biomass growth per substrate consumption (g g^{-1})
μ_{max}	Maximum specific growth rate (ratio between initial volumetric rate of biomass growth (r_p) and initial biomass concentration X_0) (h^{-1})

4.2 INTRODUCTION

Lactobacillus species are often together with *Streptococcus* being used as acid and flavour producers in the dairy industry (Hofvendahl and Hahn-Hägerdal 2000). In addition to their occurrence in plant material and food products, lactobacilli also inhabit the gastrointestinal tract of healthy mammals, and they are the most common members of indigenous microflora of the urogenital tract (Velraeds *et al.* 1996a). *Lactobacillus* and *Streptococcus* species have been shown to be able to displace adhering uropathogenic *Enterococcus faecalis* from hydrophobic and hydrophilic substrata in a parallel plate flow chamber, possibly through biosurfactant production (Velraeds *et al.* 1996b).

Biosurfactants are biological surface-active compounds released by microorganisms that can have some influence on interfaces. With regard to an anti-adhesive effect of biosurfactants, hypotheses have been forwarded in which adsorption of biosurfactants to a substratum surface alters the hydrophobicity of the surface and causes interference in microbial adhesion and desorption processes (Desai and Banat 1997). Biosurfactants have also been reported to have strong antibacterial, antifungal and antiviral activity (Singh and Cameotra 2004). Interest in biosurfactants has increased considerably in recent years, as they are potential candidates for many commercial applications in the petroleum, pharmaceuticals, biomedical and food processing industries (Desai and Banat 1997). Dairy *Streptococcus thermophilus* strains, for example, can produce biosurfactants that cause their own desorption (Busscher *et al.* 1994) and it was found that a biosurfactant obtained from *S. thermophilus* A showed a significant antimicrobial activity against several microorganisms that contribute to the premature failure of voice prostheses as can be seen in Chapter 6 (Rodrigues *et al.* 2004a). In another study, described in Chapter 5, the use of a biosurfactant from *Lactococcus lactis* 53 as antimicrobial and/or anti-adhesive agent and its ability to inhibit adhesion in a parallel plate flow chamber of various microorganisms isolated from explanted voice prostheses has been demonstrated (Rodrigues *et al.* 2004b).

Depending upon the nature of the biosurfactant and the producing microorganisms, several patterns of biosurfactant production by fermentation are possible. Velraeds *et al.* (1996b) showed that biosurfactant release by lactobacilli is maximum for cells in the stationary phase, and growth-associated biosurfactant production has been described for the release of biodispersan by *Acinetobacter calcoaceticus* (Desai and Desai 1993). In addition, biosurfactant production may occur, or be stimulated, by growing the microbial cells under growth-limiting conditions. *Pseudomonas aeruginosa* shows an overproduction of

rhamnolipid when the culture reaches the stationary growth phase due to limitation of the nitrogen source (Desai and Desai 1993).

The biosurfactant production by growing cells has been reported to be affected by environmental factors (Hofvendahl and Hahn-Hägerdal 2000), for example, the carbon source plays an important role. A good substrate for biosurfactant production is lactic whey, as it is composed of high levels of lactose (75% dry matter), 12-14% dry matter protein, organic acids and vitamins. Whey is a waste product from cheese production that represents a major pollution problem for countries depending on dairy economics and is normally used as animal feed. For instance, sophorolipids production using whey was reported by Otto and his co-workers (1999).

The aims of this study were to screen a number of *Lactobacillus* strains for biosurfactant production by blood agar method and surface tension determination, and to model the biosurfactant production as well as the time courses of sugar (glucose or lactose) consumption and biomass growth. The relation between cellular growth and surface-activity of the biosurfactant in time (as a measure of its production) was determined for all the strains.

4.3 MATERIALS AND METHODS

4.3.1 Strains and Culture Conditions

Several *Lactobacillus* strains were investigated in this study. The bacterial strains *Lactobacillus casei* CECT-5275, *Lactobacillus rhamnosus* CECT-288, *Lactobacillus pentosus* CECT-4023 and *Lactobacillus coryniformis* subsp *torquens* CECT-25600 obtained from the Spanish Collection of Type Cultures (Valencia, Spain) were stored at –20ºC in MRS broth (medium introduced by De Man, Rogosa and Sharpe for cultivation of *Lactobacillus* species, OXOID, Basingstoke, England) containing 15% (v/v) glycerol solution. From frozen stock, bacteria were streaked on MRS agar plates and incubated at the optimum temperature for each strain for further culturing.

4.3.2 Growth Curves

Growth curves for the *Lactobacillus* strains were determined because biosurfactant production may be influenced by the growth phase of the organisms (Desai and Desai 1993).

The bacterial strains were cultured in shake flasks without baffles with 100 ml MRS broth (OXOID, Basingstoke, England) and growth was measured by determining the optical density at 600 nm during different time intervals up to 72 h. The biomass concentrations (g dry weight l^{-1}) were determined using a calibration curve. The calibration curve was calculated for each strain using dilutions of a biomass suspension with known optical density. A fixed volume of the dilutions was filtered (0.22 µm) and dried at 105ºC for 24 h. All the filters were weighed before filtration and after drying. Thus, a relationship between biomass concentration (g l^{-1}) and optical density (600 nm) can be determined (Cbiomass (g l^{-1}) = (OD600nm $\times$ 0.506) + 0.036; r^2 = 0.9998).

4.3.3 Blood Agar Screening Method

The blood agar method is widely used to screen for biosurfactant production and several studies where this method was employed are reported in the literature (Moran *et al.* 2002, Johnson and Boese-Marrazzo 1980, Carrillo *et al.* 1996, Banat 1993). Briefly, each strain was streaked onto blood agar plates and incubated for 48 h at 37ºC or 31ºC. The plates were visually inspected for zones of clearing around colonies. The diameter of the clear zones depends on the concentration of the crude biosurfactant. The zones of clearing were scored as follows: '-', no haemolysis; '+', incomplete haemolysis; '++', complete haemolysis with a diameter of lysis < 1 cm; '+++', complete haemolysis with a diameter of lysis > 1 cm but < 3 cm; and '++++', complete haemolysis with a diameter of lysis > 3 cm and green colonies.

4.3.4 Biosurfactant Production

The bacterial strains were cultured in 100 ml MRS broth and grown for 72 h, at 31ºC for *L. pentosus* and 37ºC for all the other *Lactobacillus* strains. For extracellular biosurfactant determination, at different time intervals samples were taken to assay the surface activity of the media broth.

For cell-bound biosurfactant determination, at the end of the experiments (72 h) cells were harvested by centrifugation (10000 $\times$ g, 5 min, 10ºC), washed twice with demineralized water, and resuspended in 20 ml of phosphate-buffered saline (PBS: 10 mM KH_2PO_4/K_2HPO_4 and 150 mM NaCl with pH adjusted to 7.0) (Velraeds *et al.* 1996a). The bacteria were left at room temperature up to 24 h with gentle stirring for biosurfactant release. During extraction process, samples were taken at different time intervals, bacteria were removed by centrifugation and the remaining supernatant liquid was tested for surface activity.

For *L. pentosus* the biosurfactant production was also assayed growing the strain in whey at 31ºC. Commercial whey was prepared as follows: after adjusting the pH to 4.5 with 5N HCl, it was heated at 121ºC for 15 min to denature the proteins. The precipitates were removed by centrifugation at 4ºC and 8000 × g for 10 minutes. The supernatants were adjusted to pH 6.3, sterilized at 121ºC for 15 min and used as culture media. The supernatant contained 56 g l^{-1} of lactose.

4.3.5 Analytical Methods

Sugars were determined by high performance liquid chromatography (Agilent, model 1100, Palo Alto, CA) using ION-300 column (Transgenomic Inc., San Jose, CA) with refractive index detector. The mobile phase was 0.01 N H_2SO_4 with a flow rate of 0.4 ml min^{-1}.

4.3.6 Surface-activity Determination

The surface activity of biosurfactants produced by the bacterial strains was determined by measuring the surface tension of the samples with the Ring method (Kim *et al.* 2000).

The surface tension of the culture broth samples, of the PBS extraction samples and also of the whey fermentation experiment with *L. pentosus* was measured by the Ring method (Kim *et al.* 2000) using a KRUSS Tensiometer equipped with a 1.9 cm De Noüy platinum ring at room temperature. Tensiometers determine the surface tension with the help of an optimally wettable ring suspended from a precision balance. In the Ring method the liquid is raised until contact with the surface is registered. The sample is then lowered again so that the liquid film produced beneath the ring is stretched. As the film is stretched a maximum force is experienced, the force is measured and used to calculate the surface tension. To increase the accuracy an average of triplicates was used for this study.

The biosurfactant concentrations (g l^{-1}) were determined for each *Lactobacillus* strain using a calibration curve (Surface Tension (mN m^{-1}) = - 8.6465 Concentration (g l^{-1}) + 76.984, r^2 = 0.9729). The calibration curve was calculated for a commercial biosurfactant produced by several *Bacilli* (surfactin) using different concentrations of biosurfactant solution, below the critical micelle concentration, with known surface tension. In this biosurfactant concentration range the decrease of surface tension is linear and it is possible to establish a relationship between the biosurfactant concentration and the surface tension (Kim *et al.* 2000). Nevertheless, to estimate biosurfactant concentration it was necessary sometimes to dilute the culture broth under the critical micelle concentration.

4.3.7 Glucose Consumption and Biosurfactant production - *Fitting of Data*

Experimental data were fitted to proposed models using commercial software (Solver of Microsoft Excel 2002) by nonlinear regression using the least-squares method. A number of mathematical models have been reported in the literature to express the kinetics of lactic acid fermentation on glucose. These models represent the lactic acid fermentation process using different species of microorganisms. Although they have all been able to successfully simulate the corresponding process under the conditions applied, the simple logistic model proposed by Mercier *et al.* (1992) for lactic acid production was chosen to adjust the experimental data and adequately represent the concentrations of biomass, biosurfactant and substrate. Thus, biosurfactant production was mathematically modelled following the equation:

$$\frac{dP}{dt} = P_r P\left(1 - \frac{P}{P_{max}}\right) \qquad \text{Equation 4.1}$$

where t is time (h), P is biosurfactant concentration (g l^{-1}), P_{max} is maximum concentration of biosurfactant (g l^{-1}), and P_r is the ratio between the initial volumetric rate of product formation (r_p) and the initial product concentration P_0 (g l^{-1}). Equation 4.1 can be directly solved to give the Equation 4.2:

$$P = \frac{P_0 P_{max} e^{P_r t}}{P_{max} - P_0 + P_0 e^{P_r t}} \qquad \text{Equation 4.2}$$

From the series of experimental data biosurfactant concentration/time, the model parameters P_0, P_{max}, and P_r can be calculated for each *Lactobacillus* strain growing in MRS broth, and also for *L. pentosus* growing on whey.

Also biomass production was mathematically modelled and can be interpreted by Equation 4.3:

$$X = \frac{X_0 X_{\max} e^{\mu \max t}}{X_{\max} - X_0 + X_0 e^{\mu \max t}}$$

Equation 4.3

where t is time (h), X is biomass concentration (g l^{-1}), X_{max} is maximum concentration of biomass (g l^{-1}), and μ_{max} (h^{-1}) is the ratio between the initial volumetric rate of biomass formation and the initial biomass concentration X_0 (g l^{-1}). The model parameters X_0, X_{max}, and μ_{max} can be calculated from the series of experimental data biomass concentration/time.

Glucose consumption by the *Lactobacillus* strains can be interpreted by the Equation 4.4:

$$S = S_0 - \frac{1}{Y_{P/S}}(P - P_0) - \frac{1}{Y_{X/S}}(X - X_0)$$

Equation 4.4

where $Y_{P/S}$ (g g^{-1}) and $Y_{X/S}$ (g g^{-1}) are the product yield for biosurfactant and biomass respectively, P and P_0 are the final and initial biosurfactant concentrations (g l^{-1}), X and X_0 are the final and initial biomass concentrations (g l^{-1}), and finally S_0 is the initial glucose concentration (g l^{-1}). The model parameters $Y_{P/S}$, $Y_{X/S}$ and S_0 (g l^{-1}) were calculated for each *Lactobacillus* strain from the series of experimental data glucose concentration/time and the Equations 4.2 and 4.3.

The mathematical model proposed by Mercier *et al.* (1992) was chosen because it fairly describes biomass growth, substrate consumption and product accumulation kinetic pattern, and is reasonable to predict that this mathematical model will adjust the biosurfactant production results with statistical significance of the parameters determined.

4.4 RESULTS

4.4.1 Blood Agar Screening Method

All of the tested *Lactobacillus* strains showed zones of clearing in the blood agar with scores corresponding to complete haemolysis with a diameter < 1 cm (++), as can be seen in Figure 4.1.

Table 4.1 shows that all these strains allowed for a surface tension reduction between 8 and 10.5 mN m^{-1} when compared with the control MRS broth (50 mN m^{-1}) consistent with their ability to produce biosurfactants.

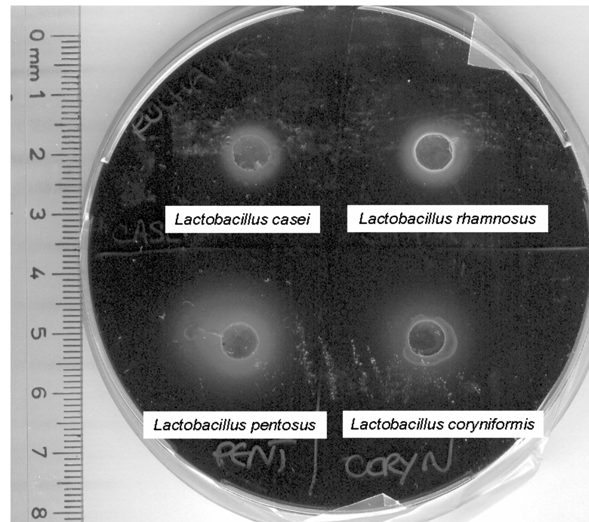


Figure 4.1 Blood agar screening method results for *L. casei* CECT-5275 (up-left spot), *L. rhamnosus* CECT-288 (up-right spot), *L. pentosus* CECT-4023 (down-left spot) and *L. coryniformis* subsp. *torquens* CECT-25600 (down-right spot).

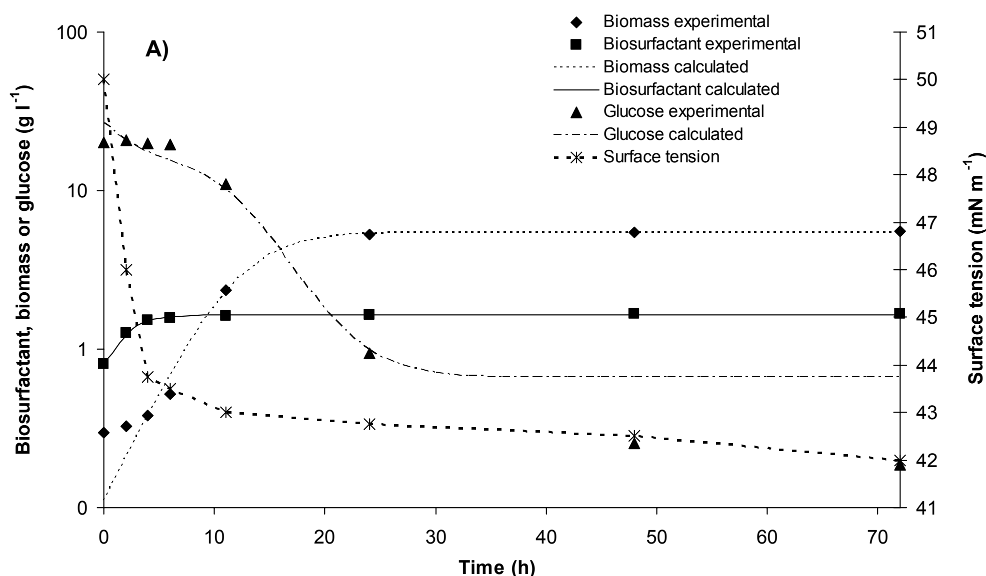
Table 4.1 Surface tension values (mN m^{-1}) obtained for the culture broth supernatants during the 72 h fermentation (the control surface tension (MRS) was 50 mN m^{-1}) and from the biosurfactant extraction with PBS during 24 h (the control surface tension (PBS) was 72 mN m^{-1}). Results are expressed as means ± standard deviations of values from triplicate experiments.

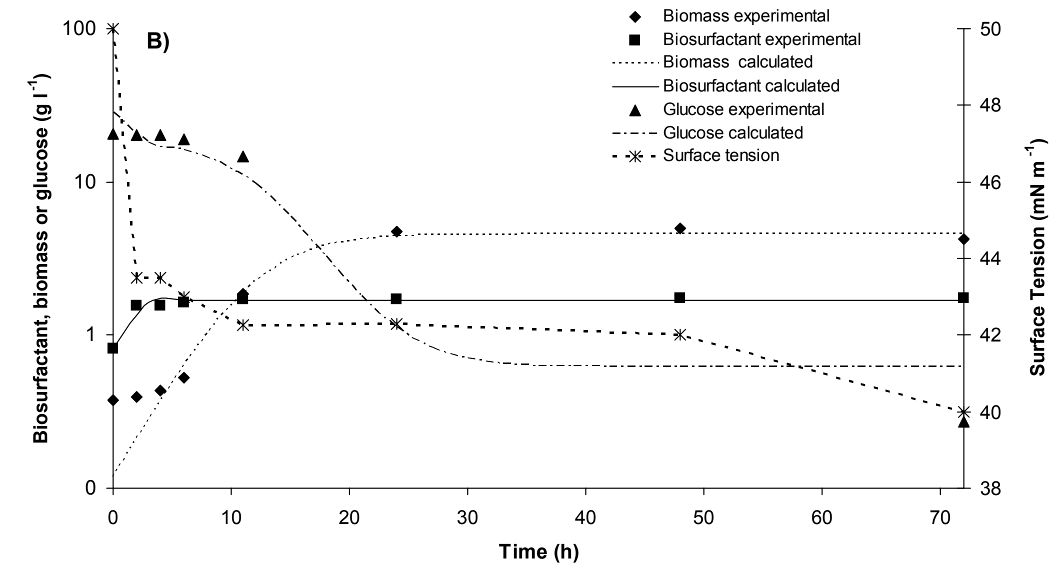
***Lactobacillus* strains**	**Surface tension of culture broth supernatants (mN m^{-1})**					
	0 h	**4 h**	**10 h**	**24 h**	**48 h**	**72 h**
L. casei	50.0 ± 0.0	44.0 ± 0.5	43.0 ± 0.1	43.0 ± 0.4	43.0 ± 0.3	42.0 ± 0.2
L. rhamnosus	50.0 ± 0.1	43.5 ± 1.0	42.3 ± 0.4	42.3 ± 0.1	42.0 ± 0.7	40.0 ± 0.9
L. pentosus	50.0 ± 0.0	45.0 ± 0.3	43.8 ± 0.8	43.3 ± 0.0	42.3 ± 0.1	39.5 ± 0.1
L. coryniformis	50.0 ± 0.1	43.0 ± 0.8	42.5 ± 0.2	41.5 ± 0.6	41.0 ± 0.0	40.5 ± 0.3

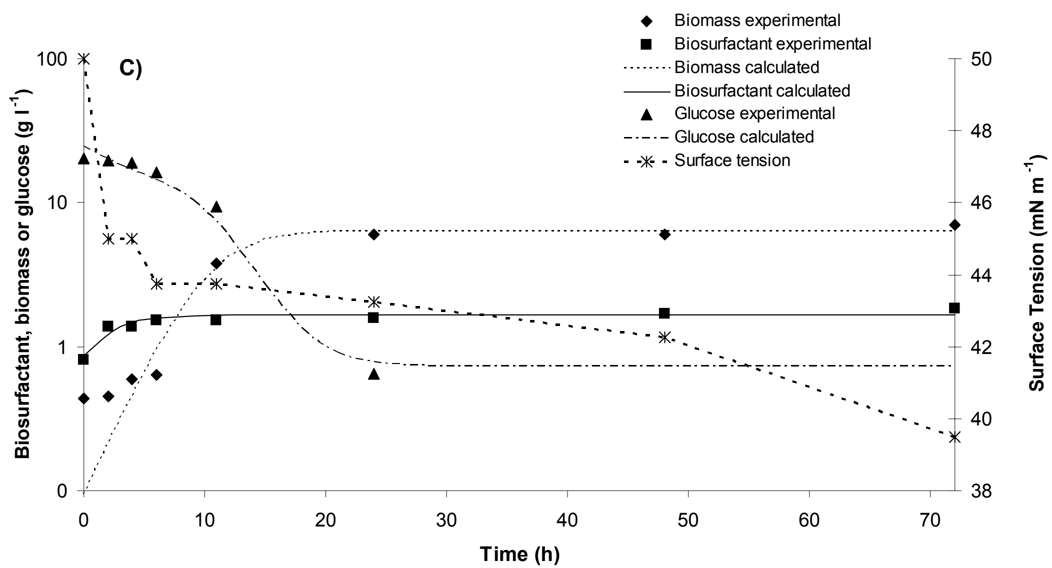
***Lactobacillus* strains**	**Surface tension of biosurfactant extracted with PBS (mN m^{-1})**			
	0 h	**0.5 h**	**8 h**	**24 h**
L. casei	70.0 ± 0.1	55.0 ± 0.2	53.0 ± 0.3	53.0 ± 0.1
L. rhamnosus	70.0 ± 0.2	56.0 ± 0.0	52.0 ± 0.1	51.5 ± 0.2
L. pentosus	70.0 ± 0.3	56.0 ± 0.1	51.0 ± 0.2	50.5 ± 0.1
L. coryniformis	72.0 ± 0.1	60.0 ± 0.4	55.0 ± 0.5	55.0 ± 0.1

4.4.2 Biosurfactant Production

Growth curves were obtained for the four *Lactobacillus* strains in order to establish the relation between cell growth and surface-activity of the biosurfactant in time as can be seen in Figure 4.2 (A,B, C and D) and Table 4.1. For all the strains the biosurfactant production occurs mainly in the first hours (4 h) where cell growth is almost inexistent and the substrate consumption is very low. However, the biosurfactant production continues during all 72 hours of fermentation but at a very slow production rate. As the decreases in the surface tension exceeded 8 mN m^{-1} (Busscher *et al.* 1994, Velraeds *et al.* 1996a), all four strains were found to produce biosurfactants. The surface tension decreases were compared with the surface tension of MRS broth (control) to correct for lower initial surface tension values as a result of the medium ingredients that can have surface-active characteristics themselves.




B)
Biomass experimental
Biosurfactant experimental
Biomass calculated
Biosurfactant calculated
Glucose experimental
Glucose calculated
Surface tension
Biosurfactant, biomass or glucose (g l-1)
Surface Tension (mN m-1)
Time (h)
100
10
1
0
50
48
46
44
42
40
38
0
10
20
30
40
50
60
70


C)
Biomass experimental
Biosurfactant experimental
Biomass calculated
Biosurfactant calculated
Glucose experimental
Glucose calculated
Surface tension
Biosurfactant, biomass or glucose (g l-1)
Surface Tension (mN m-1)
Time (h)
100
10
1
0
50
48
46
44
42
40
38
0
10
20
30
40
50
60
70

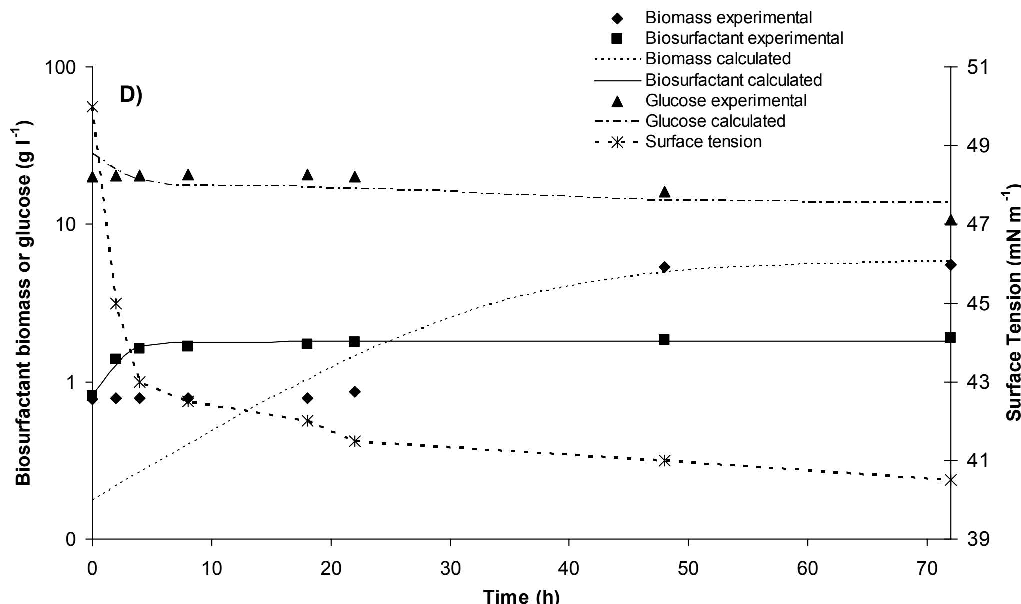


Figure 4.2 Representation of the surface tension variation (--*--), experimental data and calculated time courses of biomass (◆, -----), glucose (▲, — - —) and biosurfactant concentrations (■,——) during fermentations carried out with MRS broth using **(A)** *L. casei* CECT-5275, **(B)** *L. rhamnosus* CECT-288, **(C)** *L. pentosus* CECT-4023, **(D)** *L. coryniformis* subsp. *torquens* CECT-25600. Results represent the average of three independent experiments.

4.4.3 Biosurfactant Extraction with PBS - Fitting of Data

Reduction of surface tension during the PBS extraction of cells in stationary phase were fitted to proposed models using commercial software (Table Curve Windows v1.11). For all the *Lactobacillus* strains an exponential fit was possible according with the following equation:

$$y = a + b \times e^{(-\frac{x}{c})} \quad \text{Equation 4.5}$$

where y is surface tension (mN m^{-1}) and x is the extraction time (h). The equation parameters obtained were very similar for all the tested strains, with a = 52 ± 1.5; b = 18 ± 1.2; c = 0.4 ± 0.14 and r^2 = 0.991. Table 4.1 compiles the surface tension values decrease along the 24 h extraction procedure with PBS and it was found that all the *Lactobacillus* strains released cell-bound biosurfactants. These strains allowed a surface tension reduction between 17 and 21.5 mN m^{-1} when compared with the control PBS (72 mN m^{-1}) and the results were found to be statistically similar. Nevertheless, as the lowest surface tension value was achieved for *L. pentosus* this strain was considered the best biosurfactant producer.

4.4.4 Fermentation in MRS Broth

Fermentation runs were carried out using the fully supplemented medium MRS broth for all *Lactobacillus* strains. Figure 4.2 shows the experimental data as well as the predicted values calculated by Equations 4.2, 4.3 and 4.4 using the regression parameters listed in Table 4.2. All experiments show a kinetic pattern fairly described by the mathematical models with $r^2 >$ 0.891, 0.907 and 0.907 for glucose consumption, biomass and biosurfactant production, respectively. It can be noted that *L. coryniformis* presents the highest P_{max} (1.8 g of biosurfactant l^{-1}) followed by *L. rhamnosus*, *L. pentosus* and *L. casei*. Regarding the $Y_{P/S}$ all the *Lactobacillus* strains present the values between 0.08 and 0.09 g g^{-1}. With regard to the regression parameters listed in Table 4.2, the most remarkable finding was that the product yields calculated for all strains was roughly the same, which means that all the four strains showed a similar behaviour concerning biosurfactant production. The r_p/X values listed in Table 4.2 reflect the activity of the microorganisms concerning biosurfactant production. It can be seen that *L. rhamnosus* presents the highest r_p/X value (0.217 g l^{-1} h^{-1}) followed by *L. casei*, *L. coryniformis* and *L. pentosus*.

4.4.5 *L. pentosus* Fermentation in Whey

The lowest value of surface tension was achieved in the stationary phase (45 mN m^{-1}) and the reduction in the surface tension exceeded 8 mN m^{-1} (Busscher *et al.*1994, Velraeds *et al.* 1996a). The surface tension decreases were compared with the surface tension of whey broth control (54 mN m^{-1}).

Fermentation was carried out using whey as culture broth for *L. pentosus* strain because this was the strain that showed the best results concerning simultaneously cell growth and biosurfactant production. Nothing else besides whey was used for media preparation. Figure 4.3 shows the experimental data as well as the predicted values calculated by Equations 4.2, 4.3 and 4.4 using the regression parameters listed in Table 4.3. The experiment show a kinetic pattern reasonably described by the mathematical model with 0.959 and 0.990 for biomass and biosurfactant production, respectively. The r^2 value obtained for lactose consumption was not so good (r^2 = 0.698) and this could be explained by the fact that not all the lactose was consumed during cell growth. Moreover, the lactose consumption at certain time of fermentation becomes constant while biomass growth is still increasing maybe due to the fact that *L. pentosus* metabolizes other medium ingredients rather than lactose, thus very high residual sugar content was left at the end of fermentation. It was achieved P_{max} = 1.4 g of biosurfactant l^{-1}, P_r = 0.353 h^{-1} and r_p/X = 0.093 g l^{-1} h^{-1}. Regarding the $Y_{P/S}$ the value obtained was 0.09 g g^{-1} and X_{max} = 1.5 g l^{-1} with a μ_{max} of 0.05 h^{-1}.

Table 4.2 Results obtained by regression of biosurfactant, biomass and glucose concentration data in MRS broth fermentations [(a)].

Lactobacillus strains	Biosurfactant production						Biomass growth					Glucose consumption				
	P_0 (g l^{-1})	P_{max} (g l^{-1})	P_r (h^{-1})	r_p / X (g l^{-1} h^{-1})	r^2	*F* value	X_0 (g l^{-1})	X_{max} (g l^{-1})	μ_{max} (h^{-1})	r^2	*F* value	S_0 (g l^{-1})	$Y_{P/S}$ (g g^{-1})	$Y_{X/S}$ (g g^{-1})	r^2	*F* value
L. casei	0.8	1.6	0.612	0.091	0.993	286 [(f)]	0.11	5.5	0.324	0.998	858 [(f)]	26.7	0.08	0.34	0.955	43[(d)]
L. rhamnosus	0.8	1.7	1.215	0.217	0.969	63 [(d)]	0.12	4.6	0.299	0.984	145 [(e)]	28.4	0.09	0.25	0.939	31 [(d)]
L. pentosus	0.9	1.7	0.506	0.069	0.891	16 [(b)]	0.10	6.4	0.409	0.990	204 [(f)]	24.5	0.09	0.41	0.977	86 [(d)]
L. coryniformis	0.8	1.8	0.637	0.090	0.974	76 [(d)]	0.18	5.9	0.107	0.907	19 [(c)]	28.1	0.09	1.38	0.956	43 [(d)]

[(a)] Parameters defined in the Nomenclature Table; [(b)], significance level >90%; [(c)], significance level >95%; [(d)], significance level >97.5%; [(e)], significance level >99%; [(f)], significance level >99.5%.

Table 4.3 Results obtained by regression of biosurfactant, biomass and lactose concentration data for *L. pentosus* in whey broth fermentation [(a)].

Biosurfactant production						Biomass growth					Lactose consumption				
P_0 (g l^{-1})	P_{max} (g l^{-1})	P_r (h^{-1})	r_p / X (g l^{-1} h^{-1})	r^2	*F* value	X_0 (g l^{-1})	X_{max} (g l^{-1})	μ_{max} (h^{-1})	r^2	*F* value	S_0 (g l^{-1})	$Y_{P/S}$ (g g^{-1})	$Y_{X/S}$ (g g^{-1})	r^2	*F* value
0.4	1.4	0.353	0.093	0.990	195 [(d)]	0.2	1.5	0.05	0.959	46 [(c)]	55.6	0.09	3.1	0.698	5 [(b)]

[(a)] Parameters defined in the Nomenclature Table; [(b)], significance level >90%; [(c)], significance level >95%; [(d)], significance level >99%.

From Figure 4.3 it is possible to observe that this strain did not grow very well maybe because not all its nutritional requirements were fulfilled, although similar concentrations of biosurfactant were achieved if compared to those obtained with MRS medium. Comparing the kinetic parameters obtained with the two tested media, it was possible to notice that a lower μ_{max} (10% less than with synthetic medium) was obtained with whey medium, as well as a lower X_{max} (approximately one third of the value obtained with synthetic medium).

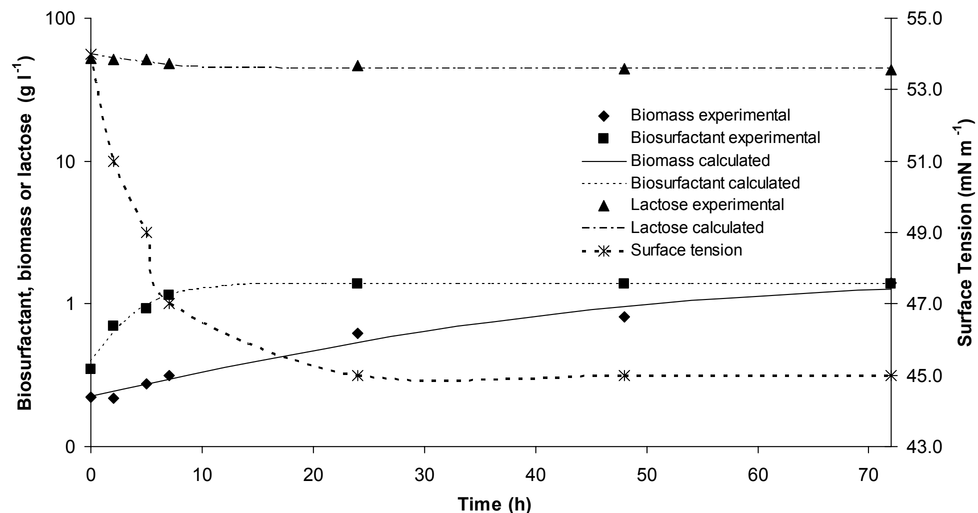


Figure 4.3 Representation of the surface tension variation (--*--), experimental data and calculated time courses of biomass (◆, -----), glucose (▲, — - —) and biosurfactant concentrations (■,——) during fermentations carried out with whey broth using *L. pentosus* CECT-4023. Results represent the average of three independent experiments.

4.5 DISCUSSION

The lactic acid bacteria *L. casei* CECT-5275, *L. rhamnosus* CECT-288, *L. pentosus* CECT-4023 and *L. coryniformis* subsp *torquens* CECT-25600 were found to be biosurfactant-producing strains. Depending upon the nature of the biosurfactant and the producing microorganisms, several patterns of biosurfactant production by fermentation are possible (Desai and Desai 1993). In this study, the biosurfactant production occurs mainly in the first hours (4 h) where cell growth is almost inexistent and the substrate consumption is very low. However, for all strains the biosurfactant production continues during all 72 hours of fermentation but at a very slow production rate. This slow production rate can be a consequence of product inhibition and pH reduction. The pH reduction results of simultaneous

production of lactic acid that changes drastically the media conditions and can be responsible for the biosurfactant production inhibition. The lowest values of surface tension were obtained in the end of fermentation, therefore, our present observation that biosurfactant release by the selected lactobacilli strains is maximal for cells in the stationary phase is in accordance with the general notion on this point in the literature (Velraeds et al. 1996a,b, Desai and Desai 1993). Four *Lactobacillus* strains were screened for biosurfactant production, by surface tension determination, and the biosurfactant production as well as the time courses of glucose consumption and biomass growth modelled. The approach used in this study allowed the determination of the fermentation parameters as well as a way to deduce the biosurfactant extraction with PBS for the four strains studied. From the PBS extraction results it was found that the best biosurfactant production strain tested was *L. pentosus* allowing a surface tension reduction of 21.5 mN m^{-1} when compared to the PBS control (72 mN m^{-1}). Comparing results obtained in Table 4.1 it is possible to conclude that with the accumulation of biosurfactant in the culture broth lower surface tensions are achieved and also for the extracellular biosurfactant results *L. pentosus* showed the higher reduction in the surface tension. The effectiveness of a surfactant is determined by its ability to lower the surface tension. For example, a good surfactant can lower the surface tension of water (air-water interface) from 72 mN m^{-1} to 35 mN m^{-1} (Mulligan and Gibbs 1993). In accordance with literature the biosurfactants produced by *P. aeruginosa* and *Bacillus subtilis* lower the surface tension of water 29 mN m^{-1} and 27 mN m^{-1}, respectively (Mulligan and Gibbs 1993).

For all four *Lactobacillus* strains, suitable models were found to describe the response of the experiments pertaining to glucose consumption, cell growth and biosurfactant production. The models were validated by comparing the observed and predicted values, and a deviation of about 5% was found. The modelling procedure allowed a better characterization of the biosurfactant production by the determination of the fermentation parameters and a reasonable fitting with a significance level over 90% was observed. Additionally, the blood agar method was included in this study since it is widely used to screen for biosurfactant production, and in some cases, it is the sole method used (Banat 1993). None of the studies reported in the literature (Johnson *et al.* 1980, Banat 1993, Carrillo *et al.* 1996, Moran *et al.* 2002) mention the possibility of biosurfactant production without a haemolytic activity. However, despite *Lactobacillus* species are not known to produce haemolysin, Andreu and co-workers (1995) demonstrated that some species agglutinate blood cells. Furthermore, the biosurfactants obtained in this study were not purified, thus consist of a mixture of several compounds other than biosurfactants that may cause haemolysis. The haemolytic activity of biosurfactants was first discovered when Bernheimer and Avigad (1970) reported that the biosurfactant produced

by *B. subtilis*, surfactin, lysed red blood cells. Blood agar lysis has been used to quantify surfactin (Moran *et al.* 2002) and rhamnolipids (Johnson *et al.* 1980) and has been used to screen for biosurfactant production by new isolates (Banat 1993, Carrillo *et al.* 1996). Carrillo *et al.* (1996) found an association between haemolytic activity and surfactant production, and they recommended the use of blood agar lysis as a primary method to screen for biosurfactant activity. However, only 13.5% of the haemolytic strains lowered the surface tension of water below 40 mN m^{-1}. In addition, other microbial products such as virulence factors lyse blood agar and biosurfactants that are poorly diffusible may not lyse blood cells. Thus, as not all biosurfactants have a haemolytic activity and compounds other than biosurfactants may cause haemolysis it is not clear whether blood agar lysis should be used exclusively to screen for biosurfactant production and surface tension can then be used to confirm the results if required.

Velraeds *et al.* (1996b) screened 15 *Lactobacillus* isolates for biosurfactant production and found that *Lactobacillus acidophilus* RC14 and *Lactobacillus fermentum* B54 were strongly biosurfactant-producing strains. Moreover, they found that biosurfactant layers of several *Lactobacillus* strains inhibited the adhesion of uropathogenic *E. faecalis* 1131 strain to glass in a parallel plate flow chamber for 4 h, however the inhibition of the uropathogen by the several strains tested was not the same. This indicates that there are different aspects of adherence on the part of the pathogen and that it should not be expected that the products of different *Lactobacillus* strains would produce equivalent results for any given pathogen. Other biomedical applications of the biosurfactants were found, namely the use of biosurfactants obtained from *L. lactis* 53 and from *S. thermophilus* A to prevent the microbial colonization of silicone rubber voice prostheses (Chapter 5 and 6). In this study, as the decreases in the surface tension exceeded 8 mN m^{-1}, all four strains were found to produce biosurfactants after reaching the stationary growth phase and from all the above can be used for further investigation. The reference surface tension value (8 mN m^{-1}) was previously described in the literature (Busscher *et al.* 1994), and although it must be emphasized that the criterion of 8 mN m^{-1} is of course somewhat arbitrarily chosen and must not be considered as strict as suggested here, it represents twice the variation in surface tension of a suspension with a non-producing strain and is therefore "on the safe side".

Finally, *L. pentosus* was assayed for biosurfactant production using whey as the culture medium. Comparing with the results obtained for this strain growing in MRS it was possible to see that *L. pentosus* did not grow well on whey medium probably due to some lack of nutrients, and although similar biosurfactant concentrations were achieved these results could be further improved. The lack of fit observed for the lactose consumption is probably caused by the first

experimental data point that is the one that is worst fitted by the model. However, the model has to be seen as a balance between biomass growth, lactose consumption and biosurfactant production, thus the model was considered adequate.

4.6 CONCLUSIONS

Concluding, a model could be established to follow the biosurfactant production at any fermentation time for all the tested strains with a significance level over 90%. The results obtained for *L. pentosus* CECT-4023 showed that this is a strong biosurfactant producer strain and that whey can be used as an alternative medium for biosurfactant production.

4.7 REFERENCES

Andreu, A., Stapleton, A.E., Fennell, C.L., Hillier, S.L. and Stamm, W.E. (1995) Hemmagglutination, adherence, and surface properties of vaginal *Lactobacillus* species. *Journal of infectious Diseases*. 171: 1237-1243.

Banat, I.M. (1993) The isolation of a thermophilic biosurfactant-producing *Bacillus* species. *Biotechnology Letters*. 15: 591-594.

Bernheimer, A. and Avigad, L. (1970) Nature and properties of a cytolytic agent produced by *Bacillus subtilis*. *Journal of General Applied Microbiology*. 61: 361-369.

Busscher, H.J., Neu, T. and Van der Mei, H.C. (1994) Biosurfactant production by thermophilic dairy streptococci. *Applied Microbiology and Biotechnology*. 41: 4-7.

Carrillo, P., Mardaraz, C., Pitta-Alvarez, S. and Giulietti, A. (1996) Isolation and selection of biosurfactant-producing bacteria. *World Journal of Microbial Biotechnology.* 12: 82-84.

Desai, J. and Banat, I.M. (1997) Microbial production of surfactants and their commercial potential. *Microbiology and Molecular Biology Reviews*. 61: 47-64.

Desai, J. and Desai, A. (1993) Production of biosurfactants. In: *Biosurfactants: Production, Properties and Applications*. Ed. Kosaric, N. New York: Marcel Dekker. Pp 65-97.

Hofvendahl, K. and Hahn-Hägerdal, B. (2000) Factors affecting the fermentative lactic acid production from renewable resources. *Enzyme and Microbial Techonology*. 26: 87-107.

Johnson, M. and Boese-Marrazzo, D. (1980) Production and properties of heat stable extracellular haemolysin from *Pseudomonas aeruginosa*. *Infectious Immunology*. 29: 1028-1033.

Kim, S., Lim, E., Lee, S., Lee, J. and Lee, T. (2000) Purification and characterization of biosurfactants from *Nocardia* sp. L-417. *Biotechnology and Applied Biochemistry.* 31: 249-253.

Mercier, P., Yerushalmi, L., Rouleau, D. and Dochain, D. (1992) Kinetics of lactic acid fermentation on glucose and corn by *Lactobacillus amylophilus*. *Journal of Chemical Technology and Biotechnology*. 55: 111-121.

Moran, A., Alejandra Martinez, M. and Sineriz, F. (2002) Quantification of surfactin in culture supernatant by haemolytic activity. *Biotechnology Letters*. 24: 177-180.

Mulligan, C. and Gibbs, B. (1993) Factors influencing the economics of biosurfactants. In: *Biosurfactants: Production, Properties and Applications*. Ed. Kosaric, N. New York: Marcel Dekker. Pp. 329-371.

Otto, R.T., Daniel, H.J., Pekin, G. and Muller-Decker, K. (1999) Production of sophorolipids from whey II. - Product composition, surface active properties, cytotoxicity and stability against hydrolases by enzymatic treatment. *Applied Microbiology and Biotechnology*. 52: 495-501.

Rodrigues, L.R., Van der Mei, H.C., Teixeira, J. and Oliveira, R. (2004a) Influence of biosurfactants from probiotic bacteria on formation of biofilms on voice prostheses. *Applied and Environmental Microbiology.* 70: 4408-4410.

Rodrigues, L.R., Van der Mei, H.C., Teixeira, J. and Oliveira, R. (2004b) Biosurfactant from *Lactococcus lactis* 53 inhibits microbial adhesion on silicone rubber. *Applied Microbiology and Biotechnology.* 66: 306-311.

Singh, P. and Cameotra, S. (2004) Potential applications of microbial surfactants in biomedical sciences. *Trends in Biotechnology*. 22: 142-146.

Velraeds, M.M., Van der Mei, H.C., Reid, G. and Busscher, H.J. (1996a) Physico-chemical and biochemical characterization of biosurfactants released by *Lactobacillus* strains. *Colloid Surface B Biointerfaces*. 8:51-61.

Velraeds, M.M., Van der Mei, H.C., Reid, G. and Busscher, H.J. (1996b) Inhibition of initial adhesion of uropathogenic *Enterococcus faecalis* by biosurfactants from *Lactobacillus* isolates. *Applied and Environmental Microbiology.* 62:1958-1963.

CHAPTER 5

BIOSURFACTANT FROM *LACTOCOCCUS LACTIS* 53 INHIBIT MICROBIAL ADHESION ON SILICONE RUBBER

"A handful of patience is worth more than a bushel of brains".

Dutch Proverb.

The ability of the biosurfactant obtained from the probiotic bacterium, *Lactococcus lactis* 53, to inhibit adhesion of four bacterial and two yeast strains isolated from explanted voice prostheses to silicone rubber with and without an adsorbed biosurfactant layer in a parallel plate flow chamber was discussed in this chapter. The results obtained showed that the biosurfactant was effective in decreasing the initial deposition rates of *Staphylococcus epidermidis* GB 9/6, *Streptococcus salivarius* GB 24/9 and *Staphylococcus aureus* GB 2/1, allowing for a 90% reduction of the deposition rates. The deposition rates of *Rothia dentocariosa* GBJ 52/2B, *Candida albicans* GBJ 13/4A and *Candida tropicalis* GB 9/9 were far less reduced in the presence of the biosurfactant as compared with the other strains. This study constitutes a step ahead in developing strategies to prevent the microbial colonization of silicone rubber voice prostheses.

5.1 INTRODUCTION

5.2 MATERIALS AND METHODS

5.2.1 Biosurfactant Production

5.2.2 Microbial Strains and Growth Conditions

5.2.3 Contact Angle Measurements and Surface Free Energy Calculation

5.2.4 Parallel Plate Flow Chamber and Image Analysis

5.3 RESULTS

5.3.1 Microbial Cell Surface and Silicone Rubber Characterization

5.3.2 Microbial Adhesion

5.4 DISCUSSION

5.5 CONCLUSIONS

5.6 REFERENCES

5.1 INTRODUCTION

Silicone rubber is used for a wide variety of biomedical applications due to its good mechanical properties, combined with a hydrophobic surface. For instance, silicone rubber has been used for voice prostheses (Neu *et al.* 1993), urinary catheters (Farber and Wolff 1993) and contact lens materials (Holly 1983). Patients who have undergone a laryngectomy due to a malignant laryngeal tumour need to breath through a tracheostoma and receive voice prostheses for speech rehabilitation (Van der Mei *et al.* 1999). The major drawback of silicone rubber voice prostheses is the concurrent microbial colonization of the devices (Neu *et al.* 1993) that cause leakage of food and liquid, or blocking of the valve increasing the airflow resistance (Mahieu *et al.* 1986), thus forcing their replacement on average every 3-4 months. It has been suggested by laryngectomized patients that the consumption of dairy products may be influential on the lifetime of their prostheses (Van der Mei *et al.* 1999). There is anecdotal evidence among patients that the consumption of buttermilk, which contains antimycotic-releasing *Lactococcus lactis* spp., prolongs the lifetime of indwelling voice prostheses. This suggestion has been confirmed in an artificial throat model, in which the effects of daily buttermilk consumption on biofilm formation on silicone rubber voice prostheses have been simulated (Busscher *et al.* 1998).

The formation of an infectious biofilm on biomaterials appears to involve several sequential steps, and includes adsorption of host conditioning film onto the device and adhesion of the infectious microorganisms anchoring by exopolymer production, growth and spread of the organisms in large clumps separated by water channels (Bos *et al.* 1999, Busscher *et al.* 1996, Reid 1999). When microorganisms and biomaterials surfaces are in an aqueous environment, in which organic matter is present (e.g. sea water, milk, saliva, urine or blood), macromolecular components adsorb on biomaterial surfaces to form a conditioning film and may change the electrical properties and the hydrophobicity of the surface. If it was possible to weaken or break this link, for example under the influence of fluctuating shear forces or using surfactants (Velraeds *et al.* 1996), it could be feasible for the entire biofilm to detach, thereby aiding and abetting the eradication of infection.

Biosurfactants are surface-active compounds released by microorganisms that have some influence on interfaces, most notably on the surface tension of liquid-vapour interfaces. With regard to an anti-adhesive effect of biosurfactants, hypotheses have been forwarded in which adsorption of biosurfactants to a substratum surface alters the hydrophobicity of the surface

and causes interference in microbial adhesion and desorption processes (Desai and Banat 1997).

The aim of the present study was to compare the extent of adhesion of a large variety of different bacterial and yeast strains isolated from explanted voice prostheses to silicone rubber with and without an adsorbed biosurfactant layer. The tested biosurfactant was obtained from the probiotic bacterium *L. lactis* 53.

5.2 MATERIALS AND METHODS

5.2.1 Biosurfactant Production

The lactic acid strain *L. lactis* 53 was cultured in 600 ml optimized MRS broth and grown overnight (18 h) in ambient air. The growth medium used for the production of this biosurfactant was previously optimized to obtain higher yields of biosurfactant production as described in Chapter 3 (Rodrigues *et al.* 2003, 2005). Cells were harvested by centrifugation (10000 × g, 5 min, 10ºC), washed twice with demineralized water, and resuspended in 100 ml of phosphate-buffered saline (PBS: 10 mM KH_2PO_4/K_2HPO_4 and 150 mM NaCl with pH adjusted to 7.0). The bacteria were left at room temperature for 2 h with gentle stirring for biosurfactant release. Subsequently, the bacteria were removed by centrifugation and the remaining supernatant liquid was filtered through a 0.22 μm pore-size filter (Millipore). The supernatant was dialyzed against demineralized water at 4ºC in a Spectrapor membrane tube (molecular weight cut off 6000 to 8000, Spectrum Medical Industries Inc., CA) and freeze-dried, and the biosurfactant was used for further studies.

5.2.2 Microbial Strains and Growth Conditions

Four bacterial strains, *Staphylococcus epidermidis* GB 9/6, *Streptococcus salivarius* GB 24/9, *Staphylococcus aureus* GB 2/1, and *Rothia dentocariosa* GBJ 52/2B and two yeast strains: *Candida albicans* GBJ 13/4A and *Candida tropicalis* GB 9/9 isolated from explanted voice prostheses were used in this study. All strains were first grown overnight at 37°C in ambient air on an agar plate from a frozen stock, and this agar plate was kept at 4°C, never longer than two weeks. Several colonies were used to inoculate 10 ml of brain heart infusion broth (BHI, OXOID, Basingstoke, England) for all the bacterial and yeast strains in use. This pre-culture was incubated at 37°C in ambient air for 24 h and used to inoculate a second culture

of 200 ml that was grown for 18 h. The microorganisms from the second culture were harvested by centrifugation for 5 min at 10000 × g and washed twice with demineralized water. Subsequently, bacterial cells were suspended in 200 ml PBS solution, after sonication on ice (10 s), to a concentration of 3×10^8 bacteria ml^{-1}. The sonication procedure did not promote cell lysis. Yeasts were suspended in PBS to a concentration of 3×10^6 yeast ml^{-1}. A Bürker-Türk counting chamber was used to count the cells.

5.2.3 Contact Angle Measurements and Surface Free Energy Calculation

Advancing type contact angles with three different standard liquids (Millipore water, formamide and diiodomethane) on silicone rubber with and without an adsorbed biosurfactant layer were measured with a homemade contour monitor using the sessile drop technique. On each sample, at least five droplets were placed at different positions and results of three separately prepared surfaces with adsorbed biosurfactant were averaged. For the microorganisms the contact angles were measured on lawns of yeasts and bacteria. Briefly, microorganisms were layered onto 0.45 (bacteria) or 3 μm (yeasts) pore sized filters (Millipore) using negative pressure. The yeasts were layered from a suspension in PBS buffer and washed afterwards with demineralized water, while the bacteria were suspended in water. The filters were left to air-dry until so-called plateau contact angles could be measured (Van Oss and Gillman 1972). At least three different filters from separate cultures were used for each strain tested.

The acid-base nature of surfaces can be directly determined in terms of surface free energy components γ_{sv}^{LW} (LW, Lifshitz-Van der Waals) and γ_{sv}^{AB} (AB, acid-base) (Van Oss 1989, Van Oss *et al.* 1988, Volpe and Siboni 1997),

$$\gamma_{sv} = \gamma_{sv}^{LW} + \gamma_{sv}^{AB} \qquad \text{Equation 5.1}$$

in which the AB component equals

$$\gamma_{sv}^{AB} = 2\sqrt{\gamma_{sv}^{-} \times \gamma_{sv}^{+}} \qquad \text{Equation 5.2}$$

where γ_{sv}^{-} and γ_{sv}^{+} are the electron-donating and electron-accepting surface free energy parameters. Note that *s* stands for solid, *v* for vapour and *l* for liquid. Proper diagnostic liquids with known surface free energy components (γ_{sv}^{LW}, γ_{sv}^{AB}, γ_{sv}^{+} and γ_{sv}^{-}) should be selected and the most commonly applied are diiodomethane, water and formamide. Diiodomethane is apolar (γ_{lv}^{AB} = 0) and consequently its contact angle on a surface can be employed to calculate the apolar or Lifshitz-Van der Waals component of the surface free energy

$$\gamma_{sv}^{LW} = \left(\frac{\sqrt{\gamma_{lv}} \times (\cos\theta + 1)}{2} \right)^2$$ Equation 5.3

Water and formamide are both polar liquids and combined use of their contact angles on a surface yields γ_{sv}^{-} and γ_{sv}^{+} from the Young equation

$$\gamma_{lv} \times (\cos\theta + 1) - 2\sqrt{\gamma_{sv}^{LW}\gamma_{lv}^{LW}} = 2\sqrt{\gamma_{sv}^{-}\gamma_{lv}^{+}} + 2\sqrt{\gamma_{sv}^{+}\gamma_{lv}^{-}}$$ Equation 5.4

5.2.4 Parallel Plate Flow Chamber and Image Analysis

A parallel plate flow chamber (Sjollema *et al.* 1989) was used to study the deposition of the bacterial and yeast strains on silicone rubber with and without an adsorbed biosurfactant layer. The parallel plate flow chamber consists of a polymethylmethacrylate (PMMA) bottom plate with implant grade silicone rubber affixed to it with double sided sticky tape, and a glass top plate, both with dimensions 5.5 × 3.8 cm. The top and bottom plates were cleaned by sonicating for 3 min in a commercially available surfactant cleaning solution (2% RBS35 in water, Societé des Traitements Chimiques de Surface, Lambersat, France), rinsed thoroughly with tap water, and then rinsed with demineralized water. Top and bottom plates were subsequently mounted in the housing of the flow chamber, separated by 0.06 cm Delrin spacers. Images were taken from the silicone rubber with or without an adsorbed biosurfactant layer on the bottom plate. Deposition was observed with a CCD-MXRi camera (High Technology, Eindhoven, The Netherlands) mounted on a phase contrast microscope (Olympus BH-2) equipped with a 40 × ultra long working distance objective (Olympus ULWD-CD Plan 40 PL) for experiments with bacteria and with a 10 × objective for experiments with

yeasts. The camera was coupled to an image analyzer (TEA, Difa, Breda, The Netherlands). Each live image (512 × 512 pixels with 8 bit resolution) was obtained after summation of 15 consecutive images (time interval 1 s) in order to enhance the signal to noise ratio and to eliminate moving microorganisms from the analysis.

Silicone rubber was immersed overnight in a 5 g l^{-1} biosurfactant solution at 4ºC for the experiments done with conditioned silicone rubber. The concentration of biosurfactant used in the experiments was estimated by axisymmetric drop shape analysis by profile (ADSA-P) as the one that allowed a reasonable reduction in the surface tension (12 mN m^{-1}), which means that at this concentration the biosurfactant has a good surface activity and these results are presented in Figure 5.1 (Van der Vegt *et al.* 1991).

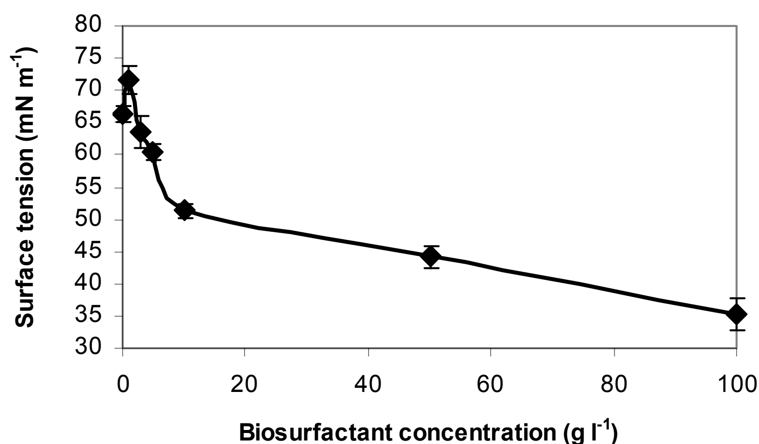


Figure 5.1 Surface tension of crude biosurfactant concentrations obtained from *L. lactis* 53 in PBS (pH 7.0) after 2 h as measured by ADSA-P. Results are averages of triplicate experiments varying within 2-7% (ANOVA) and the standard deviation represented by error bars.

Prior to each experiment, all tubes and the flow chamber were filled with PBS, while care was taken to remove air bubbles from the system. Flasks, containing microbial suspension and buffer, were positioned at the same height with respect to the chamber to ensure that immediately after the flows were started, all fluids would circulate by hydrostatic pressure through the chamber at the desired shear rate of 10 s^{-1} (0.025 ml s^{-1}), which yields a laminar flow (Reynolds number 0.6). The microbial suspension was circulated through the system for 4 h and images were obtained from silicone rubber with or without the adsorbed biosurfactant layer. The initial increase in the number of adhering microorganisms with time was expressed by a so-called initial deposition rate j_0 (microorganisms cm^{-2} s^{-1}), i.e. the number of adhering microorganisms per unit area and time. The number of adhering microorganisms after 4 h

was also determined (n_{4h}). All values presented in this work are the averages of experiments on three separate silicone rubber surfaces with or without the adsorbed biosurfactant layer, and were carried out with separately grown microorganisms.

5.3 RESULTS

5.3.1 Microbial Cell Surface and Silicone Rubber Characterization

Table 5.1 summarizes contact angles measured with water, formamide and diiodomethane on silicone rubber with and without adsorbed biosurfactant, as well as the contact angles measured on the microorganisms. The water contact angle on bare silicone rubber decreased from 109 degrees to 48 degrees after adsorption of the biosurfactant. A similar behavior was obtained with formamide. The diiodomethane contact angle decreased in a much lesser extent from 86 to 64 degrees.

The water contact angles obtained for all the microorganisms tested were between 24 and 35 degrees, except for *R. dentocariosa* GBJ 52/2B that was found to be 54 degrees, thus being the most hydrophobic bacterial strain studied. The formamide contact angle of all microorganisms is higher than their water contact angle, except for *R. dentocariosa* GBJ 52/2B. Diiodomethane contact angles were similar for all microorganisms being between 26 and 33 degrees, except for *R. dentocariosa* GBJ 52/2B that was 40 degrees.

The surface free energies calculated from the contact angles are also given in Table 5.1 and show that silicone rubber with the adsorbed biosurfactant layer is strongly electron-donating compared with the bare silicone rubber. The Lifshitz-Van der Waals component is higher for the conditioned silicone rubber compared with bare silicone rubber.

The Lifshitz-Van der Waals component of the surface free energies determined for all the microorganisms were similar and were found to be between 40 and 46 mN m^{-1}, being the lowest value obtained for *R. dentocariosa* GBJ 52/2B and the highest for *C. albicans* GBJ 13/4A. The major differences were observed in the electron-donating component, being *R. dentocariosa* GBJ 52/2B the microorganism that showed the lowest electron-donating component. The electron-accepting component was zero for all the microorganisms, except for *R. dentocariosa* GBJ 52/2B that was 1.0 mN m^{-1}.

Table 5.1 Contact angles (degrees) measured at 25ºC on silicone rubber with and without an adsorbed biosurfactant layer, and on microorganisms isolated from explanted voice prostheses, as determined by the sessile drop technique. The surface free energy (mN m^{-1}) component γ_{sv}^{LW}, electron-accepting γ_{sv}^{+} and electron-donating γ_{sv}^{-} parameters are presented. Standard deviations (±) were determined over three separate measurements.

Surface	Contact angles (degrees)			Parameter (mN m^{-1})		
	W*	F*	D*	γ_{sv}^{LW}	γ_{sv}^{+}	γ_{sv}^{-}
Silicone rubber	109 ± 2	101 ± 2	86 ± 1	14.5 ± 0.5	0.0 ± 0.0	3.3 ± 0.4
Silicone rubber + biosurfactant	48 ± 7	54 ± 4	64 ± 1	26.3 ± 0.6	0.4 ± 0.1	44.2 ± 6.9
Microorganism	**Contact angles (degrees)**			**Parameter (mN m^{-1})**		
	W*	F*	D*	γ_{sv}^{LW}	γ_{sv}^{+}	γ_{sv}^{-}
S. epidermidis GB 9/6	35 ± 2	42 ± 6	33 ± 2	42.9 ± 0.9	0.0 ± 0.0	53.0 ± 3.4
S. salivarius GB 24/9	30 ± 2	35 ± 3	30 ± 1	44.2 ± 0.4	0.0 ± 0.0	52.7 ± 0.2
S. aureus GB 2/1	24 ± 1	40 ± 5	29 ± 3	44.6 ± 1.2	0.0 ± 0.0	64.8 ±4.3
R. dentocariosa GBJ 52/2B	54 ± 4	37 ± 4	40 ± 1	39.6 ± 0.5	0.8 ± 0.2	22.3 ± 2.7
C. albicans GBJ 13/4A	25 ± 2	53 ± 5	26 ± 2	45.8 ± 0.8	0.0 ± 0.0	80.6 ± 5.0
C. tropicalis GB 9/9	31 ± 4	59 ± 5	29 ± 4	44.6 ± 1.7	0.0 ± 0.0	81.3 ± 1.9

* W – water; F – formamide; D - diiodomethane

5.3.2 Microbial Adhesion

The initial deposition rates (Figure 5.2) of *S. epidermidis* GB 9/6, *R. dentocariosa* GBJ 52/2B and *C. tropicalis* GB 9/9 on silicone rubber are relatively high, between 2000 and 2600 microorganisms cm^{-2} s^{-1}, while *S. salivarius* GB 24/9 and *S. aureus* GB 2/1 deposit slower onto silicone rubber (1200 - 1600 microorganisms cm^{-2} s^{-1}) and *C. albicans* GBJ 13/4A has by far the lowest initial deposition rate (170 microorganisms cm^{-2} s^{-1}). Adsorption of biosurfactant on the silicone rubber reduces the deposition rate about 90% for *S. epidermidis* GB 9/6, *S. salivarius* GB 24/9 and *S. aureus* GB 2/1. The deposition rates of *R. dentocariosa*

GBJ 52/2B, *C. albicans* GBJ 13/4A and *C. tropicalis* GB 9/9 are far less reduced in the presence of the biosurfactant than the deposition rates of the other strains.

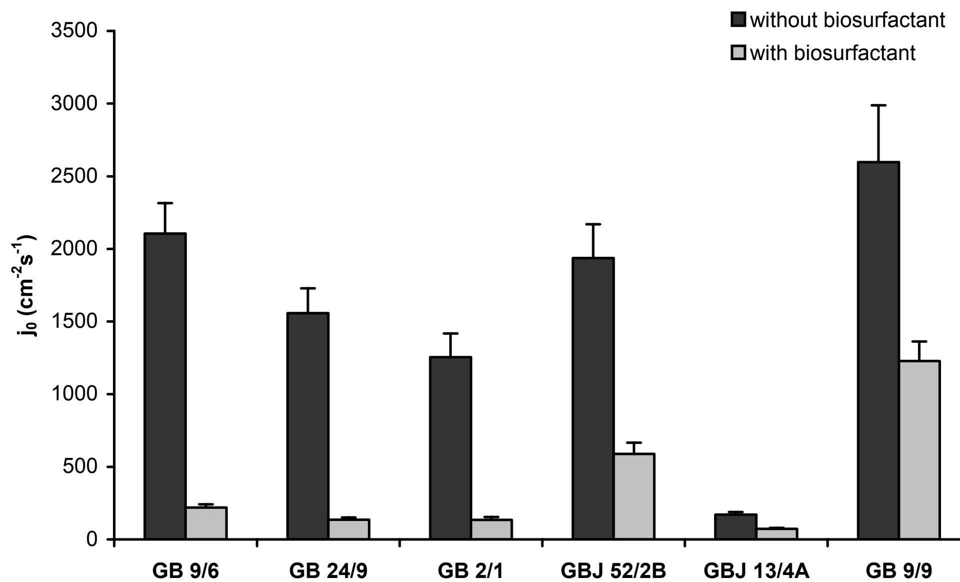


Figure 5.2 The initial deposition rates (j_0) of the bacterial strains(*Staphylococcus epidermidi*s GB 9/6, *Streptococcus salivarius* GB 24/9, *Staphylococcus aureus* GB 2/1,and *Rothia dentocariosa* GBJ 52/2B) and yeast (*Candida albicans* GBJ 13/4A and *Candida tropicalis* GB 9/9) isolated from explanted voice prostheses on silicone rubber with and without an adsorbed biosurfactant layer. Results are averages of triplicate experiments varying within 10-15% (ANOVA) and the standard deviation represented by error bars.

The numbers of microorganisms adhering to silicone rubber after 4 h, (n_{4h}, Figure 5.3) are between 15 $\times$ 10^6 and 7 $\times$ 10^6 cm^{-2} for all the microorganisms studied except for *C. albicans* GBJ 13/4A that exhibit the lowest number of adhering microorganisms (0.6 $\times$ 10^6 cm^{-2}). The number of cells adhering after 4 h on the silicone rubber conditioned with biosurfactant was reduced to 90% for *S. epidermidis* GB 9/6, *S. salivarius* GB 24/9 and *S. aureus* GB 2/1. The yeast strains *C. albicans* GBJ 13/4A and *C. tropicalis* GB 9/9, as well as the bacterial strain *R. dentocariosa* GBJ 52/2B showed a smaller decrease in the number of attached cells after 4 h, between 56% and 78%.

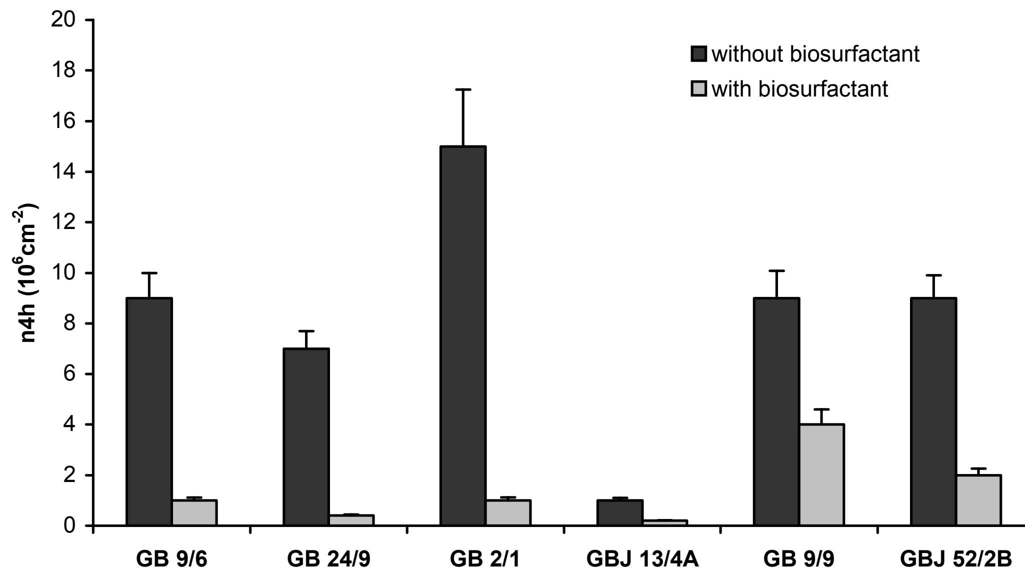


Figure 5.3 The number of microorganisms adhering after 4 h (n_{4h}) on silicone rubber with and without an adsorbed biosurfactant layer. The codification of the microorganisms is presented in Fig. 5.2. Results are averages of triplicate experiments varying within 10-15% (ANOVA) and the standard deviation represented by error bars.

5.4 DISCUSSION

In this chapter, biosurfactant obtained from the probiotic bacteria *L. lactis* 53, was studied for its ability to inhibit the adhesion of several microbial strains involved in the biofilm formation on voice prostheses. Continuous exposure to saliva, food, drinks and oropharyngeal microflora contributes to the rapid colonization by a mixed biofilm of bacteria and yeasts, leading to valve failure and frequent exchange of the implant (Neu *et al.* 1993). In this context, antifouling improvement of the silicone rubber material is desirable. In case of laryngectomized patients with voice prostheses lifetimes less than two months, there is need for employing “antibiofilm” therapy from the time of insertion of the voice prostheses, preferably without using antimycotics or antibiotics because of the risk of inducing resistant strains (Foley and Gilbert 1996, Mahieu *et al.* 1986, Van Weissenbruch *et al.* 1997). Therefore, research into the development of new methods for preventing or retarding biofilm

formation on voice prostheses is worthwhile. The use of biosurfactants as antimicrobial agents seems to be promising as a method of prolonging lifetimes of voice prostheses.

In this study, it has been demonstrated that bacterial adhesion in a parallel plate flow chamber to silicone rubber conditioned with a biosurfactant produced by *L. lactis* 53 is greatly decreased with respect to adhesion to bare silicone rubber. Similarly, adhesion of yeasts was also decreased by the presence of the biosurfactant, but not to the same extent as observed for bacteria. The mechanisms by which a biosurfactant discourages microbial adhesion are not fully elucidated, but the main physiological role of biosurfactants is to facilitate the uptake of water-immiscible substrates by lowering the surface tension at the phase boundary (Fiechter 1992, Hommel and Ratledge 1993). Involvement of biosurfactants in microbial adhesion, as demonstrated in this study, has been described previously. Dairy *Streptococcus thermophilus* strains, for example, were found to produce biosurfactants that are responsible for their own desorption (Busscher *et al.* 1994) and also for the reduction of yeasts adhesion to silicone rubber as studied in a parallel plate flow chamber system (Busscher *et al.* 1997). It has been demonstrated as well that these *S. thermophilus* strains interfere on the formation of a mixed fungal/bacterial biofilm on silicone rubber voice prostheses as studied in the modified Robbins device (Busscher *et al.* 1999), whereas oral *Streptococcus mitis* strains secrete biosurfactants inhibiting the adhesion of *Streptococcus mutans* (Pratt-Terpstra *et al.* 1989).

In the classical DLVO approach (theory advanced independently by Derjaguin & Landau and by Vervey & Overbeek), microbial adhesion is described as a balance between attractive Lifshitz-Van der Waals and repulsive or attractive electrostatic forces. An extendend DLVO analysis also considers the interfacial free energy due to Lewis acid-base interactions, ΔG^{AB}_{mws}, between the microorganisms (m) and the silicone rubber with or without an adsorbed biosurfactant layer (s) in aqueous medium (w). According to Van Oss (Van Oss 1989) evaluation of the free energy of adhesion (ΔG_{mws}) is relatively easy on the basis of the equation of state, but becomes increasingly complicated when surface free energy components are included. The free energy of adhesion, ΔG_{mws}, can be separated into an electrodynamic (LW) and an acid-base (AB) component, i.e.,

$$\Delta G_{mws} = \Delta G^{LW}_{mws} + \Delta G^{AB}_{mws} \qquad \text{Equation 5.5}$$

while

$$\Delta G_{mws}^{LW} = -2\left(\sqrt{\gamma_m^{LW}} - \sqrt{\gamma_w^{LW}}\right)\left(\sqrt{\gamma_s^{LW}} - \sqrt{\gamma_w^{LW}}\right)$$ Equation 5.6

and

$$\Delta G_{mws}^{AB} = 2\left[\left(\sqrt{\gamma_m^+} - \sqrt{\gamma_s^+}\right)\left(\sqrt{\gamma_m^-} - \sqrt{\gamma_s^-}\right) - \left(\sqrt{\gamma_m^+} - \sqrt{\gamma_w^+}\right)\left(\sqrt{\gamma_m^-} - \sqrt{\gamma_w^-}\right) - \left(\sqrt{\gamma_s^+} - \sqrt{\gamma_w^+}\right)\left(\sqrt{\gamma_s^-} - \sqrt{\gamma_w^-}\right)\right]$$ Equation 5.7

All calculations of interaction energies were made using γ_w^{LW} = 21.8, γ_w^- = γ_w^+ = 25.5 mN m^{-1} for water and the surface free energy components determined in Table 5.1 for silicone rubber and microorganisms. If the adhesion is thermodynamically favourable the values of the free energy of adhesion will be negative. From the results, summarized in Table 5.2, it can be observed that thermodynamically

(i) all microorganisms have favourable free energy of adhesion ($\Delta G < 0$) to the bare silicone rubber, being the LW interactions the most significant

(ii) all microorganisms have a unfavourable free energy of adhesion ($\Delta G > 0$) to silicone rubber conditioned with biosurfactant, except for *R. dentocariosa* GBJ 52/2B but even in this case the ΔG is less negative than the determined for bare silicone rubber

(iii) LW interactions are always favourable and AB interactions are always unfavourable ($\Delta G > 0$) for the silicone rubber with adsorbed biosurfactant.

According to the thermodynamic approach the experiments done with silicone rubber conditioned with biosurfactant show an unfavourable free energy of adhesion ($\Delta G > 0$) for all microorganisms, except for *R. dentocariosa* GBJ 52/2B that exhibit a negative value, which explains the less effective reduction in the extent of adhesion observed for this microorganism.

Table 5.2 Interfacial free energies of adhesion between the several microorganisms isolated from explanted voice prostheses and silicone rubber with an adsorbed biosurfactant layer. The total free energy (ΔG) as well as the Lifshitz-Van der Waals (ΔG^{LW}) and the acid-base (ΔG^{AB}) components are presented. Experiments were performed in triplicate and correspond within 15%.

Free energy of adhesion (mN m^{-1})						
Microorganism	**Silicone rubber**			**Silicone rubber with adsorbed biosurfactant**		
	ΔG^{LW}	**ΔG^{AB}**	**ΔG**	**ΔG^{LW}**	**ΔG^{AB}**	**ΔG**
S. epidermidis GB 9/6	-18.7	-10.1	-28.9	-13.5	35.8	22.4
S. salivarius GB 24/9	-17.8	-10.3	-28.1	-12.8	35.7	22.8
S. aureus GB 2/1	-17.5	-2.4	-19.9	-12.6	42.6	30.0
R. dentocariosa GBJ 52/2B	-21.2	-30.2	-51.3	-15.2	10.4	-4.9
C. albicans GBJ 13/4A	-16.7	7.0	-9.7	-12.0	50.8	38.8
C. tropicalis GB 9/9	-17.5	7.4	-10.1	-12.6	51.2	38.6

Preventing or retarding a biofilm formation implies that it is necessary to interfere in the weakest link, which means in the formation of the conditioning film and adhesion of the first microorganisms (Busscher *et al.* 1995). The use of biosurfactants adsorbed to silicone rubber is believed to interfere with the formation of this conditioning film as it was observed that the initial deposition rates of all the microorganisms involved in this study decreased. By consequence, biofilm formation can be influenced by adjusting the properties of the voice prostheses material or by surface modification.

All the studied bacteria are hydrophilic except for *R. dentocariosa* GBJ 52/2B that exhibit a more hydrophobic character. Normally an organism will tend to adsorb irreversibly to minimize the free energy of the system. However, charge repulsion or steric exclusion may prevent close approach, and even when this has been achieved ordered water structure associated very closely with the surface could still be a barrier. Hydrophilic surfaces may be stabilized by ordering of surface-associated water molecules, which would be disrupted by the close approach of a bacterial cell. This releases the ordered water molecules into the bulk phase, increasing their free energy. Hydrophobic surfaces should attract strongly because water molecules moving away from the surface into the bulk will decrease their free energy, as their level of hydrogen bonding will increase. Alternatively, the approach of a hydrophilic cell to a hydrophobic surface would yield a stronger interaction (Chamberlain 1992). Thus,

this could explain why the microorganisms studied adhered strongly to the bare hydrophobic silicone rubber, and hardly to the hydrophilic silicone rubber with the adsorbed biosurfactant layer.

The most encompassing theory to explain the adhesion of microorganisms to solid surfaces is the concept of interfacial free energy as the controlling force in cell adhesion. According to this theory, cell adhesion is favored when interfacial tension between the solid, the microorganism, and the suspending liquid is reduced. For most microorganisms, this translates into greater microbial adhesion to solid surfaces in direct proportion to the hydrophobicity of the surface, provided that the suspending medium is water or a simple buffer. Surface-active agents reduce hydrophobic interactions and by doing so reduce microbial adhesion to silicone rubber (Klotz 1990).

5.5 CONCLUSIONS

In conclusion, this study may be a step in developing strategies to prevent the microbial colonization of silicone rubber voice prostheses with the change of the surface properties of silicone rubber by adsorbing the biosurfactant obtained from *L. lactis* 53. Therewith, the lifetime of the voice prostheses would be extended.

5.6 REFERENCES

Bos, R., Van der Mei, H.C. and Busscher, H.J. (1999) Physico-chemistry of initial microbial adhesive interactions – its mechanisms and methods to study. *FEMS Microbiology Reviews*. 23: 179-230.

Busscher, H.J., Bos, R. and Van der Mei, H.C. (1995) Initial microbial adhesion is a determinant for the strength of biofilm adhesion. *FEMS Microbiology Letters.* 128: 229-234.

Busscher, H.J., Bruinsma, G., Van Weissenbruch, R., Leunisse, C., Van der Mei, H.C., Dijk, F. and Albers, F.W. (1998) The effect of buttermilk consumption on biofilm formation on silicone rubber voice prostheses in an artificial throat. *European Archives of Otorhinolaryngology*. 255: 410-413.

Busscher, H.J., Geertsema-Doornbusch, G.I., Everaert, E.P., Verkerke, G.J., Van de Belt-Gritter, B., Kalicharan, R. and Van der Mei, H.C. (1996) Biofilm formation and silicone rubber surface

modification in the development of a total artificial larynx. In: *Surgery and Prosthetic voice restoration after total and subtotal laryngectomy.* Ed. Algaba, J. Amesterdam: Elsevier Science. Pp 47-52.

Busscher, H.J., Neu, T.R. and Van der Mei, H.C. (1994) Biosurfactant production by thermophilic dairy streptococci. *Applied Microbiology and Biotechnology*. 41: 4-7.

Busscher, H.J., Van de Belt-Gritter, B., Westerhof, M., Van Weissenbruch, R., Albers, F.W. and Van der Mei, H.C. (1999) Microbial interference in the colonization of silicone rubber implant surfaces in the oropharynx: *Streptococcus thermophilus* against a mixed fungal/bacterial biofilm. In: *Microbial Ecology and Infectious Disease.* Ed. Rosenberg, E. Washignton D.C.: American Society for Microbiology. Pp 66-74.

Busscher, H.J., Van Hoogmoed, C.G., Geertsema-Doornbusch, G.I., Van der Kuijl-Booij, M. and Van der Mei, H.C. (1997) *Streptococcus thermophilus* and its biosurfactants inhibit adhesion by *Candida* spp. on silicone rubber. *Applied and Environmental Microbiology.* 63: 3810-3817.

Chamberlain, A.H. (1992) The role of adsorbed layers in bacterial adhesion. In: *Biofilms – Science and Technology. NATO ASI Series, Series E: Applied Sciences*. Ed. Melo, L.F, Bott, T.R., Fletcher, M. and Capdeville, B. The Netherlands: Kluwer Academic Publishers. 223: 59-67.

Desai, J. and Banat, I.M. (1997) Microbial production of surfactants and their commercial potential. *Microbiology and Molecular Biology Reviews*. 61:47-64.

Farber, B.F. and Wolff, A.G. (1993) Salicylic acid prevents the adherence of bacteria and yeast to silastic catheters. *Journal of Biomedical Materials Research.* 27: 599-602.

Fiechter, A. (1992) Biosurfactants: moving towards industrial application. *Tibtech.* 10: 208-217.

Foley, I. and Gilbert, P. (1996) Antibiotic resistance of biofilms. *Biofouling.* 10: 334-346.

Holly, F.J. and Owen, M.J. (1983) In: *Physicochemical Aspects of Polymer Surfaces*. Ed. Mittal, K.L. New York: Plenum Press.

Hommel, R.K. and Ratledge, C. (1993) Biosynthetic mechanisms of low molecular weight surfactants and their precursor molecules. In: *Biosurfactants: Production, Properties and Applications.* Ed. Kosaric, N. New York: Marcel Dekker. Pp 3-63.

Klotz, S.A. (1990) Role of hydrophobic interactions in microbial adhesion to plastics used in medical devices. In: *Microbial Cell Surface Hydrophobicity.* Ed. Doyle, R.J. and Rosenberg, M. Washington: American Society of Microbiology. Pp 107-136.

Mahieu, H.F., Van Saene, J.J., Van den Besten, J. and Van Saene, H.K. (1986) Oropharynx decontamination preventing *Candida* vegetation on voice prostheses. *Archives of Otolaryngology: Head and Neck Surgery*. 112: 1090-1092.

Neu, T.R., Van der Mei, H.C., Busscher, H.J., Dijk, F. and Verkerke, G.J. (1993) Biodeterioration of medical-grade silicone rubber used for voice prostheses: a SEM study. *Biomaterials*. 14: 459-464.

Pratt-Terpstra, I.H., Weerkamp, A.H. and Busscher, H.J. (1989) Microbial factors in a thermodynamic approach of oral streptococcal adhesion to solid substrata. *Journal of Colloid Interface Science*. 129: 568-574.

Reid, G. (1999) Biofilms in infectious disease and on medical devices. *International Journal of Antimicrobial Agents*. 11: 223-226.

Rodrigues, L.R., Teixeira, J. and Oliveira, R. (2003) Response surface optimization of the medium components for the production of biosurfactant by lactic acid bacteria. In: *Abstracts of 11th European Congress on Biotecnology*, 24-29 August, Basel, Switzerland, Pp 76.

Rodrigues, L.R., Teixeira, J., Oliveira, R. and Van der Mei, H.C. (2005) Response surface optimization of the medium components for the production of biosurfactants by probiotic bacteria. *Process Biochemistry*. *In Press*.

Sjollema, J., Busscher, H.J. and Weerkamp, A. (1989) Real-time enumeration of adhering microorganisms in a parallel plate flow cell using automated image analysis. *Journal of Microbiological Methods.* 9: 73-78.

Van der Mei, H.C., Van de Belt-Gritter, B., Van Weissenbruch, R., Dijk, F., Albers, F.W. and Busscher, H.J. (1999) Effect of consumption of dairy products with probiotic bacteria on biofilm formation on silicone rubber implant surfaces in an artificial throat. *Trans IChemE*. 77: 156-158.

Van der Vegt, W., Van der Mei, H.C., Noordmans, J. and Busscher, H.J. (1991) Assessment of bacterial biosurfactant production through axisymmetric drop shape analysis by profile. *Applied Microbiology and Biotechnology*. 35:766-770.

Van Oss, C.J. (1989) Hydrophobicity and hydrophilicity of biosurfaces. *Current Opinion on Colloid Interface Science.* 2: 503-510.

Van Oss, C.J. and Gillman, C.F. (1972) Phagocytosis as a surface phenomenon. I. Contact angles and phagocytosis of non-opsonized bacteria. *Journal Reticulo Society.* 12: 283-292.

Van Oss, C.J., Good, R. and Chaudhury, M. (1988) Additive and nonadditive surface tension components and the interpretation of contact angles. *Langmuir.* 4: 884-891.

Van Weissenbruch, R., Bouckaert, S., Remon, J., Nelis, H.J., Aerts, R. and Albers, F.W. (1997) Chemoptophylaxis of fungal deterioration of the Provox prostheses. *Annals of Otology, Rhinology and Laryngology.* 106: 329-337.

Velraeds, M.C., Van der Mei, H.C., Reid, G. and Busscher, H.J. (1996) Inhibition of initial adhesion of uropathogenic *Enterococcus faecalis* by biosurfactants from *Lactobacillus* isolates. *Applied and Environmental Microbiology.* 62: 1958-1963.

Volpe, C. and Siboni, S. (1997) Some reflections on acid-base solid surface free energy theories. *Journal of Colloid Interface Science.* 195: 121-136.

CHAPTER 6

INHIBITION OF MICROBIAL ADHESION TO SILICONE RUBBER TREATED WITH BIOSURFACTANT FROM *STREPTOCOCCUS THERMOPHILUS* A

"Persistency is the way to success".

Charles Chaplin.

Microbial adhesion of four bacterial and two yeast strains isolated from explanted voice prostheses to silicone rubber before and after conditioning with a biosurfactant obtained from the probiotic bacterium *Streptococcus thermophilus* A in a parallel plate flow chamber is described in this section. The results obtained showed that the biosurfactant was effective in decreasing the initial deposition rates, as well as the number of bacterial cells adhering after 4 h, for all microorganisms tested. Such a pretreatment with surface active compounds may constitute a promising strategy to reduce microbial colonization rate of silicone rubber voice prostheses.

6.1 INTRODUCTION

6.2 MATERIALS AND METHODS

6.2.1 Biosurfactant Production

6.2.2 Biosurfactant Surface-activity Determination

6.2.3 Microbial Strains and Growth Conditions

6.2.4 Contact Angle Measurements

6.2.5 Adhesion Experiments

6.2.6 Statistical Analysis

6.3 RESULTS

6.4 DISCUSSION

6.5 CONCLUSIONS

6.6 REFERENCES

6.1 INTRODUCTION

Patients who have undergone a laryngectomy due to a malignant laryngeal tumour need to breath through a tracheostoma and receive voice prostheses for speech rehabilitation (Van der Mei *et al.* 1999). Silicone rubber is an ideal material for manufacturing voice prostheses because of its ease of moulding and excellent mechanical properties, but in laryngectomized patients, the hydrophobic silicone rubber surfaces becomes colonized rapidly with a thick biofilm, consisting of a variety of bacterial and yeast strains (Neu *et al.* 1993). Biofilm formation poses a severe problem once the biofilm has extended towards the valve of the prosthesis leading to either blocking or leakage with the consequent loss of function of the device and inevitable need for replacement on average every 3-4 months (Busccher *et al.* 1997). It has been suggested by laryngectomized patients that consumption of dairy products may positively influence the lifetime of their prostheses (Van der Mei *et al.* 1999). Active bioyogurt containing active *Streptococcus thermophilus* has been suggested to have beneficial effects in prolonging the lifetime of indwelling voice prostheses. The mechanism by which this occurs has not been investigated, but it is hypothesized that the presence of *S. thermophilus* and *Lactobacillus bulgaricus*, two well-known probiotic bacterial strains, in active bioyogurt may interfere with the adhesion of yeast to the silicone rubber (Van der Mei *et al.* 1999, 2000). In addition, it has been demonstrated that dairy *S. thermophilus* strains, produce biosurfactants causing their own desorption (Busscher *et al.* 1994) and reduction of yeasts adhesion to silicone rubber in a parallel plate flow chamber system (Busscher *et al*. 1997), as well as interference with the formation of a mixed fungal/bacterial biofilm on silicone rubber voice prostheses in the modified Robbins device (Busscher *et al.* 1999).

Biosurfactants are biological surface-active compounds produced by some microorganisms and can have some influence on interfaces. They include a wide variety of chemical structures such as glycolipids, lipopeptides, polysaccharide-protein complexes, phospholipids, fatty acids, and neutral lipids (Desai and Banat 1997). With regard to an anti-adhesive effect of biosurfactants, hypotheses have been forwarded in which adsorption of biosurfactants to a substratum surface alters the hydrophobicity of the surface and causes interference in microbial adhesion and desorption processes (Desai and Banat 1997). Biosurfactants have also been reported to have various degrees of antimicrobial activity (Banat *et al.* 2000). Several biosurfactants have strong antibacterial, antifungal and antiviral activity (Singh and Cameotra 2004).

This chapter reports on the use of a biosurfactant produced by the probiotic bacterium *S. thermophilus* A in the reduction of the adhesion ability of four bacterial and two yeast strains isolated from explanted voice prostheses to silicone rubber.

6.2 MATERIALS AND METHODS

6.2.1 Biosurfactant Production

An overnight subculture (10 ml) of *S. thermophilus* A grown in optimized M17 broth was used to inoculate a second culture (600 ml). Cells were harvested by centrifugation (10000 × g, 5 min, 10ºC), washed twice with demineralized water and resuspended in 100 ml of phosphate-buffered saline (PBS: 10 mM KH_2PO_4/K_2HPO_4 and 150 mM NaCl with pH adjusted to 7.0). Crude biosurfactant extract was produced by gently stirring this suspension for 2 h at room temperature. Subsequently, the bacteria were removed by centrifugation and the remaining supernatant was filtered through a 0.22 μm pore-size filter (Millipore). To recover the biosurfactant, the supernatant was dialyzed against demineralized water at 4ºC in a Spectrapor membrane tube (molecular weight cut off 6000 to 8000, Spectrum Medical Industries Inc., CA) and freeze-dried.

6.2.2 Biosurfactant Surface-activity Determination

Axisymmetric drop shape analysis by profile (ADSA-P) is a technique for determining liquid surface tensions based on the shape of an axisymmetric droplet on a solid substratum. This technique was used as an indirect yet a reliable estimation of the presence of a suitable biosurfactant concentration. In order to measure the surface-activity of the biosurfactant by ADSA-P, a 100 μl droplet of a biosurfactant solution was placed on fluoroethylene-propylene (FEP)-Teflon (Fluorplast, The Netherlands) in a closed chamber to prevent evaporation from the droplet. The shape of the droplet was monitored for 2 h at room temperature and the surface tension of the droplet was calculated from its shape as a function of time as described by Van der Vegt *et al.* (1991). All values presented are the averages of at least triplicate measurements.

6.2.3 Microbial Strains and Growth Conditions

Four bacterial strains, *Staphylococcus epidermidis* GB 9/6, *Streptococcus salivarius* GB 24/9, *Staphylococcus aureus* GB 2/1, and *Rothia dentocariosa* GBJ 52/2B and two yeast strains: *Candida albicans* GBJ 13/4A and *Candida tropicalis* GB 9/9 isolated from explanted voice prostheses were used in this study. All strains were first grown overnight at 37°C in ambient air on an agar plate from a frozen stock. The agar plate was kept at 4°C for a maximum of two weeks. Several colonies were used to inoculate 10 ml of brain heart infusion broth (BHI, OXOID, Basingstoke, England) for all the bacterial and yeast strains in use. This pre-culture was incubated at 37°C in ambient air for 24 h and used to inoculate a second culture of 200 ml that was grown for 18 h. The microorganisms from the second culture were harvested by centrifugation for 5 min at 10000 × g and washed twice with demineralised water. Subsequently, bacterial cells were suspended in 200 ml PBS solution, after sonication on ice (10 s), to a concentration of 3×10^8 ml^{-1}. Yeasts were suspended in PBS to a concentration of 3×10^6 ml^{-1}. A Bürker-Türk counting chamber was used to count the cells.

6.2.4 Contact Angle Measurements

Advancing type contact angles with Millipore water on silicone rubber with and without an adsorbed biosurfactant layer were measured with a locally manufactured contour monitor using the sessile drop technique (Van Oss *et al.* 1988). On each sample, at least five droplets were placed at different positions and results of three separately prepared surfaces with adsorbed biosurfactant were averaged.

6.2.5 Adhesion Experiments

The flow chamber and image analysis system have been described in detail before (Sjollema *et al.* 1989). Images were taken from the bottom plate (5.5 × 3.8 cm) of the parallel plate flow chamber that consisted of a thin sheet of silicone rubber (with or without an adsorded biosurfactant layer) affixed to a polymethylmethacrylate (PMMA) plate. The top plate of the chamber was made of glass.

Conditioned silicone rubber was prepared through overnight immersion in a 3 g l^{-1} biosurfactant solution at 4ºC. This concentration was estimated by axisymmetric drop shape analysis by profile (ADSA-P) as described by Van der Vegt *et al.* (1991) as the one that allowed for a reasonable reduction in the surface tension (18.6 mN m^{-1}) and that would be expected to interfere in adhesion.

Prior to each experiment, all tubes and the flow chamber were filled with PBS, while care was taken to remove air bubbles from the system. Flasks, containing microbial suspension and buffer, were positioned at the same height with respect to the chamber to ensure that immediately after the flows were started, all fluids would circulate by hydrostatic pressure through the chamber at the desired shear rate of 10 s^{-1} (0.025 ml s^{-1}), which yields a laminar flow (Reynolds number 0.6). The microbial suspension was circulated through the system for 4 h and images were obtained from silicone rubber with or without the adsorbed biosurfactant layer. The initial increase in the number of adhering microorganisms with time was expressed by a so-called initial deposition rate j_0 (microorganisms cm^{-2} s^{-1}), i.e. the number of adhering microorganisms per unit area and time. The number of adhering microorganisms after 4 h was also determined (n_{4h}). All values presented in this work are the averages of at least triplicate measurements on silicone rubber surfaces with or without the adsorbed biosurfactant layer, and were carried out with separately grown microorganisms.

6.2.6 Statistical Analysis

All the adhesion experiments were compared using one-way analysis of variance (ANOVA) by applying the Levene's test of homogeneity of variances, the Tukey multiple-comparisons test, and also paired samples t-test, using SigmaPlot 8.0 software. Student's *t*-test was applied to all the experimental data, for rejection of some experimental values. All tests were performed with a confidence level of 95%.

6.3 RESULTS

The water contact angle measured at 25ºC on untreated silicone rubber decreased from 109 ± 2 degrees to 58 ± 5 degrees after adsorption of the biosurfactant, which means that the biosurfactant turns the silicone rubber surface less hydrophobic.

The liquid surface tensions of the freeze-dried biosurfactant were measured by ADSA-P as a dilution series from 50 to 0.1 g l^{-1}. The surface tension measured after 2 h is plotted against the biosurfactant concentration in Figure 6.1.

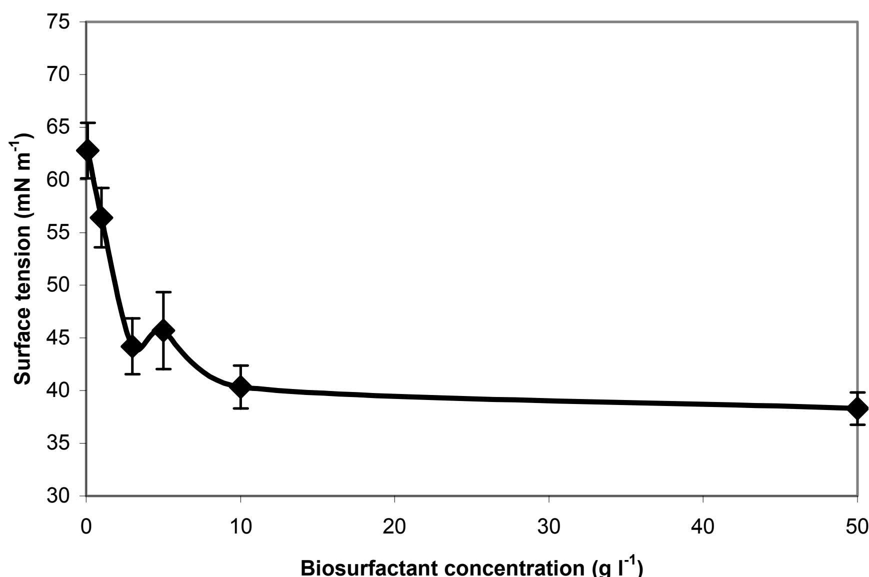


Figure 6.1 Surface tension of crude biosurfactant concentrations obtained from *S. thermophilus* A in PBS (pH 7.0) after 2 h as measured by ADSA-P. Results are averages of triplicate experiments varying within 3-8% (ANOVA) and the standard deviation represented by error bars.

The biosurfactant reached the lowest surface tension of 40 mN m^{-1} with a concentration of 10 g l^{-1}. Although the surface tension measurements give a clear indication of the surface activity of the compound it is not sufficient to determine whether it would produce an anti-adhesive effect against the selected microorganisms. Therefore, previous results presented on Chapter 8 of an inhibition test (Rodrigues *et al.* 2004a) were considered for the selection of the biosurfactant concentration to use in the flow experiments. It was evaluated the antimicrobial activity of the biosurfactant at several concentrations against a variety of bacterial and yeast strains isolated from explanted voice prostheses, and it was found that the biosurfactant is an antimicrobial agent, but depending on the microorganism there are different effective concentrations (Rodrigues *et al.* 2004a). Thus, in the adhesion experiments a biosurfactant concentration of 3 g l^{-1} was used because a surface tension decrease of 19 mN m^{-1} was assumed to be enough to give an effect in adhesion.

The initial deposition rates (Figure 6.2) of *R. dentocariosa* GBJ 52/2B, *S. epidermidis* GB 9/6 and *C. tropicalis* GB 9/9 on silicone rubber were relatively high between 1937 ± 194, 2105 ± 234 and 2598 ± 227 microorganisms cm^{-2} s^{-1}, while *S. salivarius* GB 24/9 and *S. aureus* GB 2/1 deposit at a slower rate onto silicone rubber (1200 - 1600 microorganisms cm^{-2} s^{-1}) and *C. albicans* GBJ 13/4A has by far the lowest initial deposition rate (172 ± 22 microorganisms

cm^{-2} s^{-1}). Adsorption of biosurfactant on the silicone rubber reduces the initial deposition rate about 86% for *R. dentocariosa* GBJ 52/2B and *S. aureus* GB 2/1. A reduction of about 75% on average for *S. salivarius* GB 24/9 and *C. tropicalis* GB 9/9 was achieved. The initial deposition rates of *S. epidermidis* GB 9/6 and *C. albicans* GBJ 13/4A are far less reduced (43% and 65% respectively) in the presence of the biosurfactant than the initial deposition rates of the other strains.

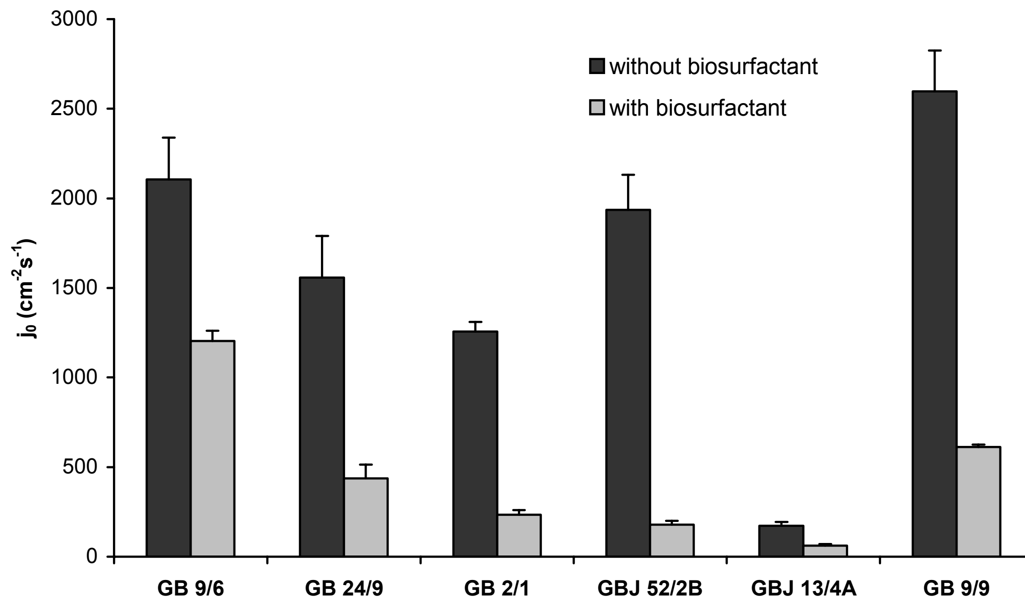


Figure 6.2 The initial deposition rates (j_0) of the bacterial strains (*Staphylococcus epidermidi*s GB 9/6, *Streptococcus salivarius* GB 24/9, *Staphylococcus aureus* GB 2/1 and *Rothia dentocariosa* GBJ 52/2B) and yeast (*Candida albicans* GBJ 13/4A and *Candida tropicalis* GB 9/9) isolated from explanted voice prostheses on silicone rubber with and without an adsorbed biosurfactant layer. Results are averages of triplicate experiments varying within 10-15% (ANOVA) and the standard deviation represented by error bars.

The numbers of microorganisms adhering to silicone rubber after 4 h, (n_{4h}, Figure 6.3) varied between 7×10^6 and 15×10^6 cm^{-2} for all the microorganisms studied except for *C. albicans* GBJ 13/4A that exhibit the least number of adhering microorganisms (1×10^6 cm^{-2}). The numbers of bacterial and yeast cells, adhering to silicone rubber treated with biosurfactant after 4 h were significantly reduced by 89 to 97% and 67 to 70%, respectively.

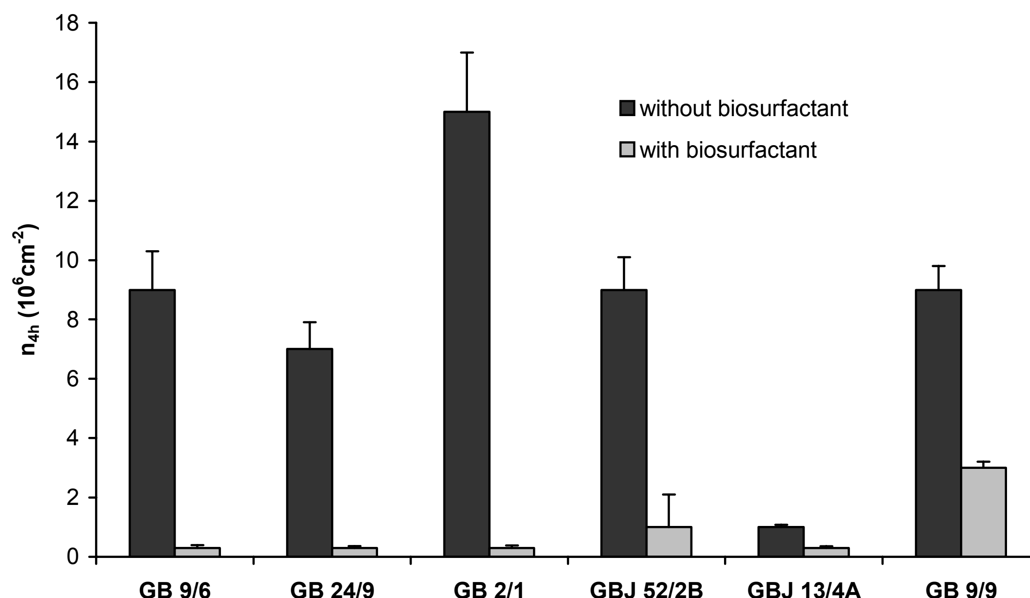


Figure 6.3 The number of microorganisms adhering after 4 h (n_{4h}) on silicone rubber with and without an adsorbed biosurfactant layer. The microorganisms used are as described in Fig. 6.2. Results are averages of triplicate experiments varying within 10-15% (ANOVA) and the standard deviation represented by the error bars.

The replicates of adhesion experiments with and without adsorbed biosurfactant for each strain were compared and found to be statistically similar ($p<0.05$, ANOVA and Tukey's multiple comparison test). Differences in mean values by group that resulted in p-values < 0.05 were considered statistically significant. The extent of adherence of all the strains studied was significantly different ($p<0.05$, ANOVA and Tukey's multiple comparison test), when comparing the number of adhered cells after 4 h and also the initial deposition rate, for experiments done with and without adsorbed biosurfactant. The extent of adhesion (j_0, n_{4h}) on silicone rubber was several times higher than the extent of adhesion on silicone rubber with adsorbed biosurfactant ($p< 0.05$, paired samples t-test).

Furthermore, the extent of adhesion of all the strains studied was significantly different when comparing experiments done with and without adsorbed biosurfactant, being the F-values 5 fold higher than the critical F-values (critical value extracted from the f-distribution in statistical tables).

6.4 DISCUSSION

The aim of this study was to evaluate the extent of adhesion of several microbial strains, involved in the biofilm formation on voice prostheses, to silicone rubber in the absence and presence of an adsorbed biosurfactant layer obtained from *S. thermophilus* A by using a parallel plate flow chamber system.

The two main reasons for the frequent replacement of silicone rubber voice prostheses are patients complaints about leakage of esophageal contents into the trachea and increasing efforts needed to produce phonation. Both are signs of failure of voice prostheses and are due to biofilm formation (Neu *et al.* 1993). An antifouling improvement of the silicone rubber material is therefore a highly desirable outcome. In the case of laryngectomized patients, the voice prostheses' lifetimes are usually less than two months and employing "antibiofilm" therapy at the time of insertion of the voice prostheses therefore is usually necessary. Using antimycotics or antibiotics is undesirable due to the risk of inducing the production of resistant strains (Mahieu *et al.* 1986, Van Weissenbruch *et al.* 1997). Research into the development of new methods for preventing or retarding biofilm formation on voice prostheses is therefore increasing.

Biosurfactants can exhibit antimicrobial activity against various microbes (Singh and Cameotra 2004). One of the earliest noted antimicrobial activities of biosurfactants was that of iturin A, a potential antifungal lipopeptide produced by strains of *Bacillus subtilis*. Surfactin, a cyclic lipopeptide produced by *B. subtilis* strains, is another biosurfactants with well-known antimicrobial properties (Ahimou *et al.* 2000). A new antibiotic from *Pseudomonas fluorescens*, with surface active properties different from those of the known biosurfactant viscosin from the same species, was later identified and named viscosinamide, which was also found to have antifungal properties (Singh and Cameotra 2004). Interestingly, as can be seen in Chapter 8, it was found that a biosurfactant obtained from *S. thermophilus* A showed a significant antimicrobial activity against *C. tropicalis* GB 9/9 at low biosurfactant concentrations (Rodrigues *et al.* (2004a) and it has been demonstrated that *C. tropicalis* contributes to the premature failure of the prostheses (Elving *et al.* 2002). The use of biosurfactants as antimicrobial agents therefore may represent a promising technique for prolonging the lifetime of voice prostheses.

In the present study, it has been demonstrated that the extent of bacterial adhesion to silicone rubber conditioned with a biosurfactant produced by *S. thermophilus* A in a parallel plate flow chamber during the first four hours was significantly decreased with respect to

untreated silicone rubber. Similarly the number of adhered yeast cells was also decreased in the presence of the biosurfactant to a lesser extent in comparision to bacteria. This reduction in the extent of adhesion may have been improved if higher concentrations of biosurfactant were used. The ADSA-P results demonstrated that a 23 mN m^{-1} reduction on surface tension is possible with a biosurfactant concentration of 10 g l^{-1} compared with the 19 mN m^{-1} reduction obtained with the concentration (3 g l^{-1}) used in this experiments. The involvement of biosurfactants in microbial adhesion, as demonstrated in this study, has been described previously. The use of a biosurfactant from *Lactococcus lactis* 53 as antimicrobial and/or anti-adhesive agent and its ability to inhibit adhesion in a parallel plate flow chamber of various microorganisms isolated from explanted voice prostheses has been demonstrated by Rodrigues *et al.* (2004b), as can be seen in Chapter 5. Regarding the exposure time, in adhesion experiments we only considered 4 hours as the aim of this study was to evaluate the inhibition of initial adhesion and that happens in the first hours. However, in a previous work performed in the artificial throat model the exposure time was up to 8 days (Chapter 8, Rodrigues *et al.* 2004a) and the cycle of nutrients provided during this experiment was essential to grow biofilms with features as found on explanted prostheses. The performance of the biosurfactant in the artificial throat model confirmed the results presented in this study and although the biosurfactant was not tested for exposure times longer than 8 days the results achieved allow us to conclude that this is a coating that resists to body fluids as no biological degradation was observed.

Biosurfactants have the potential to be used as a preventive strategy to delay the onset of biofilm growth on catheters and other implant materials, thus decreasing the large number of hospital infections without increased use of synthetic drugs or chemicals. They can also be used in pulmonary immunotherapy and incorporated into probiotic preparations to combat urogenital tract infections (Singh and Cameotra 2004). Biofilm formation on solid surfaces, for most microorganisms, occurs in direct proportion to the hydrophobicity of the surface, provided that the suspended medium is a simple buffer. Surface-active agents reduce hydrophobic interactions and by doing so reduce microbial adhesion to silicone rubber (Klotz 1990). The use of preadsorbed biosurfactants to silicone rubber is believed to interfere with the formation of the conditioning film and adhesion of the first microorganisms, thus preventing or retarding the biofilm formation (Busscher *et al.* 1998, Rodrigues *et al.* 2004b). Consequently, biofilm formation can be influenced by adjusting the properties of the voice prostheses material or by surface modification.

6.5 CONCLUSIONS

In conclusion, it has been demonstrated in this study that the adsorption of the biosurfactant from *S. thermophilus* A to silicone rubber significantly reduces the water contact angle, thus reducing the hydrophobic interactions and the ability of microbial adhesion to silicone rubber and its general surface properties. As a consequence, the lifetime of treated silicone rubber voice prostheses may be lengthened which would directly benefit laryngectomized patients.

6.6 REFERENCES

Ahimou, F., Jacques, P. and Deleu, M. (2000) Surfactin and iturin A effects on *Bacillus subtilis* surface hydrophobicity. *Enzyme Microbiology and Technology*. 27: 749-754.

Banat, I.M., Makkar, S. and Cameotra, S. (2000) Potential commercial application of microbial surfactants - A review article. *Applied Microbiology and Biotechnology*. 53: 495-508.

Busscher, H.J., Bos, R. and Van der Mei, H.C. (1995) Initial microbial adhesion is a determinant for the strength of biofilm adhesion. *FEMS Microbiology Letters.* 128: 229-234.

Busscher, H.J., Neu, T.R. and Van der Mei, H.C. (1994) Biosurfactant production by thermophilic dairy streptococci. *Applied Microbiology and Biotechnology*. 41: 4-7.

Busscher, H.J., Van de Belt-Gritter, B., Westerhof, M., Van Weissenbruch, R., Albers, F.W. and Van der Mei, H.C. (1999) Microbial interference in the colonization of silicone rubber implant surfaces in the oropharynx: *Streptococcus thermophilus* against a mixed fungal/bacterial biofilm. In: *Microbial Ecology and Infectious Disease.* Ed. Rosenberg, E. Washigton D.C.: American Society for Microbiology. Pp 66-74.

Busscher, H.J., Van Hoogmoed, C.G., Geertsema-Doornbusch, G.I., Van der Kuijl-Booij, M. and Van der Mei, H.C. (1997) *Streptococcus thermophilus* and its biosurfactants inhibit adhesion by *Candida* spp. on silicone rubber. *Applied and Environmental Microbiology.* 63: 3810-3817.

Desai, J. and Banat, I.M. (1997) Microbial production of surfactants and their commercial potential. *Microbiology and Molecular Biology Reviews*. 61:47-64.

Elving, G.J., Van der Mei, H.C., Busscher, H.J., Van Weissenbruch, R. and Albers, F.W.J. (2002) A comparison of the microbial composition of voice prosthetic biofilms from patients requiring frequent *versus* infrequent replacements. *Annals of Otology, Rhinology and Laryngology*. 111: 200-203.

Klotz, S.A. (1990) Role of hydrophobic interactions in microbial adhesion to plastics used in medical devices. In: *Microbial Cell Surface Hydrophobicity.* Ed. Doyle, R.J. and Rosenberg, M. Washington: American Society of Microbiology. Pp 107-136.

Mahieu, H.F., Van Saene, J.J., Van den Besten, J. and Van Saene, H.K. (1986) Oropharynx decontamination preventing *Candida* vegetation on voice prostheses. *Archives of Otolaryngology: Head and Neck Surgery*. 112: 1090-1092.

Neu, T.R., Van der Mei, H.C., Busscher, H.J., Dijk, F. and Verkerke, G.J. (1993) Biodeterioration of medical-grade silicone rubber used for voice prostheses: a SEM study. *Biomaterials*. 14: 459-464.

Rodrigues, L.R., Van der Mei, H.C., Teixeira, J. and Oliveira, R. (2004a) Influence of biosurfactants from probiotic bacteria on formation of biofilms on voice prostheses. *Applied and Environmental Microbiology.* 70: 4408-4410.

Rodrigues, L.R., Van der Mei, H.C., Teixeira, J. and Oliveira, R. (2004b) Biosurfactant from *Lactococcus lactis* 53 inhibits microbial adhesion on silicone rubber. *Applied Microbiology and Biotechnology*. 66: 306-311.

Singh, P. and Cameotra, S. (2004) Potential applications of microbial surfactants in biomedical sciences. *Trends in Biotechnology*. 22, 142-146.

Sjollema, J., Busscher, H.J. and Weerkamp, A. (1989) Real-time enumeration of adhering microorganisms in a parallel plate flow cell using automated image analysis. *Journal of Microbiological Methods.* 9: 73-78.

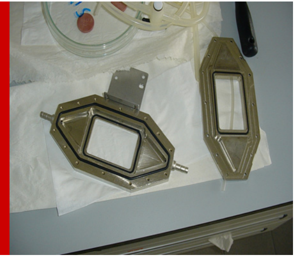
Van der Mei, H.C., Free, R.H., Elving, G.J., Van Weissenbruch, R., Albers, F.W.J. and Busscher, H.J. (2000) Effect of probiotic bacteria on prevalence of yeasts in oropharyngeal biofilms on silicone rubber voice prostheses *in vitro*. *Journal of Medical Microbiology*. 49: 713-718.

Van der Mei, H.C., Van de Belt-Gritter, B., Van Weissenbruch, R., Dijk, F., Albers, F.W. and Busscher, H.J. (1999) Effect of consumption of dairy products with probiotic bacteria on biofilm formation on silicone rubber implant surfaces in an artificial throat. *Trans IChemE*. 77: 156-158.

Van der Vegt, W., Van der Mei, H.C., Noordmans, J. and Busscher, H.J. (1991) Assessment of bacterial biosurfactant production through axisymmetric drop shape analysis by profile. *Applied Microbiology and Biotechnology*. 35:766-770.

Van Oss, C.J., Good, R. and Chaudhury, M. (1988) Additive and nonadditive surface tension components and the interpretation of contact angles. *Langmuir.* 4: 884-891.

Van Weissenbruch, R., Bouckaert, S., Remon, J., Nelis, H.J., Aerts, R. and Albers, F.W. (1997) Chemoptophylaxis of fungal deterioration of the Provox prostheses. *Annals of Otology, Rhinology and Laryngology*. 106: 329-337.



CHAPTER 7

INTERFERENCE IN ADHESION OF BACTERIA AND YEASTS ISOLATED FROM EXPLANTED VOICE PROSTHESES TO SILICONE RUBBER BY RHAMNOLIPIDS

"Nothing will work unless you do".

Maya Angelou.

The effects and extent of adhesion of microbial strains isolated from explanted voice prostheses to silicone rubber with and without an adsorbed rhamnolipid biosurfactant layer obtained from *Pseudomonas aeruginosa* DS10-129 are described in this chapter. The rhamnolipid showed an anti-adhesive activity against all strains and an effective reduction in the initial deposition rates, as well as in the number of bacterial cells adhering after 4 h.

7.1 INTRODUCTION
7.2 MATERIALS AND METHODS
7.2.1 Biosurfactant Production and Extraction
7.2.2 Derivatization and HPLC Analysis of Rhamnolipid
7.2.3 Surface-activity Determination
7.2.4 Contact Angle Measurements
7.2.5 Microbial Strains and Growth Conditions
7.2.6 Adhesion Assay in 96 Wells Plate
7.2.7 Adhesion Experiments in the Parallel Plate Flow Chamber
7.2.8 Detachment Protocol
7.2.9 Statistical Analysis
7.3 RESULTS
7.3.1 Surface-activity and Biosurfactant Concentration
7.3.2 Anti-adhesive Activity of the Rhamnolipid
7.3.3 Microbial Adhesion in the Parallel Plate Flow Chamber
7.3.4 Detachment Protocol
7.4 DISCUSSION
7.5 CONCLUSIONS
7.6 REFERENCES

7.1 INTRODUCTION

Silicone rubber has several advantageous properties that led to its use in voice prostheses (Neu *et al.* 1993), urinary catheters (Farber and Wolff 1993) and contact lens materials (Holly 1983). Patients who have undergone a laryngectomy due to a malignant laryngeal tumour need to breathe through a tracheostoma and receive voice prostheses for speech rehabilitation (Neu *et al.* 1993). A serious problem in laryngectomized patients with a voice prosthesis inserted is the limited lifetime of the prosthesis of about 3-4 months. Voice prostheses which are covered with a biofilm (Neu *et al.* 1993) cause leakage of food and liquid or an increased airflow resistance (Mahieu *et al.* 1986) that result in the frequent replacement of the prosthesis. This biofilm formation immediately starts once the device has been placed in the fistula and is usually composed of various bacterial and yeast strains. The type of environment of the neopharynx and the presence of a tracheostoma are ideal for microbial adherence to the surface of the voice prosthesis (Busscher *et al.* 2000). Frequent replacement of the prosthesis is uncomfortable, costly, time consuming and may lead to damage of the shunt with scar tissue formation, insufficiency or stenosis. Less frequent replacements of voice prosthesis therefore would be advantageous and has resulted in general interest in finding techniques to inhibit biofilm formation and prolonging the lifetime of voice prostheses (Elving *et al.* 2000, Rodrigues *et al.* 2004a,b, Busscher *et al.* 2000). Everaert *et al.* (1999) studied, *in vivo*, the influence of perfluoro-alkylsiloxane (PA) surface modification of silicone rubber voice prostheses on biofouling, with 18 consecutive patients with laryngectomies, and found that chemisorption of long-chain PAs reduces biofilm formation. Additionally, as described in Chapter 8, Rodrigues *et al.* (2004a) developed a promising strategy to lengthen the voice prostheses lifetime as it was demonstrating that the use of biosurfactants obtained from probiotic bacteria inhibit biofilm formation and the occurrence of increased airflow resistances.

Surfactants of microbial origin, referred to as biosurfactants, are surface-active compounds that have some influence on interfaces. With regard to an anti-adhesive effect of biosurfactants, hypotheses have been forwarded in which adsorption of biosurfactants to a substratum surface alters the hydrophobicity of the surface and causes interference in microbial adhesion and desorption processes (Desai and Banat 1997, Bai *et al.* 1997). Rhamnolipid biosurfactants mainly produced by *Pseudomonas aeruginosa* are the most well-known and characterized biosurfactant molecules (Banat *et al.* 2000, Rahman *et al.* 2002a,b, 2003a,b). Due to their diversity, environmentally friendly nature, possibility of large-scale

production, selectivity, effectiveness under extreme conditions and at low concentrations, in addition to the possibility for production on renewable sources, they have been essentially applied in environmental protection (Banat *et al.* 2000, Rahman *et al.* 2002a,b, 2003a,b). Despite their potential only a few studies were done on application in biomedical sciences. Moreover, since they are biological and safe (Benincasa *et al.* 2004; Flasz *et al.* 1998; Makkar and Cameotra 2002), rhamnolipid biosurfactant are a suitable alternative to synthetic medicines and antimicrobial agents, and may be used as safe and effective therapeutic agents (Singh and Cameotra 2004).

The aim of this study was to investigate the effects and extent of adhesion of four different bacterial and two yeast strains isolated from explanted voice prostheses to silicone rubber with and without an adsorbed rhamnolipid biosurfactant layer in a parallel plate flow chamber. Additionally, the influence of the biosurfactant on the detachment of these microorganisms adhering to silicone rubber was studied. In order to determine the best dilution of the rhamnolipid with an anti-adhesive effect a quick screening was performed in a 96 wells plate, before the adhesion experiments in the parallel plate flow chamber were started.

7.2 MATERIALS AND METHODS

7.2.1 Biosurfactant Production and Extraction

Biosurfactants were produced in batch culture in 250 ml conical flasks containing 100 ml of medium with the following composition in g l^{-1} Na_2HPO_4, 2.2; KH_2PO_4, 1.4; $MgSO_4.7H_2O$, 0.6; $(NH_4)_2SO_4$, 3.0, glucose, 2.0 and 1 ml trace element solution (Banat *et al*. 1991). The culture medium pH was maintained at 7.5. The flasks were inoculated with *P. aeruginosa* DS10-129 and incubated in a shaker at 200 rpm at 30ºC. Glycerol was added to the culture at 10 g l^{-1} after 48 h growth to induce biosurfactant production (Rahman *et al.* 2002b) and flasks re-incubated for another 48-72 h. After removing the cells by centrifugation (8000 × g, 15 min) the whole culture broth was sterilized (121ºC, 15 min) and used to extract the rhamnolipid biosurfactant. The supernatant was shaken with a chloroform:methanol (2:1) solution and allowed to stand for 2 h. Subsequently, the chloroform solvent layer containing the rhamnolipid was separated and left at 40°C overnight to complete evaporation. The quantity of rhamnolipid obtained after chloroform:methanol extraction varied between 2-4 g per liter of culture broth.

7.2.2 Derivatization and HPLC Analysis of Rhamnolipid

The derivatization and HPLC analysis were carried out using a modified method described by Schenk *et al*. (1995) and Mata-Sandoval *et al*. (1999) to determine the rhamnolipid-containing solution concentration. One milliliter rhamnolipid-containing water solution was added to 1 ml acetonitrile containing 2-bromoacetophenone and triethylamine at a molar ratio 1:8:2 (glycolipid:2-bromoacetophenone:triethylamine). The mixture was incubated at 80ºC for 1 h. The mixture was filtered with Minisart 0.22 μm syringe filters prior to HPLC analysis. The derivatized phenacyl esters were separated by HPLC using an isocratic mobile phase of CH_3CN – 3.3 mM H_3PO_4 (80:20 (v/v)) at a flow rate of 1 ml min^{-1}. The column used was a phenomenex Kromasil C-18 (150 $\times$ 4.6 mm) and the detection was done by absorbance measured at 254 nm. The rhamnolipid-containing solution used in further studies had a 4 g l^{-1} rhamnolipid concentration.

7.2.3 Surface-activity Determination

The surface activity of the rhamnolipid was determined by measuring the surface tension by the Ring method (Kim *et al.* 2000) using a KRUSS Tensiometer equipped with a 1.9 cm De Noüy platinum ring at room temperature (25 $\pm$ 1ºC). The concentration at which micelles began to form was represented as the *cmc* (critical micelle concentration). The *cmc* was determined by plotting the surface tension as a function of the biosurfactant concentration. Dilutions of the rhamnolipid-containing solution were prepared with phosphate saline buffer (PBS: 10 mM KH_2PO_4/K_2HPO_4 and 150 mM NaCl with pH adjusted to 7.0). Measurements were done in triplicate.

7.2.4 Contact Angle Measurements

Advancing type contact angles with Millipore water on silicone rubber with and without an adsorbed rhamnolipid layer at several concentrations were measured with an optical contact-measuring KRUSS device using the sessile drop technique. On each sample, at least six droplets were placed at different positions and results of three separately prepared surfaces with adsorbed biosurfactant were averaged. Conditioned silicone rubber was prepared through overnight immersion in a rhamnolipid solution (several rhamnolipid dilutions were tested) at 4ºC. In a second set of experiments after conditioning the silicone rubber with rhamnolipid it was rinsed in PBS solution for half an hour. All the samples were left to air dry before contact angle measurements.

7.2.5 Microbial Strains and Growth Conditions

Four bacterial strains, *Staphylococcus epidermidi*s GB 9/6, *Streptococcus salivarius* GB 24/9, *Staphylococcus aureus* GB 2/1, and *Rothia dentocariosa* GBJ 52/2B and two yeast strains: *Candida albicans* GBJ 13/4A and *Candida tropicalis* GB 9/9 isolated from explanted voice prostheses were used in this study (Elving *et al.* 2002). All strains were first grown overnight at 37°C in ambient air on agar plates from frozen stocks, the agar plates were kept at 4°C, never longer than two weeks. Several colonies were used to inoculate 10 ml of brain heart infusion broth (BHI, OXOID, Basingstoke, England) for all the bacterial and yeast strains in use. This pre-culture was incubated at 37°C in ambient air for 24 h and used to inoculate a second culture of 200 ml that was grown for 18 h. The microorganisms from the second culture were harvested by centrifugation for 5 min at 10,000 × *g* and washed twice with demineralized water. Subsequently, bacterial cells were suspended in 200 ml of PBS solution (PBS: 10 mM KH_2PO_4/K_2HPO_4 and 150 mM NaCl with pH adjusted to 7.0), after sonication on ice (10 s), to a concentration of 3×10^8 ml^{-1}. The sonication procedure did not promote cell lysis. Yeasts were suspended in PBS to a concentration of 3×10^6 ml^{-1}. A Bürker-Türk counting chamber was used to count the cells.

7.2.6 Adhesion Assay in 96 Wells Plate

The anti-adhesive activity of the rhamnolipid biosurfactant against four bacterial strains, *S. epidermidi*s GB 9/6, *S. salivarius* GB 24/9, *S. aureus* GB 2/1, and *R. dentocariosa* GBJ 52/2B and two yeast strains: *C. albicans* GBJ 13/4A and *C. tropicalis* GB 9/9 isolated from explanted voice prostheses was quantified according to a previously reported adhesion assay (Heinemann *et al*. 2000, Stepanovic *et al*. 2000). Briefly, the wells of a sterile 96-well flat-bottomed plastic tissue culture plate with a lid were filled with 200 μl of the rhamnolipid solution to be tested for anti-adhesive activity. Several rhamnolipid dilutions were tested. The undiluted rhamnolipid-containing solution used had a 4 g l^{-1} rhamnolipid concentration. The plate was incubated for 18 h at 4ºC and subsequently washed twice with phosphate-buffered saline (PBS). Control wells contained buffer (PBS) only. An aliquot of 200 μl of a washed bacterial or yeast suspension was added and incubated in the wells for 4 h at 4ºC. Unattached organisms were removed by washing the wells three times with PBS. The adherent microorganisms were fixed with 200 μl of 99% methanol per well, and after 15 min the plates were emptied and left to dry. Then the plates were stained for 5 min with 200 μl of 2% crystal violet used for Gram staining per well. Excess stain was rinsed out by placing the plate under running tap water. After the plates were air dried, the dye bound to the adherent microorganisms was resolubilized with 200 μl of 33% (v/v) glacial acetic acid per well and the

optical density readings of each well were taken at 595 nm. The microtiter-plate anti-adhesion assay allows the estimation of the rhamnolipid concentration that is effective in inhibiting adhesion of the microorganisms studied and constitutes a quick screening for the dilution to use in the flow experiments.

7.2.7 Adhesion Experiments in the Parallel Plate Flow Chamber

The flow chamber and image analysis system have been described in detail before (Sjollema *et al*. 1989). The parallel plate flow chamber consists of a thin sheet of silicone rubber (with or without an adsorded rhamnolipid layer) affixed to a polymethylmethacrylate (PMMA) bottom plate and a top plate made of glass, both with dimensions 5.5 × 3.8 cm. The top and bottom plates were cleaned by sonicating for 3 min in a commercial detergent (2% Sonazol Pril in water, Portugal), rinsed thoroughly with tap water, and then rinsed with demineralized water. Top and bottom plates were subsequently mounted in the housing of the flow chamber, separated by 0.06 cm spacers. Images were taken from the bottom plate with a 3CCD video camera (Axiocam HRC, Zeiss, Germany) mounted on a phase-contrast microscope (Zeiss Axioscop, Zeiss, Germany) equipped with a 40 × ultra-long working distance objective for experiments with bacteria and with a 10 × objective for experiments with yeasts. The camera was coupled to an image analyzer (Image Pro Plus, Media Cybernetics, USA). Each life image (768 x 576 pixels with 8 bit resolution) was obtained after summation of 5 consecutive images (time interval 500 ms) in order to eliminate moving bacteria and to enhance the signal-to-noise ratio and to eliminate moving microorganisms from the analysis.

Conditioned silicone rubber was prepared through overnight immersion in a rhamnolipid solution at 4ºC. Two different sets of experiments were performed, the first with rhamnolipid solution without any dilution and the second with a 1:1000 dilution.

Prior to each experiment, all tubes and the flow chamber were filled with PBS, while care was taken to remove air bubbles from the system. Flasks, containing microbial suspension and buffer, were positioned at the same height with respect to the chamber to ensure that immediately after the flow was started, all fluids would circulate (each fluid at the time) by hydrostatic pressure through the chamber at the desired shear rate of 10 s^{-1} (0.025 ml s^{-1}), which yields a laminar flow (Reynolds number 0.6). The microbial suspension was circulated through the system for 4 h and images were obtained from silicone rubber with or without the adsorbed rhamnolipid. The number of microorganisms per unit area was plotted *versus* time and the initial linear increase in the number of adhering microorganisms with time was expressed by a so-called initial deposition rate j_0 (microorganisms cm^{-2} s^{-1}), i.e. the number of adhering microorganisms per unit area and time. The number of adhering microorganisms

after 4 h was also determined (n_{4h}). All values presented in this work are the averages of triplicate measurements on silicone rubber surfaces with or without the adsorbed rhamnolipid layer and were carried out with separately grown microorganisms.

7.2.8 Detachment Protocol

After the 4 h adhesion period, flow was switched for 30 min to PBS in order to remove non-adhering cells from the tubes and chamber. Then, 15 ml of the rhamnolipid solution (dilution 1:15) was perfused once through the flow chamber, at a flow rate of 0.025 ml s^{-1}, by a second pump connected to the system by a valve downstream of the flow chamber, followed by 30 ml of PBS to clean the system from rinsing components. The time required to pass one dosage (15 ml) of test agent through the system was nearly 4 min. Subsequently the number of organisms still adhering to the silicone rubber was determined. Finally, the flow was switched back to PBS for 1 h in order to remove possibly detached cells and the chamber was emptied by hydrostatic pressure, so that a liquid-air interface could pass over the surface. Finally, the number of adhering organisms withstanding this extremely high removal force was counted again. All experiments were done in duplicate with separate microbial cultures at room temperature.

7.2.9 Statistical Analysis

All the adhesion experiments were compared using one-way analysis of variance (ANOVA) by applying the Levene's test of homogeneity of variances, the Tukey multiple-comparisons test, and also paired samples *t*-test, using SigmaPlot 8.0 software. Student's *t*-test was applied to all the experimental data, for rejection of some experimental values. All tests were performed with a confidence level of 95% (Altman, 1991).

7.3 RESULTS

7.3.1 Surface-activity and Biosurfactant Concentration

Surface tension measurements at ten fold dilution factors up to 10^{-9} of the rhamnolipid containing solution measured by the Ring method are shown in Figure 7.1. The rhamnolipid reached the lowest surface tension of 32 $\pm$ 0.5 mN m^{-1} with a dilution up to 10^{-5} which means that the solution had a strong effect on surface tension at low concentrations. The critical micelle concentration (*cmc*) is achieved with a 10^{-5} dilution of the rhamnolipid solution

therefore, no matter how much we reduce the dilution factor, the surface tension obtained will be nearly the same.

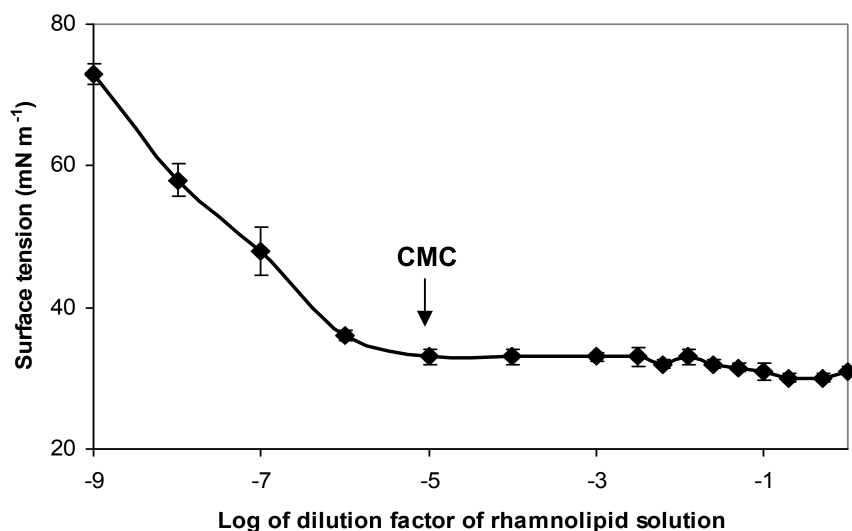


Figure 7.1 Surface tension of several rhamnolipid dilutions in PBS (pH 7.0) as measured by the Ring method. Results are averages of triplicate experiments varying within 2-7% (ANOVA) and the standard deviation represented by error bars.

7.3.2 Anti-adhesive Activity of the Rhamnolipid

The anti-adhesive activity of the rhamnolipid was evaluated at several dilutions and compared against a variety of bacterial and yeast strains isolated from explanted voice prostheses (Table 7.1). The biosurfactant had an anti-adhesive effect against all microorganisms tested but it depends on the dilution factor and microorganism tested. The rhamnolipid biosurfactant showed the highest anti-adhesive activity against *R. dentocariosa* GBJ 52/2B and worked even at a dilution of 100000. *R. dentocariosa* GBJ 52/2B, *S. epidermidis* GB 9/6 and *S. salivarius* GB 24/9 were inhibited till more than 50%. The combined results from surface tension measurements and microtiter-plate anti-adhesion assay permitted the selection of the rhamnolipid concentration to use in the flow experiments. In the adhesion experiments with the parallel-plate flow chamber an undiluted rhamnolipid solution and at 1:1000 dilution were used. In the detachment protocol a 1:15 dilution of the rhamnolipid solution was used.

Table 7.1 Microbial inhibition percentages obtained from the microtiter-plate anti-adhesion assay with several rhamnolipid concentrations. The dilution factor is a direct measure of the rhamnolipid concentration. The undiluted rhamnolipid-containing solution used had a 4 g l^{-1} rhamnolipid concentration. PBS was used as control and set at 0 % as no microbial inhibition occurs. Experiments were carried out in triplicate and correspond within 15%.

Microorganism	**Microbial inhibition (%)**							
	Rhamnolipid dilution factor							
	PBS control	undiluted	2	5	10	100	1000	100000
S. epidermidis GB 9/6	0.0	53.1	48.8	28.2	21.0	16.9	2.7	0.6
S. salivarius GB 24/9	0.0	58.2	55.2	45.1	36.8	19.0	14.3	1.4
S. aureus GB 2/1	0.0	33.8	25.1	22.5	21.5	19.0	4.8	1.3
R. dentocariosa GBJ 52/2B	0.0	60.9	55.2	54.6	49.7	48.1	42.0	31.0
C. albicans GBJ 13/4A	0.0	38.2	32.0	26.4	24.8	23.3	17.1	7.1
C. tropicalis GB 9/9	0.0	35.3	16.9	14.3	13.3	11.8	8.7	2.7

7.3.3 Microbial Adhesion in the Parallel Plate Flow Chamber

Initial deposition rates of the tested bacterial and yeast strains on silicone rubber, before and after coating with the 1:1000 or undiluted rhamnolipid solution, as measured in buffer (PBS) in a parallel plate flow chamber are presented in Figure 7.2. For the microbial strains *R. dentocariosa* GBJ 52/2B, *S. epidermidis* GB 9/6 and *C. tropicalis* GB 9/9 the initial deposition rates on silicone rubber were relatively high between 1716 ± 206, 1793 ± 179 and 2312 ± 254 microorganisms cm^{-2} s^{-1}, respectively, while *S. salivarius* GB 24/9 and *S. aureus* GB 2/1 had a lower deposition rate (1167 ± 105 to 1398 ± 168 microorganisms cm^{-2} s^{-1}). *C. albicans* GBJ 13/4A has the lowest initial deposition rate (163 ± 13 microorganisms cm^{-2} s^{-1}). The adsorption of undiluted rhamnolipid solution on the silicone rubber reduces the initial deposition rate about 40% for *S. epidermidis* GB 9/6, *S. aureus* GB 2/1, *R. dentocariosa* GBJ 52/2B and *C. albicans* GBJ 13/4A, while a higher reduction of about 66% on average for *S. salivarius* GB 24/9 and *C. tropicalis* GB 9/9 was achieved. When coating the silicone rubber with the 1:1000 rhamnolipid dilution a minor effect was observed.

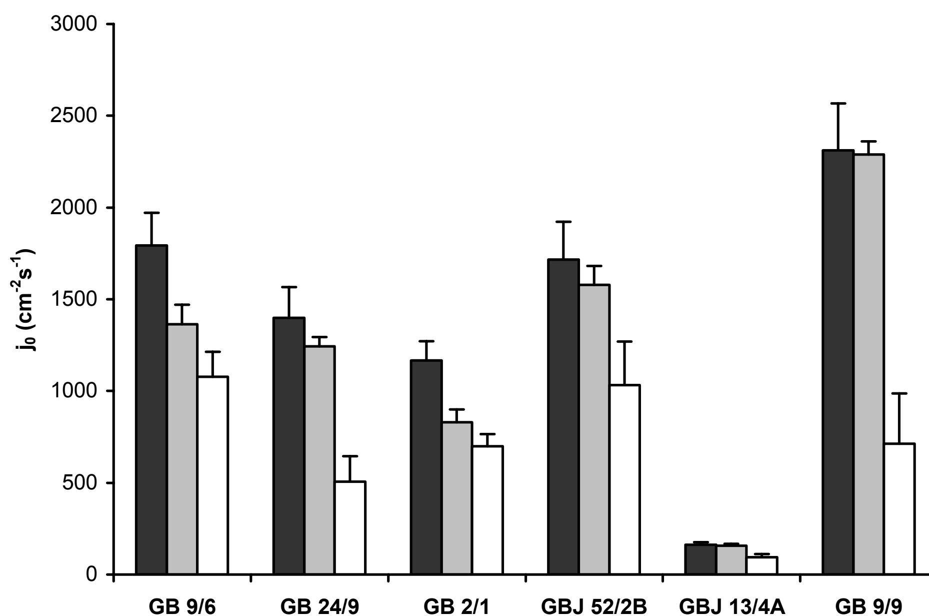


Figure 7.2 The initial deposition rates (j_0) of the bacterial (*S. epidermidis* GB 9/6, *S. salivarius* GB 24/9, *S. aureus* GB 2/1, and *R. dentocariosa* GBJ 52/2B) and yeast (*C. albicans* GBJ 13/4A and *C. tropicalis* GB 9/9) strains isolated from explanted voice prostheses on silicone rubber with and without an adsorbed rhamnolipid layer. The undiluted rhamnolipid-containing solution used had a 4 g l^{-1} rhamnolipid concentration. Black column – without rhamnolipid; grey column – with rhamnolipid dilution 1:1000 and white column – with undiluted rhamnolipid. Results are averages of triplicate experiments varying within 10-15% (ANOVA) and the standard deviation represented by the error bars.

The number of attached microorganisms after 4 h on biosurfactant-coated and uncoated silicone rubber is shown in Figure 7.3. The number of microorganisms adhering to bare silicone rubber after 4 h, (n_{4h}) was between 7×10^6 and 13×10^6 cm^{-2} for all microorganisms studied except for *C. albicans* GBJ 13/4A that exhibit the lowest number of adhering microorganisms (1×10^6 cm^{-2}). Coating the silicone rubber with a 1:1000 dilution of rhamnolipid solution resulted in a number of adhered cells after 4 h similar to the control. Reductions of 50% on the number of cells adhering after 4 h on the silicone rubber conditioned with undiluted rhamnolipid solutions was achieved for *S. epidermidis* GB 9/6, *S. salivarius* GB 24/9, *S. aureus* GB 2/1 and *C. tropicalis* GB 9/9. The yeast strain *C. albicans* GBJ 13/4A as well as the bacterial strain *R. dentocariosa* GBJ 52/2B showed a lower decrease in the number of attached cells after 4 h (20-28%).

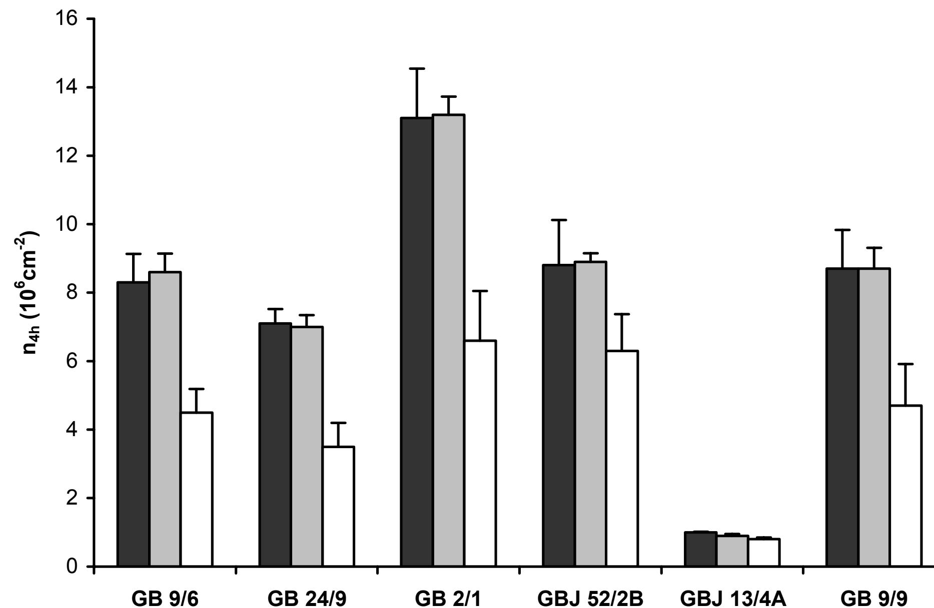


Figure 7.3 The number of microorganisms adhering after 4 h (n_{4h}) on silicone rubber with and without an adsorbed rhamnolipid layer (1:1000 dilution and undiluted rhamnolipid). The codification of the microorganisms is presented in Fig. 7.2 The undiluted rhamnolipid-containing solution used had a 4 g l^{-1} rhamnolipid concentration. Black column – without rhamnolipid; grey column – with rhamnolipid dilution 1:1000 and white column – with undiluted rhamnolipid. Results are averages of triplicate experiments varying within 10-15% (ANOVA) and the standard deviation represented by the error bars.

Although the surface tension measurements give a clear indication of the surface activity of the compound it is not sufficient to determine whether it would produce an anti-adhesive effect against the selected microorganisms. Table 7.2 summarizes contact angles measured with water on silicone rubber with and without an adsorbed rhamnolipid layer at several concentrations. The water contact angle on bare silicone rubber decreased from 115 $\pm$ 1 degrees to 35 $\pm$ 1 degrees after adsorption of the undiluted rhamnolipid. The adsorption of a rhamnolipid dilution up to 10^{-5} decreased the silicone rubber contact angle in a much lesser extent to 89 $\pm$ 1 degrees. From Table 7.2 it is possible to observe a concentration effect as the water contact angle of silicone rubber increases with the dilution factor of the rhamnolipid solution. Moreover, higher water contact angles values were achieved when a rinsing of the conditioned silicone rubber in PBS was performed. The adsorption of dilutions from 10^{-5} to

10^{-2} followed by a PBS rinsing did not produce any effect on the silicone rubber contact angle. The water contact angles results permitted the confirmation of the rhamnolipid concentration to be used in the flow experiments.

Table 7.2 Contact angles (degrees) measured at 25ºC on silicone rubber with and without an adsorbed rhamnolipid layer as determined by the sessile drop technique. Standard deviations (±) were determined over three separate measurements.

Surface	**Contact angles (degrees)**	
	Without rinsing in PBS	**With rinsing in PBS**
Silicone rubber	115 ± 1	115 ± 1
Silicone rubber + PBS	113 ± 1	113 ± 1
Silicone rubber + undiluted rhamnolipid	35 ± 1	55 ± 1
Silicone rubber + rhamnolipid (1:2 dilution)	39 ± 1	59 ± 1
Silicone rubber + rhamnolipid (1:5 dilution)	45 ± 1	86 ± 1
Silicone rubber + rhamnolipid (1:10 dilution)	57 ± 1	89 ± 1
Silicone rubber + rhamnolipid (1:15 dilution)	60 ± 1	94 ± 1
Silicone rubber + rhamnolipid (1:100 dilution)	65 ± 1	113 ± 2
Silicone rubber + rhamnolipid (1:1000 dilution)	71 ± 1	113 ± 2
Silicone rubber + rhamnolipid (1:100000 dilution)	89 ± 1	113 ± 1

The adherence of all the strains studied to the silicone rubber conditioned with the rhamnolipid biosurfactant was significantly different ($p<0.05$, ANOVA and Tukey's multiple comparison test) from the adhesion to the silicone rubber without the rhamnolipid, as proved by the *F*-values that are 5 fold higher than the critical *F*-values (critical value extracted from the *f*-distribution in statistical tables). The replicates of adhesion experiments with and without adsorbed rhamnolipid for each strain were compared and found to be statistically similar. Adhesion (j_0, n_{4h}) on silicone rubber occurred in a larger extent and was several times faster than the adhesion on silicone rubber with adsorbed rhamnolipid ($p< 0.05$, paired samples *t*-test).

7.3.4 Detachment Protocol

Figure 7.4 shows the deposition kinetics of all the microbial strains tested and the subsequent effects of perfusing the rhamnolipid solution (1:15 dilution) through the parallel plate flow chamber and then followed by the passage of a liquid-air interface. Perfusion of the rhamnolipid resulted in 81% desorption on average of adhered cells for *S. epidermidis* GB 9/6, *S. salivarius* GB 24/9, *C. albicans* GBJ 13/4A and *C. tropicalis* GB 9/9. The bacterial strains *S. aureus* GB 2/1 and *R. dentocariosa* GBJ 52/2B showed a lower reduction in the number of adhering cells after 4 h, 21% and 63%, respectively. The combined effect of rinsing with the rhamnolipid solution and passing a liquid-air interface through the flow chamber produced maximum desorption percentages (96%) for all microorganisms studied, except for *S. aureus* GB 2/1 which had the lowest percentage (67%).

Statistical analysis was conducted to compare adhesion experiments with and without rinsing rhamnolipid solution through the flow chamber followed by the passage of a liquid-air interface and it was found that the differences obtained are statistically significant (*F*-values > critical *F*-values), as proved by the *F*-values that are 3 fold higher than the critical *F*-values. Additionally, the comparison of duplicate experiments using one-way analysis of variance (ANOVA) resulted in a 10-15% variation in the results.

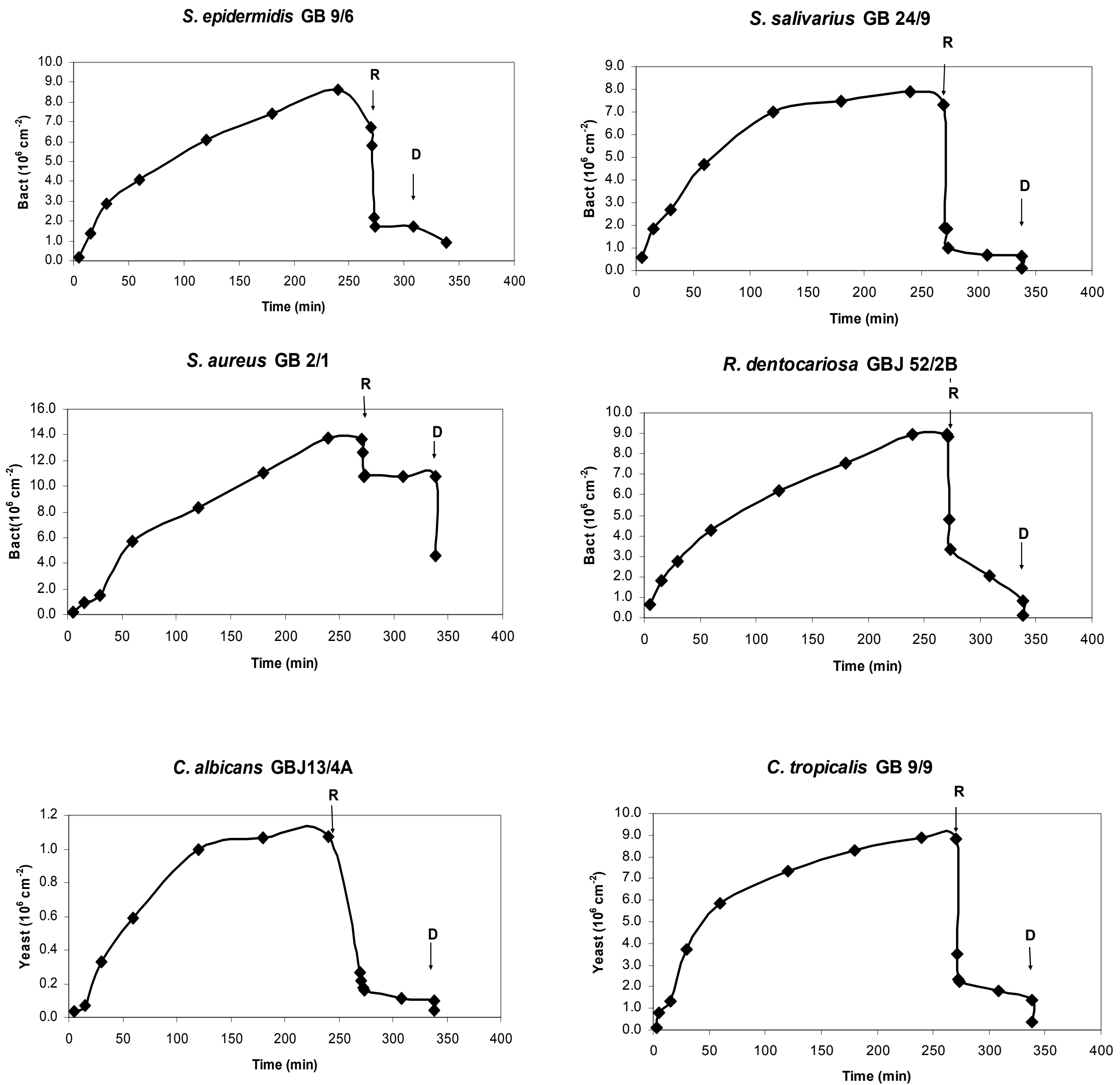


Figure 7.4 Deposition kinetics observed for the microbial strains isolated from explanted voice prostheses adhering to silicone rubber, and the subsequent effects of perfusing a rhamnolipid solution (dilution 1:15) through the chamber followed by the passage of a liquid-air interface. R, denotes the perfusion of the flow chamber with the rhamnolipid solution. D, denotes the passage of a liquid-air interface. Results are averages of duplicates experiments varying within 10-15% (ANOVA).

7.4 DISCUSSION

The extent of adhesion of several microbial strains, associated with biofilm formation on voice prostheses to silicone rubber in the presence and absence of an adsorbed rhamnolipid layer obtained from *P. aeruginosa* DS10-129 were compared using a parallel plate flow chamber system under defined hydrodynamic shear conditions. It is well known that the voice prostheses major weakness relies on the fact that the hydrophobic silicone rubber surface becomes rapidly colonized with a thick biofilm and in this perspective the antifouling improvement of the silicone rubber material is desirable. New technologies for preventing or retarding biofilm formation on voice prostheses have to be developed since the long term use of antimycotic or antibiotic agents may induce the growth of resistant strains with all associated risks. The use of biosurfactants as antimicrobial agents seems to be promising as a method of prolonging lifetimes of voice prostheses (Rodrigues *et al*. 2004a,b). Biosurfactants antimicrobial activity against various microbes has been described before. Surfactin, for example, a cyclic lipopeptide produced by *B. subtilis* strains, is a biosurfactant with well-known antimicrobial properties (Ahimou *et al.* 2000). A new antibiotic from *Pseudomonas fluorescens*, with surface active properties different from those of the known biosurfactant viscosin from the same species, was also reported to have antifungal properties (Singh and Cameotra 2004). Additionally, a biosurfactant obtained from *S. thermophilus* A showed a significant antimicrobial activity against *C. tropicalis* GB 9/9 at low biosurfactant concentrations as presented in Chapter 8 (Rodrigues *et al*. 2004a) and it has been reported that *C. tropicalis* is implicated in premature failure of the prostheses (Elving *et al*. 2002).

In the present case, the extent of bacterial adhesion to silicone rubber conditioned with a rhamnolipid produced by *P. aeruginosa* DS10-129 in a parallel plate flow chamber was significantly reduced in comparison to the untreated silicone rubber. However, coating the silicone rubber with a 1:1000 dilution of the rhamnolipid solution resulted in a lower reduction in the initial deposition rates (lower than 26%) and the number of adhered cells was not affected. From the surface tension measurements it was expected that a 1:1000 rhamnolipid dilution would have an enormous anti-adhesive effect since the lowest surface tension of 32 ± 0.5 mN m^{-1} was achieved, however in the parallel plate flow chamber we did not observe this effect. The number of microorganisms adhering after coating the silicone rubber with undiluted rhamnolipid solution was reduced between 40 - 66% for all strains studied, which is less effective than the biosurfactant from *Lactococcus lactis* 53 described previously in Chapter 5 (Rodrigues *et al*. 2004b) which inhibited 90%

the microbial adhesion for all strains studied, except for *R. dentocariosa* GBJ 52/2B, *C. albicans* GBJ 13/4A and *C. tropicalis* GB 9/9, which had an inhibitory effect between 56-78%.

Although a very low concentration of the rhamnolipid solution was necessary to produce a high reduction in the surface tension it was observed that a relative high concentration is needed to coat the silicone rubber in order to achieve an effect in the adhesion process. These results are believed to be a consequence of a washing out of the rhamnolipid solution layer adsorbed to the silicone rubber, since the rhamnolipid is bond to the surface by Lifshitz-Van der Waals forces that are relatively weak. This conclusion is based on the results obtained from the water contact angle measurements, as clearly demonstrated a concentration effect, as a result of the rhamnolipid adsorption onto the silicone rubber surface, by the lowering of the contact angle with the rhamnolipid concentration increase. Moreover, a rinsing effect was observed as it is possible to see that for all rhamnolipid concentrations studied, the rinsing procedure produced higher contact angles values than before.

The initial deposition rates and numbers of microorganisms adhering in a stationary state are determined by a complicated interplay of hydrophobicity (interfacial free energies), electrostatic interactions, the relative prevalence of specific receptor sites on the microbial cell surfaces and possible biosurfactants produced (Landa *et al*. 1996). Often, high initial deposition rates of a given strain are found on hydrophobic substrates (for example, silicone rubber), presumably due to easy removal of interfacial water from the interacting surfaces, facilitating close approach and adhesion of an organism. Biofilm formation on solid surfaces, for most microorganisms, occurs in direct proportion to the hydrophobicity of the surface, provided that the suspended medium is a simple buffer. Surface-active agents reduce hydrophobic interactions and by doing so reduce microbial adhesion to silicone rubber (Klotz 1990). Clearly, in the detachment protocol, rinsing the flow chamber with the rhamnolipid solution appears effective in disrupting the bond between initially adhering microorganisms and the silicone rubber. Interestingly, the combined effect of rinsing with the rhamnolipid solution and passing a liquid-air interface through the flow chamber produced a 96% reduction on the number of adhered cells for five out of six microorganisms studied, except for *S. aureus* GB 2/1 that exhibit only 67%. It has been demonstrated that the effect of passing an air-liquid interface through adherent cells in the parallel flow chamber induces a shear force of about 10^{-7} N, which is in the range of the adhesion strength evaluated by atomic force microscopy that results in removal of a large proportion of the adherent organisms (Fang *et al*. 2000).

Microbial detachment stimulated by the rhamnolipid solution is more likely due to a proper detergent system rather than an antimicrobial effect. Surface active agents as incorporated into oral hygiene products have not received the attention they merit and have mostly been included into oral hygiene products for their ability to create foam (Landa *et al*. 1996). Rhamnolipids are among the best known biosurfactants and it is well known that there are different structural variants of rhamnose lipids, as the type produced depends on the *Pseudomonas* strain, the carbon source used and the strategy of production. Also, rhamnolipids are known as potent antimicrobial compounds against several microorganisms. In the cosmetic industry, rhamnolipids have been useful because they are compatible with skin and produce extremely low irritation (Haba *et al*. 2000). Benincasa *et al*. (2004) have demonstrated that the rhamnolipid obtained from *P. aeruginosa* LBI is non toxic and has antimicrobial activity against several microorganisms, thus suitable for application in the pharmaceutical and cosmetic industries. Additionally, rhamnolipids are food grade as they are a source of stereospecific L-rhamnose which is used commercially in the production of high-quality flavour compounds (Makkar and Cameotra 2002). Nevertheless, some rhamnolipids have been implicated as virulence factors and influence the immune response (Cosson *et al.* 2002), thus further work has to be done in order to guarantee that this rhamnolipid will not produce undesirable effects in the oropharyngeal environment. Since the detachment results obtained in this study were very promising it would be advantageous to evaluate the biocompatibility of the rhamnolipid for therapeutic application.

7.5 CONCLUSIONS

In conclusion, biosurfactants may have the potential to be used as one of the preventive strategies to delay the onset of biofilm growth on catheters and other implant materials, thus decreasing the large number of hospital infections without increased use of synthetic drugs or chemicals. They may also be used in pulmonary immunotherapy and incorporated into probiotic preparations to combat urogenital tract infections. It has been demonstrated in this study that using a rinse of rhamnolipid from *P. aeruginosa* DS10-129 through a flow chamber significantly reduces the adhesion of a variety of bacterial and yeast strains isolated from explanted voice prostheses to silicone rubber, and therefore may be used as a biodetergent solution for prostheses cleaning, prolonging their lifetime and directly benefiting laryngectomized patients.

7.6 References

Ahimou, F., Jacques, P. and Deleu, M. (2000) Surfactin and iturin A effects on *Bacillus subtilis* surface hydrophobicity. *Enzyme Microbiology and Technology*. 27: 749-754.

Altman, D.G. (1991) In: *Practical statistics for medical research*. Ed. Altman, D.G. London: Chapman & Hall.

Bai, G., Brusseau, M.L. and Miller, R.M. (1997) Influence of a rhamnolipid biosurfactant on the transport of bacteria through a sandy soil. *Applied and Environmental Microbiology*. 63: 1866-1873.

Banat, I.M., Makkar, S. and Cameotra, S. (2000) Potential commercial application of microbial surfactants - A review article. *Applied Microbiology and Biotechnology*. 53: 495-508.

Banat, I.M., Samarah, N., Murad, M., Horne, R. and Banergee, S. (1991) Biosurfactant production and use in oil tank clean-up. *World Journal of Microbiology and Biotechnology*. 7: 80-88.

Benincasa, M., Abalos, A., Oliveira, I. and Manresa, A. (2004) Chemical structure, surface properties and biological activities of the biosurfactant produced by *Pseudomonas aeruginosa* LBI from soapstock. *Antonie Van Leeuwenhoek.* 85: 1–8.

Busscher, H.J., Free, R.H., Van Weissenbruch, R., Albers, F.W.J. and Van der Mei, H.C. (2000) Preliminary observations on influence of dairy products on biofilm removal from silicone rubber voice prostheses *in vitro*. *Journal of Dairy Science.* 83: 641-647.

Cosson, P., Zulianello, L., Join-Lambert, O., Faurisson, F., Gebbie, L., Benghezal, M., Van Delden, C., Curty, L. and Kohler, T. (2002) *Pseudomonas aeruginosa* virulence analyzed in a *Dictyostelium discoideum* host system. *Journal of Bacteriology*. 184: 3027-3033.

Desai, J. and Banat, I.M. (1997) Microbial production of surfactants and their commercial potential. *Microbiology and Molecular Biology Reviews*. 61:47-64.

Elving, G.J, Van der Mei, H.C., Busscher, H.J., Nieuw Amerongen, A., Veerman, E.C., Van Weissenbruch, R. and Albers, F.W.J. (2000) Antimicrobial activity of synthetic salivary peptides against voice prosthetic microorganisms. *Laryngoscope*. 110: 321-324.

Elving, G.J., Van der Mei, H.C., Busscher, H.J., Van Weissenbruch, R. and Albers, F.W.J. (2002) A comparison of the microbial composition of voice prosthetic biofilms from patients requiring frequent *versus* infrequent replacements. *Annals of Otology, Rhinology and Laryngology*. 111: 200-203.

Everaert, E.P.J.M., Mahieu, H.F., Van de Belt-Gritter, B., Peeters, J.G.E., Verkerke, G.J., Van der Mei, H.C. and Busscher, H.J. (1999) Biofilm formation *in vivo* on perfluoro-alkylsiloxane-modified voice prostheses. *Archives of Otolaryngology: Head & Neck Surgery.* 125: 1329-1332.

Fang, H.H., Chan, K.Y. and Xu, L.C. (2000) Quantification of bacterial adhesion forces using atomic force microscopy (AFM). *Journal of Microbiological Methods.* 40: 89-97.

Farber, B.F. and Wolff, A.G. (1993) Salicylic acid prevents the adherence of bacteria and yeast to silastic catheters. *Journal of Biomedical Materials Research*. 27: 599-602.

Flasz, A., Rocha, C.A., Mosquera, B. and Sajo, C. (1998) A comparative study of the toxicity of a synthetic surfactant and one produced by *Pseudomonas aeruginosa* ATCC 55925. *Medical Science Research.* 6: 181-185.

Haba, E., Espuny, M.J., Busquets, M. and Manresa, A. (2000) Screening and production of rhamnolipids by *Pseudomonas aeruginosa* 47T2 NCIB 40044 from waste frying oils. *Journal of Applied Microbiology.* 88: 379-387.

Heinemann, C., Van Hylckama, V.J.E., Janssen, D.B., Busscher, H.J., Van der Mei, H.C. and Reid, G. (2000) Purification and characterization of a surface-binding protein from *Lactobacillus fermentum* RC-14 that inhibits adhesion of *Enterococcus faecalis* 1131. *FEMS Microbiology Letters.* 40: 175-179.

Holly, F. and Owen, M. (1983) In: *Physicochemical Aspects of Polymer Surfaces*. Ed. Mittal, K. New York: Plenum Press.

Kim, S.H., Lim, E.J., Lee, S.O., Lee, J.D. and Lee, T.H. (2000) Purification and characterization of biosurfactants from *Nocardia* sp. L-417. *Biotechnology and Applied Biochemistry.* 31: 249-253.

Klotz, S.A. (1990) Role of hydrophobic interactions in microbial adhesion to plastics used in medical devices. In: *Microbial Cell Surface Hydrophobicity.* Ed. Doyle, R.J. and Rosenberg, M. Washington: American Society of Microbiology. Pp 107-136.

Landa, A.S., Van der Mei, H.C. and Busscher, H.J. (1996) A comparison of the detachment of an adhering oral streptococcal strain stimulated by mouthrinses and a pre-brushing rinse. *Biofouling*. 9: 327-339.

Mahieu, H.F., Van Saene, J.J., Van den Besten, J. and Van Saene, H.K. (1986) Oropharynx decontamination preventing *Candida* vegetation on voice prostheses. *Archives of Otolaryngology: Head and Neck Surgery*. 112: 1090-1092.

Makkar, R.S. and Cameotra, S.S. (2002) An update on the use of unconventional substrates for biosurfactant production and their new applications. *Applied Microbiology and Biotechnology.* 58: 428–434.

Mata-Sandoval, J.C., Karns, J. and Torrents, A. (1999) High-performance liquid-chromatography method for the characterization of rhamnolipids mixtures produced by *Pseudomonas aeruginosa* UG2 on corn oil. *Journal of Chromatography A*. 864: 211-220.

Neu, T.R., Van der Mei, H.C., Busscher, H.J., Dijk, F. and Verkerke, G.J. (1993) Biodeterioration of medical-grade silicone rubber used for voice prostheses: a SEM study. *Biomaterials*. 14: 459-464.

Rahman, K.S., Rhaman, T.J, McClean, S., Marchant, R. and Banat, I.M. (2002b) Rhamnolipid biosurfactant production by strains of *Pseudomonas aeruginosa* using low-cost raw materials. *Biotechnology Progress.* 18: 1277-1281.

Rhaman, K.S, Banat, I.M., Thahira, J., Thayumanavan, T. and Lakshmanaperumalsamy, P. (2002a) Bioremediation of gasoline contaminated soil by a bacterial consortium amended with poultry litter, coir pith and rhamnolipid biosurfactant. *Bioresource Technology.* 81: 25-32.

Rhaman, K.S, Rahman, T.J., Kourkoutas, Y., Petsas, I., Marchant, R. and Banat, I.M. (2003b) Enhanced bioremediation of n-alkane in petroleum sludge using bacterial consortium amended with rhamnolipid and micronutrients. *Bioresource Technology*. 90: 159-168.

Rhaman, K.S, Rahman, T.J., Lakshmanaperumalsamy, P., Marchant, R. and Banat, I.M. (2003a) The potential of bacterial isolates for emulsification with a range of hydrocarbons. *Acta Biotechnologica.* 23: 335-345.

Rodrigues, L.R., Van der Mei, H.C., Teixeira, J. and Oliveira, R. (2004a) Influence of biosurfactants from probiotic bacteria on formation of biofilms on voice prostheses. *Applied and Environmental Microbiology.* 70: 4408-4410.

Rodrigues, L.R., Van der Mei, H.C., Teixeira, J. and Oliveira, R. (2004b) Biosurfactant from *Lactococcus lactis* 53 inhibits microbial adhesion on silicone rubber. *Applied Microbiology and Biotechnology*. 66: 306-311.

Schenk, T., Schuphan, I. and Schmidt, B. (1995) High-performance liquid-chromatographic determination of the rhamnolipids produced by *Pseudomonas aeruginosa*. *Journal of Chromatography A.* 693: 7-13.

Singh, P. and Cameotra, S. (2004) Potential applications of microbial surfactants in biomedical sciences. *Trends in Biotechnology*. 22, 142-146.

Sjollema, J., Busscher, H.J. and Weerkamp, A. (1989) Real-time enumeration of adhering microorganisms in a parallel plate flow cell using automated image analysis. *Journal of Microbiological Methods.* 9: 73-78.

Stepanovic, S., Vukovic, D., Dakic, I., Savic, B. and Svabic-Vlahovic, M. (2000) A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *Journal of Microbiological Methods*. 40: 175-179.

THE INFLUENCE OF BIOSURFACTANTS FROM PROBIOTIC BACTERIA ON THE FORMATION OF BIOFILMS ON VOICE PROSTHESES

"There are no such things as applied sciences, only applications of science".

Pasteur.

In this Chapter the influence of biosurfactants from probiotic bacteria on the formation of voice prosthetic biofilms is evaluated. Biofilms were grown on pre-conditioned voice prostheses with biosurfactants obtained from *Lactococcus lactis* 53 and *Streptococcus thermophilus* A, in an artificial throat model. Both biosurfactants greatly reduced microbial numbers on prostheses and also induced a decrease in the airflow resistance of voice prostheses after biofilm formation. The results achieved represent a promising strategy for prolonging the lifetime of voice prostheses.

8.1 INTRODUCTION
8.2 MATERIALS AND METHODS
8.2.1 Biosurfactant Production
8.2.2 Microbial Growth Inhibition Test
8.2.3 Voice Prostheses
8.2.4 Biofilm Formation
8.2.5 Measurement of Airflow Resistance
8.2.6 Evaluation of Biofilms
8.3 RESULTS
8.3.1 Biosurfactant Antimicrobial Activity
8.3.2 Biofilms Evaluation and Airflow Resistance
8.4 DISCUSSION
8.5 CONCLUSIONS
8.6 REFERENCES

8.1 INTRODUCTION

Voice rehabilitation after laryngectomy (Figure 8.1 A, B and D) using voice prostheses is generally considered to be superior to esophageal speech for most patients. A major drawback, however, involves colonization of the prostheses within several weeks by a thick biofilm that consists of a variety of adhering yeast and bacterial strains that eventually cause leakage of food and liquid, or blocking of the valve increasing the airflow resistance (Neu *et al.* 1993, Everaert *et al.* 1997). Especially adhesion of yeasts is troublesome, as they have the tendency to grow into the silicone rubber, therewith their detachment by naturally occurring shear forces in the oropharyngeal cavity is impeded (Busscher *et al.* 1994a, Everaert *et al.* 1997). As a consequence, indwelling silicone rubber voice prostheses, for example the Groningen button, have to be replaced on average every 3-4 months. However, some patients require more frequent replacements, within 1 or 2 weeks, which can be harmful to the tracheoesophageal fistula. Especially for these patients, antifouling improvement of the silicone rubber material is desirable, and various other pathways have been attempted to retard biofilm formation on indwelling voice prosthesis (Mahieu *et al.* 1986, Van Weissenbruch *et al.* 1997, Elving *et al.* 2000).

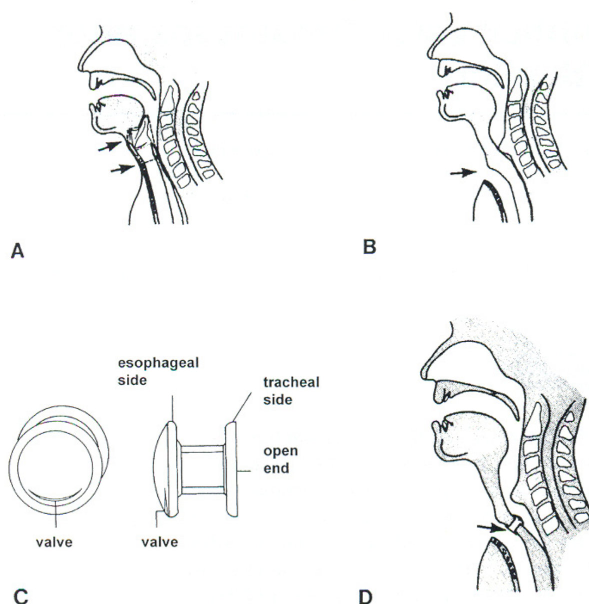


Figure 8.1 (Adapted from Neu *et al.* 1994) **A)** Anatomy of the esophageal region before laryngectomy (arrows indicate parts to be removed). **B)** Anatomy after laryngectomy showing the separation of the airway and the digestive tract (arrow indicates tracheostoma). **C)** Schematic drawing of the Groningen button silicone rubber voice prosthesis. **D)** Groningen button inserted into the tracheo-esophageal shunt (arrow).

Prevention of colonization was partly achieved *in vivo* using antifungal amphotericin B lozenges (10 mg four times daily), which significantly prolonged the lifetime and therewith reduced the airflow of the voice prostheses (Mahieu *et al.* 1986). The use of a buccal bioadhesive slow-release tablet containing antimycotic agents, such as miconazole-nitrate was also reported (Elving *et al.* 2000). Prolonged administration of antifungal agents to patients is, however, uneconomical, undesirable because of the danger to induce resistance, and the compliance of long-term medication is low. Moreover, once infection of polymer devices becomes well established, antimicrobial therapy is usually futile and the only effective course of action is removal of the device. Therefore it would be much more effective if deterioration of the prostheses by microorganisms could be prevented by changing material or its surface characteristics.

As described in previous chapters several researchers attempted to retard biofilm formation on indwelling voice prostheses with varying degrees of success by daily intake of 2 litters of Turkish yoghurt or Kephir containing *Streptococcus thermophilus* and *Lactobacillus bulgaricus* (Van der Mei *et al.* 1999, 2000, Busscher *et al.* 2000). Moreover, laryngectomized patients have suggested that the consumption of buttermilk, containg antimycotic-releasing *Lactococcus lactis*, positively affects the lifetime of voice prostheses. This suggestion has been confirmed in an artificial throat model, in which the effects of daily buttermilk consumption on biofilm formation onto silicone rubber voice prostheses have been simulated (Busscher *et al.* 1998).

All these methods are especially applied in laryngectomized patients with prostheses lifetimes of less than two months, because there is need for employing antifouling therapy from the time of insertion of the voice prostheses, preferably without using antimycotics or antibiotics (Mahieu *et al.* 1986, Foley and Gilbert 1996, Van Weissenbruch *et al.* 1997). The use of biosurfactants from probiotic bacteria as antimicrobial and/or anti-adhesive agents seems to be promising as a method of prolonging lifetimes of voice prostheses as it was demonstrated in Chapter 5 and 6, in a parallel plate flow chamber, their ability to inhibit adhesion of various microorganisms isolated from explanted voice prostheses (Rodrigues *et al.* 2004).

Biofilm formation on surfaces is a natural phenomenon and will arise wherever suitable conditions of moisture, temperature and nutrition exist. It is well accepted that all biofilms originate from the same sequence of events (Busscher *et al.* 1996, Bos *et al.* 1999). The formation of a biofilm is usually considered to start with the adsorption of conditioning film components, transport and adhesion of microorganisms, attachment and growth, possibly

followed by ingrowth of selected organisms, most notably *Candida tropicalis* and *Candida albicans* in case of silicone rubber voice prostheses (Gristina 1987). Although voice prostheses will become covered by a conditioning film of adsorbed salivary components prior to adhesion of bacteria or yeasts, experiments in the human oral cavity have demonstrated that the properties of this conditioning film are determined by the material itself (Busscher *et al.* 1997a). The first link, namely the film deposition, is the anchor from which further formation of a biofilm takes place. The bond represents the link between the solid surface and the growing biofilm (Busscher *et al.* 1995, Reid 1999). If it was possible to weaken or break this link, for example under the influence of fluctuating shear forces or using surfactants (Velraeds *et al.* 1996), it could be feasible for the entire biofilm to detach, thereby aiding and abetting the eradication of infection.

Biosurfactants are surface-active compounds released by microorganisms that have some influence on interfaces, most notably on the surface tension of liquid-vapor interfaces. With regard to an anti-adhesive effect of biosurfactants, hypotheses have been forwarded in which adsorption of biosurfactants to a substratum surface alters the hydrophobicity of the surface and causes interference in microbial adhesion and desorption processes (Neu 1996, Desai and Banat 1997). Microbial adhesion to surfaces is an interplay of the hydrophobicity and charge properties of the interacting surfaces. Therefore, modification of the surface properties of silicone rubber might reduce the adhesion and ingrowth of microorganisms to voice prostheses and therewith improve their lifetime (Mozes *et al.* 1988).

The aim of the present study was to evaluate the extent of biofilm formation by a mixture of several bacterial and yeast strains, isolated from explanted voice prostheses, onto silicone rubber voice prostheses with an adsorbed biosurfactant layer. The two tested biosurfactants were obtained from the probiotic bacteria *L. lactis* 53 and *S. thermophilus* A. A microbial growth inhibition test was first performed in order to estimate the concentration of biosurfactant necessary in the artificial throat experiments. To this end, biofilms are grown on voice prostheses in the artificial throat model (Leunisse *et al.* 1999). In addition to biofilm evaluation, the effects of biosurfactant adsorption to voice prostheses on airflow resistances were determined.

8.2 MATERIALS AND METHODS

8.2.1 Biosurfactant Production

For the selected probiotic bacterial strains *L. lactis* 53 and *S. thermophilus* A, 600 ml cultures in optimized MRS and M17 broth respectively were grown overnight (18 h). The growth medium used for the production of these biosurfactants were previously optimized to obtain higher yields of production (Optimized MRS medium: 38.6 g l^{-1} peptone, 10 g l^{-1} meat extract, 5 g l^{-1} yeast extract, 43 g l^{-1} lactose, 1.08 g l^{-1} Tween®80, 2 g l^{-1} KH_2PO_4, 2 g l^{-1} CH_3COONa, 2 g l^{-1} ammonium citrate, 0.2 g l^{-1} $MgSO_4.7H_2O$ and 0.05 g l^{-1} $MnSO_4.4H_2O$. Optimized M17 medium: 5.0 g l^{-1} peptone, 5 g l^{-1} meat extract, 2.5 g l^{-1} yeast extract, 5.7 g l^{-1} lactose, 5 g l^{-1} soya peptone, 0.25 g l^{-1} $MgSO_4.7H_2O$, 26.4 g l^{-1} sodium glycerophosphate and 0.5 g l^{-1} ascorbic acid) (Chapter 3, Rodrigues *et al.* 2005). Cells were harvested by centrifugation (10000 $\times$ g, 5 min, 10ºC), washed twice with demineralized water, and resuspended in 100 ml of phosphate-buffered saline (PBS: 10 mM KH_2PO_4/K_2HPO_4 and 150 mM NaCl with pH adjusted to 7.0). The bacteria were left at room temperature for 2 h with gentle stirring for biosurfactants production. Subsequently, the bacteria were removed by centrifugation and the remaining supernatant liquid was filtered through a 0.22 μm pore-size filter (Millipore). The supernatant was dialyzed against demineralized water at 4ºC in a Spectrapor membrane tube (molecular weight cut off 6000 to 8000, Spectrum Medical Industries Inc., CA) and freeze-dried. Both biosurfactants were used for further studies, being the biosurfactant 1 obtained from *L. lactis* 53 and the biosurfactant 2 obtained from *S. thermophilus* A.

8.2.2 Microbial Growth Inhibition Test

Four bacterial strains, *Staphylococcus epidermidis* GB 9/6, *Streptococcus salivarius* GB 24/9, *Staphylococcus aureus* GB 2/1, and *Rothia dentocariosa* GBJ 52/2B were used in this study, together with two yeast strains: *Candida albicans* GBJ 13/4A and *Candida tropicalis* GB 9/9 isolated from explanted voice prostheses. The microorganisms were grown aerobically in brain heart infusion broth (OXOID, Basingstoke, England) at 37ºC. Yeasts and bacteria cultured overnight under appropriate conditions were harvested by centrifugation and diluted in reduced transport fluid (NaCl 0.9 g l^{-1}, $(NH_4)_2SO_4$ 0.9 g l^{-1}, KH_2PO_4 0.45 g l^{-1}, $MgSO_4$ 0.19 g l^{-1}, K_2HPO_4 0.45 g l^{-1}, Na_2EDTA 0.37 g l^{-1}, L-Cysteine HCl 0.2 g l^{-1}, pH 6.8) to a concentration allowing confluent growth when plated with a cotton swab on the agar. Yeasts were plated on MRS agar (De Man, Rogosa and Sharpe, Merck), while bacteria were plated on brain heart infusion agar. Agar plates were dried for 20 min at room temperature and 5 μl biosurfactant 1

and 2 solutions of several concentrations (3, 5, 10, 25, 50 and 100 g l^{-1}), were spotted onto the surface of the agar plate. After overnight incubation, the agar plates were screened for growth inhibition zones around the biosurfactant spots. The spectrum of susceptible microorganisms was determined qualitatively. The experiments were scored as positive (+) when growth inhibition was observed; a (±) sign indicated some colonies formed within the zones; and no growth inhibition was marked as negative (-).

8.2.3 Voice Prostheses

"Low Resistance" Groningen button voice prostheses were supplied by Médin Instruments and Supplies (Groningen, The Netherlands). The Groningen button voice prosthesis (Figure 8.1 C) consists of a shaft with two flanges with a semicircular slit of 145º in the hat of the esophageal flange, functioning as a one-way valve. The prostheses are made of implant grade silicone rubber.

8.2.4 Biofilm Formation

Groningen button voice prostheses were placed in modified Robbins devices or artificial throats (Leunisse *et al.* 1999), made of stainless steel (Figure 8.2).

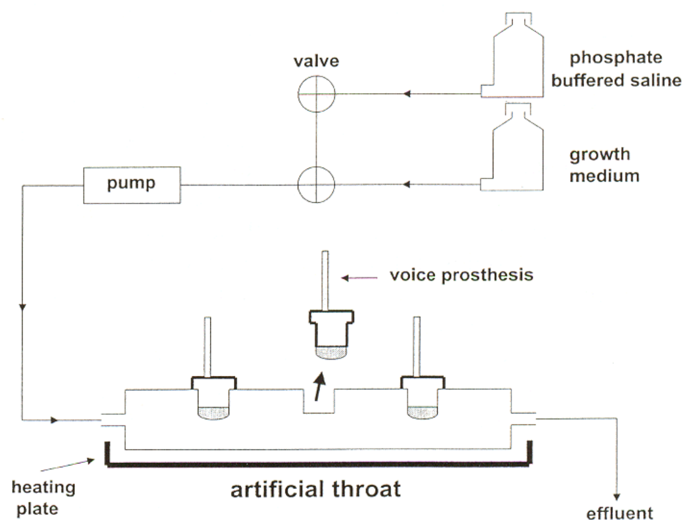


Figure 8.2 Schematic presentation of the artificial throat, equipped with three Groningen button voice prostheses.

Artificial throats were autoclaved before use, equipped with three voice prostheses and maintained at 37ºC on average, as in a laryngectomized patient. In each artificial throat, one voice prostheses was used as a control as no treatment was applied, and the other two voice prostheses were pre-conditioned overnight at 4ºC with 100 g l^{-1} of biosurfactant 1 and 2. To mimic biofilms found in laryngectomized patients, artificial throats were inoculated for five hours with a combination of bacterial and yeast strains, previously isolated from explanted Groningen button voice prostheses from different patients and lifetimes varying from 1-29 months. This combination comprised *S. epidermidi*s GB 9/6, *S. salivarius* GB 24/9, *S. aureus* GB 2/1, *R. dentocariosa* GBJ 52/2B, *C. albicans* GBJ 13/4A and *C. tropicalis* GB 9/9 and was cultured in a mixture of 30% brain heart infusion broth and 70% defined yeast medium (per liter: 7.5 g glucose, 3.5 g $(NH_4)_2SO_4$, 1.5 g L-asparagine, 10 mg L-histidine, 20 mg DL-methionine, 20 mg DL-tryptophane, 1 g KH_2PO_4, 500 mg $MgSO_4.7H_2O$, 500 mg NaCl, 500 mg $CaCl_2.2H_2O$, 100 mg yeast extract, 500 μg H_3BO_3, 400 μg $ZnSO_4.7H_2O$, 120 μg Fe(III)Cl_3, 200 μg $Na_2MoO_4.2H_2O$, 100 μg KI, 40 μg $CuSO_4.5H_2O$). After inoculation, a biofilm was allowed to grow on the voice prostheses during three days by filling the devices with growth medium. From day four till day seven the artificial throats were perfused three times a day with 250 ml of PBS. After each perfusion with PBS, the prostheses were left in the moist environment of the artificial throats. At the end of each day the devices were filled with growth medium during half an hour and left overnight in the moist environment of the drained artificial throats. The tracheal sides of the prostheses were left in ambient air, similar to the situation with a stoma. Previously, this cycle of feast and famine has been demonstrated essential to grow biofilms with features as found on explanted prostheses (Leunisse *et al.* 1999). After establishing the best treatment to apply to the voice prostheses, all experiments were carried out in triplicate.

8.2.5 Measurement of Airflow Resistances

Compressed air was blown through each voice prosthesis prior to biofilm formation and as covered with a 7-day-old biofilm. Air pressures were varied from 10 to 20 cm H_2O and the resulting airflow (l s^{-1}) through the prostheses was measured using a flow head, calibrated with a Brooks flow meter. The pressure was measured just before the valve of the voice prostheses with a pressure transducer, calibrated against a water manometer. The pressure range applied corresponds to clinically relevant conditions during tracheoesophageal shunt speech and yielded a linear relationship between air pressure and flow. For all the prostheses the pressure was measured three times and the mean was calculated. From the linear trajectory between air pressure and flow, an airflow resistance (cm H_2O s l^{-1}) was

calculated by regression analysis (Verkerke *et al.* 2001). As the airflow resistance of individual voice prostheses prior to biofilm formation differed due to manufacturing, all airflow resistances measured were expressed relative to the airflow resistance of the same prosthesis prior to biofilm formation.

8.2.6 Evaluation of Biofilms

On the eighth day of an experiment, voice prostheses were removed from the artificial throats after a final perfusion with 250 ml of PBS. After measuring the airflow resistances of the prostheses as described above, biofilm formation on the valve side of the prostheses was used to determine the number of colony forming units (CFU's). Biofilms were removed by scraping and sonication (ice, 10 s) and subsequently serially diluted. After plating bacteria on blood agar and yeast on MRS agar, plates were stored at 37ºC in an aerobic incubator for three days prior to enumeration. The number of bacterial and yeast CFU's on each prosthesis was determined separately and expressed as a percentage with respect to the control.

8.3 RESULTS

8.3.1 Biosurfactant Antimicrobial Activity

Table 8.1 summarizes the antimicrobial activities of the biosurfactants evaluated at several concentrations against a variety of bacterial and yeast strains isolated from explanted voice prostheses. Both biosurfactants are antimicrobial agents but, depending on the microbial strain, there are different effective concentrations that must be employed. All concentrations of both biosurfactants were effective against *C. tropicalis* GB 9/9. At the highest concentration tested (100 g l^{-1}) both biosurfactants were active against all bacterial and yeast strains involved in this study, except for *R. dentocariosa* GBJ52/2B, which formed some colonies within the biosurfactant spots. From the results of the microbial growth inhibition test a concentration of 100 g l^{-1} of biosurfactant solutions was chosen for pre-conditioning the voice prostheses.

Table 8.1 Antimicrobial activity of biosurfactants with different concentrations against several bacterial and yeast strains isolated from explanted voice prostheses. Biosurfactant 1 was obtained from *L. lactis* 53 and biosurfactant 2 from *S. thermophilus* A. The experiments were scored as positive (+) when growth inhibition was observed; a (±) sign indicated some colonies formed within the zones; and no growth inhibition was marked as negative (-).

Biosurfactant 1					
Microorganism	**5 g l^{-1}**	**10 g l^{-1}**	**25 g l^{-1}**	**50 g l^{-1}**	**100 g l^{-1}**
S. epidermidis GB 9/6	±	±	+	+	+
S. salivarius GB 24/9	-	-	±	±	+
S. aureus GB 2/1	±	±	±	+	+
R. dentocariosa GBJ 52/2B	-	-	±	±	±
C. albicans GBJ 13/4A	-	±	±	+	+
C. tropicalis GB 9/9	+	+	+	+	+
Biosurfactant 2					
Microorganism	**3 g l^{-1}**	**5 g l^{-1}**	**10 g l^{-1}**	**50 g l^{-1}**	**100 g l^{-1}**
S. epidermidis GB 9/6	±	±	±	+	+
S. salivarius GB 24/9	-	-	±	±	+
S. aureus GB 2/1	-	-	±	±	+
R. dentocariosa GBJ 52/2B	-	-	±	±	±
C. albicans GBJ 13/4A	-	-	±	+	+
C. tropicalis GB 9/9	+	+	+	+	+

8.3.2 Biofilms Evaluation and Airflow Resistance

Table 8.2 summarizes the percentage of bacteria and yeasts on the esophageal side of Groningen button voice prostheses. Both biosurfactants 1 and 2 showed a significant decrease of the amount of bacteria in the biofilm to 4% and 13% of the control, respectively. Also, yeasts prevalence was diminished in biofilms formed upon voice prostheses treated with biosurfactant solutions as compared with the control. Biosurfactant 1 reduced the amount of yeasts in the biofilm to 15% of the control, while biosurfactant 2 reduced the yeasts to 26%.

Table 8.2 The percentage of viable bacteria and yeasts isolated from the voice prostheses, with and without adsorbed biosurfactants, after biofilm formation in the artificial throat. Both for bacteria and yeasts, the number of organisms found after using PBS as a control was set at 100%. Also included are the decreases in airflow resistance caused by biofilms influenced by biosurfactants, compared with the effects of PBS as a control. The relative decrease in airflow resistance caused by the control was set as 0 cm $H_2O.s\ l^{-1}$. Biosurfactant 1 was obtained from *L. lactis* 53 and biosurfactant 2 from *S. thermophilus* A. All experiments were carried out in triplicate with separately cultured strains.

Agents tested	Percentage of bacteria[a]	Percentage of yeasts[a]	Decrease in airflow resistance (cm H_2O s l^{-1})[a]
Biosurfactant 1	4 ± 2	15 ± 3	-16 ± 6
Biosurfactant 2	13 ± 2	26 ± 1	-22 ± 6
PBS	100[b]	100[b]	0[c]

[a] Significant differences from the control (paired Student *t* test, $p<0.05$). Standard deviations represented by (±).

[b] The number of bacterial and yeast colony forming units of the control amounts respectively 6.7×10^6 and 1.4×10^5 per cm^2 on the esophageal surface of the "Low Resistance" Groningen button voice prosthesis.

[c] PBS causes an increase in airflow resistance of 31 ± 9 cm H_2O s l^{-1}.

Figure 8.3 shows pressure-flow diagrams of Groningen button voice prostheses prior to and after a 7-day-old biofilm formation for voice prostheses pre-conditioned with biosurfactant 1 and 2. The airflow resistance increases slightly after the formation of a biofilm, as indicated by the slope of the line. However, a 7–day old biofilm in the control voice prostheses yield a higher increase in airflow resistance than both biosurfactants.

In Table 8.2 the airflow resistance of biofilms as influenced by the different biosurfactants tested is summarized. The airflow resistance of the Groningen button voice prostheses used prior to biofilm formation amounted 66 ± 7 cm H_2O s l^{-1}, as averaged over all 9 prostheses involved in this study. The airflow resistances of prostheses increased on average by 31 ± 9 cm H_2O s l^{-1} after 7 days of biofilm formation and perfusion of the artificial throats with PBS (control). Both biosurfactants 1 and 2 caused significant decreases (Student *t*-test, $p<0.05$) in airflow resistance of 16 and 22 cm H_2O s l^{-1} respectively, as compared with the control.

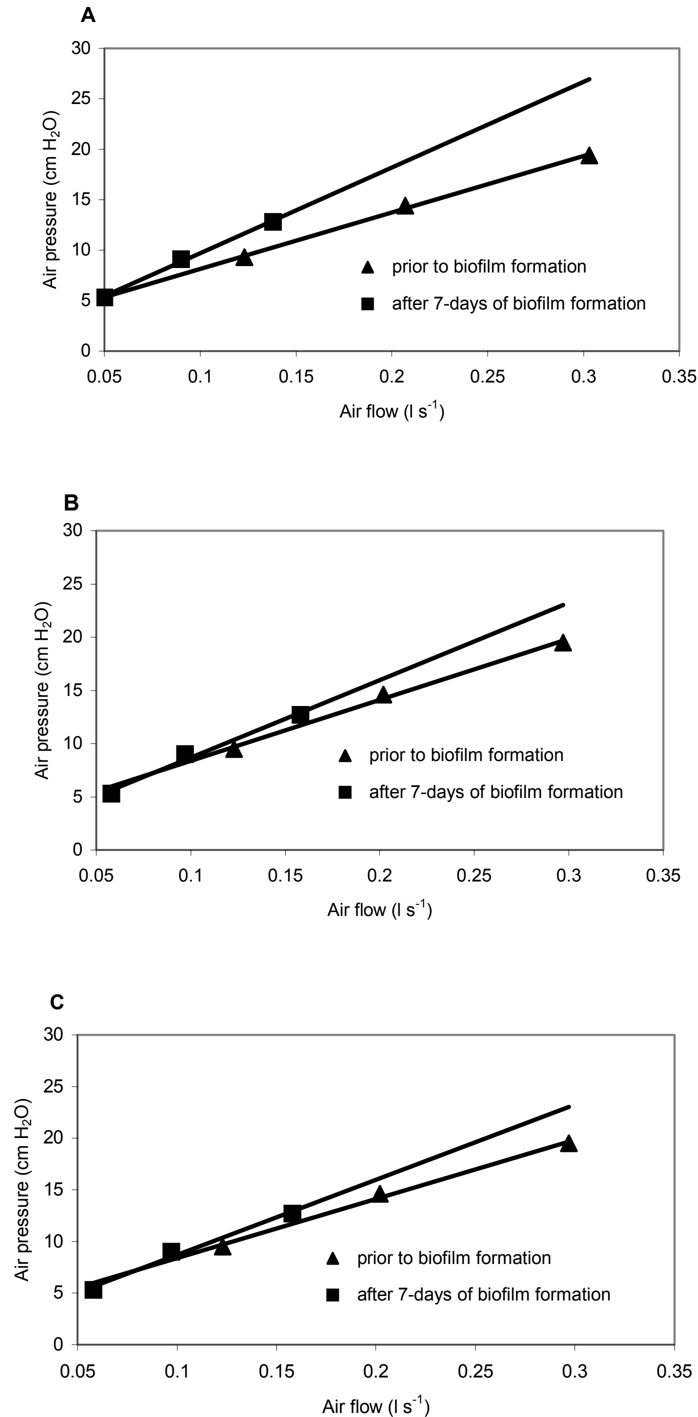


Figure 8.3 Examples of pressure-flow diagrams of “Low-Resistance” Groningen button voice prostheses prior and after 7-days of biofilm formation in the artificial throat: (A) control voice prosthesis; (B) voice prosthesis pre-conditioned with biosurfactant 1; (C) voice prosthesis pre-conditioned with biosurfactant 2.

8.4 DISCUSSION

The two major reasons for replacement of silicone rubber voice prostheses are patient's complaints about leakage of esophageal contents into the trachea or increasing efforts to produce phonation. These signs of failure of voice prostheses are both due to biofilm formation (Neu *et al.* 1993). In the present study, biosurfactants obtained from the probiotic bacteria *L. lactis* 53 (biosurfactant 1) and *S. thermophilus* A (biosurfactant 2), were studied for their ability to influence the biofilm formation on the esophageal side of Groningen button voice prostheses, as well as their airflow resistances. Moreover, the antimicrobial activity was determined of the biosurfactants against the bacterial and yeast strains isolated from explanted voice prostheses. These biosurfactants have been used for this study, because it was demonstrated in Chapter 5 and 6 that the extent of adhesion of several bacterial and yeast strains, isolated from explanted voice prostheses, to silicone rubber with adsorbed biosurfactants from probiotic bacteria in a parallel plate flow chamber was greatly decreased with respect to adhesion to bare silicone rubber (Rodrigues *et al.* 2004). However, in the parallel plate flow chamber the effects of biosurfactants were carried out using a single strain, while in the artificial throat model, a combination of those single strains is used in the biofilm formation to mimic biofilms found in laryngectomized patients.

The mechanisms by which a biosurfactant discourages microbial adhesion are not fully elucidated, but the main physiological role of biosurfactants is to facilitate the uptake of water-immiscible substrates by lowering the surface tension at the phase boundary, emulsification, and enabling the microbial cell to adhere to the organic compounds (Fiechter 1992, Hommel and Ratledge 1993). Biosurfactants can also exhibit antimicrobial activity against various microbes (Pratt-Terpstra *et al.* 1989, Jenny *et al.* 1993, Kitamoto *et al.* 1993) and this item is of great importance since laryngectomized patients with tracheoesophageal speech by using voice prosthesis are mainly benefited by a frequent and long-term antifouling therapy. In case of patients with voice prosthesis lifetimes less than two months, there is need for employing antifouling therapy from the time of insertion of the voice prostheses, preferably without using antimycotics or antibiotics because of the risk of inducing resistant strains (Mahieu *et al.* 1986, Foley and Gilbert 1996, Van Weissenbruch *et al.* 1997). Interestingly, it was found that both biosurfactants show a high antimicrobial activity against *C. tropicalis* GB 9/9 using low biosurfactant concentrations. *C. tropicalis* (Neu *et al.* 1994, Eving *et al.* 2002) was only identified in a voice prostheses short lifetime group of patients and never in the extended lifetime group, therefore it can be concluded that this microorganism contributes for the premature failure of the prostheses. Moreover, in the

literature, there seems to be agreement that *Candida* species are mainly responsible for deterioration of silicone rubber voice prostheses (Mahieu *et al.* 1986). In order to slow down the colonization of voice prostheses by yeast, otolaryngologists frequently apply oropharyngeal yeast decontamination by using antifungal agents. However, there is no compelling evidence that the prescription of antifungal agents will prolong the lifetime of voice prostheses thus alternative approaches are needed. Unfortunately, both biosurfactants were not so effective in the inhibition of *R. dentocariosa* GBJ 52/2B growth, even using high biosurfactant concentrations. Elving *et al*. (2002) recently demonstrated that *R. dentocariosa* was the most frequently isolated bacterial strain in a short lifetime group of patients, suggesting its association with prosthesis failure. Therefore, the exclusion of this bacterial strain from the oral microflora, by selected antibiotics or salivary peptides might well be more effective than the currently applied antimycotic regime, with no proven clinical efficacy.

Preventing or retarding a biofilm formation implies that its necessary to interfere in the weakest link, which means in the formation of the conditioning film and adhesion of the first microorganisms (Rodrigues *et al.* 2004). The use of biosurfactants adsorbed to silicone rubber voice prostheses is believed to interfere with the formation of this conditioning film since both biosurfactants tested reduced the number of bacteria and yeasts found in the biofilms. By consequence, biofilm formation can be influenced by adjusting the properties of the voice prosthesis material.

A reduction in the number of yeasts found in the biofilms may be beneficial, as proliferation of yeasts on the prosthesis surface is generally considered to be the main cause of device failure. Amongst both the biosurfactants studied, biosurfactant 1 (obtained from *L. lactis* 53) caused the greatest reduction in the prevalence of yeasts in the biofilms. According to the literature, some patients have suggested that the consumption of buttermilk, containg antimycotic-releasing *L. lactis*, positively affects the lifetime of voice prostheses. This suggestion has been confirmed in an artificial throat model, in which the effects of daily buttermilk consumption on biofilm formation onto silicone rubber voice prostheses have been simulated (Busscher *et al.* 1998). On the other hand, biosurfactant 2 (obtained from *S. thermophilus*) also provided a high reduction in the number of microorganisms found in the biofilms. *S. thermophilus* releases anti-adhesive biosurfactants, that have been demonstrated to cause their own desorption (Busscher *et al.* 1994b), to interfere with the initial deposition of *C. albicans* and *C. tropicalis* to silicone rubber (Busscher *et al.* 1997b), as well as to interfere on the formation of a mixed fungal/bacterial biofilm on silicone rubber voice prostheses in the modified Robbins device (Busscher *et al.* 1999).

The decrease in airflow resistance is also suggests that the integrity of the biofilm was affected. The integrity of biofilms on voice prostheses causes unwanted increases in airflow resistance impeding speech and is ensured by extracellular polymeric substances (EPS). The EPS embedding organisms in a biofilm on biomaterials implants are generally held responsible for the poor influence antibiotics or antimycotics have on biofilm infections and the lack of impact the host immune system has on biomaterials-associated infections (Decho and Kawaguchi 1999). Elving *et al.* (Elving *et al.* 2003) suggested that the thickness of biofilms is not the most important issue in valve failure, but rather the combined presence of EPS-producing bacterial strains and yeast species.

8.5 CONCLUSIONS

Concluding, this study demonstrated that the adsorption of biosurfactants from probiotic bacteria, *L. lactis* 53 and *S. thermophilus* A, to silicone rubber voice prostheses improve their antifouling properties. The approach developed in this study is a promising strategy as it was demonstrated that the biosurfactants adsorbed inhibit biofilm formation and the occurrence of increased airflow resistances As a consequence, the lifetime of voice prostheses may be lengthened which would directly benefit laryngectomized patients.

8.6 REFERENCES

Bos, R., Van der Mei, H.C. and Busscher, H.J. (1999) Physico-chemistry of initial microbial adhesive interactions – its mechanisms and methods to study. *FEMS Microbiology Reviews.* 23: 179-230.

Busscher, H. J., Bos, R. and Van der Mei, H.C. (1995) Initial microbial adhesion is a determinant for the strength of biofilm adhesion. *FEMS Microbiology Letters.* 128: 229-234.

Busscher, H. J., Bruinsma, G., Van Weissenbruch, R., Leunisse, C., Van der Mei, H.C., Dijk, F. and Albers, F.W. (1998) The effect of buttermilk consumption on biofilm formation on silicone rubber voice prostheses in an artificial throat. *European Archives of Otorhinolaryngology*. 255: 410-413.

Busscher, H. J., Geertsema-Doornbusch, G.I. and Van der Mei, H.C. (1997) Adhesion to silicone rubber of yeasts and bacteria isolated from voice prostheses: influence of salivary conditioning films. *Journal of Biomedical Materials Research*. 34: 201-210.

Busscher, H. J., Neu, T.R. and Van der Mei, H.C. (1994a) Biosurfactant production by thermophilic dairy streptococci. *Applied Microbiology and Biotechnology.* 41: 4-7.

Busscher, H. J., Van Hoogmoed, C.G., Geertsema-Doornbusch, G.I., Van der Kuijl-Booij, M. and Van der Mei, H.C. (1997) *Streptococcus thermophilus* and its biosurfactants inhibit adhesion by *Candida* spp. on silicone rubber. Applied and Environmental Microbiology. 10: 3810-3817.

Busscher, H.J., De Boer, C.E., Verkerke, G.J., Kalicharan, R., Schutte, H.K. and Van der Mei, H.C. (1994b) *In vitro* ingrowth of yeasts into medical grade silicone rubber. *International Biodeterioration and Biodegradation.* 33: 383-390.

Busscher, H.J., Free, R.H., Van Weissenbruch, R., Albers, F.W.J. and Van der Mei, H.C. (2000) Preliminary observations on influence of dairy products on biofilm removal from silicone rubber voice prostheses *in vitro*. *Journal of Dairy Science.* 83: 641-647.

Busscher, H.J., Geertsema-Doornbusch, G.I., Everaert, E.P., Verkerke, G.J., Van de Belt-Gritter, B., Kalicharan, R. and Van der Mei, H.C. (1996) Biofilm formation and silicone rubber surface modification in the development of a total artificial larynx. *In: Surgery and Prosthetic voice restoration after total and subtotal laryngectomy.* Ed. Algaba, J. Amesterdam: Elsevier Science. Pp. 47-52.

Busscher, H.J., Van de Belt-Gritter, B., Westerhof, M. Van Weissenbruch, R., Albers,F.W. and Van der Mei, H.C (1999) Microbial interference in the colonization of silicone rubber implant surfaces in the oropharynx: Streptococcus thermophilus against a mixed fungal/bacterial biofilm. *In: Microbial ecology and infectious disease*. Ed. Rosenberg, E. Washington D.C.: American Society for Microbiology. Pp. 66-74.

Decho, A. W. and Kawaguchi, T. (1999) Confocal imaging of *in situ* natural microbial communities and their extracellular polymeric secretions using Nanoplast resin. *Biotechniques.* 27: 1246-1252.

Desai, J. and Banat, I.M. (1997) Microbial production of surfactants and their commercial potential. *Microbiology and Molecular Biology Reviews*. 61:47-64.

Elving, G. J., Van der Mei, H.C., Busscher, H.J., Van Weissenbruch, R. and Albers, F.W. (2003) Influence of different combinations of bacteria and yeasts in voice prosthetic biofilms on the airflow resistances of prostheses. *Antonie Van Leeuwenhoek.* 83: 45-55.

Elving, G. J., Van der Mei, H.C., Van Weissenbruch, R., Albers, F.W. and Busscher, H.J. (2000) Effect of antifungal agents on indwelling voice prosthetic biofilms. *Current Opinion in Otolaryngology: Head & Neck Surgery.* 8: 165-168.

Elving, G.J., Van der Mei, H.C., Busscher, H.J., Van Weissenbruch, R. and Albers, F.W.J. (2002) A comparison of the microbial composition of voice prosthetic biofilms from patients requiring frequent *versus* infrequent replacements. *Annals of Otology, Rhinology and Laryngology*. 111: 200-203.

Everaert, E. P., Mahieu, H.F., Wong Chung, R.P., Verkerke, G.J., Van der Mei, H.C. and Busscher, H.J. (1997) A new method for *in vivo* evaluation of biofilms on surface-modified silicone rubber voice prostheses. *European Archives of Otorhinolaryngology.* 254: 261-263.

Fiechter, A. (1992) Biosurfactants: moving towards industrial application. *Tibtech.* 10: 208-217.

Foley, I. and Gilbert, P. (1996) Antibiotic resistance of biofilms. *Biofouling*. 10: 334-346.

Gristina, A.G. (1987) Biomaterial-centered infection. Microbial adhesion *versus* tissue integration. *Science*. 237: 1588-1595.

Hommel, R. K. and Ratledge, C. (1993) Biosynthetic mechanisms of low molecular weight surfactants and their precursor molecules. *In: Biosurfactants: production, properties and applications*. Ed. Kosaric, N. New York: Marcel Dekker. Pp. 3-63.

Jenny K., Deltrieu, V. and Kappeli, O. (1993) Lipopeptide production by *Bacillus licheniformis*. *In: Biosurfactants: production, properties and applications*. Ed. Kosaric, N. New York: Marcel Dekker. Pp. 135-156.

Kitamoto, D., Yanagishita, H., Shinbo, T., Nakane, T., Kamisawa, C. and Nakahara, T. (1993) Surface active properties and antimicrobial activities of mannosylerythritol lipids as biosurfactants produced by *Candida antartica*. *Journal of Biotechnology.* 29: 91-96.

Leunisse, C., Van Weissenbruch, R., Busscher, H.J., Van der Mei, H.C. and Albers, F.W. (1999) The artificial throat: a new method for standardization of *in vitro* experiments with tracheo-esophageal voice prostheses. *Acta Otolaryngologica.* 119: 604-608.

Mahieu, H.F., Van Saene, J.J., Van den Besten, J. and Van Saene, H.K. (1986) Oropharynx decontamination preventing *Candida* vegetation on voice prostheses. *Archives of Otolaryngology: Head and Neck Surgery*. 112: 1090-1092.

Mozes, N., Léonard, A.J. and Rouxhet, P.G. (1988) On the relation between the elemental surface composition of yeasts and bacteria and their charge and hydrophobicity. *Biochimica et Biophysica Acta.* 945: 324-334.

Neu, T.R. (1996) Significance of bacterial surface-active compounds in interactions of bacteria with interfaces. *Microbiology Reviews.* 60: 151-166.

Neu, T.R., Van der Mei, H.C., Busscher, H.J., Dijk, F. and Verkerke, G.J. (1993) Biodeterioration of medical-grade silicone rubber used for voice prostheses: a SEM study. *Biomaterials*. 14: 459-464.

Neu, T.R., Verkerke, G.J., Herrmann, I.F., Schutte, H.K., Van der Mei, H.C. and Busscher, H.J. (1994) Microflora on explanted silicone rubber voice prostheses: taxonomy, hydrophobicity and electrophorectic mobility. *Journal of Applied Bacteriology*. 76: 521-528.

Pratt-Terpstra I. H., Weerkamp, A.H. and Busscher, H.J. (1989) Microbial factors in a thermodynamic approach of oral streptococcal adhesion to solid substrata. *Journal of Colloid Interface Science*. 129: 568-574.

Reid, G. (1999) Biofilms in infectious disease and on medical devices. *International Journal of Antimicrobial Agents.* 11: 223-226.

Rodrigues, L.R., Teixeira, J., Oliveira, R. and Van der Mei, H.C. (2005). Response surface optimization of the medium components for the production of biosurfactants by probiotic bacteria. *Process Biochemistry.* In Press

Rodrigues, L.R., Van der Mei, H.C., Teixeira, J. and Oliveira, R. (2004) Biosurfactant from *Lactococcus lactis* 53 inhibits microbial adhesion on silicone rubber. *Applied Microbiology and Biotechnology*. 66: 306-311.

Van der Mei, H.C., Free, R.H., Elving, G.J., Van Weissenbruch, R., Albers, F.W. and Busscher, H.J. (2000) Effect of probiotic bacteria on prevalence of yeasts in oropharyngeal biofilms on silicone rubber voice prostheses *in vitro*. *Journal of Medical Microbiology.* 49: 713-718.

Van der Mei, H.C., Van de Belt-Gritter, B., Van Weissenbruch, R., Dijk, F., Albers, F.W. and Busscher, H.J. (1999) Effect of consumption of dairy products with probiotic bacteria on biofilm formation on silicone rubber implant surfaces in an artificial throat. *Trans IChemE.* 77: 156-158.

Van Weissenbruch, R., Bouckaert, S., Remon, J., Nelis, H.J., Aerts, R. and Albers, F.W. (1997) Chemoptophylaxis of fungal deterioration of the Provox prosthesis. *Annals of Otology, Rhinology and Laryngology.* 106: 329-337.

Velraeds, M.C., Van der Mei, H.C., Reid, G. and Busscher, H.J. (1996) Inhibition of initial adhesion of uropathogenic *Enterococcus faecalis* by biosurfactants from *Lactobacillus* isolates. *Applied and Environmental Microbiology*. 62: 1958-1963.

Verkerke, G. J., Geertsema, A.A. and Schutte, H.K. (2001) Airflow resistance of airflow-regulating devices described by independent coefficients. *Annals of Otology, Rhinology and Laryngology*. 110: 639-645.

CHAPTER 9

LOW COST FERMENTATIVE MEDIUM FOR BIOSURFACTANT PRODUCTION BY PROBIOTIC BACTERIA

"I keep the subject of my inquiry constantly before me, and wait till the first dawning opens gradually (…) into a full and clear light ".

Isaac Newton.

In this section the potential use of alternative fermentative medium for biosurfactant production by probiotic strains is discussed. Suitable models were established to describe the response of the experiments pertaining to glucose, lactose or sucrose consumption, cell growth and biosurfactant production. When the synthetic media (MRS or M17) were replaced by cheaper alternative media (cheese whey and molasses) fermentations were carried out effectively with high yields of biosurfactant production and 60 to 80% medium preparation costs reduction. This is an attractive alternative as many of the potential applications for biosurfactants depend on whether they can be produced economically.

9.1 NOMENCLATURE

9.2 INTRODUCTION

9.3 MATERIALS AND METHODS

9.3.1 Microorganisms and Inoculums

9.3.2 Fermentation Experiments

9.3.3 Cheese Whey Preparation

9.3.4 Molasses Preparation

9.3.5 Bacterial Growth Determination

9.3.6 Sugar Analysis

9.3.7 Surface-activity Determination

9.3.8 Sugar Consumption, Biosurfactant Production and Biomass Growth

9.4 RESULTS

9.4.1 Biosurfactant Production Using Conventional Synthetic Medium

9.4.2 Biosurfactant Production Using Cheese Whey

9.4.3 Biosurfactant Production Using Molasses

9.5 DISCUSSION

9.6 CONCLUSIONS

9.7 REFERENCES

9.1 NOMENCLATURE

F value	*F* test statistical parameter
P	Biosurfactant concentration ($g\ l^{-1}$)
P_0	Initial biosurfactant concentration ($g\ l^{-1}$)
P_{max}	Maximum biosurfactant concentration ($g\ l^{-1}$)
P_r	Ratio between initial volumetric rate of biosurfactant formation (r_p) and initial biosurfactant concentration P_0 (h^{-1})
r^2	Correlation coefficient
r_p	Initial volumetric rate of biosurfactant production ($g\ l^{-1}\ h^{-1}$)
S	Substrate (glucose or lactose) concentration ($g\ l^{-1}$)
S_0	Initial substrate (glucose or lactose) concentration ($g\ l^{-1}$)
X	Biomass concentration ($g\ l^{-1}$)
X_0	Initial biomass concentration ($g\ l^{-1}$)
X_{max}	Maximum biomass concentration ($g\ l^{-1}$)
$Y_{P/S}$	Yield of biosurfactant production per substrate consumption ($g\ g^{-1}$)
$Y_{X/S}$	Yield of biomass growth per substrate consumption ($g\ g^{-1}$)
$Y_{P/X}$	Yield of biosurfactant production per biomass growth ($mg\ g^{-1}$)
μ_{max}	Maximum specific growth rate (ratio between initial volumetric rate of biomass growth (r_p) and initial biomass concentration X_0) (h^{-1})

9.2 INTRODUCTION

The interest in biosurfactants has increased considerably in recent years, as they are potential candidates for many commercial applications in the petroleum, pharmaceuticals, biomedical and food processing industries (Desai and Banat 1997). The biosurfactants have several advantages over chemical surfactants including lower toxicity and higher biodegradability, and effectiveness at extreme temperatures or pH values (Kosaric 1992, Cameotra and Makkar 1998). In spite of the advantages, fermentation must be cost competitive with chemical synthesis and many of the potential applications that have been considered for biosurfactants depend on whether they can be produced economically. Fermentation medium can represent almost 30% of the cost for a microbial fermentation (Miller and Churchill 1986, Hofvendahl and Hahn-Hägerdal 2000, Makkar and Cameotra 2002). Complex media commonly employed for growth of lactic acid bacteria are not economically attractive due to their high amount of expensive nutrients such as yeast extract, peptone and salts (Batish *et al.* 1990, Jensen and Hammer 1993, Hofvendahl and Hahn-Hägerdal 2000). Nevertheless, much effort in process optimization and at the engineering and biological levels has been done, and for some applications biosurfactants can be produced from several inexpensive waste substrates, thereby decreasing their production cost (Zhou and Kosaric 1995, Otto *et al.* 1999, Daniel *et al.* 1999, Sarrubo *et al.* 1999, Fox and Bala 2000, Makkar and Cameotra 2002, Rahman *et al.* 2002).

Biosurfactant production by probiotic strains, *Lactococcus lactis* 53 and *Streptococcus thermophilus* A, using conventional medium and its applications was reported previously in Chapters 5, 6 and 8 (Rodrigues *et al.* 2004a,b, 2005c). Rodrigues and co-workers (2005a) optimized the medium components by response surface optimization for the production of biosurfactants by probiotic bacteria and concluded that it was possible to determine optimal medium compositions to obtain a higher cellular growth, thus a higher biosurfactant production yield, as can be seen in Chapter 3. Moreover, the authors suggested that since both bacterial strains studied showed higher amounts of biosurfactant produced with the optimized medium, it would be possible to develop strategies for biosurfactant production from cheese whey. In another study described in Chapter 4 (Rodrigues *et al.* 2005b) suitable kinetic models were established for several *Lactobacillus* strains biosurfactant producers using cheese whey as an alternative medium. A great variety of alternative raw materials is currently available as nutrients for industrial fermentations, namely various agricultural and industrial by-products and waste materials. A good substrate for biosurfactant production is whey, as it is composed of high levels of lactose, protein, organic acids and vitamins. Whey is

a waste product from cheese production that represents a major pollution problem for countries depending on dairy economics and is normally used as animal feed. Sophorolipids production using whey was reported by Otto and his co-workers (1999). On the other hand, molasses is also an interesting alternative. Molasses is a by-product of the sugar cane industry and it has many applications because of its low price compared to other sources of sugar, and the presence of several other compounds besides sucrose. These include minerals, organic compounds and vitamins, which are valuable for the fermentation process (Makkar and Cameotra 1997, Patel and Desai 1997).

The aim of this study was to develop a low-cost alternative medium for biosurfactant production by *L. lactis* 53 and *S. thermophilus* A. Molasses and cheese whey heat precipitated were evaluated as alternative media and compared with the conventional synthetic media. The yields of biosurfactant production for both strains were determined for all tested media. Additionally, the time courses of biosurfactant production, glucose, sucrose or lactose consumption and biomass growth were modeled.

9.3 MATERIALS AND METHODS

9.3.1 Microorganisms and Inoculums

The strains used in this work were *L. lactis* 53 and *S. thermophilus* A obtained from Nutricia (The Netherlands) and NIZO (The Netherlands), respectively. The bacterial strains *L. lactis* 53 and *S. thermophilus* A were stored at –20ºC in conventional MRS or M17 broth (OXOID, Basingstoke, England), respectively. From frozen stock, bacteria were streaked on MRS or M17 agar plates and incubated at 37ºC for further culturing. To prepare subcultures, the respective medium was inoculated with a colony from the plate and incubated overnight under the same conditions.

9.3.2 Fermentation Experiments

To test biosurfactant production using alternative fermentation medium, batch fermentations were carried out using the compositions described in Table 9.1. The conventional synthetic medium was prepared according to the supplier instructions (OXOID, Basingstoke, England). Appropriate dilutions were made in order to adjust lactose or sucrose initial concentrations of the medium. A 1-l bioreactor fitted with agitation control, as well as temperature and pH

measurement and control were used. The temperature was maintained at 37°C, the pH at 6.7 by automatic addition of a potassium hydroxide solution, and the agitation speed was set at 150 rpm. The total working volume was 0.5 l.

Table 9.1 Medium composition used in the fermentation experiments for both tested strains.

Medium	*Lactococcus lactis 53*
A	MRS broth
B	W(50 g l^{-1} lactose content) + 3 g l^{-1} YE 5 g l^{-1} PEP
C	W (50 g l^{-1} lactose content) + 3 g l^{-1} YE + 10 g l^{-1} PEP
D	W (50 g l^{-1} lactose content) + 5.8 g l^{-1} YE + 44.8 g l^{-1} PEP
E	M (20 g l^{-1} sucrose content) + 3 g l^{-1} YE + 5 g l^{-1} PEP
F	M (20 g l^{-1} sucrose content) + 2.3 g l^{-1} YE + 18 g l^{-1} PEP
Medium	***Streptococcus thermophilus A***
G	M17 broth
H	W (50 g l^{-1} lactose content) + 3 g l^{-1} YE + 5 g l^{-1} PEP
I	W (50 g l^{-1} lactose content) + 3 g l^{-1} YE + 10 g l^{-1} PEP
J	W (50 g l^{-1} lactose content) + 22 g l^{-1} YE + 43.8 g l^{-1} PEP + 231.6 g l^{-1} NGP
L	M (20 g l^{-1} sucrose content) + 3 g l^{-1} YE + 5 g l^{-1} PEP
M	M (20 g l^{-1} sucrose content) + 8.8 g l^{-1} YE + 17.5 g l^{-1} PEP + 92.6 g l^{-1} NGP

* W – Whey; YE – Yeast extract; PEP – Peptone; M – Molasses; NGP – Sodium glycerophosphate

9.3.3 Cheese Whey Preparation

Commercial whey supplied by Sigma Aldrich contained 65% w/w lactose and 11% w/w protein and was prepared as follows: after adjusting the pH to 4.5 with 5N HCl, it was heated at 121ºC for 15 min to denature the proteins. The precipitates were removed by centrifugation at 4ºC and 8000 × g for 10 min. The supernatants were adjusted to pH 6.7, sterilized at 121ºC for 15 min and used as culture media. The supernatant contained approximately 50 g l^{-1} of lactose. Yeast extract and peptone were added in suitable concentrations according to Table 9.1. In previous published results (Chapter 3, Rodrigues *et al.* 2005a) peptone and sodium glycerophosphate were found to be significant factors for biosurfactant production by *L. lactis* 53 and *S. thermophilus* A, respectively. Thus, proportions of yeast extract, peptone and sodium glycerophosphate used in media D, F, J and M were defined according to this previous study.

9.3.4 Molasses Preparation

Molasses, by-product of the sugar cane industry, supplied by RAR (Porto, Portugal), contained 45% w/v sucrose, 20% w/v fructose and 10% w/v glucose. Molasses was diluted to a concentration of 20 g l^{-1} sucrose and supplemented with yeast extract and peptone as described in Table 9.1. The pH of the medium was adjusted to 6.7 prior to autoclaving (15 min at 121ºC).

9.3.5 Bacterial Growth Determination

Bacterial growth was measured by determining the optical density at 600 nm during different time intervals up to 30 h. The biomass concentrations (g dry weight l^{-1}) were determined using a calibration curve. The calibration curve was calculated for each strain using dilutions of a biomass suspension with known optical density. A fixed volume of the dilutions was filtered (0.22 μm) and left to dry at 105ºC for 24 h. All the filters were weighed before filtration and after drying. Thus, a relationship between biomass concentration (g l^{-1}) and optical density (600 nm) can be determined for each strain.

9.3.6 Sugar Analysis

Sugar concentrations were determined by high performance liquid chromatography (Agilent, model 1100, Palo Alto, CA) using ION-300 column (Transgenomic Inc., San Jose, CA) with refractive index detector.

The mobile phase was 0.01 N H_2SO_4 at a flow rate of 0.4 ml min^{-1}.

9.3.7 Surface-activity Determination

The surface activity of biosurfactants produced by the bacterial strains was determined by measuring the surface tension of the broth samples by the Ring method (Kim *et al.* 2000) using a KRUSS Tensiometer equipped with a 1.9 cm De Noüy platinum ring at room temperature. To increase the accuracy an average of triplicates was used for this study.

The biosurfactant concentrations (g l^{-1}) were determined for each strain using a calibration curve (Surface Tension (mN m^{-1}) = - 8.6465 Concentration (g l^{-1}) + 76.984, r^2 = 0.9729). The calibration curve was calculated for a commercial biosurfactant produced by several *Bacilli* (surfactin) using different concentrations of biosurfactant solution, below the critical micelle concentration, with known surface tension. In this biosurfactant concentration range the decrease of surface tension is linear and it is possible to establish a relationship between the biosurfactant concentration and the surface tension (Kim *et al.* 2000). Nevertheless, to estimate biosurfactant concentration it was necessary sometimes to dilute the culture broth under the critical micelle concentration.

9.3.8 Sugar Consumption, Biosurfactant Production and Biomass Growth - Fitting of Data

Experimental data were fitted to proposed models using commercial software (Solver of Microsoft Excel 2002) by nonlinear regression using the least-squares method. Biosurfactant production was mathematically modeled following the equation proposed by Mercier *et al.* (1992) for lactic acid production,

$$\frac{dP}{dt} = P_r P\left(1 - \frac{P}{P_{\max}}\right)$$ Equation 9.1

where t is time (h), P is biosurfactant concentration (g l^{-1}), P_{max} is maximum concentration of biosurfactant (g l^{-1}), and P_r is the ratio between the initial volumetric rate of product formation (r_p) and the initial product concentration P_0 (g l^{-1}). Equation 9.1 can be directly solved to give the Equation 9.2:

$$P = \frac{P_0 P_{\max} e^{P_r t}}{P_{\max} - P_0 + P_0 e^{P_r t}}$$ Equation 9.2

From the series of experimental data biosurfactant concentration/time, the model parameters P_0, P_{max}, and P_r can be calculated for each strain growing in the several tested fermentation medium.

Also biomass production was mathematically modeled and can be interpreted by Equation 9.3:

$$X = \frac{X_0 X_{\max} e^{\mu \max t}}{X_{\max} - X_0 + X_0 e^{\mu \max t}}$$ Equation 9.3

where t is time (h), X is biomass concentration (g l^{-1}), X_{max} is maximum concentration of biomass (g l^{-1}), and μ_{max} (h^{-1}) is the ratio between the initial volumetric rate of biomass formation and the initial biomass concentration X_0 (g l^{-1}). The model parameters X_0, X_{max}, and μ_{max} can be calculated from the series of experimental data biomass concentration/time.

Sugar consumption can be interpreted by the Equation 9.4:

$$S = S_0 - \frac{1}{Y_{P/S}}(P - P_0) - \frac{1}{Y_{X/S}}(X - X_0)$$ Equation 9.4

where $Y_{P/S}$ (g g^{-1}) and $Y_{X/S}$ (g g^{-1}) are the product yield for biosurfactant and biomass respectively, P and P_0 are the final and initial biosurfactant concentrations (g l^{-1}), X and X_0 are the final and initial biomass concentrations (g l^{-1}), and finally S_0 is the initial glucose, lactose or sucrose concentration (g l^{-1}). The model parameters $Y_{P/S}$, $Y_{X/S}$ and S_0 (g l^{-1}) were calculated for each strain from the series of experimental data glucose, lactose or sucrose concentration/time and the Equations 9.2 and 9.3.

The mathematical model proposed by Mercier *et al.* (1992) was chosen because it fairly describes biomass growth, substrate consumption and product accumulation kinetic pattern, and is reasonable to predict that this mathematical model will adjust the biosurfactant production results with statistical significance of the parameters determined.

9.4 RESULTS

9.4.1 Biosurfactant Production Using Conventional Synthetic Medium

Fermentation control runs were carried out using the conventional synthetic medium MRS or M17 broth (A and G as defined in Table 9.1) for *L. lactis* 53 and *S. thermophilus* A, respectively. Experimental data were fitted to proposed models by nonlinear regression using the least-squares method. Table 9.2 and Table 9.3 show the kinetic and regression parameters as well as the biosurfactant production yields. Both experiments show a kinetic pattern fairly described by the mathematical models with r^2 > 0.952, 0.996 and 0.983 for glucose or lactose consumption, biomass growth and biosurfactant production, respectively. It can be noted that *S. thermophilus* A presents a higher P_{max} (0.8 g of biosurfactant l^{-1}) compared to *L. lactis* 53 (0.7 g of biosurfactant l^{-1}). Regarding the $Y_{P/S}$ both strains present the same value (0.05 g g^{-1}). The $Y_{P/X}$ values listed in Table 9.2 and Table 9.3 reflect the amount of biosurfactant produced (mg) per amount of dry cells (g). The $Y_{P/X}$ values obtained for both strains growing in control medium was 163 mg g^{-1} and 116 mg g^{-1} for *L. lactis* 53 and *S. thermophilus* A, respectively.

Table 9.2 Results obtained by regression of glucose, lactose or sucrose, biomass and biosurfactant concentration data in several fermentation media for *Lactococcus lactis* 53 [(a)].

Medium	Sugar consumption						Biomass growth					Biosurfactant production				
	S_0 (g l^{-1})	$Y_{P/S}$ (g g^{-1})	$Y_{X/S}$ (g g^{-1})	$Y_{P/X}$ (mg g^{-1})	r^2	*F* value	X_0 (g l^{-1})	X_{max} (g l^{-1})	μ_{max} (h^{-1})	r^2	*F* value	P_0 (g l^{-1})	P_{max} (g l^{-1})	P_r (h^{-1})	r^2	*F* value
A	29.0	0.05	0.30	163	0.958	45 [(c)]	0.068	4.244	0.405	0.996	461 [(f)]	0.030	0.693	0.640	0.983	116 [(e)]
B	55.0	0.04	0.22	200	0.936	29 [(c)]	0.064	5.963	0.315	0.997	766 [(g)]	0.259	1.054	0.222	0.948	37 [(c)]
C	52.0	0.04	0.22	159	0.940	31 [(c)]	0.062	6.023	0.196	0.921	23 [(c)]	0.097	0.919	0.419	0.979	92 [(d)]
D	57.0	0.06	0.23	240	0.961	49 [(c)]	0.036	5.680	0.372	0.984	119 [(e)]	0.154	1.379	0.429	0.977	83 [(d)]
E	26.3	0.07	0.36	197	0.997	632 [(g)]	0.579	5.992	0.103	0.944	34 [(c)]	0.053	1.041	0.338	0.958	46 [(c)]
F	35.0	0.12	0.43	281	0.901	18 [(b)]	0.064	5.990	0.202	0.959	46 [(c)]	0.116	1.735	0.294	0.917	22 [(c)]

[(a)] Parameters defined in the Nomenclature Table; [(b)], significance level >90%; [(c)], significance level >95%; [(d)], significance level >97.5%; [(e)], significance level >99%; [(f)], significance level >99.5%; [(g)], significance level >99.9%.

Table 9.3 Results obtained by regression of glucose, lactose or sucrose, biomass and biosurfactant concentration data in several fermentation medium for *Streptococcus thermophilus* A [(a)].

Medium	**Sugar consumption**						**Biomass growth**					**Biosurfactant production**				
	S_0 (g l^{-1})	$Y_{P/S}$ (g g^{-1})	$Y_{X/S}$ (g g^{-1})	$Y_{P/X}$ (mg g^{-1})	r^2	F value	X_0 (g l^{-1})	X_{max} (g l^{-1})	μ_{max} (h^{-1})	r^2	F value	P_0 (g l^{-1})	P_{max} (g l^{-1})	P_r (h^{-1})	r^2	F value
G	35.0	0.05	0.41	116	0.952	40 [(c)]	0.103	7.065	0.341	0.997	565 [(g)]	0.060	0.828	0.862	0.988	171 [(e)]
H	54.0	0.04	0.20	176	0.942	24 [(c)]	0.255	5.246	0.201	0.929	26 [(c)]	0.338	1.110	0.078	0.913	21 [(c)]
I	52.4	0.04	0.26	155	0.991	227 [(f)]	0.130	5.785	0.290	0.995	388 [(f)]	0.040	0.914	0.725	0.990	206 [(f)]
J	45.0	0.06	0.27	222	0.973	71 [(d)]	0.035	6.079	0.447	0.968	61 [(d)]	0.658	1.366	0.359	0.980	99 [(d)]
L	20.0	0.08	0.49	164	0.989	177 [(e)]	0.410	5.173	0.152	0.997	665 [(g)]	0.031	1.017	0.152	0.986	140 [(e)]
M	26.0	0.13	0.48	272	0.975	77 [(c)]	1.099	5.941	0.084	0.991	212 [(f)]	0.119	1.401	0.257	0.982	107 [(d)]

[(a)] Parameters defined in the Nomenclature Table; [(b)], significance level >90%; [(c)], significance level >95%; [(d)], significance level >97.5%; [(e)], significance level >99%; [(f)], significance level >99.5%; [(g)], significance level >99.9%.

9.4.3 Biosurfactant Production Using Cheese Whey

Fermentations were carried out using whey supplemented with yeast extract and peptone as culture broth for both studied strains. Different sets of medium composition in yeast extract and peptone were evaluated. Figure 9.1 A and 9.2 A show the experimental data as well as the predicted values calculated by Equations 9.2, 9.3 and 9.4 using the regression parameters listed in Table 9.2 and Table 9.3 for *L. lactis* 53 and *S. thermophilus* A growing in medium D and J (as defined in Table 9.1), respectively. For both strains growing in all the tested cheese whey medium (B, C, D, H, I, J as defined in Table 9.1), the experiments show a kinetic pattern reasonably described by the mathematical model with $r^2 > 0.936$, 0.921 and 0.913 for lactose consumption, biomass growth and biosurfactant production, respectively. P_{max} values achieved with all cheese whey media were higher than the observed for the control experiments. P_{max} values between 0.9 and 1.4 g of biosurfactant l^{-1} were obtained for both strains. Regarding the P_r the values obtained were between 0.22 and 0.429 h^{-1} for *L. lactis* 53; and between 0.078 and 0.725 h^{-1} for *S. thermophilus* A. Moreover, the $Y_{P/S}$ values obtained were similar for both strains and between 0.04 and 0.06 g g^{-1}; and X_{max} between 5.2 and 6.1 g l^{-1} with a μmax between 0.196 and 0.447 h^{-1}.

Comparing the kinetic parameters obtained with the cheese whey medium experiments and control, it was possible to notice that higher $Y_{P/X}$ values were obtained. A mass of produced biosurfactant (mg per g cell dry weight) 1.5 times higher compared to MRS control medium was obtained for *L. lactis* 53 growing in medium D (as defined in Table 9.1). Similarly, for *S. thermophilus* A growing in medium J (as defined in Table 9.1) it was achieved an increase 1.9 times in the $Y_{P/X}$ values.

9.4.4 Biosurfactant Production Using Molasses

In another set of experiments, fermentations were carried out using molasses supplemented with yeast extract and peptone as culture broth for both studied strains. Also, different set of medium composition in yeast extract and peptone were evaluated. Figure 9.1 B and 9.2 B show the experimental data and predicted values for *L. lactis* 53 and *S. thermophilus* A growing in medium F and M (as defined in Table 9.1), respectively.

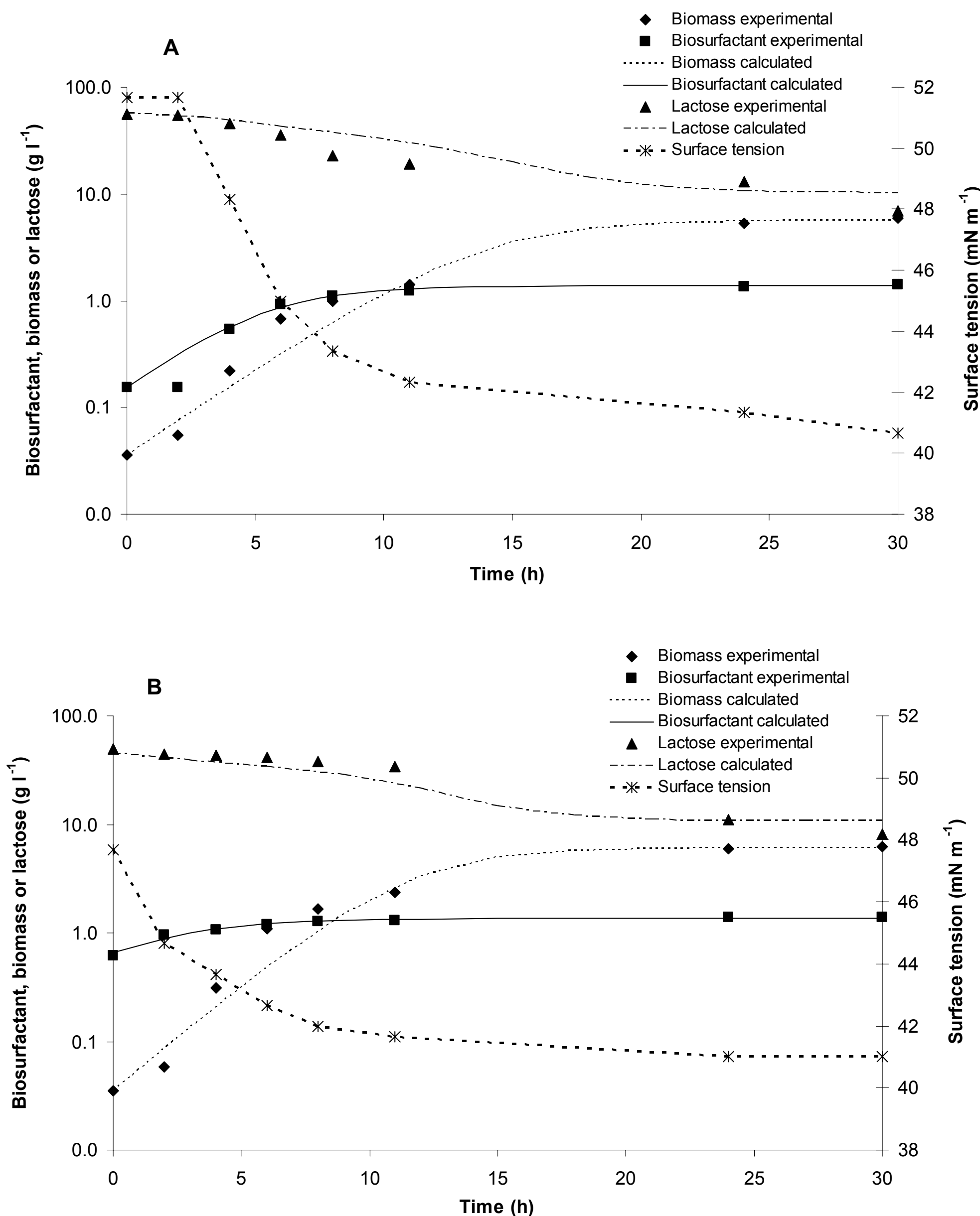


Figure 9.1 Representation of the surface tension variation (-----), experimental data and calculated time courses of biomass (◆, -----), lactose (▲, — - —) and biosurfactant concentrations (■, ——) during fermentations carried out with medium D (whey (50 g l^{-1} lactose content) + 5.8 g l^{-1} yeast extract + 44.8 g l^{-1} peptone) or medium J (whey (50 g l^{-1} lactose content) + 22 g l^{-1} yeast extract + 43.8 g l^{-1} peptone + 231.6 g l^{-1} sodium glycerophosphate) for **(A)** *L. lactis* 53 or **(B)** *S. thermophilus* A, respectively. Results represent the average of three independent experiments.

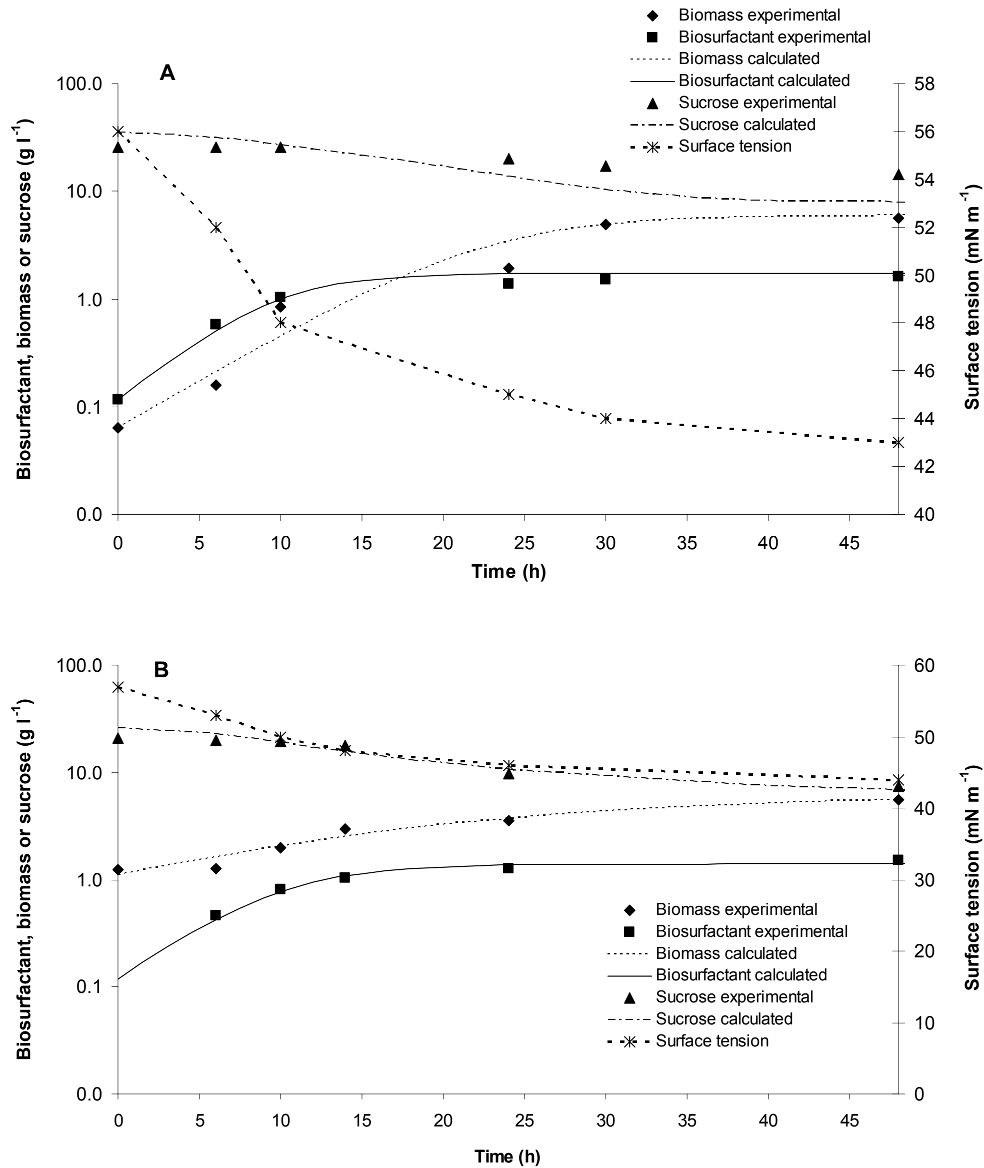


Figure 9.2 Representation of the surface tension variation (--*--), experimental data and calculated time courses of biomass (◆, -----), lactose (▲, — - —) and biosurfactant concentrations (■, ——) during fermentations carried out with medium F (molasses (20 g l^{-1} sucrose content) + 2.3 g l^{-1} yeast extract + 18 g l^{-1} peptone) or medium M (molasses (20 g l^{-1} sucrose content) + 8.8 g l^{-1} yeast extract + 17.5 g l^{-1} peptone + 92.6 g l^{-1} sodium glycerophosphate) for **(A)** *L. lactis* 53 or **(B)** *S. thermophilus* A, respectively. Results represent the average of three independent experiments.

For both strains growing in all the tested molasses media (E, F, L, M as defined in Table 9.1), the mathematical model describes realistically the experimental data with r^2 > 0.901, 0.944 and 0.917 for sucrose consumption, biomass growth and biosurfactant production, respectively. P_{max} values between 1.0 and 1.7 g of biosurfactant l^{-1} and P_r values between 0.152 and 0.338 h^{-1}were obtained for both strains. Additionally, the $Y_{P/S}$ values obtained were similar for both strains and between 0.07 and 0.13 g/g; and X_{max} between 5.2 and 6.0 g l^{-1} with a μmax between 0.08 and 0.202 h^{-1}.

The higher $Y_{P/X}$ values were obtained for both strains compared whether to control or cheese whey medium experiments. A mass of produced biosurfactant (mg per g cell dry weight) 1.7 times higher compared to MRS control medium was obtained for *L. lactis* 53 growing in medium F (as defined in Table 9.1). Similarly, for *S. thermophilus* A growing in medium M (as defined in Table 9.1) it was achieved an increase 2.3 times in the $Y_{P/X}$ values.

9.5 DISCUSSION

This study focused on the potential use of alternative fermentative medium formulations for biosurfactant production. For *L. lactis* 53 and *S. thermophilus* A, suitable models were established to describe the response of the experiments pertaining to glucose, lactose or sucrose consumption, cell growth and biosurfactant production. The models were validated by comparing the observed and predicted values, and a deviation of about 5% was found. The modelling procedure allowed a better characterization of the biosurfactant production by the determination of the fermentation parameters and it was observed a reasonable fitting with a significance level over 90%.

The success of biosurfactant production depends on the development of cheaper processes and the use of low cost raw materials, which account for 10-30% of the overall cost (Makkar and Cameotra 2002). A great variety of agro-industrial wastes have been studied as potential substrates for biosurfactant production. Starch-rich wastes from potato-processing industries were successfully used for surfactant production (Fox and Bala 2000); molasses from sugar industry were assessed for biosurfactant production by *Bacillus* strains (Makkar and Cameotra 1997); and distillery and whey wastes were found to produce better results than conventional medium for rhamnolipid production (Sudhakar Babu *et al.* 1996, Dubey and Juwarkar 2001). Another good substrate for biosurfactant production is cheese whey, Daniel and co-workers (1999) achieved production of high concentrations of sophorolipids from

Candida bombicola ATCC 22214 and *Cryptococcus curvatus* ATCC 20509, using a two-stage fed batch process.

Whey is produced in large amounts by the cheese industry and is a huge waste disposal problem (Palma Revillion *et al.* 2003), being estimated a worldwide annual amount of about 4×10^7 tons. Cultivation of microorganisms on cheese whey has been proposed as an alternative to reduce waste disposal problem since it can reduce 90-95% of its biochemical oxygen demand (BOD), resulting in high-added value bio-ingredients for food industry. Several studies have been reported on the use of cheese whey for lactic acid production (Amrane *et al.* 1997, 2001, Kulozik and Wilde 1999, Kwon *et al.* 2000, Téllez-Luis *et al.* 2003, Bustos *et al.* 2004). Also, cheese whey was used for the production of dextran and fructose by *Leuconostoc mesenteroides* NRRL B512 (f) (Santos *et al.* 2005); production of ethanol (Domingues *et al.* 2001) and for the production of yeast extract by *Kluyveromyces marxianus* (Palma Revillion *et al.* 2003). Hinted by a previous work presented in Chapter 3 (Rodrigues *et al.* 2005a) and the fact that probiotic bacteria, especially *L. lactis* 53 and *S. thermophilus* A, have been used for the production of biosurfactants (Rodrigues *et al.* 2004a, 2005b,c, Busscher *et al.* 1994, Velraeds *et al.* 1996, Reid *et al.* 1999), three different sets of medium conditions using cheese whey were tested to see their potentials for biosurfactant fermentation. In the present study it was achieved an increase about 1.5 to 1.9 times in the mass of produced biosurfactant per gram cell dry weight, for *L. lactis* 53 and *S. thermophilus* A, respectively. From the different proportions of yeast extract, peptone and sodium glycerophosphate supplemented to cheese whey it was possible to observe that the best results were achieved with the medium D (50 g l^{-1} lactose content, supplemented with 5.8 g l^{-1} yeast extract and 44.8 g l^{-1} peptone) for *L. lactis* 53, and with the medium J (50 g l^{-1} lactose content, supplemented with 22 g l^{-1} yeast extract, 43.8 g l^{-1} peptone and 231.6 g l^{-1} sodium glycerophosphate) for *S. thermophilus* A; which is in accordance with previous published results (Rodrigues *et al.* 2005a) where peptone and sodium glycerophosphate were found to be significant factors for biosurfactant production by *L. lactis* 53 and *S. thermophilus* A, respectively. Despite a higher biosurfactant production yield was achieved with medium D (50 g l^{-1} lactose content, supplemented with 5.8 g l^{-1} yeast extract and 44.8 g l^{-1} peptone) for *L. lactis* 53, an increase of 40% in the medium preparation costs comparing with the synthetic MRS medium was estimated due to the high amounts of peptone supplemented; thus a compromise situation must be established to obtain higher biosurfactant production yields with lower medium preparation costs. With medium B (50 g l^{-1} lactose content, supplemented with 3 g l^{-1} yeast extract and 5 g l^{-1} peptone) the mass of produced biosurfactant (mg) per gram cell dry weight increased 1.2 times with an estimated 60% decrease in the medium

preparation costs comparing with the synthetic MRS medium. Similar conclusions were established for *S. thermophilus* A, where the use of medium H (50 g l^{-1} lactose content, supplemented with 3 g l^{-1} yeast extract and 5 g l^{-1} peptone) resulted in a biosurfactant production yield 1.5 times higher with an estimated 60% reduction in the medium preparation costs comparing with the synthetic M17 medium.

A by-product of the sugar cane industry, molasses, represents an alternative for the biosurfactant production process as it is a relatively inexpensive raw material compared to other substrate sources, and it possesses other valuable compounds for the fermentation process. This alternative medium was also studied in the present work for biosurfactant production by probiotic bacteria. The biosurfactant production yields achieved with supplemented molasses medium were higher than the obtained whether with conventional or supplemented cheese whey medium. Although higher amounts of biosurfactant were produced with medium F (20 g l^{-1} sucrose content, supplemented with 2.3 g l^{-1} yeast extract and 18 g l^{-1} peptone) and M (20 g l^{-1} sucrose content, supplemented with 8.8 g l^{-1} yeast extract, 17.5 g l^{-1} peptone and 92.6 g l^{-1} sodium glycerophosphatee) for *L. lactis* 53 and *S. thermophilus* A, respectively; resembling what was observed for cheese whey medium, a better compromise between good yields and low costs is achievable with medium where peptone and yeast extract amounts are lower (20 g l^{-1} sucrose content, supplemented with 3 g l^{-1} yeast extract and 5 g l^{-1} peptone). Thus, an increase about 1.2 to 1.4 times in the mass of produced biosurfactant per gram cell dry weight and a 80% medium preparation costs reduction comparing with the synthetic MRS or M17 medium were achieved, for both strains.

Lactic acid bacteria ferment sugars via different pathways and are also capable of forming other products, e.g. flavours such as diacetyl and acetoin, bacteriocins or biosurfactants. The different carbon sources give varying amounts of by-products (Hofvendahl and Hahn-Hägerdal 2000, Kleerebezem *et al.* 2000). Hence, it can be speculated that the use of lactose or sucrose as carbon source instead of glucose induced the cells to use another metabolic pathway, and therefore the amount of mass of biosurfactant produced varied. Lactic acid bacteria have already proven to be ideal hosts for metabolic engineering. The efficacy of metabolic engineering of lactic acid bacteria for the increased production of biosynthetic metabolites is yet to be demonstrated, but based on the results gathered in this study it seems to be an interesting approach for developing new strategies of biosurfactant production.

9.6 CONCLUSIONS

Concluding, *L. lactis* 53 and *S. thermophilus* A showed a good performance for glucose or lactose to biosurfactant fermentation using the costly MRS or M17 broth respectively, which includes among others yeast extract and peptone. When the conventional synthetic media (MRS or M17) were replaced by cheaper alternative media, as cheese whey heat precipitated and molasses, in all cases fermentations were carried out effectively with high yields and productivities of biosurfactant. The best results, even higher than those obtained with the conventional synthetic media (MRS or M17), were obtained using molasses, thus it can be used as an alternative economical medium for biosurfactant production.

9.7 REFERENCES

Amrane, A. and Prigent, Y. (1997) Growth and lactic acid production coupling for *Lactobacillus helveticus* cultivated on supplemented whey: influence of peptidic nitrogen deficiency. *Journal of Biotechnology.* 55: 1-8.

Amrane, A. (2001) Batch cultures of supplemented whey permeate using *Lactobacillus helveticus*: unstructured model for biomass formation, substrate consumption and lactic acid production. *Enzyme and Microbial Techonology.* 28: 827-834.

Batish, V.K., Lal, R. and Chander, H. (1990) Effect of nutritional factors on the production of antifungal substance by *Lactococcus lactis* subsp. *lactis biovar diacetylactis*. *Australian Journal of Dairy Technology.* 74-76.

Busscher, H. J., Neu, T.R. and Van der Mei, H.C. (1994) Biosurfactant production by thermophilic dairy streptococci. *Applied Microbiology and Biotechnology.* 41: 4-7.

Bustos, G., Moldes, A., Alonso, J.L. and Vasquez, M. (2004) Optimization of D-lactic acid production by *Lactobacillus coryniformis* using response surface methodology. *Food Microbiology.* 21: 143-148.

Cameotra, S. and Makkar, R. (1998) Synthesis of biosurfactants in extreme conditions. *Applied Microbiology and Biotechnology.* 50: 520-529.

Daniel, H., Otto, R.T., Binder, M., Reuss, M. and Syldatk, C. (1999) Production of sophorolipids from whey: development of a two-stage process with *Cryptococcus curvatus* ATCC 20509 and

Candida bombicola ATCC 22214 using deproteinized whey concentrates as substrates. *Applied Microbiology and Biotechnology*. 51: 40-45.

Desai, J. and Banat, I.M. (1997) Microbial production of surfactants and their commercial potential. *Microbiology and Molecular Biology Reviews*. 61:47-64.

Domingues, L., Lima, N. and Teixeira, J.A. (2001) Alcohol production from cheese whey permeate using genetically modified flocculent yeast cells. *Biotechnology and Bioengineering*. 72: 507-514.

Dubey, K. and Juwarkar, A. (2001) Distillery and curd whey wastes as viable alternative sources for biosurfactant production. *World Journal of Microbiology and Biotechnology.* 17: 61-69.

Fox, S. and Bala, G. (2000) Production of surfactant from *Bacillus subtilis* ATCC 21332 using potato substrates. Bioresource Technology. 75: 235-240.

Hofvendahl, K. and Hahn-Hägerdal, B. (2000) Factors affecting the fermentative lactic acid production from renewable resources. *Enzyme and Microbial Techonology.* 26:87-107.

Jensen, P. and Hammer, K. (1993) Minimal requirements for exponential growth of *Lactococcus lactis*. *Applied and Environmental Microbiology*. 59: 4363-4366.

Kim, S., Lim, E., Lee, S., Lee, J. and Lee, T. (2000) Purification and characterization of biosurfactants from Nocardia sp. L-417. Biotechnology and Applied Biochemistry. 31: 249-253.

Kleerebezem, M. and Hugenholtz, J. (2000) Lactic acid bacteria as a cell factory: rerouting of carbon metabolism in *Lactococcus lactis* by metabolic engineering. *Enzyme and Microbial Techonology.* 26: 840-848.

Kosaric, N. (1998) Biosurfactants in industry. *Journal of American Oil Chemistry Society*. 64:1731-1737.

Kulozik, U. and Wilde, J. (1999) Rapid lactic acid production at high cell concentrations in whey ultrafiltrate by *Lactobacillus helveticus*. *Enzyme and Microbial Techonology*. 24: 297-302.

Kwon, S., Lee, P., Lee, E., Chang, Y. and Chang, N. (2000) Production of lactic acid by *Lactobacillus rhamnosus* with vitamin-supplemented soybean hydrolysate. *Enzyme and Microbial Techonology*. 26: 209-215.

Makkar, R. and Cameotra, S. (1997) Utilization of molasses for biosurfactant production by two *Bacillus* strains at thermophilic conditions. *Journal of American Oil Chemistry Society*.. 74: 887-889.

Makkar, R. and Cameotra, S. (2002) An update on the use of unconventional substrates for biosurfactant production and their new applications. *Applied Microbiology and Biotechnology*. 58:428-434.

Mercier, P., Yerushalmi, L., Rouleau, D. and Dochain, D. (1992) Kinetics of lactic acid fermentation on glucose and corn by *Lactobacillus amylophilus. Journal of Chemical Technology and Biotechnology*. 55: 111-121.

Miller, T. and Churchill, B. (1986) Substrates for large-scale fermentations. *In: Manual of Industrial Microbiology and Biotechnology.* Ed. Demain, A. and Solomon, L. Washington, D.C.: American Society for Microbiology. Pp 127-140.

Otto, R.T., Daniel, H.J., Pekin, G. and Muller-Decker, K. (1999) Production of sophorolipids from whey II. - Product composition, surface active properties, cytotoxicity and stability against hydrolases by enzymatic treatment. *Applied Microbiology and Biotechnology*. 52: 495-501.

Palma Revillion, J.P., Brandelli, A. and Ayub, Z. (2003) Production of yeast extract from whey using *Kluyveromyces marxianus*. *Brazilian Archives of Biological Technology*. 46: 121-127.

Patel, R. and Desai, A. (1997) Biosurfactant production by *Pseudomonas aeruginosa* GS3 from molasses. *Letters in Applied Microbiology*. 25: 91-94.

Rahman, K.S., Rahman, T.J., McClean, S., Marchant, R. and Banat, I.M. (2002) Rhamnolipid biosurfactant production by strains of *Pseudomonas aeruginosa* using low-cost raw materials. *Biotechnology Progress.* 18: 1277-1281.

Reid, G., Heinemann, C., Velraeds, M., Van der Mei, H.C. and Busscher, H.J. (1999) Biosurfactants produced by *Lactobacillus*. *Methods in Enzymology*. 310: 426-433.

Rodrigues, L.R., Teixeira, J., Oliveira, R. and Van der Mei, H.C. (2005a). Response surface optimization of the medium components for the production of biosurfactants by probiotic bacteria. *Process Biochemistry.* In Press

Rodrigues, L.R., Moldes, A., Teixeira, J. and Oliveira, R. (2005b). Kinetic study of fermentative biosurfactant production by *Lactobacillus* strains. *Biochemical Engineering Journal.* In Press

Rodrigues, L.R., Van der Mei, H.C., Banat, I.M., Teixeira, J. and Oliveira R. (2005c) Inhibition of microbial adhesion to silicone rubber treated with biosurfactant from *Streptococcus thermophilus* A. *FEMS Immunology and Medical Microbiology*. In Press.

Rodrigues, L.R., Van der Mei, H.C., Teixeira, J. and Oliveira, R. (2004a) Biosurfactant from *Lactococcus lactis* 53 inhibits microbial adhesion on silicone rubber. *Applied Microbiology and Biotechnology*. 66: 306-311.

Rodrigues, L.R., Van der Mei, H.C., Teixeira, J. and Oliveira, R. (2004b) Influence of biosurfactants from probiotic bacteria on formation of biofilms on voice prostheses. *Applied and Environmental Microbiology*. 70: 4408-4410.

Santos, M., Rodrigues, A. and Teixeira, J. (2005) Production of dextran and fructose from carob pod extract and cheese whey by *Leuconostoc mesenteroides* NRRL B512(f). *Biochemical Engineering Journal*. 25: 1-6.

Sarrubo, L.A., Porto, A.L. and Campos-Takaki, G.M. (1999) The use of babassu oil as substrate to produce bioemulsifiers by *Candida lypolytica*. *Canadian Journal of Microbiology*. 45: 423-426.

Sudhakar-Babu, P., Vaidya, A.N., Bal, A.S., Kapur, R., Juwarkar, A. and Khanna, P. (1996) Kinetics of biosurfactant production by *Pseudomonas aeruginosa* strain BS2 from industrial wastes. *Biotechnology Letters*. 18: 263-268.

Tellez-Luis, S.J., Moldes, A., Vasquez, M. and Alonso, J. (2003) Optimization of lactic acid production by *Lactobacillus delbrueckii* through response surface methodology. *Journal of Food Science*. 68: 1454-1458.

Velraeds, M., Van der Mei, H.C., Reid, G. and Busscher, H.J. (1996) Physicochemical and biochemical characterization of biosurfactants released by *Lactobaillus* strains. *Colloids Surfaces B: Biointerfaces*. 8: 51-61.

Zhou, Q.-H. and Kosaric, N. (1995) Utilization of canola oil and lactose to produce biosurfactant with *Candida bombicola*. *Journal of American Oil Chemical Society*. 72: 67-71.

CHAPTER 10

ISOLATION AND PARTIAL PHYSICOCHEMICAL AND FUNCTIONAL CHARACTERIZATION OF BIOSURFACTANTS PRODUCED BY PROBIOTIC BACTERIA

"As closer a man gets to its targets the greater become the difficulties ".

Goethe.

In this Chapter, isolation and identification of key components of the crude biosurfactants produced by *Lactococcus lactis* 53 and *Streptococcus thermophilus* A are presented.

10.1 INTRODUCTION
10.2 MATERIALS AND METHODS
10.2.1 Strains and Culture Conditions
10.2.2 Biosurfactant Production and Isolation
10.2.3 Hydrophobic Interaction Chromatography
10.2.4 Blood Agar Test
10.2.5 Oil Spreading Test
10.2.6 Critical Micelle Concentration
10.2.7 Anti-adhesion Assay in 96 Wells Plate
10.2.8 Antimicrobial Assay
10.2.9 Biosurfactant Desorption Assay
10.2.10 Biosurfactant Stability
10.2.11 FTIR
10.2.12 XPS
10.2.13 Mass Spectrometry
10.2.14 Quantitative Determination of Monosaccharides
10.3 RESULTS
10.3.1 Partial Purification of the Biosurfactants
10.3.2 Haemolytic Activity
10.3.3 Oil Spreading Capacity
10.3.4 Critical Micelle Concentration
10.3.5 Anti-adhesive Activity
10.3.6 Antimicrobial Activity
10.3.7 Desorption Assay
10.3.8 Biosurfactant Stability
10.3.9 FTIR
10.3.10 XPS
10.3.11 Mass Spectrometry
10.3.12 Quantitative Determination of Monosaccharides
10.4 DISCUSSION
10.5 CONCLUSIONS
10.6 REFERENCES

10.1 INTRODUCTION

Laryngectomized patients need to breath through a tracheostoma and receive voice prostheses for speech rehabilitation (Van der Mei *et al.* 1999). The main drawback of silicone rubber voice prostheses is the microbial colonization of the devices (Neu *et al*. 1993) that cause leakage of food and liquid, or blocking of the valve increasing the airflow resistance (Mahieu *et al.* 1986), thus forcing their replacement on average every 3-4 months. It has been suggested by patients that the consumption of buttermilk, containing antimycotic-releasing *Lactococcus lactis*, positively affects the lifetime of voice prostheses (Van der Mei *et al.* 1999). This suggestion has been confirmed in an artificial throat model, in which the effects of daily buttermilk consumption on biofilm formation onto silicone rubber voice prostheses have been simulated (Busscher *et al*. 1998). Similarly, Turkish yogurt containing *Streptococcus thermophilus* have been suggested to have such beneficial effects. Evaluations in the artificial throat model have furthermore indicated that the development of an oropharyngeal biofilm on silicone rubber voice prostheses can be delayed by exposure to caffeinated soft drinks (Free *et al.* 2000) or suspensions of active probiotic bacteria, such as *L. lactis* 53 and *S. thermophilus* B (Van der Mei *et al.* 2000). Biosurfactants obtained from the probiotic bacteria, *L. lactis* 53 and *S. thermophilus* A, were found to inhibit the adhesion of microbial strains isolated from explanted voice prostheses to silicone rubber with an adsorbed biosurfactant layer in a parallel plate flow chamber as described in Chapters 5 and 6 (Rodrigues *et al.* 2004a, 2005a). Further work presented in Chapter 8 was developed by the authors (Rodrigues *et al.* 2004b) to assess the influence of these biosurfactants in the growth phase of biofilm formation, and it was found that they greatly reduced microbial numbers on prostheses and also induced a decrease in the airflow resistance of voice prostheses after biofilm formation.

Microbial compounds which exhibit pronounced surface and emulsifying activities are classified as biosurfactants. A wide variety of chemical structures, such as glycolipids, lipopeptides, polysaccharide-protein complexes, protein-like substances, phospholipids, lipopolysaccharides, fatty acids and neutral lipids, have been attributed to biosurfactants (Jenny *et al.* 1993, Morikawa *et al.* 1993, Lin 1996, Velraeds *et al.* 1996, Angelova and Shmauder 1999, Ahimou *et al.* 2000). It is, therefore, reasonable to expect diverse properties and physiological functions for different groups of biosurfactants. Biosurfactants are not only useful as antibacterial, antifungal and antiviral agents, but also have potential for use as major immunomodulatory molecules, adhesive agents and even in vaccines and gene

therapy. Involvement of biosurfactants in microbial adhesion and desorption has been widely described. For example, precoating vinyl urethral catheters with surfactin obtained from *Bacillus* strains was found to decrease the amount of biofilm formed (Bechard *et al.* 1998, Mireles *et al.* 2001). In addition, there are reports of inhibition of biofilm formed by uropathogens and yeast on silicone rubber by biosurfactants produced by *Lactobacillus acidophilus* and *Lactobacillus fermentum* RC-14 (Velraeds *et al.* 1998, Reid 2000, Heinemann *et al.* 2000). Also, a dairy *S. thermophilus* strain was found to produce a biosurfactant which caused its own desorption from glass, leaving a completely non-adhesive coating (Busscher *et al.* 1994), and an oral *Streptococcus mitis* strain released a biosurfactant which reduced the adhesion of *Streptococcus mutans* (Pratt-Terpstra *et al.* 1989). Recently the authors demonstrated that when rinsing parallel plate flow chambers, designed to monitor microbial adhesion, with a rhamnolipid solution the rate of deposition and adhesion was significantly reduced for a variety of microbial strains isolated from explanted voice prostheses to silicone rubber, as can be seen in Chapter 7 (Rodrigues *et al.*, 2005b).

Information on the chemical structure of biosurfactants produced by probiotic bacteria is scarce. *Lactobacillus* biosurfactants have been reported to be glycosyldiglycerides (Gerson 1993). These glycolipids are ubiquitous in gram-positive bacteria and probably participate in cell wall polysaccharide formation as carbohydrate reservoirs or by facilitating the transfer of hexose molecules across the membrane. However, the *Lactobacillus* biosurfactants with demonstrated inhibition of enterococcal adhesion, which were isolated by Velraeds *et al.* (1996), appear to be protein-like. Heinemann and co-workers (2000) purified a protein with strong anti-adhesion activity from the crude biosurfactant mixture produced by *L. fermentum* RC-14. In addition, biosurfactants produced by *S. thermophilus* strains have not been extensively studied, but thin layer chromatograms (TLC) have indicated that the crude product is a mixture of various components, with a glycolipid-like component being the most surface active (Busscher *et al.* 1994, 1997).

The aim of this study was to isolate and identify key components of the crude biosurfactant mixtures produced by *L. lactis* 53 and *S. thermophilus* A, including their molecular composition (by Fourier transform infrared spectroscopy), elemental composition (by X-ray photoelectron spectroscopy), molecular mass (by mass spectrometry) and monosaccharide composition (by gas-liquid chromatography). Moreover, partial functional characterization was established using the following techniques: blood agar test, oil spreading test, critical micelle concentration determination, antimicrobial activity and anti-adhesion test. Finally,

desorption of biosurfactants from silicone rubber and their stability at several pH was evaluated.

10.2 MATERIALS AND METHODS

10.2.1 Strains and Culture Conditions

The strains used in this work were *L. lactis* 53 and *S. thermophilus* A obtained from Nutricia (The Netherlands) and NIZO (The Netherlands), respectively. The bacterial strains *L. lactis* 53 and *S. thermophilus* A were stored at –20ºC in conventional MRS or M17 broth (OXOID, Basingstoke, England), respectively. From frozen stock, bacteria were streaked on MRS or M17 agar plates and incubated at 37ºC for further culturing. To prepare subcultures, the respective medium was inoculated with a colony from the plate and incubated overnight under the same conditions.

10.2.2 Biosurfactant Production and Isolation

The production of crude biosurfactant 1 and 2 obtained from *L. lactis* 53 and *S. thermophilus* A, respectively, is described in Chapters 5 and 6 (Rodrigues *et al.* 2004a, 2005a). Briefly, 600 ml of MRS or M17 broth was incubated with 15 ml of an overnight *L. lactis* 53 or *S. thermophilus* A subculture and incubated for 18 h. Cells were harvested by centrifugation (10000 $\times$ g, 5 min, 10ºC), washed twice with demineralized water, and resuspended in 100 ml of phosphate-buffered saline (PBS: 10 mM KH_2PO_4/K_2HPO_4 and 150 mM NaCl with pH adjusted to 7.0). The bacteria were left at room temperature for 2 h with gentle stirring for biosurfactant release. Subsequently, the bacteria were removed by centrifugation and the remaining supernatant liquid was filtered through a 0.22 µm pore-size filter (Millipore). The supernatant was dialyzed against demineralized water at 4ºC in a Spectrapor membrane tube (molecular weight cut off 6000 to 8000, Spectrum Medical Industries Inc., CA) and freeze-dried, and the crude biosurfactant was used for further studies.

10.2.3 Hydrophobic Interaction Chromatography

Fractionation was performed on an Octyl Sepharose 4 FF Prep hydrophobic interaction column (20 ml, Pharmacia Biotech). Freeze-dried crude biosurfactants (100 g l^{-1}) dissolved in PBS+1.5 M $(NH_4)_2SO_4$ buffer (pH 7.0) were loaded (500 µl) onto the column equilibrated in the same buffer. Elution was carried out with a 60 ml linear gradient from 1 to 0 M $(NH_4)_2SO_4$

in PBS buffer (flow rate, 60 ml h^{-1}; fractions, 2.5 ml). The absorbance at 280 nm was recorded, using a L-7455 Diode-Array detector (Merck), and analysed with D-7000 HPLC System Manager (Version3.1) Software. The protein content of the eluted fractions was determined according to the Bradford method with Coomassie brilliant blue using bovine serum albumin as the standard. The total sugar content of the eluted fractions was evaluated by the phenol-sulphuric method described by Dubois *et al.* (1956) using glucose as the standard. All the fractions eluted were tested for surface activity by measuring the surface tension of the samples with the Ring method, as described in Chapter 4 (Rodrigues *et al.* 2005c). Six fractions were obtained from this fractionation procedure: fraction A, B and C from biosurfactant 1 (*L. lactis* 53), and fractions D, E and F from biosurfactant 2 (*S. thermophilus* A). The isolated fractions were dialyzed against demineralized water at 4ºC in a Spectrapor membrane tube (molecular weight cut off 6000 to 8000, Spectrum Medical Industries Inc., CA) and freeze-dried, and the fractions were used for further studies.

10.2.4 Blood Agar Test

The blood agar method is widely used to screen for biosurfactant production and several studies where this method was employed are reported in the literature (Moran *et al.* 2002, Carrillo *et al.* 1996, Banat 1993). Briefly, each sterilized (120ºC, 15 min) crude biosurfactant and isolated fractions dissolved in PBS (at concentrations ranging from 2.5-40 g l^{-1}) were streaked onto blood agar plates and incubated for 48 h at 37ºC. Also, a control plate was incubated with PBS. The plates were visually inspected for zones of clearing around colonies. The diameter of the clear zones depends on the concentration of the crude biosurfactant. The zones of clearing were scored as follows: ‘-‘, no haemolysis; ‘+’, incomplete haemolysis; ‘++’, complete haemolysis with a diameter of lysis < 1 cm; ‘+++’, complete haemolysis with a diameter of lysis > 1 cm but < 3 cm; and ‘++++’, complete haemolysis with a diameter of lysis > 3 cm.

10.2.5 Oil Spreading Test

For the oil spreading technique, 50 ml of distilled water was added to a large Petri dish (25 cm diameter) followed by addition of 20 µl of crude oil to the surface of water, 10 µl of crude biosurfactant and isolated fractions dissolved in PBS (at concentrations ranging from 2.5-40 g l^{-1}) were then added to the surface of oil. Also, a control plate was evaluated with PBS. By plotting the diameter of the clear zone on the oil surface against the concentration of biosurfactant tested is possible to establish a standard curve for each tested sample. The diameters of triplicate assays from the same sample were determined.

10.2.6 Critical Micelle Concentration (*cmc*)

The concentration at which micelles began to form was represented as the *cmc* (critical micelle concentration). The *cmc* was determined by plotting the surface tension as a function of the biosurfactant concentration. Concentrations ranging from 2.5-40 g l^{-1} of the crude biosurfactants and isolated fractions were prepared with PBS. The surface tension of each sample was determined by the Ring method (Rodrigues *et al.* 2005c) using a KRUSS Tensiometer equipped with a 1.9 cm De Noüy platinum ring at room temperature (25 ± 1ºC). Measurements were done in triplicate.

10.2.7 Anti-adhesion Assay in 96 Wells Plate

The anti-adhesive activity of the crude biosurfactant and isolated fractions against four bacterial strains, *Staphylococcus epidermidis* GB 9/6, *Streptococcus salivarius* GB 24/9, *Staphylococcus aureus* GB 2/1, and *Rothia dentocariosa* GBJ 52/2B and two yeast strains: *Candida albicans* GBJ 13/4A and *Candida tropicalis* GB 9/9 isolated from explanted voice prostheses, was quantified according to a previously reported adhesion assay (Heinemann *et al.* 2000, Stepanovic *et al.* 2000). Briefly, the wells of a sterile 96-well flat-bottomed plastic tissue culture plate with a lid were filled with 200 µl of the crude biosurfactant and isolated fractions to be tested for anti-adhesive activity. Several concentrations were tested ranging from 2.5 to 40 g l^{-1}. The plate was incubated for 18 h at 4ºC and subsequently washed twice with PBS. Control wells contained buffer (PBS) only. An aliquot of 200 µl of a washed bacterial or yeast suspension was added and incubated in the wells for 4 h at 4ºC. Unattached organisms were removed by washing the wells three times with PBS. The adherent microorganisms were fixed with 200 µl of 99% methanol per well, and after 15 min the plates were emptied and left to dry. Then the plates were stained for 5 min with 200 µl of 2% crystal violet used for Gram staining per well. Excess stain was rinsed out by placing the plate under running tap water. After the plates were air dried, the dye bound to the adherent microorganisms was resolubilized with 200 µl of 33% (v/v) glacial acetic acid per well and the optical density readings of each well were taken at 595 nm. The control wells were set at 0% as no microbial inhibition occurs, thus when negative microbial inhibition percentages are calculated it means that the adhesion is being favored and no positive effect in the inhibition is registered. The microtiter-plate anti-adhesion assay allows the estimation of the crude biosurfactant and isolated fractions concentrations that are effective in inhibiting adhesion of the microorganisms studied.

10.2.8 Antimicrobial Assay

The microorganisms used for the growth inhibition test were the same used in the anti-adhesion assay in 96 wells plate described above. The inhibition test was performed as described by Elving *et al.* (2000). Briefly, yeasts and bacteria cultured overnight under appropriate conditions were harvested by centrifugation and diluted in reduced transport fluid (RTF: 0.9 g l^{-1} NaCl, 0.9 g l^{-1} $(NH_4)_2SO_4$, 0.45 g l^{-1} KH_2PO_4, 0.19 g l^{-1} $MgSO_4$, 0.45 g l^{-1} K_2HPO_4, 0.37 g l^{-1} Na_2EDTA, 0.2 g l^{-1} L-cysteine HCl, pH 6.8) to a concentration allowing confluent growth when plated with a cotton swab on the agar. Yeasts were plated on MRS agar (OXOID, Basingstoke, England), while bacteria were plated on brain heart infusion agar (OXOID, Basingstoke, England). Agar plates were dried for 20 min at room temperature and 5 µl isolated fractions of several concentrations (ranging from 2.5-40 g l^{-1}), were spotted onto the surface of the agar plate. After overnight incubation, the agar plates were screened for growth inhibition zones around the isolated fraction spots. The antimicrobial activity of the crude biosurfactants (1 and 2) was determined in Chapter 8 (Rodrigues *et al.* 2004b).

10.2.9 Biosurfactant Desorption Assay

The desorption of biosurfactants and isolated fractions attached to silicone rubber was evaluated by advancing type contact angles determination over a period of 3 months. Advancing type contact angles with Millipore water on silicone rubber with adsorbed crude biosurfactant or isolated fraction layer at 40 g l^{-1} were measured with an optical contact-measuring KRUSS device using the sessile drop technique. On each sample, at least six droplets were placed at different positions and results of three separately prepared surfaces with adsorbed biosurfactant were averaged. Conditioned silicone rubber was prepared through overnight immersion in a biosurfactant or isolated fraction solution at 4ºC. Over a period of 3 months the silicone rubber conditioned with biosurfactant or isolated fraction was rinsed 3 times a day in PBS solution in order to simulate the same conditions used in the artificial throat experiments described in Chapter 8. All the samples were left to air dry before contact angle measurements. Bare silicone rubber was used as control.

10.2.10 Biosurfactant Stability

Crude biosurfactants and isolated fractions were prepared with a 40 g l^{-1} concentration at several pH (pH 4-10). To determine the pH stability of each crude biosurfactant and isolated fraction the surface tension was measured by the Ring method (Rodrigues *et al.* 2005c).

10.2.11 Fourier Transform Infrared Spectroscopy

For further characterization, 1 mg of freeze-dried crude biosurfactants (1 and 2) and isolated fractions (A and F) were ground with 100 mg of KBr and pressed with 7500 kg for 30 s to obtain translucent pellets. Infrared absorption spectra were recorded on a FTIR/Diffus Bomem MB spectrometer with a spectral resolution and wavenumber accuracy of 4 cm^{-1} and 0.01 cm^{-1}, respectively. All measurements consisted of 500 scans, and a KBr pellet was used as the background reference. Quantification of a spectral region of interest was obtained by normalizing the area under the absorption bands with respect to the area of the CH absorption band around 2920 cm^{-1}.

10.2.12 X-ray Photoelectron Spectroscopy

Freeze-dried crude biosurfactants (1 and 2) and isolated fractions (A and F) were dissolved in demineralized water (30 g l^{-1}) and 10 µl droplets were placed on gold-coated glass slides (1 cm × 1 cm). After air drying, glass slides were inserted into the chamber of the spectrometer (Surface Science Instruments, S-probe). The residual pressure in the spectrometer was approximately 10^{-9} Pa. A magnesium anode was used for X-ray production (10 kV, 22 mA) at a spot size of 250 µm × 1000 µm. After a scan of the overall spectrum in the binding energy range 1-1200 eV at low resolution (150 eV pass energy), peaks over a 20 eV binding energy range were recorded at high resolution (50 eV pass energy) in the following order: C_{1s}, O_{1s}, N_{1s}, P_{2p}, and C_{1s} again in order to be able to account for contamination or deterioration of the samples caused by X-radiation. The areas under the peaks, after Shirley background subtraction and correction with instrument sensitivity factors, were used to calculate the elemental surface concentration ratios N/C, O/C and P/C.

The C1s peak was decomposed by a least-squares fitting program into four Gaussian components set at 284.5 eV (C1), 285.9 eV (C2), 287.3 eV (C3) and 289.2 eV (C4) by imposing a constant full width at half maximum of 1.35 eV; these four components were thought to be representative of the carbon involved in C-C and C-H bonds, C-O and C-N bonds (including ether, alcohol, amine or amide (Boonaert and Rouxhet 2000)), (C=O)-N and (C=O)-O bonds (including amide, carbonyl, carboxylate, acetal or hemiacetal or amide (Boonaert and Rouxhet 2000)), and (C=O)-OH, respectively. The oxygen peak was split into two components at 530.33 (O1) and 531.83 (O2) eV by imposing a constant full width at half maximum of 1.70 eV and thought to be representative of oxygen involved in O=C and C-O bonds, respectively.

10.2.13 Mass spectrometry (LC-MS)

Mass spectrometry characterization and detection of the crude biosurfactants (1 and 2) and isolated fractions (A and F) under investigation was carried out using an LCQTM ion-trap mass spectrometer (Finnigan MAT, San Jose, California, USA) utilizing electrospray ionization (ESI). Samples under investigation were infused into the mass spectrometer at a flow rate of 10 µl min^{-1}. In the ESI source nitrogen sheath and auxiliary gas flows were maintained at 50 and 5, respectively, and refer to arbitrary values set by the software. The heated capillary temperature was 250ºC and the spray voltage set to 5 kV. Positive ion mode was used throughout and scans were initiated over the 50-1000 *m/z* range.

10.2.14 Quantitative Determination of Monosaccharides

2-3 mg of freeze-dried crude biosurfactants (1 and 2) and isolated fractions (A and F) were weighted into a Sovirel tube recording the exact mass; 200 µl of sulfuric acid at 72% (w/w) was added to each tube and incubated at room temperature, stirring frequently. After 3 hours, 2.2 ml distilled water was added, the tubes were stirred and incubated at 100ºC for 2 hours and a half. Neutral sugars, released by hydrolysis were analysed by gas–liquid chromatography in a Carlo Erba GC 6000 series 2, with a FID detector and a DB 225 column. The injector and detector temperatures were at 220 and 230ºC, respectively. Following injection (approximately 2µl) of the sample, the oven was maintained at 220ºC for 5 min, followed by a temperature rise of 5ºC per minute, until 230ºC. This temperature was maintained for 6 min. Hydrogen was used as a carrier gas at 78 Pa. Reference neutral sugars eluted in the following order: rhamnose, fucose, arabinose, xylose, mannose, galactose and glucose.

10.3 RESULTS

10.3.1 Partial Purification of the Biosurfactants

Fractionation was performed on an Octyl Sepharose 4 FF Prep hydrophobic interaction for biosurfactant 1 and 2 (obtained from *L. lactis* 53 and *S. thermophilus* A respectively). Figure 10.1 and 10.2 show the fractionation profile concerning total protein and total sugar content, absorbance at 280 nm and surface tension, for biosurfactant 1 and 2 respectively. For the crude biosurfactant 1 loaded onto the column it was determined a surface tension of 36 mN m^{-1}; an absorbance at 280 nm of 3.096; a total sugar content of 0.235 g l^{-1} and a total protein

content of 0.587 g l^{-1}. From fractionation experiment it were isolated three fractions with surface activity, namely fraction A (from 20 to 27.5 min), fraction B (from 35 to 40 min) and fraction C (from 42.5 to 47.5 min) with surface tensions 36, 47 and 56 mN m^{-1}, respectively. Fraction A exhibited the higher values of absorbance (0.106), sugar (0.025 g l^{-1}) and total protein content (0.046 g/l) as compared to fraction B and C. For the crude biosurfactant 2 it was determined a surface tension of 37 mN m^{-1}; an absorbance at 280 nm of 2.876; a total sugar content of 0.189 g l^{-1} and a total protein content of 0.325 g l^{-1}. Also, three distinct fractions with surface activity were isolated, fraction D (from 20 to 27.5 min), fraction E (from 32.5 to 37.5 min) and fraction F (from 42.5 to 47.5 min) with surface tensions 50, 49 and 36 mN m^{-1} respectively. All the fractions D, E and F exhibited similar contents of total sugar (0.015 g l^{-1} on average) and protein (0.027 g l^{-1} on average). All fractions obtained from both biosurfactants (1 and 2) were used for further studies.

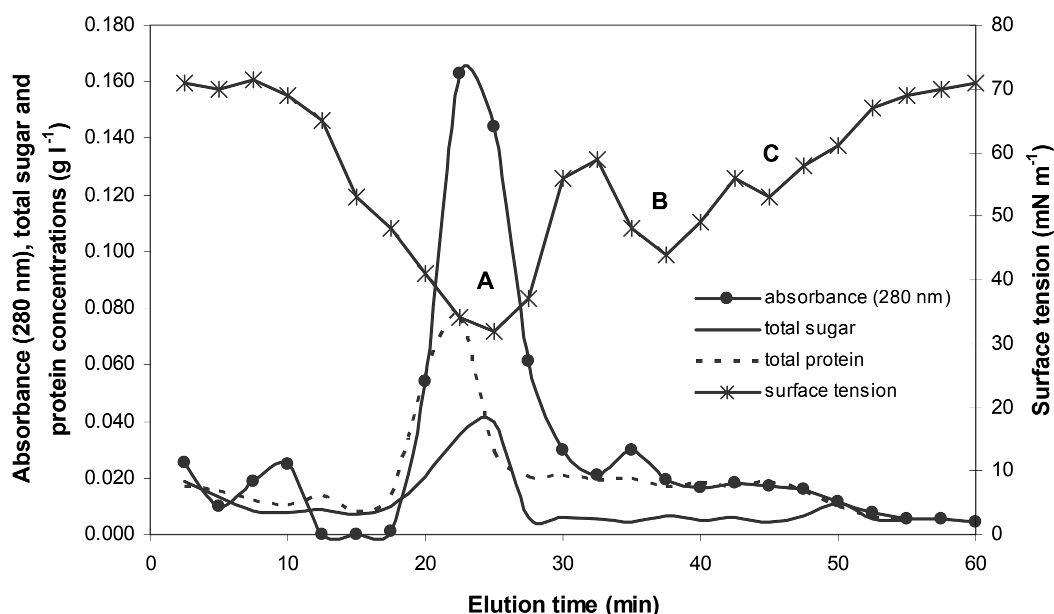


Figure 10.1 Elution profile of the crude biosurfactant obtained from *L. lactis* 53 on Octyl Sepharose 4 FF Prep column. Fractions were eluted with a linear gradient from 1 to 0 M $(NH_4)_2SO_4$ in PBS buffer. Fractions were collected and monitored by absorbance at 280 nm record, total sugars (phenol-sulfuric method), total protein (Bradford method) and surface tension (Ring method). Results represent the average of three independent experiments.

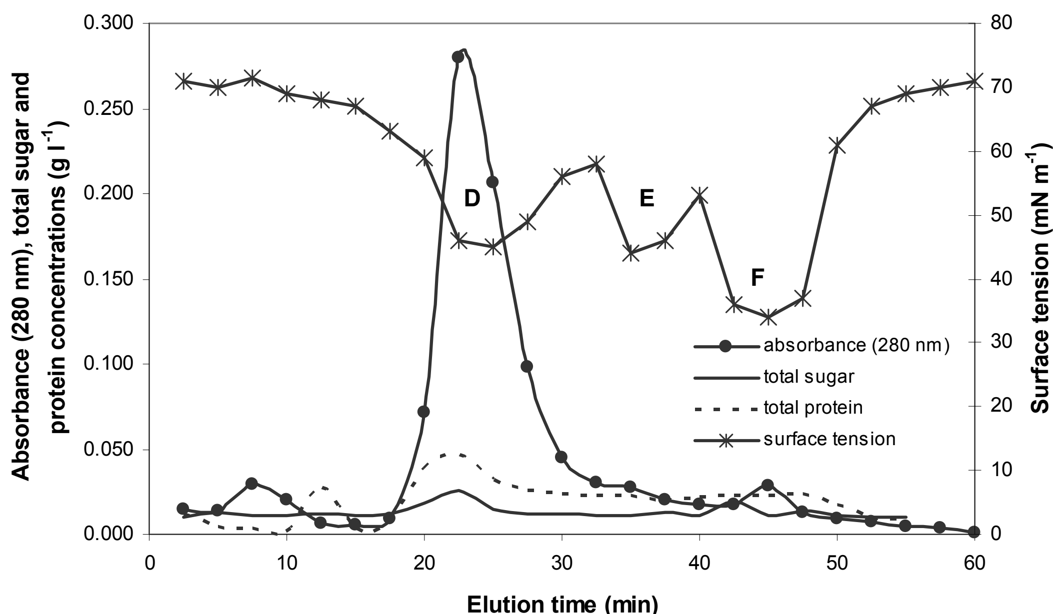


Figure 10.2 Elution profile of the crude biosurfactant obtained from *S. thermophilus* A on Octyl Sepharose 4 FF Prep column. Fractions were eluted with a linear gradient from 1 to 0 M $(NH_4)_2SO_4$ in PBS buffer. Fractions were collected and monitored by absorbance at 280 nm record, total sugars (phenol-sulfuric method), total protein (Bradford method) and surface tension (Ring method). Results represent the average of three independent experiments.

10.3.2 Haemolytic Activity

All the tested concentrations (ranging from 2.5 to 40 g l^{-1}) of crude biosurfactants (1 and 2) and isolated fractions (A, B, C, D, E and F) showed no zones of clearing in the blood agar with scores corresponding to no haemolysis (-), as determined by the blood agar test.

10.3.3 Oil Spreading Capacity

Table 10.1 illustrates the relation between crude biosurfactants (1 and 2) and isolated fractions (A, B, C, D, E and F) concentrations with the zones of clearing on the oil surface. For each tested sample a linear relationship between concentration and diameter of the clearing zone could be observed at concentrations above 5 g l^{-1}. The clearing zones are an indirect measure of the surface activity of the tested samples against hydrocarbons, thus the higher diameters represent the most surface active samples. Comparing crude biosurfactants

with isolated fractions it was possible to observe that fraction A has a higher surface activity than biosurfactant 1, and fraction F than biosurfactant 2. The fractions B, C, D and E show lower surface activities as lower diameters were found. At concentrations below 5 g l^{-1} small clear zones were observed for all tested samples.

Table 10.1 Diameters (mm) of the clearing zones on the oil surface obtained from oil spreading assay with several crude biosurfactants (1 and 2) and isolated fractions concentrations. PBS was used as control with a diameter of 0.0 mm as no clearing zone occurs. Standard deviations (±) were determined over three separate measurements.

Sample	Diameter of the clearing zone (mm)					
	Crude biosurfactants or isolated fractions concentrations (g l^{-1})					
	PBS control	2.5	5	10	20	40
Crude biosurfactant 1	0.0	1.2 ± 0.09	4.5 ± 0.03	7.2 ± 0.12	8.8 ± 0.07	11.5 ± 0.02
Fraction A	0.0	2.1 ± 0.14	6.9 ± 0.02	8.5 ± 0.05	9.6 ± 0.05	12.4 ± 0.07
Fraction B	0.0	0.1 ± 0.02	3.6 ± 0.08	4.9 ± 0.01	7.3 ± 0.03	8.8 ± 0.07
Fraction C	0.0	0.1 ± 0.01	2.9 ± 0.09	4.2 ± 0.09	6.1 ± 0.05	7.8 ± 0.15
Crude biosurfactant 2	0.0	1.5 ± 0.16	4.6 ± 0.02	5.6 ± 0.10	7.2 ± 0.08	8.7 ± 0.02
Fraction D	0.0	0.8 ± 0.07	0.9 ± 0.11	2.1 ± 0.02	4.6 ± 0.11	7.8 ± 0.09
Fraction E	0.0	0.7 ± 0. 03	1.0 ± 0.03	1.9 ± 0.06	3.4 ± 0.14	7.5 ± 0.11
Fraction F	0.0	1.9 ± 0.04	5.7 ± 0.03	7.3 ± 0.13	8.4 ± 0.02	9.9 ± 0.01

10.3.4 Critical Micelle Concentration (*cmc*)

The critical micelle concentration (*cmc*) for each crude biosurfactant (1 and 2) and isolated fractions is represented in Figure 10.3. Fraction A and F were found to be the most surface active samples, with *cmc* of 10 g l^{-1} and 5 g l^{-1} respectively, thus similar to the crude biosurfactants (1 and 2). Fractions B, D and E showed small decreases in the surface tensions despite of the increasing concentrations, and for Fraction C almost no decrease was observed.

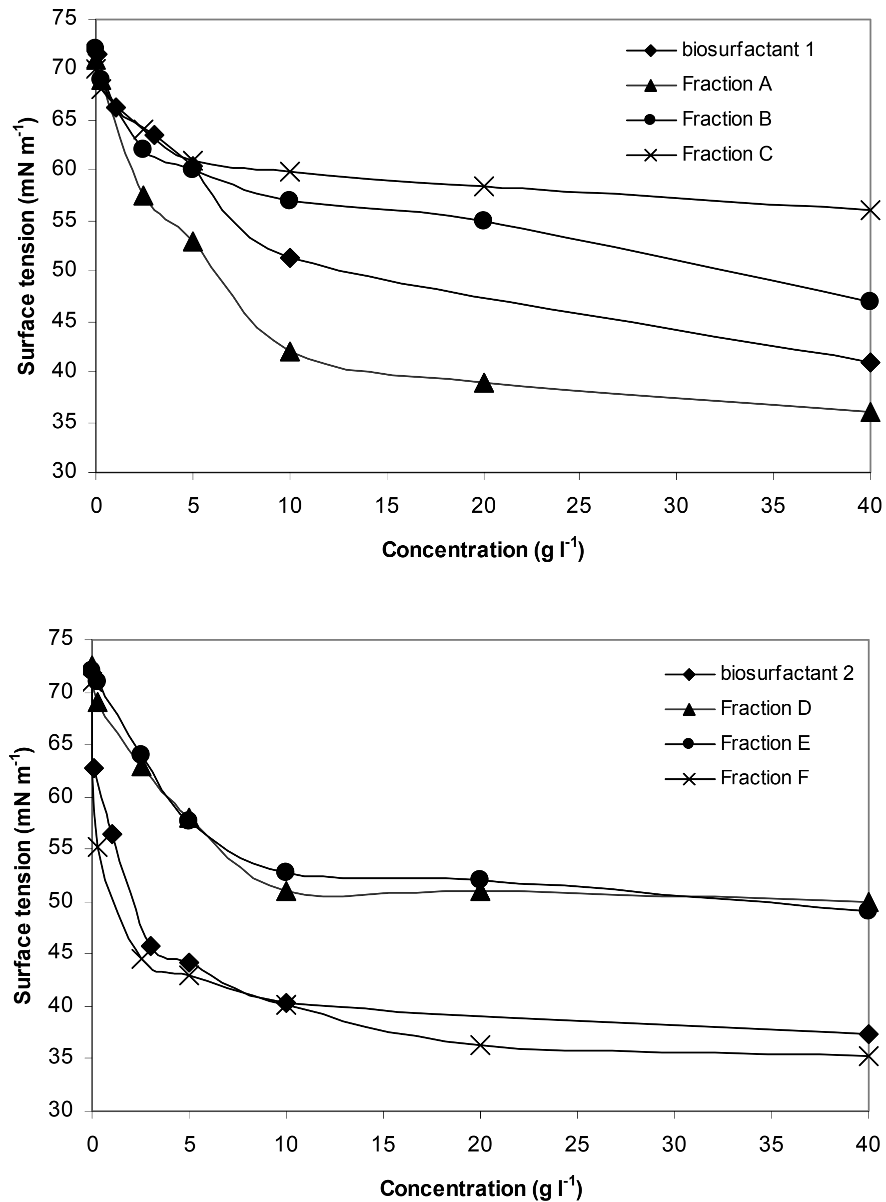


Figure 10.3 Surface tension *versus* concentrations of the crude biosurfactants (1 and 2) and isolated fractions obtained from **(A)** *L. lactis* 53 or **(B)** *S. thermophilus* A, respectively. Results represent the average of three independent experiments.

10.3.5 Anti-adhesive Activity

The anti-adhesive activity of the crude biosurfactants (1 and 2) and isolated fraction was evaluated at several concentrations and compared against a variety of bacterial and yeast strains isolated from explanted voice prostheses (Table 10.2 and 10.3). The crude biosurfactants (1 and 2) and fractions A and F had an anti-adhesive effect against all

microorganisms tested but the anti-adhesive effect depends on the concentration and microorganism tested. No inhibition of yeast strains was observed, for fractions B, C, D and E. Both the crude biosurfactant 1 and fraction A showed the highest anti-adhesive activity against *S. epidermidis* GB 9/6 and *S. aureus* GB 2/1 with inhibition percentages till 70% and worked even at a concentration of 2.5 g l^{-1}. Fractions B and C showed far less inhibition percentages when compared to the crude biosurfactant 1. For the crude biosurfactant 2 and fraction F the highest anti-adhesive activity was found against *S. epidermidis* GB 9/6, *S. salivarius* GB 24/9 and *S. aureus* GB 2/1 with inhibition percentages between 53 and 77%. Fractions D and E showed lower inhibition percentages against bacterial strains between 15 and 44% for the highest concentration tested.

Table 10.2 Microbial inhibition percentages obtained from the microtiter-plate anti-adhesion assay with several crude biosurfactant 1 and correspondent isolated fractions (A, B and C) concentrations (g l^{-1}). PBS was used as control and set at 0% as no microbial inhibition occurs. Experiments were carried out in triplicate and correspond within 20%.

Microorganism	Microbial adhesion inhibition (%)										
	Control	Crude biosurfactant 1					Fraction A				
	PBS	2.5	5	10	20	40	2.5	5	10	20	40
S. epidermidis GB 9/6	0.0	21	39	43	50	63	31	58	65	65	72
S. salivarius GB 24/9	0.0	-6	4	18	33	34	0	6	24	35	42
S. aureus GB 2/1	0.0	13	34	37	53	62	15	34	44	58	69
R. dentocariosa GBJ 52/2B	0.0	-10	9	17	19	20	-3	13	17	22	39
C. albicans GBJ 13/4A	0.0	-57	-38	-12	-4	2	-5	2	7	13	18
C. tropicalis GB 9/9	0.0	-44	-9	-4	1	4	-2	4	11	15	21

Microorganism	Microbial adhesion inhibition (%)										
	Control	Fraction B					Fraction C				
	PBS	2.5	5	10	20	40	2.5	5	10	20	40
S. epidermidis GB 9/6	0.0	19	22	25	33	36	18	20	25	32	36
S. salivarius GB 24/9	0.0	-2	2	8	14	23	-3	2	7	11	20
S. aureus GB 2/1	0.0	11	20	21	30	30	9	12	15	17	18
R. dentocariosa GBJ 52/2B	0.0	-11	1	6	11	13	-11	-4	1	2	4
C. albicans GBJ 13/4A	0.0	-57	-39	-29	-26	-19	-67	-43	-34	-31	-30
C. tropicalis GB 9/9	0.0	-49	-17	-14	-11	-9	-56	-34	-26	-16	-12

Table 10.3 Microbial inhibition percentages obtained from the microtiter-plate anti-adhesion assay with several crude biosurfactant 2 and correspondent isolated fractions (D, E and F) concentrations (g l^{-1}). PBS was used as control and set at 0% as no microbial inhibition occurs. Experiments were carried out in triplicate and correspond within 15%.

Microorganism	**Microbial adhesion inhibition (%)**										
	Control	**Crude biosurfactant 2**					**Fraction D**				
	PBS	2.5	5	10	20	40	2.5	5	10	20	40
S. epidermidis GB 9/6	0.0	28	40	62	69	70	-2	3	12	33	44
S. salivarius GB 24/9	0.0	-5	24	48	56	53	-8	2	16	26	32
S. aureus GB 2/1	0.0	11	22	42	55	56	-1	3	12	29	34
R. dentocariosa GBJ 52/2B	0.0	-70	12	20	31	35	-77	-43	7	15	17
C. albicans GBJ 13/4A	0.0	-100	-67	-21	4	11	-123	-21	-19	-14	-4
C. tropicalis GB 9/9	0.0	-43	-23	-7	8	18	-73	-31	-31	-12	-10
Microorganism	**Microbial adhesion inhibition (%)**										
	Control	**Fraction E**					**Fraction F**				
	PBS	2.5	5	10	20	40	2.5	5	10	20	40
S. epidermidis GB 9/6	0.0	34	57	63	60	43	34	46	65	71	77
S. salivarius GB 24/9	0.0	-10	2	12	20	32	2	35	51	59	62
S. aureus GB 2/1	0.0	-2	3	13	22	32	16	22	41	52	56
R. dentocariosa GBJ 52/2B	0.0	-89	-33	-3	14	15	-55	16	21	32	36
C. albicans GBJ 13/4A	0.0	-123	-33	-17	-15	-3	-88	-43	-12	6	15
C. tropicalis GB 9/9	0.0	-74	-43	-36	-22	-11	-34	-15	3	11	23

10.3.6 Antimicrobial Activity

The antimicrobial activities of the isolated fractions were evaluated at several concentrations and compared to a variety of bacterial and yeast strains isolated from explanted voice prostheses (Table 10.4 and 10.5). Both fraction A and F are antimicrobial agents but, depending on the microorganism, there are different effective concentrations. It was found that both fractions show a high antimicrobial activity against *C. tropicalis* GB 9/9 even at low concentrations. At the highest concentration tested (40 g l^{-1}) both fractions were active against all bacterial and yeast strains involved in this study, except for *R. dentocariosa* GBJ52/2B which formed some colonies within the fraction spots. From results on Table 10.4, it was observed that fractions B and C showed no antimicrobial activity against *R. dentocariosa* GBJ52/2B and low activity against *S. aureus* GB 2/1 and *C. albicans* GBJ 13/4A even at the higher concentration tested. Results found for fractions D and E (Table

10.5) showed that these fractions had no antimicrobial activity against *S. epidermidis* GB 9/6, *R. dentocariosa* GBJ52/2B and *C. tropicalis GB* 9/9 at 40 g l^{-1}. The antimicrobial activities of the crude biosurfactants were presented on Chapter 8 (Table 8.1) and it was seen that both crude biosurfactants were effective at concentrations of 100 g l^{-1}, thus the isolated fractions A and F are effective at lower concentrations than the crude preparations.

Table 10.4 Antimicrobial activities of fractions isolated from crude biosurfactant 1 with different concentrations against several bacterial and yeast strains isolated from explanted voice prostheses. The experiments were scored as positive (+) when growth inhibition was observed (no colonies formed); a (±) sign indicated some colonies formed within the zones; and no growth inhibition was marked as negative (-).

Fraction A					
Microorganism	**2.5 g l^{-1}**	**5 g l^{-1}**	**10 g l^{-1}**	**20 g l^{-1}**	**40 g l^{-1}**
S. epidermidis GB 9/6	±	+	+	+	+
S. salivarius GB 24/9	-	-	±	±	+
S. aureus GB 2/1	+	+	+	+	+
R. dentocariosa GBJ 52/2B	-	-	-	±	±
C. albicans GBJ 13/4A	-	-	±	±	+
C. tropicalis GB 9/9	+	+	+	+	+
Fraction B					
Microorganism	**2.5 g l^{-1}**	**5 g l^{-1}**	**10 g l^{-1}**	**20 g l^{-1}**	**40 g l^{-1}**
S. epidermidis GB 9/6	±	±	±	±	+
S. salivarius GB 24/9	-	-	±	+	+
S. aureus GB 2/1	-	-	-	-	±
R. dentocariosa GBJ 52/2B	-	-	-	-	-
C. albicans GBJ 13/4A	-	-	-	-	±
C. tropicalis GB 9/9	-	-	-	-	+
Fraction C					
Microorganism	**2.5 g l^{-1}**	**5 g l^{-1}**	**10 g l^{-1}**	**20 g l^{-1}**	**40 g l^{-1}**
S. epidermidis GB 9/6	±	+	+	+	+
S. salivarius GB 24/9	±	+	+	+	+
S. aureus GB 2/1	-	-	-	±	±
R. dentocariosa GBJ 52/2B	-	-	-	-	-
C. albicans GBJ 13/4A	-	-	-	±	±
C. tropicalis GB 9/9	-	-	±	+	+

Table 10.5 Antimicrobial activities of fractions isolated from crude biosurfactant 2 with different concentrations against several bacterial and yeast strains isolated from explanted voice prostheses. The experiments were scored as positive (+) when growth inhibition was observed (no colonies formed); a (±) sign indicated some colonies formed within the zones; and no growth inhibition was marked as negative (-).

Fraction D					
Microorganism	**2.5 g l^{-1}**	**5 g l^{-1}**	**10 g l^{-1}**	**20 g l^{-1}**	**40 g l^{-1}**
S. epidermidis GB 9/6	-	-	-	-	-
S. salivarius GB 24/9	±	±	±	±	+
S. aureus GB 2/1	-	-	±	±	±
R. dentocariosa GBJ 52/2B	-	-	-	-	-
C. albicans GBJ 13/4A	-	-	±	±	+
C. tropicalis GB 9/9	-	-	-	-	-
Fraction E					
Microorganism	**2.5 g l^{-1}**	**5 g l^{-1}**	**10 g l^{-1}**	**20 g l^{-1}**	**40 g l^{-1}**
S. epidermidis GB 9/6	-	-	-	-	-
S. salivarius GB 24/9	-	-	-	±	+
S. aureus GB 2/1	-	-	±	±	+
R. dentocariosa GBJ 52/2B	-	-	-	-	-
C. albicans GBJ 13/4A	-	-	-	±	±
C. tropicalis GB 9/9	-	-	-	-	-
Fraction F					
Microorganism	**2.5 g l^{-1}**	**5 g l^{-1}**	**10 g l^{-1}**	**20 g l^{-1}**	**40 g l^{-1}**
S. epidermidis GB 9/6	±	±	+	+	+
S. salivarius GB 24/9	-	±	±	±	+
S. aureus GB 2/1	±	+	+	+	+
R. dentocariosa GBJ 52/2B	-	-	-	±	±
C. albicans GBJ 13/4A	-	±	+	+	+
C. tropicalis GB 9/9	+	+	+	+	+

10.3.7 Desorption Assay

Table 10.6 summarizes contact angles measured over a period of 3 months with water on silicone rubber with adsorbed crude biosurfactants (1 and 2) and isolated fractions layers at 40 g l^{-1}. The water contact angle on bare silicone rubber decreased from 109 ± 2 degrees to

40 ± 1 and 43 ± 1 degrees after adsorption of the crude biosurfactant 1 and 2 respectively. Fraction A and F decreased the bare silicone rubber contact angle in a higher extent, 27 ± 1 and 36 ± 2 degrees respectively. For all the tested samples no decrease in bare silicone rubber contact angle was found after 3 months experiment, as the contact angle value achieved after this period is the same as the value determined on bare silicone rubber (109 ± 2 degrees) which means that all samples were desorbed from the silicone surface. The best results were achieved for fraction A and F which stayed adsorbed onto the silicone surface for a 2 and 1 month period respectively. For the crude biosurfactants it was found that after 15 days they were desorbed from the silicone surface. Fraction C and E stayed adsorbed only for 4 days.

Table 10.6 Contact angles (degrees) measured over a period of 3 months at 25ºC on silicone rubber with adsorbed crude biosurfactants (1 and 2) or isolated fractions layers as determined by sessile drop technique. Bare silicone rubber was used as control with a contact angle of 109 ± 2 degrees. Standard deviations (±) were determined over three separate measurements.

Time	Contact angles (degrees)			
	Crude biosurfactant 1	**Fraction A**	**Fraction B**	**Fraction C**
4 hours	40 ± 1	27 ± 1	42 ± 2	54 ± 2
24 hours	48 ± 2	28 ± 1	49 ± 1	61 ± 3
4 days	49 ± 1	29 ± 2	54 ± 1	80 ± 1
8 days	54 ± 3	30 ± 1	68 ± 3	103 ± 2
15 days	68 ± 1	45 ± 2	87 ± 3	105 ± 2
1 month	79 ± 4	56 ± 3	93 ± 1	110 ± 3
2 months	92 ± 2	67 ± 2	97 ± 2	112 ± 5
3 months	104 ± 3	92 ± 1	109 ± 2	113 ± 4
Time (h)	**Contact angles (degrees)**			
	Crude biosurfactant 2	**Fraction D**	**Fraction E**	**Fraction F**
4 hours	43 ± 1	62 ± 2	60 ± 1	36 ± 2
24 hours	46 ± 1	62 ± 1	70 ± 2	37 ± 2
4 days	47 ± 2	69 ± 3	86 ± 2	38 ± 1
8 days	49 ± 2	94 ± 2	91 ± 1	43 ± 1
15 days	58 ± 3	108 ± 1	93 ± 1	51 ± 1
1 month	87 ± 1	110 ± 2	105 ± 1	67 ± 2
2 months	106 ± 1	113 ± 3	114 ± 3	87 ± 1
3 months	108 ± 2	114 ± 3	114 ± 2	91 ± 3

10.3.8 Biosurfactant Stability

The surface tension reducing activity of the crude biosurfactants (1 and 2) and fractions A and F was relatively stable to pH changes (Table 10.7). The surface tensions remained stable over a pH range from 5 to 9, although there was a slight increase at pH 4 and 10. All the tested biosurfactants and fractions became turbid below pH 4 due to precipitation. For fractions B, C, D and E the surface tensions were only stable between pH 6 and 8.

Table 10.7 Surface tension measurements of crude biosurfactants (1 and 2) or isolated fractions with a concentration of 40 mg/ml at several pH values, as determined by the Ring method. Experiments were carried out in triplicate and correspond within 18%.

pH	Surface tension (mN m^{-1})			
	Crude biosurfactant 1	**Fraction A**	**Fraction B**	**Fraction C**
4	45.6	38.4	66.7	72.1
5	41.1	35.3	58.9	71.8
6	39.8	36.1	47.8	64.7
7	41.0	36.1	47.2	56.4
8	39.9	36.2	47.4	57.4
9	42.2	37.4	60.7	62.2
10	47.2	43.2	71.2	65.4
pH	**Surface tension (mN m^{-1})**			
	Crude biosurfactant 2	**Fraction D**	**Fraction E**	**Fraction F**
4	39.4	69.8	71.8	36.2
5	38.1	68.8	64.3	35.8
6	37.2	51.5	48.7	35.2
7	37.3	50.2	49.3	35.3
8	37.1	55.9	56.9	34.9
9	38.3	71.2	55.7	35.1
10	41.4	69.9	65.8	35.9

10.3.9 Fourier Transform Infrared Spectroscopy

The molecular composition of the crude biosurfactants (1 and 2) and isolated fractions (A and F) was evaluated by Fourier transform infrared spectroscopy (FTIR). Figure 10.4 presents the spectra of the freeze-dried samples. All of the spectra showed essentially the same adsorption bands, and only the relative areas under the various absorption bands differed.

The most important bands were located at 2920 cm^{-1} (CH band: CH_2-CH_3 stretching), 1655 cm^{-1} (AmI band: C=O stretching in proteins), 1551 cm^{-1} (AmII band: N-H bending in proteins), 1254 cm^{-1} (PI band: phosphates), and 1065 cm^{-1} (PII band: polysaccharides). Table 10.8 shows the absorption band ratios of AmI, AmII, PI and PII with respect to the CH band around 2920 cm^{-1} for the tested samples. Both crude biosurfactants (1 and 2) spectra showed that these biosurfactants consist of a mixture of several compounds. The crude biosurfactant obtained from *L. lactis* 53 (biosurfactant 1) appeared to contain more protein than the biosurfactant obtained from *S. thermophilus* A (biosurfactant 2). The fractionation procedure for crude biosurfactant 1 resulted in a fraction (fraction A) with similar protein and less polysaccharide and phosphate contents. Both crude biosurfactant 1 and fraction A showed a pronounced absorption band at 2500 cm^{-1}, probably indicating the presence of protonated amines. Regarding fraction F, the spectra showed that AmI and AmII absorption bands indicative of proteins were not detected. The absorption bands between 1000 and 1200 cm^{-1} were attributed to ethereal and hydroxilic C-O stretch vibrations and absorption bands present around 2965, 2920, 2860 and 1398 cm^{-1} that include (-CH_3) symmetric deformation vibrations, (C-H) bending vibrations of CH_3 and CH_2 groups and CH_2-CH_3 stretching vibrations, are characteristic of polysaccharides.

Table 10.8 Chemical composition data of crude biosurfactants (1 and 2) and isolated fractions (A and F) expressed as infrared absorption band ratios by Fourier transform infrared spectroscopy.

Compound	Fourier transform infrared spectroscopy band ratios			
	AmI/CH	**AmII/CH**	**PI/CH**	**PII/CH**
Crude biosurfactant 1	1.3	1.0	0.9	2.1
Fraction A	1.0	0.9	0.5	1.8
Crude biosurfactant 2	1.0	0.9	0.9	1.6
Fraction F	0.0	0.0	0.1	1.0

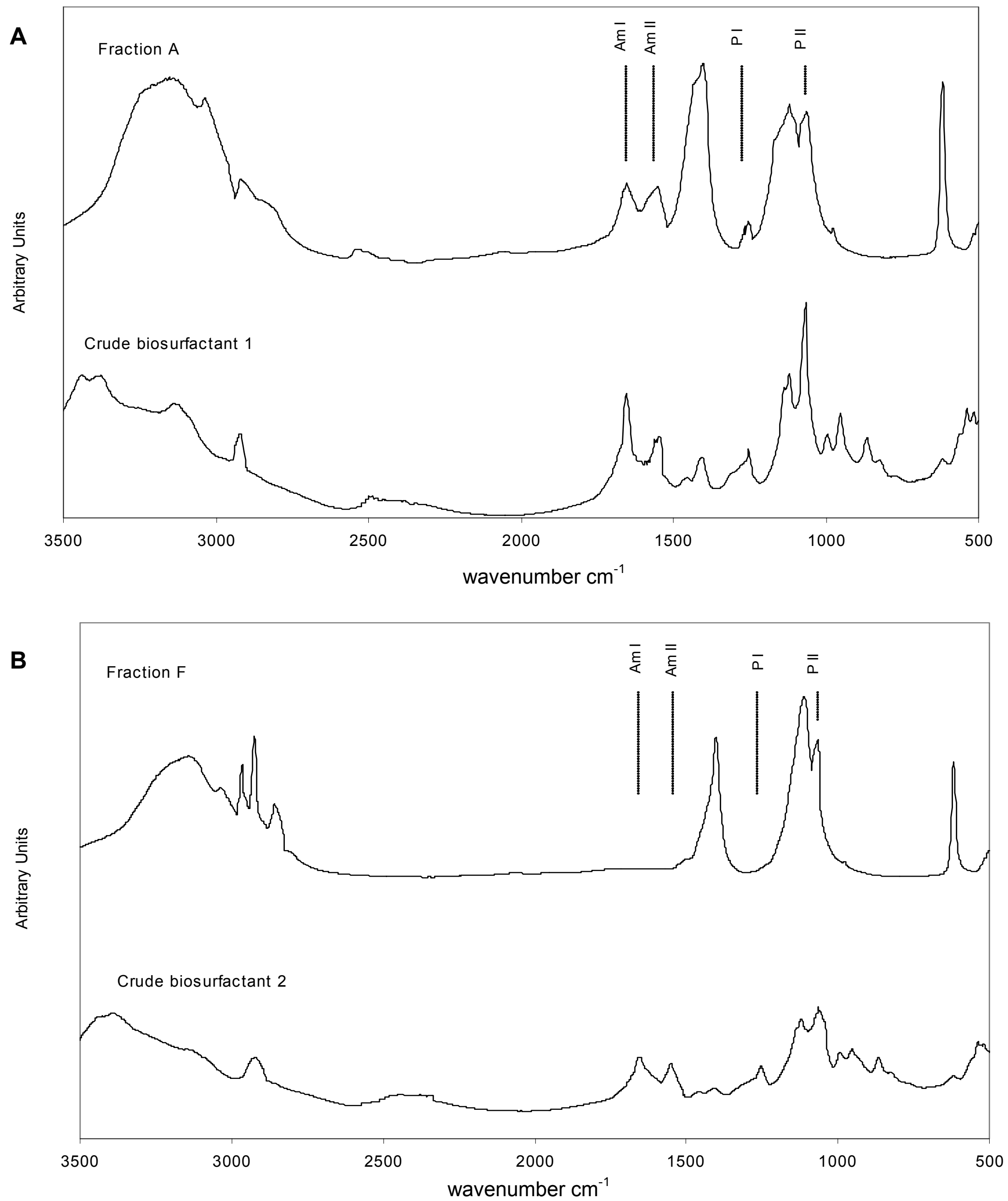


Figure 10.4 Fourier transform infrared absorption spectra of crude biosurfactants (1 and 2) and respective isolated fractions (A and F) obtained from **(A)** *L. lactis* 53 or **(B)** *S. thermophilus* A. The absorption bands used for quantification are indicated.

10.3.10 X-ray Photoelectron Spectroscopy

X-ray photoelectron spectroscopy (XPS) of the freeze-dried crude biosurfactants (1 and 2) and fractions A and F yielded the elemental surface concentration ratio N/C, O/C, P/C and

the components of the C_{1s} and O_{1s} peaks (Table 10.9). From the N/C ratio and C3+C4 peak fractions, it can be concluded that crude biosurfactant 1 and fraction A were rich in protein. The fractionation of crude biosurfactant 1 resulted in a fraction (A) where the high N/C and O/C ratios together with the higher percentage of carbon involved in C1 bonds and oxygen O1 bonds indicates that the material probably contains glycoproteins. For crude biosurfactant 2 and fraction F, although samples contained sizeable amounts of nitrogen, the N/C ratios measured were too low to be identified as pure protein. The O/C ratios were also too high to indicate the presence of pure proteins and point to the presence of polysaccharides. This was in agreement with the FTIR spectra presented in Figure 10.4, where the AmI and AmII absorption bands indicative of proteins were not detected. The XPS data demonstrated that fraction F contained a higher percentage of carbon involved in C2 bonds and oxygen in O2 bonds, while nitrogen was absent. In fact the percentage of oxygen involved in O2 was too high for the material to be identified as a phospholid, but on the basis of the XPS data and infrared absorption bands the material probably contains glycolipids.

Table 10.9 Chemical composition data by XPS of crude biosurfactants (1 and 2), isolated fractions (A and F) and reference compounds (for comparison).

Compound	Elemental composition			Fractions of C and O atoms					
	N/C	**O/C**	**P/C**	**C1**	**C2**	**C3**	**C4**	**O1**	**O2**
Crude biosurfactant 1	0.16	0.61	0.06	0.29	0.44	0.18	0.10	0.41	0.59
Fraction A	0.20	0.65	0.04	0.70	0.22	0.05	0.03	0.62	0.38
Crude biosurfactant 2	0.12	0.63	0.08	0.51	0.34	0.13	0.02	0.66	0.34
Fraction F	0.00	0.60	0.03	0.37	0.41	0.19	0.03	0.41	0.59
Reference compounds									
Glycosidic residue[a]	0.00	0.83	0.00	0.00	0.83	0.17[f]		0.00	1.00
Phospholipid[b]	0.01	0.21	0.03	0.82	0.13	0.05	0.01	0.37	0.63
Cholesterol	0.00	0.03	0.00	0.87	0.12	0.00	0.00	0.00	1.00
LTA[c]	0.03	0.63	0.07	0.41	0.44	0.44	0.05	0.24	0.76
Protein[d]	0.27	0.32	0.00	0.41	0.32	0.28[f]		0.86	0.14
Salivary glycoprotein[e]	0.18			0.52	0.32	0.16[f]			

[a] Glycosidic residue $C_6H_{10}O_5$ (Busscher *et al.* 1997)
[b] L-α-Phosphatidyl-DL-glycerol dimyristoyl (Busscher *et al.* 1997)
[c] Lipoteichoic acid from *S. mutans* (Sigma)
[d] Average protein, calculated for a collection of bacterial, fungal and mammalian proteins (Busscher *et al.* 1997)
[e] Data from Velraeds *et al.* 1996
[f] C3 and C4 together

10.3.11 Mass Spectrometry (LC-MS)

Figure 10.5 and 10.6 represent the mass spectra obtained for crude biosurfactants (1 and 2) and isolated fractions (A and F) as determined by mass spectrometry. From Figure 10.5 A and 10.6 A it is shown that crude biosurfactants (1 and 2) consist of a mixture of all sort of compounds, which is in accordance with FTIR and XPS results. Crude biosurfactants are mainly negatively charged, the smaller molecules may be sugar compounds while the larger ones mainly peptides. Despite fractionation of the crude biosurfactants provided more simple mass spectra (Figure 10.5 B and 10.6 B), they are still to complex to identify pure compounds. Nevertheless, fraction A showed two major peaks at 256.3 and 663.2 *m/Z*; and fraction F showed four major peaks at 284.3, 663.3, 690.2 and 716.2 *m/Z*. Further preliminary MS-MS assays on the main peak (663 *m/Z*) obtained for both isolated fractions (A and F) were carried out (data not shown). The peak at 663 *m/Z* is a singly charged compound that originates from a larger molecule peak at 680 *m/Z*, being the difference one water molecule. When 663 *m/Z* is fragmented it gives the following peaks 607, 551, 495, 439, 384, 327 and finally 251 *m/Z*. It can be assumed that 251 *m/Z* is the base molecule that all others are added to, yet no further identification was possible.

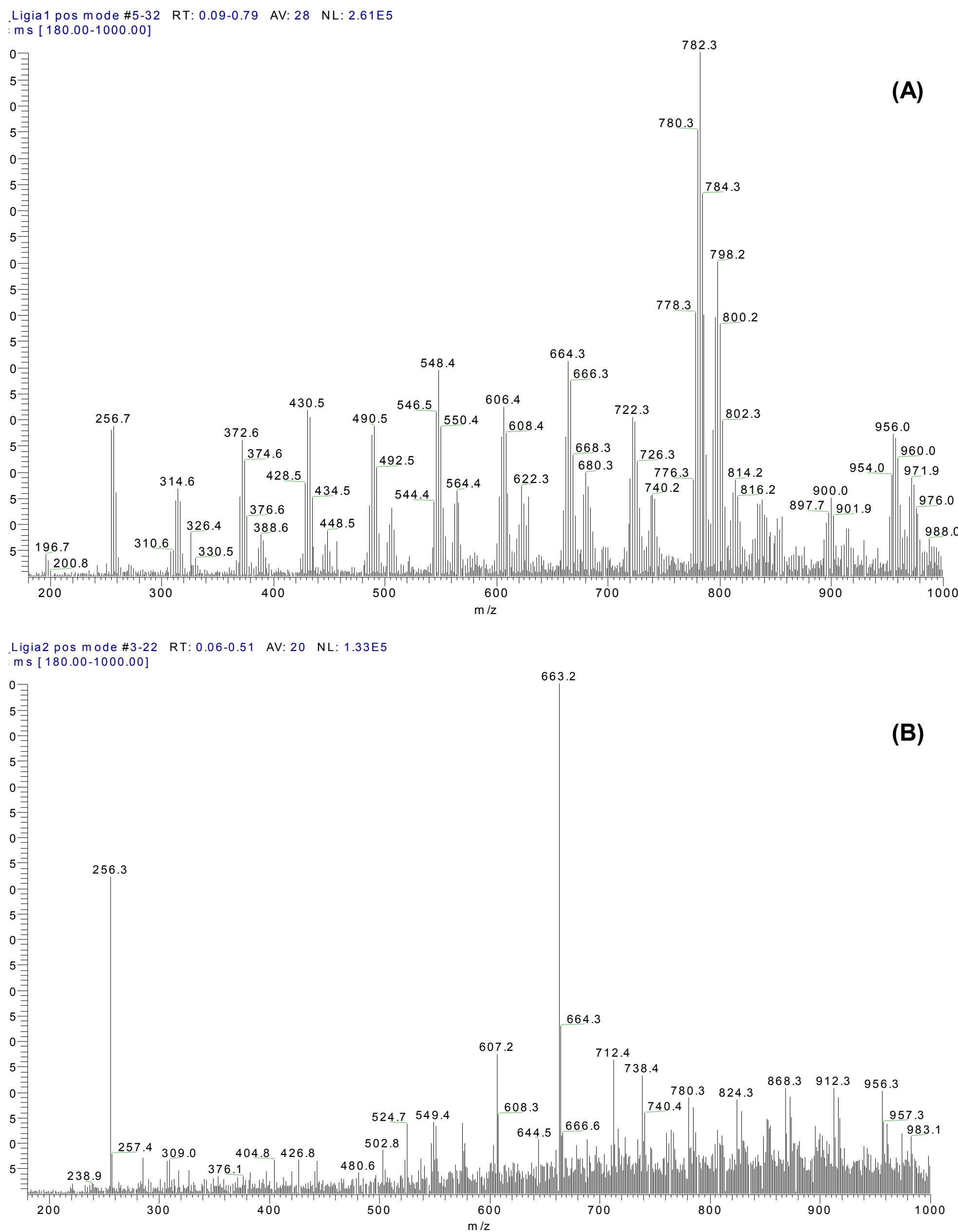
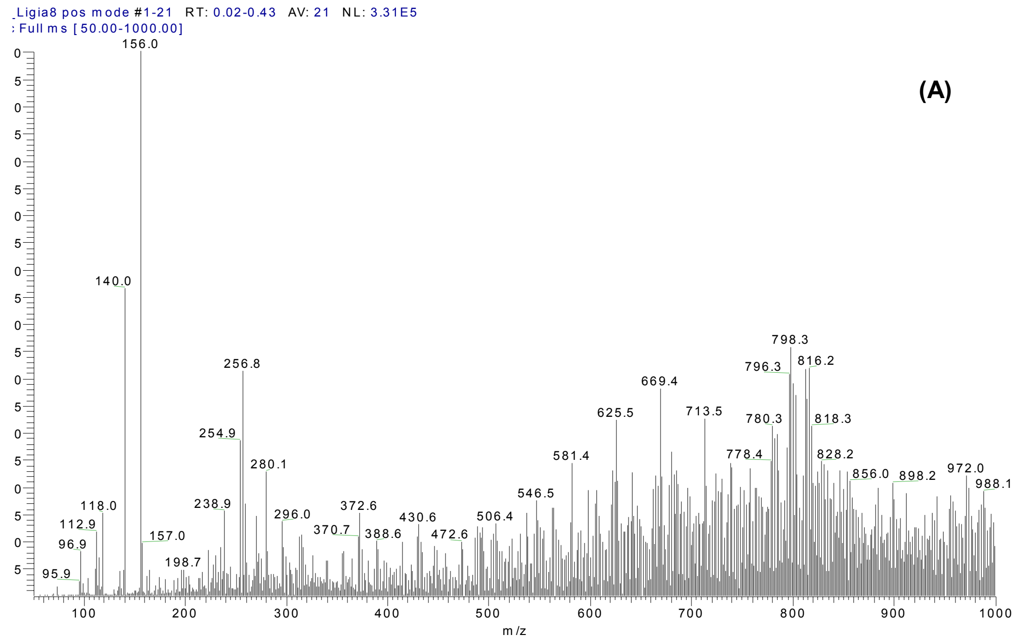


Figure 10.5 Mass spectrum of crude biosurfactant obtained from *L. lactis* 53 **(A)** and respective isolated fraction **(B)**.



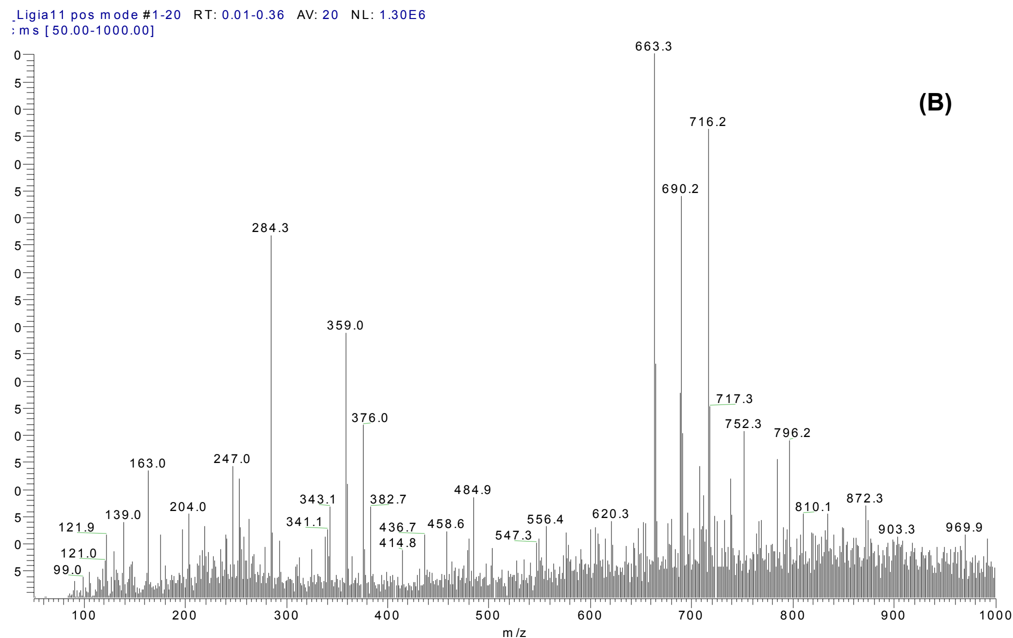


Figure 10.6 Mass spectrum of crude biosurfactant obtained from *S. thermophilus* A **(A)** and respective isolated fraction **(B)**.

10.3.12 Quantitative Determination of Monosaccharides

Crude biosurfactants (1 and 2) and isolated fractions (A and F) main sugars were quantified by gas-liquid chromatography (Table 10.10). The main sugars found for crude biosurfactant 1 were rhamnose (48 mol%), fucose (20 mol%), manose (14 mol%) and glucose (17.7 mol%). Comparing with the monosaccharide composition of the isolated fraction (A) it was found that fractionation procedure resulted in smaller amounts of glucose (7.1 mol%) and rhamnose (40.2 mol%) and higher amounts of fucose (23.9 mol%) and manose (28.8 mol%). Although the total sugars obtained for isolated fraction (24.2 mg g^{-1}) was smaller than the obtained for crude biosurfactant (42.1 mg g^{-1}), rhamnose was the most representative neutral sugar found for both crude biosurfactant and isolated fraction sugar compositions. Regarding the crude biosurfactant 2 higher total sugar content was achieved (128.2 mg g^{-1}) comparing to the isolated fraction F (31.1 mg g^{-1}). The main sugars found for fraction F were rhamnose (62.1 mol%) and fucose (23.7 mol%).

Table 10.10 Monosaccharide composition of the crude biosurfactants (1 and 2) and isolated fractions (A and F) as determined by gas-liquid chromatography.

Compound	Neutral sugars (mol%)							
	Rha*	Fuc*	Ara*	Xyl*	Man*	Gal*	Glc*	Total sugars (mg g^{-1})**
Crude biosurfactant 1	48.3	20.0	0.0	0.0	14.0	0.0	17.7	42.1
Fraction A	40.2	23.9	0.0	0.0	28.8	0.0	7.1	24.2
Crude biosurfactant 2	54.2	18.2	0.0	0.0	7.0	0.0	20.6	128.2
Fraction F	62.1	23.7	0.0	0.0	8.5	0.0	5.7	31.1

* Rha – rhamnose; Fuc – fucose; Ara – arabinose; Xyl – xylose; Man – manose; Gal – galactose; Glc - glucose
** Values are expressed as milligram anhydro sugar per gram

10.4 DISCUSSION

Crude biosurfactants obtained from *L. lactis* 53 and *S. thermophilus* A were physicochemically and biochemically characterized as multicomponent biosurfactants, consisting of protein and polysaccharides which possibly contained bound phosphate groups. Fractions with surface activity were obtained from the crude biosurfactants by hydrophobic interaction chromatography. Hydrophobic interaction chromatography, originally introduced

as an effective one-step purification technique for lipid microamphiphiles, was found to be a versatile procedure for species separation and analysis (Fischer 1996). Binding to the hydrophobic matrix of octyl sepharose occurs through the hydrocarbon chains and therefore requires the monomeric form. The dissociation of micelles and other supramolecular structures into monomers is apparently facilitated by salt addition (>0.05 M) which is necessary for binding biosurfactants to octyl sepharose. As a consequence, individual molecules are absorbed, this is a pre-requisite to separate them according to increasing hydrophobicity. For both crude biosurfactants three distinct fractions with increasing hydrophobicities were isolated as surface active compounds, as can be seen in Figure 10.1 and 10.2. For crude biosurfactant 1 the most surface active fraction isolated was fraction A which was eluted with a high content of salt (0.6-0.7 M) which means that this compound exhibited a high hydrophilic character. On the other hand, fraction F isolated from crude biosurfactant 2 (0.15-0.25 M salt) as the most surface active fraction exhibited mainly a hydrophobic character.

Further functional characterization was evaluated both for crude biosurfactants and isolated fractions. The blood agar method was included in this study since it is widely used to screen for biosurfactant production, and in some cases, it is the sole method used (Banat 1993). The haemolytic activity of biosurfactants was first discovered when Bernheimer and Avigad (1970) reported that the biosurfactant produced by *Bacillus subtilis*, surfactin, lysed red blood cells. Blood agar lysis has been used to quantify surfactin (Moran *et al.* 2002) and rhamnolipids (Johnson and Boese-Marrazzo 1980) and has been used to screen for biosurfactant production by new isolates (Banat 1993, Carrillo *et al.* 1996). Nevertheless, Carrillo *et al.* (1996) found that not all biosurfactants have a haemolytic activity. Hence, our present observation that crude biosurfactants and isolated fractions from *L. lactis* 53 and *S. thermophilus* A not lyses blood cells is in accordance with the general notion on this point in the literature.

Morikawa *et al.* (2000) showed that the oil spreading technique is a reliable method to detect biosurfactant production, as the area of displacement by a surfactant-containing solution is directly proportional to the concentration of arthrofactin and surfactin (lipopeptide biosurfactants). The diameter of the clear zone linearly increased with the concentration of surfactin over a concentration range of 50-400 mg l^{-1}. Since the concentration of biosurfactant that exceeds the critical micelle concentration will not result in further decreases in surface tension, the oil spreading technique has a larger dynamic range than surface tension. Table 10.1 presents the clear zones diameters of the crude biosurfactants and isolated fractions at

several concentrations. It was found a linear relationship for all tested samples for concentrations above 5 g l^{-1}, being the most surface active samples fractions A and F.

Figure 10.3 presents the surface tensions achieved at several concentrations for crude biosurfactants and isolated fractions, allowing the determination of the critical micelle concentrations (*cmc*). Of all samples tested, the fraction A and F were the most surface active, with a *cmc* of 10 g l^{-1} and 5 g l^{-1}, and a corresponding surface tension of 36 and 35 mN m^{-1} respectively. Crude biosurfactants and fractions A and F isolated in this study are efficient in comparison with synthetic surfactants, as sodium dodecylsulphate for example, with a *cmc* of 2.9 g l^{-1} and surface tension 37 mN m^{-1} (Mulligan and Gibbs 1993). Although the isolated fractions (A and F) were not as effective as many of biosurfactants described in the literature, for example sophorolipids obtained from *Torulopsis bombicola* with a *cmc* of 0.082 g l^{-1} and surface tension 37 mN m^{-1} (Mulligan and Gibbs 1993), it should be noted that biosurfactants studied here were not as much purified as the ones described in the literature.

Despite the surface tension measurements together with the oil displacement activity give a clear indication of the surface activity of the compound it is not sufficient to determine whether it would produce an anti-adhesive effect against the selected microorganisms. Table 10.2 and 10.3 summarizes anti-adhesive activities of the crude biosurfactants and isolated fractions evaluated in a 96 wells microtiter-plate anti-adhesion assay at several concentrations. The best results were achieved for crude biosurfactants and fraction A and F, which were found to exhibit an anti-adhesive effect against a variety of bacterial and yeast strains isolated from explanted voice prostheses. A role for biosurfactants as defense weapons in post-adhesion competition with other strains or species has been suggested for biosurfactants released by *S. mitis* strains against *S. mutans* adhesion and for biosurfactants released by lactobacilli against adhesion of uropathogens (Pratt-Terpstra *et al.* 1989).

It is well known that the voice prostheses major weakness relies on the fact that the hydrophobic silicone rubber surface becomes rapidly colonized with a thick biofilm and in this perspective the antifouling improvement of the silicone rubber material is desirable. The use of biosurfactants as antimicrobial agents seems to be promising as a method of prolonging lifetimes of voice prostheses (Rodrigues *et al*. 2004a, b). Biosurfactants antimicrobial activity against various microbes has been described before. Surfactin, for example, a cyclic lipopeptide produced by *B. subtilis* strains, is a biosurfactant with well-known antimicrobial properties (Ahimou *et al* 2000). A new antibiotic from *Pseudomonas fluorescens*, with surface active properties different from those of the known biosurfactant viscosin from the same species, was also reported to have antifungal properties (Singh and Cameotra 2004). Additionally, crude biosurfactants obtained from *L. lactis* 53 and *S. thermophilus* A showed a

significant antimicrobial activity against *C. tropicalis* GB 9/9 at low biosurfactant concentrations (Rodrigues *et al*. 2004b) and it has been reported that *C. tropicalis* is implicated in premature failure of the prostheses (Elving *et al*. 2002). The antimicrobial activities of the isolated fractions were evaluated at several concentrations and compared to a variety of bacterial and yeast strains isolated from explanted voice prostheses (Table 10.4 and 10.5). It was found that fractions A and F show a high antimicrobial activity against all microbial strains tested.

Conditioning silicone rubber voice prostheses with biosurfactants to achieve specific antimicrobial and anti-adhesive properties seems to be a pursuing strategy to increase their lifetimes. However, the applicability of biosurfactants as coating agents is dependent on whether they can stay adsorbed onto silicone rubber surfaces, and also on their stability at different pHs as saliva can exhibit pH variations according to the patient treatments. To evaluate how long the crude biosurfactants and isolated fractions stayed adsorbed to the silicone rubber surface, water contact angles were measured over a period of 3 months on silicone rubber with adsorbed crude biosurfactants (1 and 2) and isolated fractions layers (Table 10.6). As fractions A and F stayed adsorbed over a period of 2 and 1 month respectively and at the tested concentration are active against microbial colonization, an increase in the lifetime of voice prostheses is achievable. The crude biosurfactants and isolated fractions pH stability is shown in Table 10.7. Data obtained are in accordance with the described in the literature for other biosurfactants and synthetic surfactants (Kim *et al.* 2002). The surface tension reducing activity of fraction A and F were stable to pH changes between 5 and 9, as with sodium dodecylsulphate and mannosylerythritol lipid obtained from *Candida antarctica* SY16.

As the best results were achieved for fractions A and F isolated from crude biosurfactants further characterization was carried out only for these four samples. Fourier transform infrared absorption spectra, X-ray photoelectron spectroscopy, mass spectrometry and monosaccharide composition data obtained for both crude biosurfactants showed that they consist of a mixture of several compounds. In the literature, release of glycosyldiglycerides as biosurfactants from *Lactobacillus* has been reported (Gerson 1993). It is also known that lactobacilli, amongst other species, secrete lipoteichoic acid into the culture medium during exponential growth (Pollack *et al.* 1992). The cell surface of *L. lactis* subsp. *lactis* bv. diacetilactis was found to be very poor in hydrocarbonlike compounds (less than 15%), and essentially made of proteins and polysaccharides (Boonaert and Rouxhet 2000). Table 10.9 includes X-ray photoelectron spectroscopy data for reference compounds protein, salivary glycoprotein and lipoteichoic acid and, by comparison with the data for fraction A, it can be

concluded that this fraction is consistent neither with the data for lipoteichoic acid nor with glycosyldiglycerides for which the N/C ratio would be 0. The relatively high N/C ratio observed cannot solely be attributed to the presence of protein but is probably due to other nitrogen-containing compounds with $>NH_2^+$, $>(NH^+)-$ or $=(NH^+)-$ groups, which can explain the adsorption band at 2500 cm^{-1} in the infrared absorption spectra. Therefore, the high N/C and O/C ratios together with the higher percentage of carbon involved in C1 bonds and oxygen O1 bonds indicate that this fraction probably contains glycoproteins. The obtained fraction A was richer in protein and contained less polysaccharides and phosphate groups than the crude biosurfactant obtained from *L. lactis* 53. Proteinaceous biosurfactants, released by *Lactobacillus* species and named surlactin were also described by Velraeds *et al.* (1996). Moreover, monosaccharide composition showed that the purification procedure resulted in a fraction with smaller amounts of glucose and rhamnose and higher amounts of fucose and manose. Nevertheless, it was interesting to find that rhamnose was the most representative sugar. Further structural analysis should be developed in order to clarify the exact chemical structure of both fraction A and F.

The fraction isolated (F) from crude biosurfactant obtained from *S. thermophilus* A showed different molecular and elemental characteristics as compared to fraction A. Proteins were not detected in the infrared absorption spectra, and several stretch vibrations and absorption bands characteristic of polysaccharides were observed. In addition, the N/C and O/C ratios measured were in accordance with the FTIR data, pointing to the presence of polysaccharides and no proteins. The higher percentage of carbon involved in C2 bonds and oxygen in O2 bonds leads to the suggestion that the material probably contains glycolipids. The main neutral sugar determined for fraction F was also rhamnose. Busscher *et al.* (1997) described the production of crude biosurfactant by *S. thermophilus* B as mixtures of various components, with a glycolipid-like component being the most surface active.

From mass spectrometry data (Figure 10.5 and 10.6) it was possible to conclude that the fractionation yielded purer fractions from both crude biosurfactants, nevertheless these fractions still need further purification steps. A compromise situation must be achieved as further purification steps although yielding purer compounds, most of the times are not considered, especially since the amounts of biosurfactants obtained become smaller with each purification step to a degree that research becomes virtually impossible. Moreover, fractions A and F demonstrated major anti-adhesive and antimicrobial activities against microbial strains isolated from explanted voice prostheses, even at low concentrations.

10.5 CONCLUSIONS

The two fractions (A and F) isolated by hydrophobic interaction chromatography from crude biosurfactants obtained from *L. lactis* 53 and *S. thermophilus* A showed a good performance as anti-adhesive and antimicrobial agents against several microbial strains isolated from explanted voice prostheses. Moreover, both fractions were found to be stable to pH changes and delay the microbial colonization onto silicone rubber surfaces approximately 2 months. Molecular and elemental compositions suggest that fraction A is rich in glycoproteins, while fraction F is rich in glycolipids. Further purification steps should be carefully analyzed as each purification step will increase the costs and decreases the amounts of biosurfactants recovered.

10.6 REFERENCES

Ahimou, F., Jacques, P. and Deleu, M. (2001) Surfactin and iturin A effects on *Bacillus subtilis* surface hydrophobicity. *Enzyme and Microbial Technology*. 27: 749-754.

Angelova, B. and Schmauder, H.-P. (1999) Lipophilic compounds in biotechnology - interactions with cells and technological problems. *Journal of Biotechnology*. 67: 13-32.

Banat, I.M. (1993) The isolation of a thermophilic biosurfactant-producing *Bacillus* species. *Biotechnology Letters.* 15: 591-594.

Bechard, J., Eastwell, K., Sholberg, P., Mazza, G. and Skura, B. (1998) Isolation and partial chemical characterization of an antimicrobial peptide produced by a strain of *Bacillus subtilis*. *Journal of Agriculture and Food Chemistry*. 46: 5355-5361.

Bernheimer, A. and Avigad, L. (1970) Nature and properties of a cytolytic agent produced by *Bacillus subtilis*. *Journal of General Microbiology*. 61: 361-369.

Boonaert, C. J. and Rouxhet, P.G. (2000) Surface of lactic acid bacteria: relationships between chemical composition and physicochemical properties. *Applied and Environmental Microbiology.* 66: 2548-2554.

Busscher, H. J., Van Hoogmoed, C.G., Geertsema-Doornbusch, G., Van der Kuijl-Booij, M. and Van der Mei, H.C. (1997) *Streptococcus thermophilus* and its biosurfactants inhibit adhesion by *Candida* spp on silicone rubber. *Applied and Environmental Microbiology.* 63: 3810-3817.

Busscher, H.J., Bruinsma, G., Van Weissenbruch, R., Leunisse, C., Van der Mei, H.C., Dijk, F. and Albers, F.W.J. (1998) The effect of buttermilk consumption on biofilm formation on silicone rubber voice prostheses in an artificial throat. *European Archives Otorhinolaryngology*. 255: 410-413.

Busscher, H.J., Neu, T. and Van der Mei, H.C. (1994) Biosurfactant production by thermophilic dairy *streptococci*. *Applied Microbiology and Biotechnology*. 41: 4-7.

Carrillo, C., Mardaraz, C., Pitta-Alvarez, S. and Giulietti, A. (1996) Isolation and selection of biosurfactant-producing bacteria. *World Journal Microbial Biotechnology*. 12: 82-84.

Dubois, M., Gilles, K., Hamilton., J., Rebers, P. and Smith, F. (1956) Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*. 28: 350-356.

Elving, G., Van der Mei, H.C., Busscher, H.J., Amerongen, A. and Veerman, E. (2000) Antimicrobial activity of synthetic salivary peptides against voice prosthetic microrganisms. *The Laryngoscope*. 110: 321-324.

Elving, G.J., Van der Mei, H.C., Busscher, H.J., Van Weissenbruch, R. and Albers, F.W. (2002) Comparison of the microbial composition of voice prosthesis biofilms from patients requiring frequent *versus* infrequent replacement. *Annals of Otology, Rhinology & Laryngology*. 111: 200-203.

Fischer, W. (1996) Molecular analysis of lipid macroamphiphiles by hydrophobic interaction chromatography. *Journal of Microbiological Methods*. 25: 129-144.

Free, R.H., Elving, G., Van der Mei, H.C., Van Weissenbruch, R., Albers, F.W.J. and Busscher, H.J. (2000) Caffeinated soft drinks reduce bacterial prevalence in voice prosthetic biofilms. *Biofouling* 16: 69-76.

Gerson, D.F. (1993) In: *Biosurfactants: Production, Properties and Applications*. Ed. Kosaric N. New York: Marcel Dekker. Pp. 269-286.

Heinemann, C., Van Hylckama, V., Janssen, D., Busscher, H.J., Van der Mei, H.C. and Reid, G. (2000) Purification and characterization of a surface-binding protein from *Lactobacillus fermentum* RC-14 that inhibits adhesion of *Enterococcus faecalis* 1131. *FEMS Microbiology Letters*. 190: 177-180.

Jenny, K., Kappeli, O. and Fietcher, A. (1991) Biosurfactants from *Bacillus licheniformis*: structural analysis and characterization. *Applied Microbiology and Biotechnology*. 36: 5-13.

Johnson, M. and Boese-Marrazzo, D. (1980) Production and properties of heat stable extracellular hemolysin from *Pseudomonas aeruginosa*. *Infectious Immunology*. 29: 1028-1033.

Kim, H.-, Jeon, J.-, Kim, S.-, Oh, H.-, Kwon, T.-, Yoon, B.- and Fischer, W. (2002) Surface and physico-chemical properties of a glycolipid biosurfactant, mannosylerythritol lipid, from *Candida antarctica*. *Biotechnology Letters*. 24: 1637-1641.

Lin, S. (1996) Biosurfactants: recent advances. *Journal of Chemical Technology and Biotechnology*. 66: 109-120.

Mahieu, H., Van Saene, H., Rosingh, H. and Schutte, H. (1986) *Candida* vegetations on silicone voice prostheses. *Archives of Otolaryngology Head Neck Surgery*. 112: 321-325.

Mireles, J.R., Toguchi, A. and Harshey, R.M. (2001) *Salmonella enterica serovar typhimurium* swarming mutants with altered biofilm formation abilities: surfactin inhibits biofilm formation. *Journal of Bacteriology.* 183: 5848-5854.

Moran, A., Alejandra Martinez, M. and Sineriz, F. (2002) Quantification of surfactin in culture supernatant by hemolytic activity. *Biotechnology Letters*. 24: 177-180.

Morikawa, M., Daido, H., Takao, T., Murata, S., Shimonishi, Y. and Imanaka, T. (1993) A new lipopeptide biosurfactant produced by *Arthrobacter* sp. strain MIS38. *Journal of Bacteriology*. 175: 6459-6466.

Morikawa, M., Hirata, Y. and Imanaka, T. (2000) A study on the structure-function relationship of lipopeptide biosurfactants. *Biochimica et Biophysica Acta*. 1488: 211-218.

Mulligan, C. and Gibbs, B. (1993) In: *Biosurfactants: Production, Properties and Applications*. Ed. Kosaric N. New York: Marcel Dekker. Pp. 329-371.

Neu, T., Van der Mei, H.C., Busscher, H.J., Dijk, F. and Verkerke, G. (1993) Biodeterioration of medical-grade silicone rubber used for voice prostheses: a SEM study. *Biomaterials.* 14: 459-464.

Pollack, J.H., Ntamere, A.S. and Neuhaus, F.C. (1992) D-alanyl-lipoteichoic acid in *Lactobacillus casei*: secretion of vesicles in response to benzylpenicillin. *Journal of General Microbiology.* 138: 849-859.

Pratt-Terpstra, I.H., Weerkamp, A.H. and Busscher, H.J. (1989) Microbial factors in a thermodynamic approach of oral streptococcal adhesion to solid substrata. *Journal Colloid Interface Science.* 129: 568-574.

Reid, G. (2000) *In vitro* testing of *Lactobacillus acidophilus* NCFM as a possible probiotic for the urogenital tract. *International Dairy Journal*. 10: 415-419.

Rodrigues, L.R., Banat, I.M., Van der Mei, H.C., Teixeira, J. and Oliveira, R. (2005b) Interference in adhesion of bacteria and yeasts isolated from explanted voice prostheses to silicone rubber by rhamnolipid biosurfactants. *Journal of Applied Microbiology*. In press.

Rodrigues, L.R., Moldes, A., Teixeira, J. and Oliveira, R. (2005c) Kinetic study of fermentative biosurfactant production by *Lactobacillus* strains. *Biochemical Engineering Journal*. In press.

Rodrigues, L.R., Van der Mei, H.C., Banat, I.M., Teixeira, J. and Oliveira, R. (2005a) Inhibition of microbial adhesion to silicone rubber treated with biosurfactant from *Streptococcus thermophilus* A. *FEMS Immunology and Medical Microbiology*. In press.

Rodrigues, L.R., Van der Mei, H.C., Teixeira, J. and Oliveira, R. (2004a) Biosurfactant from *Lactococcus lactis* 53 inhibit microbial adhesion on silicone rubber. *Applied Microbiology and Biotechnology*. 66: 306-311.

Rodrigues, L.R., Van der Mei, H.C., Teixeira, J. and Oliveira, R. (2004b) Influence of biosurfactants from probiotic bacteria on formation of biofilms on voice prostheses. *Applied and Environmental Microbiology*. 70: 4408-4410.

Singh, P. and Cameotra, S. (2004) Potential applications of microbial surfactants in biomedical sciences. *Trends in Biotechnology*. 22: 142-146.

Stepanovic, S., Vukovic, D., Dakic, I., Savic, B. and Scabic-Vlahovic, M. (2000) A modified microtiter-plate test for quantification of *staphylococcal* biofilm formation. *Journal of Microbiological Methods*. 40: 175-179.

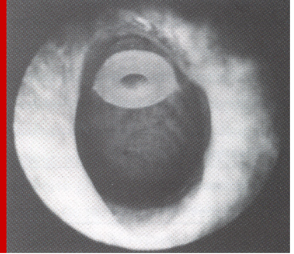
Van der Mei, H.C., Free, R.H., Elving, G.J, Van Weissenbruch, R., Albers,F.W.J. and Busscher, H.J. (2000) Effect of probiotic bacteria on prevalence of yeasts in oropharyngeal biofilms on silicone rubber voice prostheses *in vitro*. *Journal of Medical Microbiology*. 49: 713-718.

Van der Mei, H.C., Van de Belt-Gritter, B., Van Weissenbruch, R., Dijk, F., Albers, F.W.J. and Busscher, H.J. (1999) Effect of the consumption of dairy products with probiotic bacteria on biofilm formation on silicone rubber implant surfaces in an artificial throat. *Trans IChemE*. 77C: 156-158.

Velraeds, M., Van de Belt-Gritter, B., Van der Mei, H.C., Reid, G. and Busscher, H.J. (1998) Interference in initial adhesion of uropathogenic bacteria and yeasts to silicone rubber by a *Lactobacillus acidophilus* biosurfactant. *Journal of Medical Microbiology*. 47: 1081-1085.

Velraeds, M., Van der Mei, H.C., Reid, G. and Busscher, H.J. (1996) Physicochemical and biochemical characterization of biosurfactants released by *Lactobacillus* strains. *Colloids and Surfaces B: Biointerfaces*. 8: 51-61.

CHAPTER 11



GENERAL CONCLUSIONS AND RECOMMENDATIONS

"A whole is that which has beginning, middle and end".

Aristotle.

In this chapter, the major conclusions extracted from the present thesis are addressed. More detailed conclusions can be found at the end of each individual chapter. Also, some suggestions for further research in this field are given.

11.1 CONCLUSIONS

11.2 RECOMMENDATIONS

11.1 CONCLUSIONS

The aim of the present thesis was to optimize the fermentation conditions for the production of biological antifouling agents namely biosurfactants from probiotic bacteria, in order to develop new strategies for the prevention of microbial colonization of silicone rubber voice prostheses. For covering these aims, several subjects were studied and different strategies were successfully implemented. The main conclusions that can be derived are the following:

- Several lactobacilli were found to be biosurfactant-producing strains and a kinetic model allowed following the biosurfactant production at any fermentation time with a significance level over 90%.
- Probiotic bacteria *Lactococcus lactis* 53 and *Streptococcus thermophilus* A were also found to be biosurfactant-producing strains.
- Experimental factorial design and response surface analysis allowed the determination of optimal composition of synthetic medium to obtain a higher cellular growth, thus a higher biosurfactant production yield. The validity of the model was proven by fitting the values of the variables in the model equation and by actually carrying out the experiments at those values of the variables. It was achieved an increase about 2 times in the mass of produced biosurfactant.
- The change in the carbon source, from glucose to lactose, induced the cells to produce higher amounts of biosurfactants, possibly due to the use of another metabolic pathway by the bacteria.
- Economical alternatives were pursed using non conventional low cost raw materials such as molasses or cheese whey instead of synthetic medium. Similar biosurfactant production yields were achieved if molasses or cheese whey were supplemented in the same composition ratios found in the experimental factorial design; however these medium compositions were found to be too expensive. On the other hand, simpler supplementation of molasses or cheese whey resulted in reasonable improvement of biosurfactant production yields with 60 to 80% medium preparation costs reduction, respectively. This is an attractive alternative as many of the potential applications that have been considered for biosurfactants depend on whether they can be produced economically.

- Biosurfactants produced by both *L. lactis* 53 and *S. thermophilus* A appeared capable of inhibiting the adhesion of various microbial strains isolated from explanted voice prostheses to silicone rubber. Both deposition rates and number of microorganisms adhering after 4 h were reduced about 90% for most of the tested strains using biosurfactant obtained from *L. lactis* 53 with the exception of *R. dentocariosa* GBJ 52/2B, and yeast strains. Also for biosurfactant obtained from *S. thermophilus* A the adhesion inhibition was more pronounced for bacteria than for yeasts. However, this biosurfactant proved to be much more efficient against *R. dentocariosa* GBJ 52/2B that is the most frequently isolated bacteria in the group of patients whose prostheses fail after a short time of use forcing replacement.
- Rhamnolipid biosurfactant utility as antifouling agent was also assessed as this is one of the most well-known biosurfactants. Using a rinse of rhamnolipid from *Pseudomonas aeruginosa* DS10-129 through a flow chamber followed by the passage on an air-liquid interface significantly reduces (96%) the extent of adhesion of a variety of microbial strains isolated from explanted voice prostheses to silicone rubber; and therefore may be used as a biodetergent solution for prostheses cleaning, prolonging their lifetime and directly benefiting laryngectomized patients. Nevertheless, some rhamnolipids have been implicated as virulence factors and influence the immune response, thus further work has to be done in order to guarantee that this rhamnolipid will not produce undesirable effects in the oropharyngeal environment.
- Biofilms were grown on pre-conditioned voice prostheses with biosurfactants obtained from probiotic bacteria *L. lactis* 53 and *S. thermophilus* A, in an artificial throat model. Both biosurfactants greatly reduced microbial numbers on prostheses and also induced a decrease in the airflow resistance of voice prostheses after biofilm formation representing a promising strategy for prolonging the lifetime of voice prostheses.
- Based on the fractionation and physicochemical characterization of the crude biosurfactant mixture obtained from *L. lactis* 53 the most active fraction was found to be rich in glycoproteins. The most active fraction obtained from *S. thermophilus* A was found to be rich in glycolipids. Nevertheless, the costs involved in a purification process of these biosurfactant mixtures can be very high and laborious, thus once a cheap production fermentative process can be established the purification steps must be evaluated as it was found that the crude biosurfactant mixtures are also potent surfactants.

- Both crude biosurfactant mixtures and active fractions are anti-adhesive and antimicrobial agents against several bacterial and yeast strains isolated from explanted voice prostheses even at low concentrations.
- The pH stability of biosurfactants for application as coatings in silicone rubber voice prostheses is crucial as in the oropharyngeal cavity of a laryngectomized a great number of pH changes can occur due to prophylactic treatments and also food and drinks ingestion. The crude biosurfactants and active fractions studied in this work are stable at pH changes between 5 and 9.
- Fractions isolated from the crude biosurfactant mixtures obtained from *L. lactis* 53 and *S. thermophilus* A stay adsorbed to silicone rubber surfaces up to 2 months at effective concentrations against microbial colonization. Therefore by using these fractions as coating agents an increase in voice prostheses lifespan is achievable, as well as the consequent reduction of the health costs associated with prostheses replacement.

11.2 RECOMMENDATIONS

- The results achieved in this work gave very interesting perspectives on the use of biosurfactants produced by probiotic bacteria as antifouling agents. However, if the biosurfactants are cell wall components by varying the fermentation conditions their production may change in terms of quantity and composition. Information can be gained from evaluation of the cell wall surface properties of the probiotic bacteria before and after biosurfactant production.
- It is also possible that the biosurfactant production is an active gene-encoded process in which case isolation of the involved gene(s) seems worthwhile. Lactic acid bacteria have already proven to be ideal hosts for metabolic engineering. The efficacy of metabolic engineering of lactic acid bacteria for the increased production of biosynthetic metabolites is yet to be demonstrated, but based on the results gathered in this work it seems to be an interesting approach for developing new strategies of biosurfactant production.
- Another point of interest is the composition of the biosurfactants. They have been characterized as multi-component biosurfactants. Although the most active components have been fractionated they are still not in a pure state and further

purification and structural characterization steps are required. If researchers manage to isolate it, these components may possibly be synthesized.

- The stability of the biosurfactants under clinical conditions, where we are dealing with the presence of several different microbial strains at a time, varying pHs and a body temperature of 37ºC is also a question of concern. Applying higher concentrations of biosurfactant or establishing a stronger adsorption to the silicone rubber voice prostheses, might improve the efficacy of the biosurfactant against microbial adhesion. Nevertheless, the therapeutic application of biosurfactants obtained from probiotic bacteria requires an evaluation of their biocompatibility.
- Since the results achieved for the biosurfactant production using non conventional low cost raw materials as medium for fermentation were very promising it would be advantageous to evaluate other environmental factors and growth conditions such as pH, temperature, agitation, oxygen availability, reactor design and mode of operation.
- Surface and bulk modification techniques, laser-induced surface grafting and the sequential method for interpenetrating polymer networks could be exploited as ways of establishing stronger links of the biosurfactants with the silicone rubber surfaces.
- Biosurfactants can play an important role in the development of anti-adhesive and antimicrobial coatings for silicone rubber and it would be gainful to evaluate them as well in other insertional biomaterials.

The results of this thesis are promising and have led to interesting new questions that warrant further research.