Universidade do Minho
Escola de Engenharia

Eduardo José Valença Cordeiro Pires

Reduction of startup time and maintenance periods in continuous beer fermentation using immobilized yeast onto brewer's spent grains

February, 2014

Reduction of startup time and maintenance periods in continuous beer fermentation using immobilized yeast onto brewer's spent grains
Eduardo José Valença Cordeiro Pires
UMinho | 2014

Universidade do Minho
Escola de Engenharia

Eduardo José Valença Cordeiro Pires

Reduction of startup time and maintenance periods in continuous beer fermentation using immobilized yeast onto brewer's spent grains

Doctoral Dissertation for PhD degree in Chemical and Biological Engineering

Supervisor of the thesis
Professor António Augusto Martins de Oliveira Soares Vicente

February, 2014

Nome: Eduardo José Valença Cordeiro Pires

Endereço electrónico: eduardojvc@gmail.com

Título tese:

"Reduction of startup time and maintenance periods in continuous beer fermentation using immobilized yeast onto brewer's spent grains"

Orientador:

Professor António Augusto Martins de Oliveira Soares Vicente

Ano de conclusão: 2014

Designação do Doutoramento:

Programa Doutoral em Engenharia Química e Biológica

Universidade do Minho, 4 de fevereiro de 2014.

Assinatura: _____

"Fermentation is life without oxygen"

- Louis Pasteur

à minha família e esposa

Acknowledgments

First of all, I would like to express my highest gratitude to my supervisor, Professor António Augusto Vicente, to accept me (a foreign, unknown student) with arms wide open; to believe in my scientific capabilities; to give all the support I needed to finish this project; and yet, to be a friend above the mentor he is. I would also like to thank in my highest for the friendship and scientific contribution of Professor José António Teixeira. Thank you!

Furthermore I would also like to acknowledge with much appreciation the crucial role of my wife, Tânia Ferreira, for her love, kindness and support during the past two years, which were critical for the finishing of this thesis. Not less important was the help and love of my entire family, especially of my parents: Cleyder Cordeiro Pires and Edilene Valença Correa; and godparents: Romulo José Valença Correa and Angélica Maria Araújo Correa.

I am highly indebted to Fundação para a Ciência e Tecnologia (FCT) for the whole financial support given in the course of the 48 months taken to accomplish this project through the PhD scholarship SFRH / BD / 61777 / 2009.

All material and support offered by UNICER Bebidas de Portugal, S.A. (S. Mamede de Infesta, Portugal) are gratefully acknowledged. I could not forget to thank with my highest appreciation the opportunity to improve the quality of this work by the doctoral internship carried out at UNICER attained by invitation of R&D´s Director – Dr. Tiago Brandão. The internship would not be accomplished without the crucial help of Sónia Meireles, Helena Cunha and all staff of UNICER´s central laboratory and for that I am forever indebted.

Apart from my efforts, the success of any project depends largely on teamwork, encouragement and guidelines of many others. I take this opportunity to show my gratitude to the people who have been instrumentally relevant in the successful conclusion of this project. I would like to show my greatest appreciation to Professor Tomáš Brányik from the Department of Biotechnology of the Institute of Chemical Technology (Prague, Czech Republic), to accept and support my staying in Prague during my doctoral internship, for the friendship and kindness as well as for the valuable scientific contribution to this project. My thanks and appreciation also goes to Professor Maria Manuela Côrte-Real from the Department of Biology (University of Minho) for the kindness support and scientific input to the current project. The technical support, kindness and friendship provided by Manuel Santos, Maria Madalena Vieira, Cristina Ribeiro, Marcel Karabín, Milan Bittner, Gita Procházková and Jaromír Fiala, are eternal acknowledged.

I could not forget to thank all my laboratory mates from Laboratory of Industry and Process (LIP) for the friendship and collaboration through the last four years. I take this opportunity to pay homage for those friends who made the difference, who made me feel at home where I had no family: André Mota and family; and Luis Flávio Machado, my dearest "paixão" – I will never forget you. Thank you!

This thesis was financially supported by a PhD scholarship from Fundação para a Ciência e Tecnologia (Ref. number: SFRH/BD/61777/2009), inserted in the Programa Potencial Humano Quadro de Referência Estratégico Nacional (POPH – QREN) - Tipologia 4.1 - Formação Avançada. The POPH-QREN is co-financed by Fundo Social Europeu (FSE) and by Ministério da Ciência, Tecnologia e Ensino Superior (MCTES). It was also supported by UNICER Bebidas de Portugal, S.A. (S. Mamede de Infesta, Portugal).

Abstract

The current project aimed at solving technical problems involved on the implementation of a bioreactor (airlift) for continuous primary beer fermentation. In order to operate in continuous mode, the reactor must be capable of retaining a large amount of immobilized biomass. The first strategy taken towards this achievement was to effectively obtain a yeast carrier from Brewer´s Spent Grains (BSG) by suggesting several different chemical treatments. The ideal yeast carrier was obtained from a 40 min caustic treatment and had about 56% of cellulose content. This material was then used for yeast immobilization in two continuous beer fermentations. In these fermentations, foam fractionation was suggested as a method for continuous skimming non-viable biomass out from the bioreactor. The suggested setup presented good performance (*rs* = 2.95-3.62 $g.L^{-1}.h^{-1}$; and *rp* = 1.33-1.73 $g.L^{-1}.h^{-1}$) on continuous beer fermentation and a viability difference between yeast subpopulations from foam and remaining yeast in the reactor has been observed suggesting the feasibility of foam fractionation for dead biomass skimming. Yet, it has also been observed that flocculated biomass surrounding the biocatalysts were contributing in a large extent for the total immobilized biomass in the reactor. Thus, an assay testing the feasibility of flocculation as sole way of biomass immobilization in an airlift reactor was carried out. This new and simpler setup has shown much better performance than carriers' based setups for biomass loading capacity (25 $g_{cell\ dry\ wt.}.L^{-1}$), presenting very similar fermentation rates (*rs* = 3.4 $g.L^{-1}.h^{-1}$; and *rp* = 1.54 $g.L^{-1}.h^{-1}$). Then, two other continuous primary beer fermentations were carried out in order to test the feasibility and quality of beer produced using similar flocculation-based setups. In the first and longer fermentation up to 52 $g_{cell\ dry\ wt.}.L^{-1}$ were attained through flocculation. Fermentation rates were higher than any other setup suggested by the literature for continuous beer fermentation (*rs* = 8.7 $g.L^{-1}.h^{-1}$; and *rp* = 3.7 $g.L^{-1}.h^{-1}$). This experiment was also very relevant as it was possible to correlate many other variables (such as FAN consumption rate influencing higher alcohols and diacetyl formation) contributing for the success of the suggested system. The last experiment was carried out at the laboratories of a large commercial brewery where some important parameters could be better controlled (fast feedback of results by automated beer analyzer, fresh wort always available, etc.). The results were consistent with the ones observed in the last mentioned experiment, replicating its outstanding performance (*rs* = 9.43 $g.L^{-1}.h^{-1}$; and *rp* = 3.75 $g.L^{-1}.h^{-1}$). Moreover, the beer from flocculated based setup was of excellent quality, confirmed by a professional panel of beer specialists.

Resumo

Este projeto teve como principal objetivo resolver problemas técnicos associados à implementação de um bioreactor (do tipo *airlift*) para fermentação primária e contínua de cerveja. Para operar em contínuo, o reator deve ser capaz de reter uma grande quantidade de biomassa imobilizada. A primeira estratégia tomada foi obter suportes para imobilização da levedura a partir de *drêche* cervejeira através de tratamentos químicos. O suporte ideal foi obtido por um tratamento cáustico de 40 min e tinha aproximadamente 56% de pureza em celulose. Este material foi utilizado na imobilização de leveduras em duas fermentações contínuas. Nestas fermentações, o fracionamento pela espuma foi sugerido como método para escumar a biomassa morta do reactor. A configuração sugerida apresentou bom desempenho (*rs* = 2,95-3,62 $g.L^{-1}.h^{-1}$; and *rp* = 1,33-1,73 $g.L^{-1}.h^{-1}$) ao mesmo tempo que foi possível observar uma diferença de viabilidade entre as sub-populações de levedura presentes na espuma e que permaneciam no reactor. Ainda foi possível observar que a biomassa floculada em redor dos biocatalizadores estavam a contribuir em grande parte para biomassa total imobilizada no *airlift*. Assim, foi realizado um experimento para testar a viabilidade de utilizar a floculação como única forma de imobilização de leveduras no *airlift*. Esta nova configuração demonstrou não só um desempenho muito superior ao anterior em relação à capacidade de imobilização celular (25 $g_{peso\ seco\ de\ células}.L^{-1}$), mas também apresentou uma capacidade fermentativa muito semelhante (*rs* = 3,4 $g.L^{-1}.h^{-1}$; and *rp* = 1,54 $g.L^{-1}.h^{-1}$). A seguir, duas outras fermentações foram realizadas no intúito de validar a nova configuração e avaliar a qualidade da cerveja produzida. Na primeira e mais longa fermentação, um total de até 52 $g_{peso\ seco\ de\ células}.L^{-1}$ foi obtido por floculação e as taxas de fermentação foram superiores todas configurações apresentadas na literatura envolvendo a fermentação contínua de cerveja (*rs* = 8,7 $g.L^{-1}.h^{-1}$; and *rp* = 3,7 $g.L^{-1}.h^{-1}$). Ainda foi possível correlacionar muitas variáveis (como o consumo de FAN influenciando na formação de alcoois superiores e diacetil) contribuindo para o sucesso desta nova configuração. O último experimento foi realizado nos laboratórios de uma grande indústria cervejeira, aonde foi possível controlar de forma mais rigorosa alguns parâmetros (rápido retorno de resultados através de equipamentos automatizados, mosto fresco sempre disponível, etc.). Os resultados do sistema foram consistentes com o do experimento anterior, constatando-se novamente seu excelente desempenho (*rs* = 9,43 $g.L^{-1}.h^{-1}$; and *rp* = 3,75 $g.L^{-1}.h^{-1}$). Ainda, a cerveja produzida pelo sistema baseado em floculação foi de excelente qualidade, comprovada por um painel profissional de especialistas cervejeiros.

Contents

Chapter 1

Motivation and Outline 1

1.1 Motivation 2
1.2 Research Aims 3
1.3 Thesis Outline 3
1.4 References 4

Chapter 2

Beer Production 7

2.1 A brief history of brewing 8
2.2 The ingredients 9
2.2.1 Water 10
2.2.2 Malted barley and adjuncts 10
2.2.3 Hops 11
2.2.4 Yeast 12
2.3 Wort production 13
2.3.1 Milling 13
2.3.2 Mashing 13
2.3.3 Boiling 14
2.4 Fermentation 14
2.4.1 Batch fermentation 15
2.4.2 Continuous fermentation 16
2.5 References 25

Chapter 3

Yeast: the soul of beer´s aroma - a review of flavour-active esters and higher alcohols produced by the brewing yeast 31

3.1 Abstract 32
3.2 Introduction 33
3.3 Higher Alcohols 34
3.3.1 Biosynthesis of higher alcohols 35
3.3.2 Regulation of higher alcohols 38
3.3.3 Anabolic pathway 39
3.4 Esters 39
3.4.1 Biosynthesis of acetate esters 40
3.4.2 Biosynthesis of ethyl esters 42
3.4.3 Ester regulation 44
3.4.4 Esters on aging beer 44
3.5 Yeast response to fermentation parameters 45
3.5.1 Yeast strain 45
3.5.2 Temperature 45
3.5.3 Hydrostatic pressure 46

3.5.4 Wort composition 46
3.5.5 Oxygen and unsaturated fatty acids (UFAs) 48
3.6 Conclusions 49
3.7 Acknowledgements 50
3.8 References 50

Chapter 4

A new approach on brewers´ spent grains treatment and potential use as lignocellulosic yeast cells carriers 59

4.1 Abstract 60
4.2 Introduction 61
4.3 Materials and methods 62
4.3.1 Raw material 62
4.3.2 Lignocellulosic yeast carrier (LCYC) preparation 62
4.3.3 Chemical treatments 62
4.3.4 Physico-chemical characterization of LCYC 63
4.3.5 Statistical analyses 65
4.4 Results and Discussion 65
4.4.1 Carriers' yield 65
4.4.2 BSG and LCYCs´ chemical analyses 66
4.4.3 The LCYC 68
4.5 Conclusions 72
4.6 Acknowledgements 72
4.7 References 72

Chapter 5

Maintaining yeast viability in continuous primary beer fermentation 75

5.1 Abstract 76
5.2 Introduction 77
5.3 Material and Methods 78
5.3.1 Brewing Yeast 78
5.3.2 Beer wort 78
5.3.3 Brewers´ Spent Grains (BSG) 78
5.3.4 Continuous fermentation 79
5.3.5 Biomass Measurements 80
5.3.6 Flow Cytometric Assays 80
5.3.7 Epifluorescence Microscopy 81
5.3.8 Wort sugars and ethanol determination 81
5.4 Results and Discussion 81
5.4.1 Biomass growth and biofilm formation 81
5.4.2 Sugars consumption and ethanol production 83
5.4.3 LCYC losses and yeast viability 86
5.5 Conclusions 89
5.6 Acknowledgements 89

5.7 References 89

Chapter 6

Carrier-free, continuous primary beer fermentation 93

6.1 Abstract 94
6.2 Introduction 95
6.3 Material and Methods 95
6.3.1 Brewing Yeast 95
6.3.2 Beer wort 96
6.3.3 Continuous fermentation 96
6.3.4 Biomass measurements 97
6.3.5 Sugars and ethanol measurements 97
6.3.6 Flavor-active compounds measurements 97
6.4 Results and Discussion 98
6.4.1 Biomass buildup 98
6.4.2 Sugars consumption and ethanol yield 99
6.4.3 Volatile profile 101
6.5 Conclusions 104
6.6 Acknowledgements 104
6.7 References 105

Chapter 7

High-gravity primary continuous beer fermentation by flocculated biomass 109

7.1 Abstract 110
7.2 Introduction 111
7.3 Material and Methods 112
7.3.1 Brewing Yeast 112
7.3.2 Beer wort 112
7.3.3 Continuous fermentation 113
7.3.4 Biomass measurements 114
7.3.5 Yeast viability 114
7.3.6 Beer Maturation 115
7.3.7 Beer and wort analysis 115
7.4 Results and Discussion 116
7.4.1 Yeast growth and viability 116
7.4.2 SLR, FLR, nutrient consumption and productivities 118
7.4.3 Beer analysis 122
7.5 Conclusions 126
7.6 Acknowledgements 127
7.7 References 127

Chapter 8

Continuous beer fermentation – what is missing? 131

8.1 Abstract 132

8.2 Introduction 133
8.3 Material and Methods 134
8.3.1 Brewing Yeast 134
8.3.2 Wort 134
8.3.3 Continuous fermentation 134
8.3.4 Beer maturation 135
8.3.5 Cell count 136
8.3.6 Beer and wort analysis 136
8.4 Results and Discussion 137
8.4.1 High pitching rates – is it really a threat to beer quality? 137
8.4.2 Diacetyl – Still a villain! 141
8.5 Conclusions 143
8.6 Acknowledgements 143

Chapter 9

Conclusions and perspectives 147

9.1 Conclusions 148
9.2 Perspectives 149

List of publications

This thesis is based on original publications in peer-reviewed journals as follows:

Pires E. J., Ruiz, H. A., Teixeira, J. A., and Vicente, A. A. (2012) **A New Approach on Brewer's Spent Grains Treatment and Potential Use as Lignocellulosic Yeast Cells Carriers.** *Journal Agricultural Food and Chemistry* 60, 5994-5999.

Pires E. J., Teixeira, J. A., Brányik, T., and Vicente, A. A. (2014) **Yeast: the soul of beer´s aroma – a review of flavour-active esters and higher alcohols produced by the brewing yeast.** *Applied Microbiology and Biotechnology, DOI: 10.1007/s00253-013-5470-0.*

Pires E. J., Teixeira, J. A., Brányik, T., Côrte-Real, M., and Vicente, A. A. (2014) **Maintaining yeast viability in beer continuous primary fermentation.** *Journal of the Institute of Brewing* 120, 52-59.

Pires E. J., Teixeira, J. A., Brányik, T., and Vicente, A. A. (2014) **Carrier-free, continuous primary beer fermentation**. Submitted to the *Journal of the Institute of Brewing*.

Pires E. J., Teixeira, J. A., Brányik, T., Côrte-Real, M., Brandão, T. and Vicente, A. A. (2014) **High-gravity primary continuous beer fermentation by flocculated biomass.** Submitted to the *Journal of the Institute of Brewing*.

Pires E. J., Teixeira, J. A., Brányik, T., Brandão, T., and Vicente, A. A. (2014) **Continuous beer fermentation – what is missing?** Submitted to the *Journal of the Institute of Brewing.*

List of Figures

Figure 2.1 - Photomicrograph of the brewing yeast biofilm growth onto Brewer´s Spent Grains (BSG). Adapted from (*26*) with authors´ permission. 17

Figure 2.2 – Amplification of dried brewer´s spent grains (A) and yeast carriers obtained from double caustic treatment (*43*) (B). 18

Figure 2.3 – Schematic view of NewFlo phenotype interfering with the brewing yeast flocculation (A,B); and the crucial role of Ca++ triggering the biding between sugars and flocculins – if absent, flocculation will not occur (C). 21

Figure 2.4 – Internal loop airlift bioreactor. 23

Figure 3.1 - The Ehrlich Pathway and the main genes involved in the synthesis of enzymes catalyzing each reaction. The reversible transamination reaction uses different BAT-encrypted enzymes – while BAT2 catalyze the transfer of the amino group from the amino acid to α-ketoglutarate (AKG), BAT1 is usually required on the reverse transamination for amino acid biosynthesis. 37

Figure 3.2 – A schematic overview of the main metabolic routes inside brewing yeast cell contributing to higher alcohols and esters synthesis when inserted in the fermenting wort. 41

Figure 3.3 – A scheme of the chemical reactions involving acetate esters (A) and medium-chain fatty acid ethyl esters (B) biosynthesis. The main genes involved in each reaction are presented above the reaction arrows. 43

Figure 4.1 - Long Chain Fatty Acids Profile from Brewers´ Spent Grains. The LCFAs´ concentrations in grams per liter of liquid oil extracted are : Lauric Acid 0.06 g/L, Myristic Acid 0.77 g/L, Palmitic Acid 20.99 g/L, Palmitoleic Acid 1.38 g/L, Stearic Acid 5.53 g/L , Oleic Acid 0.01 g/L and Linoleic Acid 35.86 g/L. 68

Figure 4.2 – Average contact angle between pure water, non-treated barley husks from washed BSG and carriers. From the left to the right: Non-treated barley husks from washed BSG; Caustic *(11)* treatment applied on washed BSG (C*); Double Caustic 3 & 1% (DC 3.1); Acid-Caustic (11) treatment applied on washed BSG; Double Caustic 3 & 6% (DC 3.6); Fast Caustic and Acid (FCA); Double Caustic 3 & 3% (DC 3.3); Double Caustic 0.5% (DC 0.5); and Fast Caustic (FC). 69

Figure 4.3 – Changes of the total surface tension of carriers as a function of their content in (A) lignin and (B) cellulose. 70

Figure 4.4 – Zeta potential of carriers and non-treated barley husks. From left to right: Acid-Caustic (AC) *(11)*; Fast caustic and acid (FCA); Double Caustic 3 and 6% (DC 3.6); Double Caustic 3 and 3% (DC 3.3); Fast Caustic (FC); Double Caustic 3 and 1% (DC 3.1); Double Caustic 0.5% (DC 0.5); Non-treated barley husks from washed BSG. 71

Figure 5.1 – Immobilized yeast reactor system used in the current work: 1 wort barrel; 2 peristaltic pump; 3 gas-lift reactor; 4 air filter; 5 flow mass controller; 6 pressurized air; 7 CO_2 bottle; I gas outflow and foam sampling point; II green beer outflow/sampling point; III yeast (biocatalyst, flocculated and free biomass) sampling point; IV wort inlet; V gas sparger. 79

Figure 5.2 – Composition of the green beer and total free biomass concentration in the bioreactor for both foaming (a) and non-foaming (b) experiments. (a) The initial phase (I) of foaming experiment, marked by a great loss of yeast carriers, was followed by a stabilization phase (II) associated with a steady-state consumption of sugars and ethanol production from 15 ºP wort. (b) The start-up phase of non-foaming experiment (III) carried out with 5 ºP wort was associated with a slight increase in biomass and followed by a high increase in ethanol and

biomass (IV) after 11 ºP wort supply. (SLR) Saccharide loading rate; (Σr_s) total saccharide consumption rate. 82

Figure 5.3 – Photomicrographs of yeast biofilm on the lignocellulosic yeast carriers. (a) Double staining with FDA (green cells with esterase activity) and PI (red cells with compromised plasma membrane) of a biocatalyst at the end of foaming experiment. (b) Biofilm visualization using an optical microscope. 83

Figure 5.4 – The lignocellulosic yeast carrier distribution and loss during both foaming (a) and non-foaming (b) experiments. The fluidized population represents the amount of carriers still suspended in the reactor at the end of the fermentation. Outflow and sampling, represents the losses of carriers by these means. 86

Figure 5.5 – Immobilized biomass concentration measured during experiments. 87

Figure 6.1 - Immobilized yeast bioreactor system used in the current work: 1 wort barrel; 2 peristaltic pump; 3 gas-lift reactor; 4 air filter; 5 flow mass controller; 6 pressurized air; 7 CO_2 bottle; I green beer outflow/sampling point; II biomass sampling point; III wort inlet; IV gas sparger. 96

Figure 6.2 - Specific Saccharide load and Consumption (r_s), Ethanol Productivity (r_p) and total immobilized biomass inside the fermenting reactor through the primary continuous fermentation. 101

Figure 6.3 – Correlations between X_{tot} present in the gas-lift reactor and the outlet contents of total esters and higher alcohols. 104

Figure 7.1 - Immobilized yeast bioreactor system used in the current work: 1 - wort barrel; 2 - peristaltic pump; 3 - gas-lift reactor; 4 - air filter; 5 - flow mass controllers; 6 - pressurized air; 7 - CO_2 bottle; I - green beer outflow/sampling point; II - biomass sampling point; III - wort inlet; IV - gas sparger. 113

Figure 7.2 - Biomass growth rate (μ), biomass productivity rate (*BPR*), biomass washout rate (*BWR*), and yeast viability assessed by flow cytometry during the continuous fermentation. *OF* F+P-: Viable cells from outflow (Fig. 7.1,I) with positive staining for fluorescein diacetate and negative staining for propidium iodide; X_{tot} F+P-: Viable cells from sampling point II (Fig. 7.1) representing the X_{tot} with positive staining for fluorescein diacetate and negative staining for propidium iodide; *OF* F-P+: Dead cells from outflow (Fig. 7.1,I) with positive staining for propidium iodide and negatively stained by fluorescein diacetate; X_{tot} F-P+: Dead cells from sampling point II (Fig. 7.1) representing the X_{tot} with positive staining for propidium iodide and negatively stained by fluorescein diacetate. The viability values presented in this figure are the average of 20.000 cells readings, carried out in triplicate. 116

Figure 7.3 – Organic loading/consumption rate of nutrients present in the feed wort and productivity rates of main components measured in the green beer from the outflow during the continuous fermentation. (A) - Saccharide loading rate (SLR); Saccharide consumption rate (*Rs*); and Ethanol productivity (*Rp* Ethanol), in the course of continuous fermentation time. (B) – Free amino nitrogen loading rate (FLR); Free amino nitrogen consumption rate (FCR); Total higher alcohols productivity (*Rp* Higher alcohols); and Total acetate esters productivity (*Rp* Esters), through fermentation time. (C) – Consumption and productivity rates during steady inlet phases of SLR/FLR and the influence of total biomass (X_{tot}). (D) – Ratios of consumption and loading rates of saccharide and free amino nitrogen. 119

Figure 7.4 – Correlation of free amino nitrogen consumption rate (FCR) influencing the productivities of higher alcohols and diacetyl. 121

Figure 8.1 - Immobilized yeast bioreactor system used in the current work: 1 - wort barrel; 2 - peristaltic pump; 3 - airlift reactor; 4 - air filter; 5 - flow mass controllers; 6 - pressurized air; 7 - CO_2 bottle; 8 - ball lock keg as green beer storage; 9 - refrigerator at 2 °C; I - green beer

outflow/sampling point; II - biomass sampling point; III - wort inlet; IV - gas sparger. 135

Figure 8.2 – Decrease observed in isoamyl acetate concentrations during Pilsner 2 maturation inside the keg. 139

Figure 8.3 – Dynamics of diacetyl reduction from the beers produced under continuous primary fermentation during regular maturation. The line at 0.1 $mg.L^{-1}$ marks the threshold of diacetyl for lager beers (*27*). (P1) Pilsner 1; (P2) Pilsner 2 142

List of Tables

Table 2.1 – Immobilized yeast cells systems proposed either by the literature or in the current thesis for beer production 24

Table 3.1 – Threshold values of most important esters and higher alcohols present in lager beer. (*14*, *17*, *18*) 34

Table 4.1 – Chemical treatments applied on Brewers´ Spent Grains (BSG) for Ligno-cellulosic yeast carrier (LCYC) preparation 63

Table 4.2 - Bulk yield of the chemical treatments 66

Table 4.3 – Average content percentage from crude, washed BSG and carriers obtained from washed BSG. 67

Table 5.1 – Average volumetric saccharide consumption (r_s) and average volumetric ethanol productivity (r_p) measured during the steady-state phase of both the foaming and non-foaming experiments. 85

Table 5.2 - Yeast viability assessed by esterase activity and preservation of plasma membrane integrity of different cells sub-populations as determined by double staining with FDA and PI. 88

Table 6.1 - Wort and green beer composition measured in the course of both 5 and 11 degree Plato (^oP) wort supply through the continuous primary fermentation. 99

Table 6.2 - Flavour-active compounds present in the young beer at different fermentation times. 103

Table 7.1 – Composition of inlet wort from the barrels 112

Table 7.2 – Average composition (± standard deviation) of the green beer (recalculated to 5.2% ethanol) taken from the outflow (Fig. 7.1,I) of the airlift reactor. 120

Table 7.3 – Profile of three continuously produced green beers and their corresponding maturated beers. Each shaded row represents a single beer, being the number above the values of green beer and the bolded numbers below the respective final value after maturation. 125

Table 8.1 - Composition of feeding wort 134

Table 8.2 – Continuous fermentation parameters 137

Table 8.3 – Main parameters of finished beer (recalculated for 5.2% of ethanol) produced under primary continuous fermentation, compared to batch fermented beers of the same type. 140

Table 8.4 – Profile of green beer sampled from the airlift outflow 141

Nomenclature

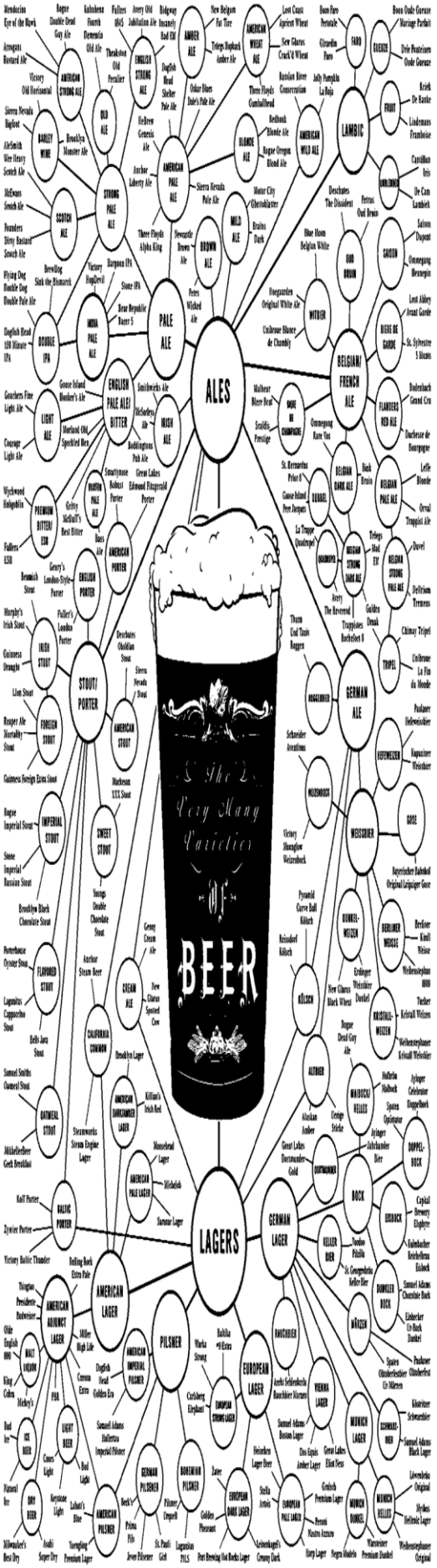
Variable	Name	Unit
μ	Specific growth rate	h^{-1}
ABV	Alcohol by volume	%
BPR	Biomass productivity rate	$g.L^{-1}h^{-1}$
BWR	Biomass washout rate	$g.L^{-1}h^{-1}$
FCR	Free-amino nitrogen consumption rate	$mg.L^{-1}h^{-1}$
FLR	Free-amino nitrogen loading rate	$mg.L^{-1}h^{-1}$
Q	Flow rate	$L.h^{-1}$
r_p	Specific productivity rate	$g.L^{-1}h^{-1}$
r_s	Specific saccharide consumption rate	$g.L^{-1}h^{-1}$
RT_{tot}	Total retention time	h
SLR	Saccharide loading rate	$g.L^{-1}h^{-1}$
X_{tot}	Total biomass	$g.L^{-1}$

Abbreviations

°P	Plato degree
A/E	Higher alcohol to esters ratio
AAT	Alcohol acetyltransferase
AD	Anno domini
AFB	Alcohol-free beer
BC	Before christ
BCAA	Branched-chain amino acids
BSG	Brewer´s spent grains
CoA	Coenzyme a
DEAE	Diethylaminoethyl
DMS	Dimethyl sulfide
EBC	European brewing convention
EU	European union
FAN	Free-amino nitrogen
FDA	Fluorescein diacetate
HPLC	High-performance liquid chromatography
ICT	Immobilized cell technology
ILV	Isoleucine-leucine-valine
LCFA	Long chain fatty acids
LCYC	Lignocellulosic yeast carriers
PI	Propidium iodide
PVA	Polyvinyl alcohol
SG	Specific gravity
SLR	Saccharide loading rate
UFA	Unsaturated fatty acid
VDK	Vicinal diketone
YPD	Yeast extract peptone dextrose

Chapter 1

Motivation and Outline



This chapter describes the purpose of the thesis as a whole. The motivational content of the work denotes not only scientific bulk, but also economic and social context. The research aims are exposed in a logical timeline so that the reader can clearly understand the main objective of the entire work. Outline section resumes every chapter of the thesis based on each objective of the project.

1.1 Motivation

The idea of continuous beer production is very recent if compared to the whole history of beer. The innovative potential involved on the application of this technology is huge. The most evident advantage in the continuous fermentation is the high volumetric productivity triggered by unusually higher pitching rates of yeast when compared to the traditional batch process. Whilst higher amount of laboring yeast will ultimately reflect in faster fermentation, continuous process also allows better vessel and space utilization. Capital and labor demands are reduced since there would be one or two fermentation lines to control instead of several fermentation tanks to regulate and periodically test. However, hygiene requirements of a continuous fermentation system are much higher than those needed for traditional batch setups. A contamination in a continuous line would imply restarting the entire process with losses of time, money and labor. Nonetheless, the advantages of a continuous fermentation process clearly overcome the disadvantages related to it, which triggers the enthusiasm in this field of research.

The European Union (EU) has a strong tradition in the brewing sector. According to the last report of Ernst & Young (2011) (*1*), only in 2010 the total of 3638 breweries produced 383 million hectoliters of beer. This share of market contributed with 50 billion euro to the EU economy in 2010, which represents 0.42% of the total gross domestic product of EU. In same year, over 2 million jobs in the EU could be attributed to the production and sale of beer. This denotes a total cut of 1% of all jobs in Europe at that time. To all that, Portugal contributes with 75000 jobs and with 1.1 billion euro to the total value added due to beer. Considering this, any technological growth in this field could help Portugal raising its share in the market.

Beyond the field of brewing, many other lines of research have been developing continuous processes. In the "gold rush" for the sustainable applicability of continuous fermentation are not only the food (*2, 3*), but also biofuels (*4-7*), waste treatments (*8-10*), and pharmaceutical (*11, 12*) industries.

There is still a lot to be discovered and studied in the field of continuous fermentation for industrial application. The maintenance of a fully functional continuous bioreactor for beer production is still very ambitious, but the applicability involved is highly encouraging. Following the trends of a competitive market for global demand, continuously operated systems will certainly win a good share of industry processes in a near future.

1.2 Research Aims

The major objective of the current work was to develop a setup for continuous beer fermentation using a gas-lift reactor. The project that initially suggested the adhesion of yeast onto treated BSG, also intended to develop an alternative way of biomass immobilization inside the bioreactor. The substitute setup suggested was flocculation as single way of biomass accumulation. It was also addressed to keep the viability of the working setup by continuous removal of non-viable biomass out of the system, without stopping the reactor. Thus, the following operational objectives were planned in order to attain these main goals:

- ✓ Develop new treatments to be applied on BSG in order to obtain different types of lignocellulosic yeast carriers (LCYC) – yields, quickness of production and chemicals needed to achieve the final carrier were taken into account when choosing the treatment that would best fit the demands of industrial production;
- ✓ Define the best conditions for biomass buildup inside the gas-lift reactor;
- ✓ Suggest operational strategies to remove aged and non-viable biomass from the system in order to maximize the working time of the continuous reactor;
- ✓ Evaluate the quality of the beer produced in the continuous system, not only by physicochemical analysis but also by acceptance of a professional tasting panel.

1.3 Thesis Outline

Following the objectives described above, the thesis was organized in nine chapters. A review of literature in traditional and continuous beer production is presented in Chapter 2. From Chapters 3 to 8, the reader will see the main published data. This means that sub-divisions were often done regarding abstract, introduction, materials and methods, results and discussion and conclusions. In the last chapter, the reader will find an overall conclusion and remaining perspectives for future works in the field of continuous beer production.

In Chapter 2 the reader will find a detailed summary of every step involved in beer production. Moreover, it makes an up to date review in continuous beer production.

A detailed review of esters and higher alcohols produced by the brewing yeast is presented in Chapter 3.

Chapter 4 is dedicated to BSG treatments for LCYC production. The proposed treatments are compared to those suggested by the literature and all carriers were chemically characterized.

Chapter 5 describes two continuous fermentations that have been carried out using a LCYC obtained by one of the treatments proposed in Chapter 4 for biomass immobilization. Fermentation performance was evaluated by sugars consumption and ethanol productivity. In this chapter foam fractionation was suggested as a continuous way for skimming old biomass from the reactor. The consequences of biocatalysts losses by foam are also discussed.

Chapter 6 evaluated the feasibility of a continuous fermentation without the use of LCYC. Biomass immobilization was carried out only by means of flocculation. Further than sugars consumption rate and ethanol productivity, in this chapter some key aromatic volatiles were measured and discussed.

In Chapter 7, a long-term fermentation was carried out using only flocculation for biomass immobilization. High-gravity wort was used and some key parameters were fine-tuned for maximum sugar attenuation under considerably high flow rate. The results of final beer were very satisfactory.

Chapter 8 addresses attention to the maturation of the green beer produced by the setup presented in Chapter 7. A short-term continuous fermentation was carried out at the facilities of UNICER and all beer parameters were assessed following the EBC standards.

Chapter 9 approaches an overall conclusion of the thesis. It also addresses what is yet to be done in the field of continuous beer fermentation.

1.4 References

1. Brink, M., Gosepa, S., Kruis, G., Oranje, E., Uyterlinde, M., Berkhout, B., and Wit, W. (2011) The Contribution made by Beer to the European Economy, Ernst & Young Tax Advisors and Regioplan Policy Research, Amsterdam.

2. Branyik, T., Silva, D. P., Vicente, A. A., Lehnert, R., e Silva, J. B., Dostalek, P., and Teixeira, J. A. (2006) Continuous immobilized yeast reactor system for complete beer fermentation using spent grains and corncobs as carrier materials, *Journal of industrial microbiology & biotechnology 33*, 1010-1018.

3. González-Sáiz, J., Garrido-Vidal, D., and Pizarro, C. (2009) Modelling the industrial production of vinegar in aerated-stirred fermentors in terms of process variables, *J Food Eng 91*, 183-196.

4. Khullar, E., Kent, A. D., Leathers, T. D., Bischoff, K. M., Rausch, K. D., Tumbleson, M. E., and Singh, V. (2013) Contamination issues in a continuous ethanol production corn wet milling facility, *World journal of microbiology & biotechnology 29*, 891-898.

5. Chen, Y., Liu, Q., Zhou, T., Li, B., Yao, S., Li, A., Wu, J., and Ying, H. (2013) Ethanol production by repeated batch and continuous fermentations by Saccharomyces cerevisiae immobilized in a fibrous bed bioreactor, *Journal of microbiology and biotechnology 23*, 511-517.

6. Silva, C. R., Zangirolami, T. C., Rodrigues, J. P., Matugi, K., Giordano, R. C., and Giordano, R. L. (2012) An innovative biocatalyst for production of ethanol from xylose in a continuous bioreactor, *Enzyme and microbial technology 50*, 35-42.

7. Crespo, C. F., Badshah, M., Alvarez, M. T., and Mattiasson, B. (2012) Ethanol production by continuous fermentation of D-(+)-cellobiose, D-(+)-xylose and sugarcane bagasse hydrolysate using the thermoanaerobe Caloramator boliviensis, *Bioresource technology 103*, 186-191.

8. Zimbron, J. A., and Reardon, K. F. (2011) Continuous combined Fenton's oxidation and biodegradation for the treatment of pentachlorophenol-contaminated water, *Water research 45*, 5705-5714.

9. Mohammed, F. M., Roberts, E. P., Hill, A., Campen, A. K., and Brown, N. W. (2011) Continuous water treatment by adsorption and electrochemical regeneration, *Water research 45*, 3065-3074.

10. Xia, S., Zhang, Y., and Zhong, F. (2009) A continuous stirred hydrogen-based polyvinyl chloride membrane biofilm reactor for the treatment of nitrate contaminated drinking water, *Bioresource technology 100*, 6223-6228.

11. Jetty, A., Potumarthi, R., Rao, A. G., Rao, B. S., and Ramakrishna, S. V. (2009) Continuous production of oxytetracycline in fluidized bed bioreactor by immobilized cells of Streptomyces varsoviensis MTCC-1537: effect of dilution and glucose loading rates, *Applied biochemistry and biotechnology 159*, 579-588.

12. Sarra, M., Casas, C., Poch, M., and Godia, F. (1999) A simple structured model for continuous production of a hybrid antibiotic by Streptomyces lividans pellets in a fluidized-bed bioreactor, *Applied biochemistry and biotechnology 80*, 39-50.

Chapter 2

Beer Production

All steps related to beer production are summarized in this chapter. The fermentation section has been divided in two parts: the traditional batch fermentation and continuous fermentation.

2.1 A brief history of brewing

Beer is perhaps the most social alcoholic beverage in the human history. It was first made, most probably by chance, thousands of years ago and it is being brewed ever since. Despite the huge technologic advances that separate the ancient brewing from today's high-tech brewing industries, the process as a whole remains quite unchanged. However, despite our ancestors could really make primitive beers from dough and cereals, they did not actually know the chemical and biological steps involved in the process.

Whereas historians suggest that beer-like beverages are consumed in China since 7000 BC (*1*), the first written records involving beer consumption date from 2800 BC in Mesopotamia. But beer history is far older than this. There is strong evidence that "beer" was born as early as 9000 BC during the Neolithic Revolution (*2*). By that time, mankind left nomadism for settling life. They began to make crops and store cereals, which ultimately could have led to "unintentional" brewing.

From Mesopotamia the beer culture starts to spread through Egypt at about 3000 BC. For the next nearly three thousand years, beer was unquestionable the beverage of choice among Egyptian people (*3*). There is when Egypt fell under Roman domain, which brought wine into their culture. But even with wine as a choice, beer endured as the sovereign beverage among Egyptian masses (*4*). Through the Roman domain wine was a drink for the high society in Egypt. In the course of their domain over Egypt, Romans have learned the knowhow of brewing. As Romans were used to drinking wine, beer started to be known as the drink of "barbarians" (*5*). In fact, before the expansion of Roman Empire, beer was the queen beverage of all Celtic peoples like France, Spain, Portugal, Belgium, Germany and Britain. Then, along with Roman conquer over these peoples, wine started to spread into their cultures (*5*). Once Romans lost control, most for Germanic conquer into European west in the 5th century AD, beer also took back its place as sovereign drink.

Before the 12th century, only monasteries produced beer in amounts that could be considered "commercial scale" (*2*). Monks started to make more beer than they could drink or give to pilgrims, poor and gests. They were also allowed to sell their beer in the monastery "pubs"(*6*). The oldest know drawings of a modern brewery setup date from 820 AD, found in the monastery of Saint Gall (*7*) in Switzerland.

The industrialization of brewing emerges in the urban centers in a context of emergent big markets for the masses. Also in this context, new patterns in the distribution of beer emerged. The brewing supplier began to provide good profits to the retailer and the independent inns became tied public houses. The percentage of pub owners depending on London breweries around 1850, exceeded 60% of all served and the loans. Thus, most of the fundamentals of manufacturing and selling beer in our times had been put in place in London by 1850 (*8*).

After the Napoleonic wars Bavaria absorbed the Franconian countries up to the central German mountains and with them a long brewing tradition, a fine barley region and the most important hop market in Germany from that time. Until the 20th century Bavaria was an agrarian state, with only few urban centers. Up to the 16th century, wine was the beverage of choice for Bavarians, but after that Bavaria became synonymous with beer. Several factors, among them climate change and state intervention, precipitated this development. Nothing exemplifies the role of the state in shaping the drinking habits of its subjects and in regulating brewing better than the famous Bavarian Purity Law – the "Reinheitsgebot", which means literally "purity order" enacted in the 23th of April 1516 (*9*). Bottom fermentation had probably been known in Bavaria since the beginning of the 15th century (*9*). From Franconia, bottom fermentation might have spread to Bavaria where it was firmly established by 1600.

Industrial-scale brewing emerged in Bavaria following British examples. However, it was not possible just to copy British technology and processes as Bavarian lager brewing significantly differed in some respects from porter and ale brewing. During the industrialization of Germany, lager beer played the role that porter had played in 18th century London. Accordingly, lager breweries came into being all over Germany.

2.2 The ingredients

It is natural that the Bavarian purity law has been adapted with time. For example, yeast was not present in the original text as it was unknown by that time. The present law (*Vorläufiges Biergesetz*) was fixed in 1993 and comprises a slightly expanded version of the *Reinheitsgebot*. In the present form, it limits water, malted barley, hops, and yeast for making lager beers; and additional different kinds of malt and sugars to make top-fermented beers. However, it is well-known that outside Bavaria, the vast majority of commercial breweries use other source of sugars as adjuncts.

2.2.1 Water

Water is the major raw material used not only as a component of beer, but also in its process for cleaning rinsing and other purposes. Thus the quality of the "liquor", which is how brewers call the water as an ingredient, will also determine the quality of beer. Thus the brewing liquor is often controlled by legislation. It has to be potable, pure, and free of pathogens, as measured by chemical and microbial analyses.

Most water has to be acclimatized previous to become the brewing liquor. This happens, for example by ion exchange using synthetic resins to get rid of unwanted minerals or by the addition of conditioning agents as brewing gypsum ($CaSO_4$). Mineral ions also influence the brewing process or taste (*10*). Sulfate, for example, can cause a hard and dry taste but favors a hop bouquet. Iron and manganese contents of more than 0.2 $mg.L^{-1}$ result in an unfavorable color and taste. Calcium protects α-amylase from early inactivation during mashing. Whilst Zinc stimulates yeast growth and fermentation, these processes are inhibited by nitrates (*11-13*).

2.2.2 Malted barley and adjuncts

After water, malted barley is the second most important ingredient for brewing. Barley germination is easily adjusted during malting. While two-row barley is preferred for the extract content, barley with more rows has less starch but higher protein content and enzymatic strength (*13*).

Alternative starch sources used as adjuncts are interesting for their availability, profitability, and their special color and aroma contribution. The most used adjuncts are unmalted barley, wheat, rice, or corn. Sometimes starch, saccharine, glucose, and corresponding syrups are also used (*14*). However, each country regulates the maximum amount of adjuncts used in their beers. As said above, the *Reinheitsgebot* forbid the use of adjuncts in Germany. The United States allows an input of unmalted cereals of up to 34% and an input of sugars or syrups of up to 2.5% of the total grist (*13*). In Brazil, unmalted cereals like corn and rice are allowed to be used in up to 45% of the recipes.

2.2.2.1 Malting

It should not be forgotten that those unmalted grains are dormant seeds of grass plants, i.e. *Hordeum spp.* (barley) and *Triticum spp.* (wheat). Through the malting process, these grains are germinated to form the corresponding malt. The correct extent of germination however, is the key parameter leading to a successful malting. Germination

is attained by wetting the grains and interrupted by drying (kilning). The embryo (small plant) metabolizes and grows, chiefly at the expense of degradation products from the starchy endosperm (*15*). As degradative and biosynthetic processes occur simultaneously, there is a net breakdown of polymeric substances (such as starch and proteins) at the same rate as there is a net migration of small sugars and amino acids to the embryo. So, as soon as the grain makes contact with developing conditions (moist and adequate temperature), all enzymatic apparatus is activated to break the reserves of starch and proteins to form a new plant. Here lies the crucial role of malting, which is enriching the malt with enzymes that will be required in the mashing step to break the starch into fermentable sugars and proteins into assimilable nitrogen sources. Thus, it is easy to understand that the optimum stage for interrupting the germination is when the malt is rich in enzymes and have consumed as less starch as possible during embryo development. After complete drying malted barley in its light color is known as Pilsener malt. All other types of malts are generated from this point by kilning at different temperatures and ways. It is good to bear in mind that the more the malt is kilned or treated, the less active enzymes it will have. So, while Pilsener malts are the richest in enzymes, chocolate malt (completely roasted) has no enzymatic activity at all.

2.2.3 Hops

Compared to water and malts, hops are lesser of the ingredients used in brewing. Yet, no doubt the amount means nothing here. In a large extent, hops are the symbol of beer characteristics. Hops are the flowers (cones, for brewers) from the *Humulus lupulus* plant. As there are different strains of this plant spread worldwide, it is normal that the quality of the flowers also vary. Thus, some hops are known as "aroma/flavour hops" while others as "bitter hops". Whilst α-acids are responsible for the bitterness strength of a given hop, the aroma is tied to essential oils from these flowers. Thus, flavor hops have lower α-acid contents but higher contents of essential oils. Bitter hops on the other hand have higher contents of α-acid but lack essential oils. Nowadays breweries rarely use cones, but pellets and hop extracts instead. Pellets are made from raw hops that are dried, ground, mixed, and pelletized. Extracts result from extraction with ethanol or carbon dioxide. After the extraction procedure, solvents are removed as far as possible. The resulting residue is a resin-like sticky substance.

2.2.4 Yeast

Yeast is often considered just as an ingredient. However, yeast is by far the most important character on brewing. Yeasts of the Genus *Saccharomyces* have always been involved on brewing since ancient times, yet for most of brewing history unwittingly. Although the first description of individual yeast cells was published in 1680 by Antonie van Leeuwenhoek, it was not before the studies carried by Louis Pasteur that conversion of wort into beer was awarded to yeast cells. Pasteur made careful microscopic examination of beer fermentations and published the results in his *Études sur la bière* (Studies about beer) (1876). He observed the growth of brewing yeast cells and demonstrated that these were responsible for fermentation.

Ale beers are produced by *Saccharomyces cerevisiae* strains. Such yeasts are known as "top-fermenting" yeasts because in traditional open fermenters they rise to the surface of the vessel, facilitating their collection by skimming. However, the hydrostatic pressure in modern cylindroconical fermenters tends to overcome this tendency bringing the yeast to the bottom, which accordingly is collected in the cone of the tank.

Lager yeasts are known as "bottom-fermenting" yeast, on account of its tendency sink in traditional open fermenters. The nomenclature of the species has evolved, passing through iterations of *S. carlsbergensis* and *S. cerevisiae* lager type to the currently accepted name, *S. pastorianus* (16). It has been known for several decades that *S. pastorianus* strains are natural, aneuploid hybrids of *S. cerevisiae* and a non-*cerevisiae Saccharomyces* species (*17*). The first complete genome sequence of a lager brewing strain was released in 2009 by Nakao and coworkers (*18*) attributing the non-*cerevisiae* part of the genome to *S. bayanus var. bayanus*. Two years later a closer look genome of *S. eubayanus* revealed that this cryotolerant yeast was actually the responsible for the non-*cerevisiae* genome of lager brewing strains of *S. pastorianus* (19).

While there are still brewers who simply repitch yeast from one fermentation to the next *ad infinitum* ("backslopping"), concerns about genetic drift and selection of variants often make most brewers pitch with yeast newly propagated from the master cultures from time to time. The frequency is typically 10 to 15 "generations" (successive fermentation batches) (16).

2.3 Wort production

2.3.1 Milling

Before mashing, malt and other grains must be milled in order to increase reactive surfaces between the enzymes and starch/proteins. Traditional breweries still use lautering for wort filtration and for those cases grains' husks should be saved because it helps as filtration layer. On the other hand, very often commercial breweries use mash filter as alternative and thus no husk or coarse pieces are really necessary. Hammer mills are normally used for fine milling. The quality of milling has an impact on mashing and lautering (if applicable), thus on quality of the final beer.

2.3.2 Mashing

The milled malt with or without other unmalted cereal is called grist. The grist is mixed with warm water to produce a slurry known as mash. During mashing the temperature may be altered (decoction mashing) or kept constant (infusion mashing) in order to attain the best conditions for different enzymes to act. The "strength" of the wort is judged by the amount of dissolved material in solution (the extract) given as a specific gravity, as if it was a sucrose solution (% w/w) with the same specific gravity. For a brewer, this "strength" is reported as Plato degree (°P). The objective of mashing is thus to obtain from the grist as much extract (with correct quality) as it is economically feasible. Better malts yield higher extracts and about 60 to 85% of wort solids are fermentable (*15*).

The enzymes catalysing the reactions during mashing are mainly hydrolases, which catalyse the breakdown of starch, proteins and other substances. There are usually changing trends in pH and temperature though the course of mashing. Substantial degradation of starch does not occur until the temperature is high enough to cause distension and gelatinization exposing the binding sites to the enzymes. As the temperature rises, enzyme-catalysed reactions accelerate, but also do the rates at which enzymes are denatured. Extended exposure to elevated temperatures inactivates (denatured) enzymes, so their catalytic properties are progressively vanished. In addition to temperature, enzyme activities and stabilities are also influenced by pH (*20*).

The conversion of starch to fermentable sugars is quantitatively the most important change occurring during mashing. Yet, for hydrolytic processes to occur in mashing, the enzymes involved must survive for a significant period.

The degradation of starch starts by action of α-amylases, which has much broader action option than β-amylases. As β-amylases can only "attack" the non-reducing ends of the dextrin chains, it depends on the "first move" of α-amylases to start working. In other words, while the smallest product of action of β-amylases is maltose (dimer of glucose); α-amylases can break the entire polymer (starch) into its monomers (glucose). Thus when dextrins are present, saccharification begins increasing the fermentability of wort. Furthermore, as β-amylase is more thermolabile than α-amylase, it is largely destroyed in 40-60 min at 65 °C (*15*). Thus, to attain adequately fermentable wort, it is necessary to have sufficient α-amylase in the mash to "liquefy" the starch rapidly, while there is still sufficient β-amylase to carry out saccharification (*15*).

2.3.3 Boiling

After separation from the residual solids (brewer´s spent grains – BSG), the hot sugary liquid (wort) is boiled with hops. Some special recipes (out of Germany) also use all kinds of "seasoning" to the wort on this step. Boiling takes normally from 90 to 120 min and is crucial not only to eliminate contaminations, but also to evaporate unwanted volatiles as dimethyl sulfide (DMS). If now boiled properly, DMS will later affect the beer with taste and aroma of sweet corn. After boiling, wort is cooled, aerated and is ready for pitching.

2.4 Fermentation

Yeasts from *Saccharomyces* genus are the most versatile of the microorganisms as are the only one capable of enter in fermentative state (if sugars are present) either in the presence of oxygen or without it and this is known as "Crabtree-effect". Irrespective of beer type or yeast strain, yeast cells will assimilate fermentable sugars, amino acids and other nutrients and minerals from the brewing wort; and excrete a wide range of molecules as ethanol, CO_2, higher alcohols, esters, etc. While on the one hand the large majority of these substances are toxins for the yeast cells, on the other hand (to a large extent) these "toxins" are the very wanted products of fermentation to the industry. The next chapter of this thesis will provide a deeper approach on the most important flavour-active compounds produced by yeast during beer fermentation – higher alcohols and esters.

2.4.1 Batch fermentation

Traditional beer fermentation by batch method uses freely suspended yeast cells to ferment the wort in static bioreactors. After cooling and aeration, the wort must be pitched as fast as possible to avoid contaminations. Common pitching involves a yeast dosage of about 15–20 x 10^6 cells.mL^{-1}. However, slightly higher dosages are used in high gravity brewing (HBG). While traditional breweries often use open fermenters, larger breweries mostly prefer closed cylindroconical tanks. These closed fermenters not only provide more sterile conditions to the fermentation but also provide to the brewer the option to regulate fermentation by CO_2 pressure.

Fermentation temperature is the decisive factor for fermentation: the higher the temperature, the faster is the fermentation, but also higher are the amounts of by-products formed (*21*). Applied pressure reduces yeast propagation and decreases formation of side products, which is very useful in HGB for recent breweries as high gravity worts also increase the formation of higher alcohols and esters. In this manner, pressure can counter balance the unfavorable by-products formation. Different gradients of temperature may also provide liquid movement in the fermentation providing good contact between wort and yeast; and also facilitate yeast sedimentation at the end of fermentation. The amount of fermentable sugars still current in a given time of the fermentation determines the wort attenuation and is the key parameter indicating the end of primary fermentation. A regular beer wort has about 80% of fermentable sugars from the total extract present. From that, green beer must have a residual fermentable extract of about 10% in order to obtain sufficient formation of dissolved CO_2 during maturation. Thus, the real desired attenuation floats around the 70% of the fermentable extract of the wort. Yet, some breweries let all fermentable sugars to be exhausted, adding more original wort for carbonation and this process is called krausening. Yeast takes about 5-7 days to finish primary beer fermentation in batch method.

Maturation further exhausts the residual extract to form CO_2, which in turn helps at removing some unwanted volatile substances as aldehydes and sulfur compounds ("CO_2 wash"). However, the main parameter fixing the end limit of maturation step is degradation of diacetyl formed in primary fermentation. Although this process can take several weeks, large breweries use selected specific yeast strains and elevated temperatures to accelerate fermentation, obtaining a finished product (from wort to beer) in about 12-15 days. After diacetyl falls below perception threshold, the

temperature of the reactor is dropped near freezing (0 to -1 ºC – "cold break") to clarify and stabilize the beer. Thereafter, beer is ready to proceed into filtering, packaging and pasteurization.

2.4.2 Continuous fermentation

Continuous beer fermentation relies on immobilized cell technology (ICT) to attain incredibly faster "transformation" of wort into beer when compared to the traditional batch process. This is possible, because ICT allows retention of a considerable amount of yeast in the reactor, which in turn provides a short residence time for wort. This feature results in high volumetric productivity, reduced space and time needed to reach the final product. Despite being very attractive, continuous fermentation of beer is still marginalized to lab benches, pilot plants and very few courageous companies like Dominion Breweries (DB) (New-Zealand), which has been continuously brewing since 1959 (*22*). A continuous fermentation setup using ICT could probably be implemented worldwide if the acquired new characteristics of the reactor resulted in a more economic system and if the new technology could be readily scaled up. Several setups have been suggested in the past decades (Table 2.1) for continuous beer fermentation covering a wide range of applications in different steps of beer production as primary fermentation, maturation and production of alcohol-free/low beers (*23*). Yet, the most challenging task is still to put together continuous primary fermentation and maturation on the production of regular alcoholic beers. As said in the previous section, a regular batch fermentation can take up to 7 days to be accomplished while in setups as the ones suggested in the last chapters of this thesis, can produce the same volume of very similar young beer as fast as within 15 h, which makes this setup very attractive.

2.4.2.1 Yeast immobilization

ICT setups often use solid carriers for cell adsorption and biofilm formation (*24-31*) or forced physical entrapment of yeast cells to solid matrixes (*32-35*) to increase cell density inside the reactor. The carrier cost is a key component for the financial viability of ICT (*36, 37*) and the composition of solid matrix may also interfere in the final beer quality and flavor profile (*23, 38*). For all these reasons, choosing the right carrier is the first step towards the development of a new setup. In this context, the carriers should fulfill some requirements such as:

- ✓ Facilitate mass and heat transfer;

- ✓ Have good mechanical (resistant to compression and abrasion) and chemical (not release any dye, flavour or odour into the fermenting beer) stability;
- ✓ Possibility to be sterilized and reused;
- ✓ Have a low-price of production/application;
- ✓ Favor cell viability;
- ✓ Be easily separated from the green/finished beer;
- ✓ Be appropriate for food applications.

2.4.2.1.1 Biofilm based carriers

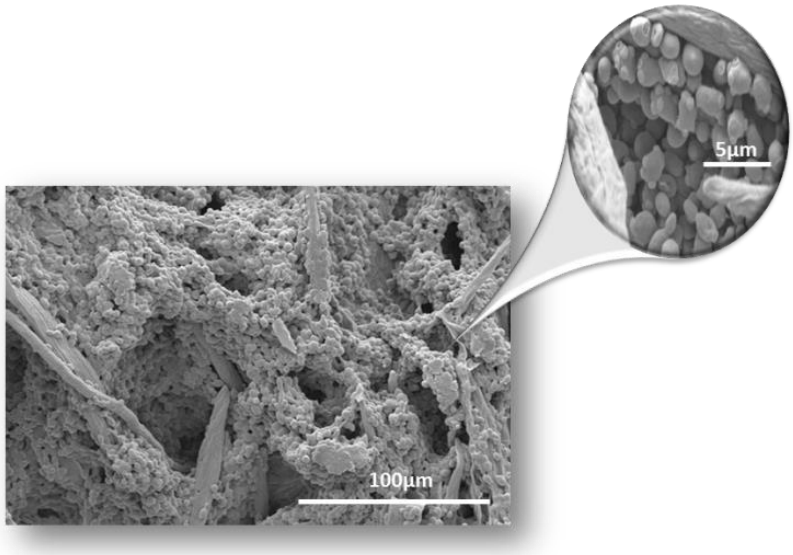


Figure 2.1 - Photomicrograph of the brewing yeast biofilm growth onto Brewer´s Spent Grains (BSG). Adapted from (*26*) with authors´ permission.

Microorganisms can attach and form biofilms on a wide range of either organic or inorganic carrier materials and yeast is not an exception. First step towards yeast adhesion occurs when long-range attractive forces (such as van der Waals) overcome the electrostatic repulsive forces. After breaking this barrier, a weak reversible attachment between cell and carrier surface is established as a result of a favorable energetic balance. Adhesion can be further intensified through short-distance forces as hydrophobic interactions, polar interactions, hydrogen bonds, and specific molecular interaction (26). Cell detachment and relocation readily occur, followed by establishment of equilibrium between adsorbed and freely suspended cells. The strength with which the cells are bonded to the carriers as well as the depth of the biofilm varies

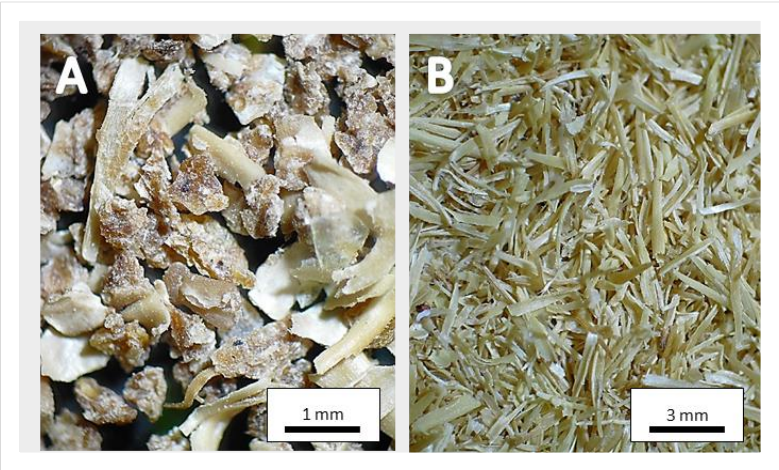


Figure 2.2 – Amplification of dried brewer´s spent grains (A) and yeast carriers obtained from double caustic treatment (*43*) (B).

from one system to another. For example, Brányik and coworkers (39) have shown that besides mechanical agitation, the damage of biofilms growing on BSG can also occur as a consequence of agitation rate, which is the driving force of the liquid circulation on an airlift reactor.

Inorganic carriers such as porous glass beads have been used successfully for primary beer fermentation by Tata and coworkers (*30*). Brányik and coworkers (*25-27*, *39*, *40*) have suggested the most promising organic based carrier for brewing yeast attachment, which consisted on the husks of malt grains obtained from treated BSG (Fig. 2.2). Other organic materials as diethylaminoethyl (DEAE) cellulose (*41*), corncobs (*25*) and wood chips (*42*) have also been applied for the same purpose. Figure 2.1 shows the biofilm formation of brewing yeast on treated BSG. As BSG is the major by-product of the brewing industry, a carrier material obtained from it, should undoubtfully be economically advantageous for its application.

2.4.2.1.2 Yeast entrapment

Entrapment involves containment of the brewing yeast within a semi-rigid matrix that permits the diffusion of nutrients (into the matrix) and products (out from the matrix). Natural polysaccharides (such as alginate (*44*, *45*), pectate (*30*) and carrageenan (*46*)) and synthetic polymers (polyvinyl alcohol (PVA) lens-shaped Lentikats (*47*)), can be solidified into semi-rigid hydrophilic matrices under mild conditions, thus allowing cell entrapment with minimal loss of viability. Entrapment based setups are claimed to obtain the best results of biomass loadings due to very high porosity of matrices and cells protection against fluid shear. However, cell growth within these matrices depends

on diffusion limitations imposed by the porosity of and available interstitial space, which decreases in time with cell propagation.

Taking the calcium alginate beads as an example, the entrapment method consists in suspending a thick yeast slurry in a sodium alginate solution, being the mixture dispersed drop by drop into a $CaCl_2$ solution. The drops immediately jellify into spherical beads with diameters ranging from about 0.3 to 5 mm. Smaller beads show better mass transfer properties of nutrients and metabolic products as the distance between the flowing wort outside the biocatalyst and the core of the beads is shorter. Moreover, reduction in bead size lowers the shear forces and may increase their long-term stability. However, small beads have larger surface-to-volume ratio compared to big particles and therefore can be more easily harmed by swelling or by exposure to oppositely charged ions (*48*). Moreover, the beads have limited mechanical stability under conditions of rapid cell growth, excessive CO_2 production, or prolonged exposure to phosphates during the maturation process (*49*).

2.4.2.1.3 Flocculation

Flocculation is a reversible process by which yeast cells adhere to each other to form aggregates. It is different from aggregates formed via budding and non-separation of daughter cells. Flocculation has a huge importance to traditional brewing as it is used to crop yeast from green beer for further repitching in batch fermentations. Flocs from top-fermenting strains will rise to the surface of traditional fermenting vessels, while flocs from lager strains will settle into the bottom. Inadequate flocculation results in poor cropping and unwanted high residual yeast counts in the green beer. Conversely, if flocculation occurs too soon, fermentation may be hindered because insufficient cells remained suspended in the fermenting wort (*50*). Thus, the ideal brewing yeast should exhibit constant flocculation characteristics through consecutive rounds of fermenting, cropping, storing and repitching. Improved and reproducible flocculation is currently important because many breweries are using increasingly high-gravity worts, which are believed to be associated with sub-optimal yeast sedimentation (*51*). Thus, the formation of flocs is an essential precursor of crop formation in traditional batch process.

The best-known mechanism of yeast flocculation is generally accepted as being lectin-mediated adhesion (*52*). The presence of lectin-like (sugar binding) proteins, such as flocculins, at the cell surface of yeast, is required for this kind of flocculation to occur.

Flocculins of one cell bind to mannose residues in the cell wall of adjacent cells, in a chain reaction that result in large clusters containing many thousands of cells (*52*). Calcium is mandatory for lectin-mediated flocculation to occur (*53*). It was first thought that Ca^{++} would change the structural conformation of flocculins (*54*). Yet, a study of crystalline and structure of flocculins through proteomics has shown that Ca^{++} is directly involved in carbohydrate binding (*55*). As flocculation is a reversible phenomenon, cell flocks can be dissociated in the presence of a Ca^{++} chelating agent or if free mannose is present, which competitively displaces cell wall mannose residues from flocculin binding sites, separating them (*55*). Stratford and Assinde (*56*) have shown that large proportion of flocculent brewing strains differs in many phenotypic characteristics from other strains of *S. cerevisiae*. These authors have demonstrated that brewing strains displayed a NewFlo phenotype that allowed other sugars (such as glucose) to compete with cell wall mannose residues, inhibiting flocculation (Fig. 2.3).

Flocculation is affected by both environmental (i.e. pH, metal ions and nutrients) and genetic factors. However, these factors should not be considered separately as the environment may influence the expression of FLO genes (*57*) and flocculins are encrypted by these genes (*58*). Hydrodynamic conditions may also help with flocculation as agitation promotes cell collision, yet strong movement may break up cell clusters. Additionally**,** concentration of yeast cells in suspension must be sufficient to cause the number of collisions necessary to form flocs (*59*). Moreover, factors that increase cell-surface hydrophobicity and that decrease the repulsive negative electrostatic charges on the cell walls cause stronger flocculation as they increase the probability of cell-cell contact (*60*). Apart from flocculation, individual yeast cells will gradually sediment if size and density overcome the Brownian motion that would keep them suspended (*52*). However, the rate of sedimentation is slow, especially when the medium is agitated, i.e. by CO_2 bubbles formed during fermentation (*61*). The sedimentation rate is also dependent on particle size: smaller particles generally settle more slowly than larger particles of the same density, because they are relatively more retarded by friction (viscosity). Therefore older yeast cells sediment faster than younger, smaller cells (*62*). However, the sedimentation of single cells is too slow to be relevant in brewery fermentations. Instead, there is a continuous exchange between cells entrapped in flocs and free cells. Single cells are constantly leaving the flocs while others cells are entrapped.

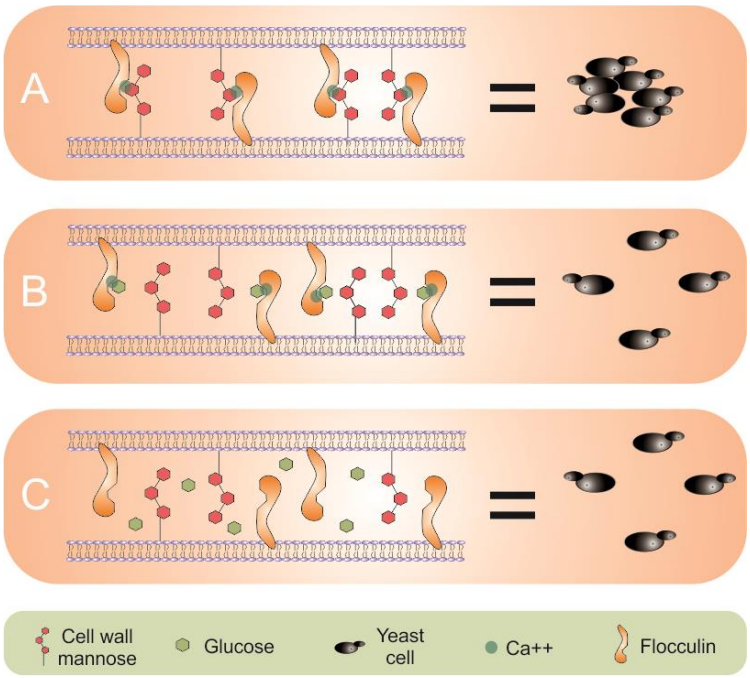


Figure 2.3 – Schematic view of NewFlo phenotype interfering with the brewing yeast flocculation (A,B); and the crucial role of Ca^{++} triggering the biding between sugars and flocculins – if absent, flocculation will not occur (C).

Despite the huge importance of self-aggregation to traditional breweries, this method seems to be poorly explored in continuous fermentation setups (Table 2.1). It should be emphasized that while all other mentioned techniques for yeast immobilization have a financial cost associated on application, flocculation is totally free of charge. In fact, one of the few existing running setups for continuous beer fermentation in industrial scale uses flocculated yeast in stirred tanks since 1959 (*22*). However, these stirred tanks do not use as high concentration of yeast as proposed by the ICT carrier-based setups mentioned in the last section. In this context, in Chapters 6, 7 and 8 the feasibility of flocculation as sole way of biomass immobilization using a gas-lift bioreactor is evaluated.

2.4.2.2 Reactor design

As important as choosing the right yeast immobilization method is the selection of the appropriate reactor type, which must also satisfy some parameters as:

- ✓ Appropriate design for the chosen carrier;
- ✓ Efficient CO_2 and biomass excess removal;
- ✓ Good mixing, heat and mass transfer rates;
- ✓ Controlled yeast growth;
- ✓ Low shear forces;

- ✓ Low investment and operation costs;
- ✓ Simple scale-up;
- ✓ Complete attenuation with desired flavour profile;
- ✓ Maintenance of sterile conditions.

ICT setups operate in batch, fed-batch, or continuous. Continuous fermentations are more productive than batch or fed-batch operations mostly because the unproductive time in processes associated with filling/emptying, cleaning and sterilization, and start-up phase of the fermentation is eliminated.

2.4.2.2.1 Fixed-bed reactors

A heavy body of literature (mainly in the early studies involving ICT) focused attention on fixed-bed (or packed-bed) bioreactors (Table 2.1). These reactors have a simpler design when compared to fluidized-bed reactors because they consist of a single column packed with immobilized yeast. Once there is no agitation in these setups, the liquid flow is very close to the inlet wort flow, reflecting in low shear forces (*63*). On the other hand, cost-benefit often weights negatively on fixed-bed reactors as they have much less advantages than disadvantages, such as:

- ✓ Heavy mass transfer restrictions;
- ✓ Flow channeling;
- ✓ CO_2 accumulation;
- ✓ Non-uniform temperature profiles;
- ✓ Dead zones.

For these reasons, the firsts setups using a fixed-bed configuration for primary beer fermentation on an industrial scale gave unsatisfactory results with respect to product quality (*64*). On the other hand, fixed-bed reactors found use in beer maturation (*25*) and production of alcohol-free beers (*65-68*).

2.4.2.2.2 Fluidized-bed reactors

In order to take advantage from continuous beer fermentation, it became clear that the "weaknesses" presented by fixed-bed bioreactors should be solved. To a large extent, the solution for most (if not all) the problems mentioned in the last section was to establish biocatalyst movement within the fermenting wort. For this purpose, fluidized-bed reactors as stirred tanks (*22*) and gas-lift reactors (*25, 29, 39, 40, 44-46, 69-75*) have been suggested (Table 2.1). The mixing provided by these systems better favors

mass and heat transfers with more uniform liquid flow throughout the reactor volume. Collisions between biocatalysts result in moderate shear stresses and abrasion, creating a need for relatively mechanically stable carriers (*63*).

The vast majority of fluidized systems for continuous beer fermentation used gas-lift bioreactors in the setups. They have an extremely simple mode of operation, which apply pneumatic agitation with no mechanical devices. Irrespective of the gas-lift design, it consists of ascendant tube (riser) through which the gas is injected and a descendant zone (downcomer). The gas in the riser decreases overall fluid density within it, creating a density difference from the degased liquid present in the downcomer, thus causing liquid circulation (Fig. 2.4). Fluid velocity will hence be

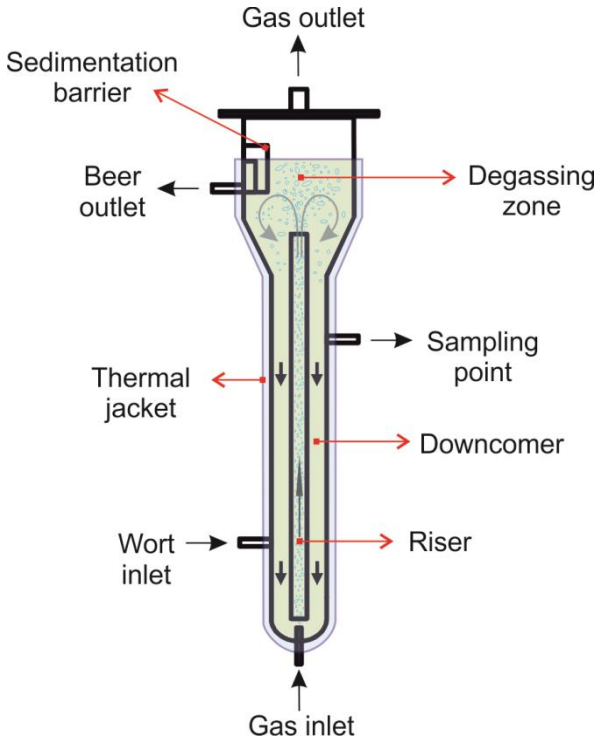


Figure 2.4 – Internal loop airlift bioreactor.

triggered by the injected gas flow, which can easily be fine-tuned for the desired flow regime. Gas-lift reactors are also routinely known as airlifts. Nedovic and coworkers (*71*) were the first to use an internal loop airlift (as the one used in the studies of this thesis) for continuous beer production. After them, many studies adopted this bioreactor design for the same purpose by varying setups and yeast carrier types (Table 2.1). The efficient mixing and low forces presented by airlifts make these reactors suitable for all types of low-density immobilization materials (*46*, *63*).

Table 2.1 – Immobilized yeast cells systems proposed either by the literature or in the current thesis for beer production

Immobilization Method	Reactor Design	Reference
Biofilms		
Ceramic beads	Fixed-bed	(*76*)
Corncobs	Fixed-bed	(*25*)
DEAE-cellulose	Fixed-bed	(*41*, *77*, *78*); (*65*, *66*)*
Gluten pellets	Fixed-bed	(*79*)
Gluten pellets	Gas-lift (EL)	(*69*)
Diatomaceous earth (Kieselguhr)	Fixed-bed	(*38*, *80*, *81*)
Polyvinyl chloride granules	Fixed-bed	(*81*)
Silicon carbide rods	Fixed-bed	(*30*, *82*, *83*); (*68*)*
Wood chips	Fixed-bed	(*42*, *84*, *85*)
Brewer´s Spent Grains	Gas-lift	(*25*, *39*, *40*); and Chapter 5 of this Thesis
Brewer´s Spent Grains	Fixed-bed	(*86*)
Stainless-steel fiber cloth	Gas-lift	(*29*)
Stainless-steel wire spheres	Fluidized-bed	(*87*)
Entrapment		
Ca-alginate beads	Fixed-bed	(*88-91*)
Ca-alginate beads	Gas-lift	(*44*, *45*, *70-73*)
k-Carrageenan beads	Gas-lift	(*46*, *74*, *75*)
Chitosan	Fluidized-bed	(*92*, *93*)
PVA beads	Gas-lift	(*31*, *47*, *91*)
Porous glass beads	Fixed-bed	(*38*, *94-96*); (*67*)*
Flocculation		
Yeast Flocs	Stirred-tank	(*22*)
Yeast Flocs	Gas-lift	Chapters 6, 7 and 8 of the current thesis.

* Alcohol-free beer; PVA – polyvinyl alcohol; DEAE – diethylaminoethyl; EL – external loop.

2.5 References

1. Bai, J., Huang, J., Rozelle, S., and Boswell, M. (2012) Beer Battles in China: The Struggle over the World's Largest Beer Market, In *The Economics of Beer* (Press, O. U., Ed.).
2. Hornsey, I. (2004) *A History of Beer and Brewing*, Vol. 1, Royal Society of Chemistry, Cambridge.
3. Geller, J. R. (1992) From prehistory to history: Beer in Egypt, In *Thefollowers of Horus* (Adams, R. F. a. B., Ed.), pp 19-26, Oxbow Books, Oxford, England.
4. Meussdoerffer, F. G. (2009) A Comprehensive History of Beer Brewing, In *Handbook of Brewing: Processes, Technology, Markets*, Eßlinger.
5. Nelson, M. (2003) The Cultural Construction of Beer among Greeks and Romans, *Syllecta Classica 14*, 101-120.
6. Rabin, D., and Forget, C. (1998) *The Dictionary of Beer and Brewing (2nd edn.)*, Fitzroy Dearborn Publishers, Chicago.
7. Horn, W., and Born, E. (1979) *The Plan of St Gall: A Study of the Architecture and Economy of, and Life in a Paradigmatic Carolingian Monastery.*, University of California Press, Berkeley, CA.
8. Mathias, P. (1959) *The Brewing Industry in England*, Cambridge University Press, Cambridge.
9. Hackel-Stehr, K. (1987) *Das Brauwesen in Bayern vom 14. bis 16. Jahrhundert*, Deutschen Brauwirtschaft, Bonn.
10. Kunze, W. (2010) Raw Materials, In *Technology Brewing and Malting* 4th International Edition ed., pp 67-82.
11. Heyse, K. U. (2000) *Praxishandbuch der Brauerei*, Behr's Verlag, Hamburg.
12. Narziss, L. (1992) Band II: Die Technologie der Würzebereitung, In *Die Bierbrauerei* 7th ed., Enke Verlag, Stuttgart.
13. Wunderlich, S., and Back, W. (2009) Overview of Manufacturing Beer: Ingredients, Processes, and Quality Criteria, In *Beer in Health and Disease Prevention* (Preedy, V. R., Ed.), pp 3-16, Elsevier, London.
14. Bamforth, C. W. (2003) *Beer: Tap into the Art and Science of Brewing*, 2 ed., Oxford University Press, NewYork
15. Briggs, D. E. (1998) *Malts and Malting*, 1st ed., Blackie Academic & Professional, London, UK.
16. Bokulich, N. A., and Bamforth, C. W. (2013) The Microbiology of Malting and Brewing, *Microbiology and Molecular Biology Reviews 77*, 157–172.
17. Bolat, I., Romagnoli, G., Zhu, F., Pronk, J. T., and Daran, J. M. (2013) Functional analysis and transcriptional regulation of two orthologs of ARO10, encoding broad-substrate-specificity 2-oxo-acid decarboxylases, in the brewing yeast Saccharomyces pastorianus CBS1483, *FEMS yeast research 13*, 505-517.
18. Nakao, Y., Kanamori, T., Itoh, T., Kodama, Y., Rainieri, S., Nakamura, N., Shimonaga, T., Hattori, M., and Ashikari, T. (2009) Genome sequence of the lager brewing yeast, an interspecies hybrid, *DNA research : an international journal for rapid publication of reports on genes and genomes 16*, 115-129.
19. Libkind, D., Hittinger, C. T., Valerio, E., Goncalves, C., Dover, J., Johnston, M., Goncalves, P., and Sampaio, J. P. (2011) Microbe domestication and the identification of the wild genetic stock of lager-brewing yeast, *Proceedings of the National Academy of Sciences of the United States of America 108*, 14539-14544.
20. Rajesh, T., Kim, Y. H., Choi, Y. K., Jeon, J. M., Kim, H. J., Park, S. H., Park, H. Y., Choi, K. Y., Kim, H., Lee, S. H., and Yang, Y. H. (2013) Identification and functional characterization of an alpha-amylase with broad temperature and pH stability from Paenibacillus sp, *Applied biochemistry and biotechnology 170*, 359-369.
21. Kunze, W. (2010) Beer Production, In *Technology Brewing and Malting*, pp 367-525.

22. Coutts, M. W. (1957) A continuous process for the production of beer, (Breweries, D., Ed.), UK.

23. Willaert, R., and Nedovic, V. A. (2006) Primary beer fermentation by immobilised yeast – a review on flavour formation and control strategies, *J Chem Technol Biotechnol 81*, 1353-1367.

24. Berlowska, J., Kregiel, D., and Ambroziak, W. (2013) Enhancing adhesion of yeast brewery strains to chamotte carriers through aminosilane surface modification, *World Journal of Microbiology and Biotechnology*.

25. Branyik, T., Silva, D. P., Vicente, A. A., Lehnert, R., e Silva, J. B., Dostalek, P., and Teixeira, J. A. (2006) Continuous immobilized yeast reactor system for complete beer fermentation using spent grains and corncobs as carrier materials, *Journal of industrial microbiology & biotechnology 33*, 1010-1018.

26. Branyik, T., Vicente, A., Oliveira, R., and Teixeira, J. (2004) Physicochemical surface properties of brewing yeast influencing their immobilization onto spent grains in a continuous reactor, *Biotechnology and bioengineering 88*, 84-93.

27. Brányik, T., Vicente, A. A., Cruz, J. M., and Teixeira, J. (2001) Spent grains – a new support for brewing yeast immobilisation, *Biotechnology Letters 23*, 1073–1078.

28. VA, N., IL, C., D, B., B, O., and B, B. (2005) New porous matrices and procedures for yeast cell immobilisation for primary beer fermentation., In *30th European Brewery Convention.*, pp 401–413, Prague.

29. Verbelen, P. J., De Schutter, D. P., Delvaux, F., Verstrepen, K. J., and Delvaux, F. R. (2006) Immobilized yeast cell systems for continuous fermentation applications, *Biotechnol Lett 28*, 1515-1525.

30. Tata, M., Bower, P., Bromberg, S., Duncombe, D., Fehring, J., Lau, V. V., Ryder, D., and Stassi, P. (1999) Immobilized yeast bioreactor systems for continuous beer fermentation, *Biotechnology progress 15*, 105-113.

31. Bezbradica, D., Obradovic, B., Leskosek-Cukalovic, I., Bugarski, B., and Nedovic, V. (2007) Immobilization of yeast cells in PVA particles for beer fermentation, *Process Biochemistry 42*, 1348–1351.

32. H, P., Margaritis, A., Mensour, N., Sobczak, J., Hancock, I., and Russell, I. (1999) Kappa-Carrageenan Gel Immobilisation of Lager Brewing Yeast, *Journal of the Institute of Brewing 105*, 398-404.

33. Almonacid, S. F., Nájera, A. L., Young, M. E., Simpson, R. J., and Acevedo, C. A. (2012) A Comparative Study of Stout Beer Batch Fermentation Using Free and Microencapsulated Yeasts, *Food and Bioprocess Technology 5*, 750-758.

34. Smogrovicova, D., Domény, Z., Gemeiner, P., Malovikova, A., and Sturdik, E. (1997) Reactors for continuous primary beer fermentation using immobilised yeast, *Biotechnology Techniques 11*, 261-264.

35. Dömény, Z., Smogrovicová, D., Gemeiner, P., Sturdík, E., Pátková, J., and Malovíková, A. (1998) Continuous secondary fermentation using immobilised yeast, *Biotechnol Lett 20*, 1041-1045.

36. Virkajärvi, I. (2001) Feasibility of Continuous Main Fermentation of Beer Using Immobilized Yeast, In *Technical Research Centre of Finland*, VTT Publications 430, Espoo.

37. Virkajärvi, I. (1999) Profiting from Immobilized Fermentation, In *Fifth Aviemore Conference on Malting, Brewing and Distilling*, pp 290-293, Institute of Brewing: London.

38. Virkajarvi, I., and Pohjala, N. (2000) Primary fermentation with immobilized yeast: Some effects of Carrier Materials on the Flavour of the Beer *J. Insi. Brew. 106*, 311-318.

39. Brányik, T., Vicente, A., Cruz, J., and Teixeira, J. (2004) Continuous primary fermentation of beer with yeast immobilized on spent grains - the effect of operational conditions., *J Am Soc Brew Chem 62*, 29-34.

40. Branyik, T., Vicente, A., Cruz, J. M., and Teixeira, J. (2002) Continuous primary beer fermentation with brewing yeast immobilized on Spent Grains, *J. Inst. Brew. 108*, 410–415.

41. Andersen, K., Bergin, J., Ranta, B., and Viljava, T. (1999) New process for the continuous fermentation of beer, In *27th European Brewery Convention Congress EBC*, pp 771-778.

42. Pajunen, E., Tapani, K., Berg, H., Ranta, B., Bergin, J., Lommi, H., and Viljava, T. (2001) Controlled beer fermentation with continuous on-stage immobilized yeast reactor., In *28th EBC Congress*, pp Chap. 49, 41-12.

43. Pires, E. J., Ruiz, H. A., Teixeira, J. A., and Vicente, A. A. (2012) A New Approach on Brewer's Spent Grains Treatment and Potential Use as Lignocellulosic Yeast Cells Carriers, *J Agr Food Chem 60*, 5994-5999.

44. Smogrovicová, D., and Dömény, Z. (1999) Beer volatile by-product formation at different fermentation temperature using immobilized yeasts., *Process Biochem 34*, 785–794.

45. Smogrovicová, D., Dömény, Z., Gemeiner, P., Malovikova, A., and Sturdik, E. (1997) Reactors for continuous primary beer fermentation using immobilised yeast, *Biotechnol Tech 11*, 261-264.

46. Mensour, N., Margaritis, A., Briens, C., Pilkington, H., and Russell, I. (1997) New developments in the brewing industry using immobilised yeast cell bioreactor systems., *J Inst Brew 103*, 363-370.

47. Smogrovicová, D., Dömény, Z., Navrátil, M., and Dvorák, P. (2001) Continuous beer fermentation using polyvinyl alcohol entrapped yeast, In *28th EBC Congress*, pp Chapter 50, pp. 51-59

48. Strand, B. L., Gaserod, O., Kulseng, B., Espevik, T., and Skjak-Baek, G. (2002) Alginate-polylysine-alginate microcapsules: effect of size reduction on capsule properties, *Journal of microencapsulation 19*, 615-630.

49. Willaert, R., and Baron, G. V. (1996) Gel entrapment and micro-encapsulation: methods, applications and engineering principles., *Rev Chem Eng 12*, 1-205.

50. Briggs, D., Brookes, P., Stevens, R., and Boulton, C. (2004) Yeast biology, In *Brewing: Science and Practice*, Woodhead Publishing Series in Food Science, Technology and Nutrition, UK.

51. Suihko, M.-L., Vilpola, A., and Linko, M. (1993) Pitching rate in high gravity brewing., *J. Inst. Brew. 99*, 341-346.

52. Stratford, M. (1992) Yeast flocculation: A new perspective, *Adv. Microbiol. Physiol. 33*, 2-71.

53. Stratford, M. (1989) Yeast flocculation: Calcium specificity, *Yeast 5*, 487-496.

54. Miki, B. L., Poon, N. H., James, A. P., and Seligy, V. L. (1982) Possible mechanism for flocculation interactions governed by gene FLO1 in Saccharomyces cerevisiae, *Journal of bacteriology 150*, 878-889.

55. Veelders, M., Bruckner, S., Ott, D., Unverzagt, C., Mosch, H. U., and Essen, L. O. (2010) Structural basis of flocculin-mediated social behavior in yeast, *Proceedings of the National Academy of Sciences of the United States of America 107*, 22511-22516.

56. Stratford, M., and Assinder, S. (1991) Yeast flocculation: Flo1 and NewFlo phenotypes and receptor structure, *Yeast 7*, 559-574.

57. Verstrepen, K. J., and Klis, F. M. (2006) Flocculation, adhesion and biofilm formation in yeast., *Mol. Microbiol 60*, 5-15.

58. Teunissen, A. W., and Steensma, H. Y. (1995) Review: the dominant flocculation genes of Saccharomyces cerevisiae constitute a new subtelomeric gene family, *Yeast 11*, 1001-1013.

59. van Hamersveld, E. H., van der Lans, R. G. J. M., and Luyben, K. C. A. M. (1997) Quantification of brewer's yeast flocculation in a stirred tank: effect of physical parameters on flocculation., *Biotechnol. Bioeng. 56*.

60. Jin, Y.-L., and Speers, R. A. (2000) Effect of environmental conditions on the flocculation of Saccharomyces cerevisiae, *J. Am. Soc. Brew. Chem. 58*, 108-116.

61. Speers, R. A., Wan, Y.-Q., Jin, Y.-L., and Stewart, R. (2006) Effect of fermentation parameters and cell wall properties on yeast flocculation., *J. Inst. Brew. 112*, 246-254.

62. Powell, C. D., Quain, D. E., and Smart, K. A. (2003) The impact of brewing yeast cell age on fermentation performance, attenuation and flocculation, *FEMS yeast research 3*, 149-157.

63. Obradovic, B., Nedovic, V., Bugarski, B., Willaert, R. G., and Vunjak-Novakovic, G. (2004) Fundamentals of cell immobilisation biotechnology, In *Focus on biotechnology* (Nedovic, V., and Willaert, R. G., Eds.), pp 411–436, Kluwer, Dordrecht.

64. Verbelen, P. J., Nedovic, V. C., Manojlovic, V., Delvaux, F., Laskošek-Čukalović, I., Bugarski, B., and Willaert, R. (2010) Bioprocess Intensification of Beer Fermentation Using Immobilised Cells, In *Encapsulation Technologies for Active Food Ingredients and Food Processing* (Zuidam, N. J., and Nedovic, V., Eds.), pp 119-146, Springer New York.

65. Collin, S., Montesinos, M., Meersman, E., Swinkels, W., and Dufour, J. P. (1991) Yeast dehydrogenase activities in relation to carbonyl compounds removal from wort and beer, In *Eur. Brew. Conv. Cong.*, pp 409–416.

66. Lommi, H. (1990) Immobilized yeast for maturation and alcohol-free beer, *Brew Dist Int 5*, 22-23.

67. Aivasidis, A., Wandrey, C., Eils, H. G., and Katzke, M. (1991) Continuous fermentation of alcohol-free beer with immobilized yeast cells in fluidized bed reactors, In *23rd EBC Cong.*, pp 569-576.

68. Van De Winkel, L., Van Beveren, P. C., and Masschelein, C. A. (1991) The application of an immobilized yeast loop reactor to the continuous production of alcohol-free beer, In *Proc. Eur. Brew. Conv. Cong.*, pp 307-314.

69. Manojlovic, V., Agouridis, N., Kopsahelis, N., Kanellaki, M., Bugarski, B., and Nedovic, V. (2008) Brewing by immobilized freeze dried cells in a novel gas flow bioreactor. , In *Joint Central Europ. Cong. Food, 6th Croat. Cong. Food Technol. Biotechnol. Nutrit.* , pp 327–334, Cavtat, Croatia.

70. Nedovic, V., Bezbradica, D., Obradovic, B., Leskosek-Cukalovic, I., and Bugarski, B. (2004) Primary beer fermentation by PVA-immobilized brewing yeast in a gas-lift bioreactor, In *World Brew. Cong.*, pp 0-63, San Diego CA.

71. Nedovic, V., Obradovic, B., Vunjak-Novakovic, G., and Leskosek-Cukalovic, I. (1993) Kinetics of beer fermentation with immobilized yeast cells in an internal-loop air-lift bioreactor., *Chem Indus*, 168-172.

72. Nedovic, V., Vunjak-Novakovic, G., Leskosek-Cukalovic, I., and Cutkovic, M. (1996) A study on considerable accelerated fermentation of beer using an airlift bioreactor with calcium alginate entrapped yeast cells. , In *5th World Cong Chem Eng*, pp 474-479.

73. Nedovic, V. C., Cukalovic, I. L., Bezbradica, D., Obradovic, B., and Bugarski, B. (2005) New porous matrices and procedures for yeast cell immobilisation for primary beer fermentation., In *30th Eur. Brew. Conv.*, pp 401-413, Prague.

74. Decamps, C., Norton, S., Poncelet, D., and Neufeld, R. J. (2004) Continuous pilot plant-scale immobilization of yeast in k-carrageenan gel beads., *AlChE J 50*, 1599-1605.

75. Mensour, N., Margaritis, A., Briens, C. L., Pilkington, H., and Russell, I. (1996) Immobilized cells: basics and applications., In *Applications of immobilized yeast cells in the brewing industry.* (Wijffels, R. H., Buitelaar, R. M., Bucke, C., and Tramper, J., Eds.), pp 661-671, Elsevier, Amsterdam.

76. Inoue, T. (1995) Development of a two-stage immobilized yeast fermentation system for continuous beer brewing, In *Eur. Brew. Conv. Cong.*, pp 25-36.

77. Kronlöf, J., Härkönen, T., Hartwall, P., Home, S., and Linko, M. (1989) Main fermentation with immobilized yeast, In *22nd Eur. Brew. Conv.*, pp 355-362, Zurich.

78. Pajunen, E., and Grönqvist, A. (1994) Immobilized yeast fermenters for continuous lager beer maturation., In *23rd Conv. Inst. Brew. Aus. N.Z. Sec.*, pp 101-103, Sydney.

79. Bardi, E., Koutinas, A. A., and Kanellaki, M. (1997) Room and low temperature brewing with yeast immobilized on gluten pellets, *Process Biochem 32*, 691–696.

80. Narziss, L., and Hellich, P. (1971) Ein Beitrag zur wesentlichen Beschleunigung der Gärung und Reifung des Bieres. 111:, *Brauwelt 111*, 1491-1500.

81. Moll, M., Durand, G., and Blachere, H. (1973) Continuous production of fermented liquids., French Patent 73/23397.

82. Van De Winkel, L., Van Beveren, P. C., Borremans, E., Goossens, E., and Masschelein, C. A. (1993) High performance immobilized yeast reactor design for continuous beer fermentation, In *24th Eur. Brew. Conv. Congr.*, pp 307-314.

83. Andries, M., Van Beveren, P. C., Goffin, O., and Masschelein, C. A. (1996) Design and application of an immobilized loop bioreactor for continuous beer fermentation., In *Immobilized cells: basics and applications* (Wijffels, R. H., Buitelaar, R. M., Bucke, C., and Tramper, J., Eds.), pp 672-678, Elsevier, Amsterdam.

84. Kronlöf, J., and Virkajärvi, I. (1999) Primary fermentation with immobilized yeast, In *27th EBC Congress*, pp 761-770.

85. Linko, M., Virkajärvi, I., Pohjala, N., Lindborg, K., Kronlöf, J., and Pajunen, E. (1997) Main fermentation with immobilized yeast - a breakthrough?, In *26th Eur. Brew. Conv.*, pp 385-394, Maastricht.

86. Kopsahelis, N., Kanellaki, M., and Bekatorou, A. (2007) Low temperature brewing using cells immobilized on brewer's spent grains, *Food Chem 104*, 480-488.

87. Cross, P. A., and Mavituna, F. (1987) Yeast retention fermentors for beer production, In *4th Eur. Cong. Biotechnol. Bioeng.*, pp 199-200, Amsterdam.

88. Ryder, D., S,, and Masschelein, C. A. (1985) The growth process of brewing yeast and the biotechnological challenge, *J Am Soc Brew Chem 43*, 66-75.

89. Onaka, T., Nakanishi, K., Inoue, T., and Kubo, S. (1985) Beer brewing with immobilized yeast, *Nat Biotechnol 3*, 467-470.

90. Shindo, S., Sahara, H., and Koshino, S. (1994) Suppression of a-acetolactate formation in brewing with immobilized yeast, *J. Inst. Brew. 100*, 69-72.

91. Manojlovic, V., Sipsas, V., Agouridis, N., Bugarski, B., Leskosek-Cukalovic, I., Kanellaki, M., and Nedovic, V. (2007) Beer fermentation by immobilized yeast in PVA/alginate beads using a catalytic multistage fixed bed tower bioreactor, In *5th Int. Cong. Food Technol.* , pp 219-222, Thessaloniki, Greece.

92. Unemoto, S., Mitani, Y., and Shinotsuka, K. (1998) Primary fermentation with immobilized yeast in a fluidized bed reactor, *MBAATechn Quart 35*, 58-61.

93. Maeba, H., Unemoto, S., Sato, M., and Shinotsuka, K. (2000) Primary fermentation with immobilized yeast in porous chitosan beads. Pilot scale trial., In *26th Conv. Inst. Brew. Aus. N.Z. Sec.* , pp 82-86, Singapore.

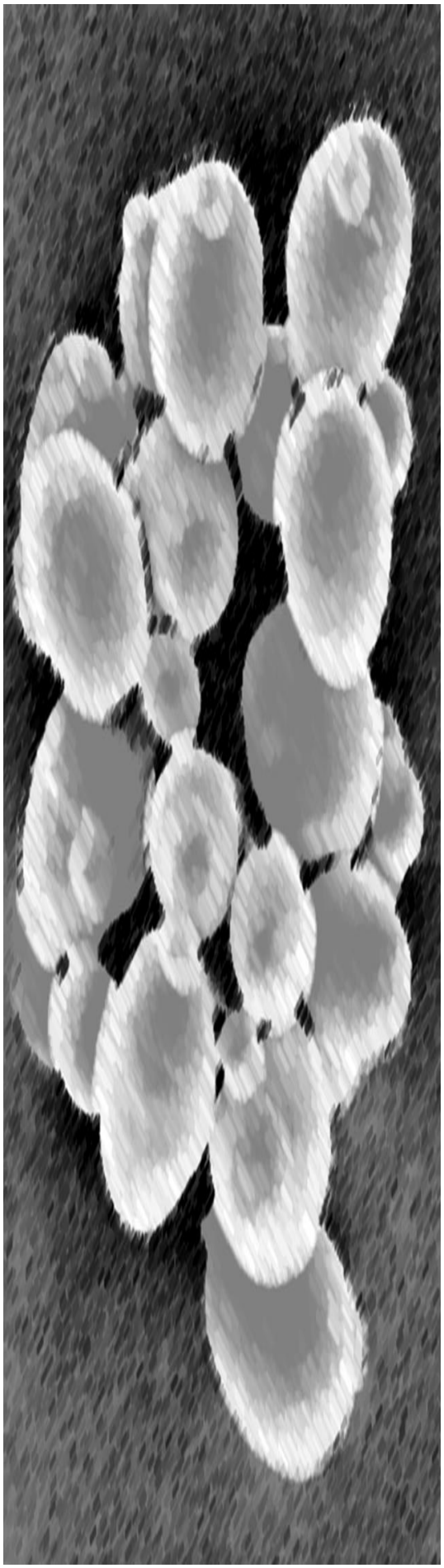
94. Virkajärvi, I., and Kronlöf, J. (1998) Long-term stability of immobilized yeast columns in primary fermentation., *J Am Soc Brew Chem 56*, 70-75.

95. Linko, M., Suihko, M.-L., Kronlöf, J., and Home, S. (1993) Use of brewer's yeast expressing a-acetolactate decarboxylase in conventional and immobilized fermentations, *MBAATechn Quart 30*, 93–97.

96. Aivasidis, A. (1996) Another look at immobilized yeast systems, *Cerevisia 21*, 27-32.

Chapter 3

Yeast: the soul of beer´s aroma - a review of flavour-active esters and higher alcohols produced by the brewing yeast



This chapter makes an up to date review of the main pleasant aromatic volatiles produced by brewing yeast during fermentation – higher alcohols and esters.

3.1 Abstract

Among the most important factors influencing beer quality is the presence of well-adjusted amounts of higher alcohols and esters. Thus, a heavy body of literature focuses on these substances and on the parameters influencing their production by the brewing yeast. Additionally, the complex metabolic pathways involved in their synthesis require special attention. More than a century of data, mainly in genetic and proteomic fields, has buildup enough information to describe in detail each step in the pathway for the synthesis of higher alcohols and their esters, but there is still place for more. Higher alcohols are formed either by anabolism or catabolism (Ehrlich pathway) of amino acids. Esters are formed by enzymatic condensation of organic acids and alcohols. The current paper reviews the up-to-date knowledge in the pathways involving higher alcohols and esters synthesis by brewing yeasts. Fermentation parameters affecting yeast response during biosynthesis of these aromatic substances are also fully reviewed.

Keywords: Higher alcohols; Fusel alcohols; Esters; Beer aroma; Brewing yeast; *Saccharomyces spp.*

3.2 Introduction

Beer is one of the most pleasant beverages in the world, the final taste/aroma of which is the resultant sum of several hundreds of flavour-active compounds produced in the course of every step of brewing. However, the great majority of these substances are produced during the fermentation phase and consist of metabolic intermediates or by-products of the main living character of brewing – the yeast. Higher alcohols, esters and vicinal diketones (VDKs) are the key elements produced by yeast, which will ultimately determine the final quality of the beer. While higher alcohols and esters are desirable volatiles constituents of a pleasant beer, VDKs are often considered as off-flavors. Together with these, yeast metabolism contributes with other three groups of chemical compounds: organic acids, sulphur compounds and aldehydes.

All flavour-active components in beer must be kept within certain limits, otherwise a single compound or group of compounds may predominate and destroy the flavour balance. Furthermore, potent odorants like esters may act in synergy with other components affecting beer flavor in concentrations well below their threshold values (*1*). However, each type of beer has its own prevailing aroma triggered either by the yeast strain (*2-5*) chosen or by parameters used during fermentation (*6-13*). For example, whilst only isoamyl acetate (banana-like aroma) concentrations are above threshold level in most lager beers, ales normally have ethyl acetate (solvent-like aroma) and ethyl hexanoate (apple-like aroma) as additional flavoring compounds with levels above the threshold (*14, 15*). However, for the vast majority of beers, other compounds like diacetyl (a vicinal diketone) should be found below the threshold values as it contributes negatively with a buttery flavor to the beer. Table 3.1 shows threshold values of the main esters and higher alcohols present in lager beer. As a full review of diacetyl formation was recently published (*16*), this paper will limit the discussion on the desirable odorants produced by the brewing yeast in the course of fermentation – higher alcohols and esters.

3.3 Higher Alcohols

Also known as fusel alcohols, higher alcohols are the most abundant organoleptic compounds present in beer. The brewing yeast absorbs amino acids present in wort from which they take the amino-group so it can be incorporated in its own structures. What is left from the amino acid (α-keto-acid) enters in an irreversible chain-reaction that will ultimately form a by-product - higher alcohols. This pathway was suggested long ago by Ehrlich (*19*), who was intrigued with the structural molecular similarities between the active amyl alcohol with isoleucine and isoamyl alcohol with leucine. This observation has led Ehrlich to investigate whether these amino acids were involved in higher alcohols synthesis or not. When supplementing the fermenting medium with those amino acids, he evidenced an increased production of fusel alcohols. This

Table 3.1 – Threshold values of most important esters and higher alcohols present in lager beer (*14, 17, 18*)

Compound	**Threshold** ($mg.L^{-1}$)	**Concentration Range** ($mg.L^{-1}$)	**Aroma Impression**
Acetate Esters			
Ethyl acetate	25-30	8-32	Fruity, Solvent
Isoamyl acetate	1.2-2	0.3-3.8	Banana
Phenylethyl acetate	0.2-3.8	0.1-0.73	Roses, honey
MCFA Ethyl Esters			
Ethyl hexanoate	0.2-0.23	0.05-0.21	Apple, fruity
Ethyl octanoate	0.9-1.0	0.04-0.53	Apple, aniseed
Higher Alcohols			
n-Propanol	600	4-17	Alcohol, sweet
Isobutanol	100	4-57	Solvent
Isoamyl alcohol	50-65	25-123	Alcoholic, Banana
Amyl alcohol	50-70	7-34	Alcoholic, solvent
2-phenylethanol	40	5-102	Roses

observation led him to state that amino acids were enzymatically hydrolyzed to form the corresponding fusel alcohols, along with ammonia and carbon dioxide. As ammonia was not detected in the medium, it was assumed to be incorporated into yeast proteins. Few years later, Neubauer and Fromherz (*20*) proposed a few intermediate steps to the Ehrlich pathway, completing the metabolic scheme as it is known until today. However, a detailed enzymatic chain reaction was only demonstrated several decades later (*21, 22*), establishing the elementary enzymatic sequence for the Ehrlich pathway: transaminase, decarboxylase, and alcohol dehydrogenase (Fig. 3.1). Although this pathway is the most studied and discussed, higher alcohols are also formed during upstream (anabolic pathway) biosynthesis of amino acids (*23-25*). The most important is the synthesis *de novo* of branched-chain amino acids (BCAA) through the isoleucine-leucine-valine (ILV) pathway (*25*).

3.3.1 Biosynthesis of higher alcohols

3.3.1.1 Transamination

The first step in Ehrlich pathway involves four enzymes encoded by the genes Bat1 (Twt1 or Eca39), Bat2 (Twt2 or Eca40), Aro8 and Aro9. These enzymes are transaminases that catalyze the transfer of amines between amino acids and their respective α-keto-acid, using glutamate/α-keto-glutarate as a donor/acceptor. While Bat1 and Bat2 encrypted enzymes are involved in the BCAA transamination (*26, 27*), Aro8p and Aro9p were first described as being aromatic amino acid aminotransferases I and II respectively (*28*). Further studies carried out by Urrestarazu and colleagues (*29*) demonstrated that Aro8 and Aro9 encoded enzymes had broader substrate specificity than just for aromatic amino acids. This was confirmed in the work performed by Boer and coworkers (*30*), who cultivated *Saccharomyces cerevisiae* using six independent nitrogen sources followed by transcriptome analysis. All phenylalanine, methionine, or leucine activated the transcription of Aro9 and Bat2 genes.

A recent study mapped almost entirely (97%) the proteome of *S. cerevisiae* (*31*). The authors organized the proteome into a network of functionally related proteins, which they called as "modules". Within these modules they highlighted one comprising of Bat1, Bat2, Rpn11, Hsp60 and Ilv2, which they termed B1B2 module. The core of this module is composed by Bat1p and Bat2p – two paralogous enzymes involved in the metabolism of the BCAA. Whilst Bat1p is manly involved in the anabolism of BCAA (amination of α-keto-acids), Bat2p is almost exclusively involved in the catabolism of

BCAA (deamination of BCAA). Thus, Bat1 and Bat2 encoded proteins catalyze the same metabolic reaction in opposite directions. Strictly related to these two proteins is the Ilv2 encrypted enzyme, which catalyzes an early step in the synthesis of BCAA from pyruvate (*31*).

The subcellular location of enzymes catalyzing the synthesis of fusel alcohols has been studied in the past (*26, 32*) and recently emphasized (*33*). Isobutanol is produced by yeast originally in the cytoplasm via Ehrlich pathway or by anabolic synthesis inside the mitochondria (*34*). Avalos and colleagues (*33*) redirected the entire enzymatic biosynthetic pathway of that fusel alcohol to the mitochondrial matrix. Compartmentalization of the Ehrlich pathway within the mitochondria increased isobutanol production by 260%, whereas overexpression of the same pathway in the cytoplasm only improved yields by 10%. These results are justified by the more favorable environmental conditions found in the mitochondria matrix, which enhanced enzymatic activity.

3.3.1.2 Decarboxylation

After transamination, the remaining α-keto-acids can be decarboxylated to form the respective aldehyde and this is a point of no return in the Ehrlich pathway (*35*). There are five genes encoding decarboxylases in *S. cerevisiae*: three encoding pyruvate decarboxylases (PDC1, PDC5 and PDC6), ARO10 and THI3 (*35-37*), with all enzymes encrypted depending on the cofactor thiamine diphosphate (TPP). From within these genes, only PDC5 and ARO10 were described to encode decarboxylases with a broad substrate-specificity (*36, 38, 39*). Dickinson and coworkers (*40*) have shown that valine is decarboxylated by any of the enzymes encrypted by PDC1, PDC5 or PDC6. In the case of isoleucine, all five decarboxylases of the family are able to produce active amyl alcohol (*41*). THI3-encoded enzyme cannot catalyze the decarboxylation of the aromatic amino acids phenylalanine and tyrosine, while all other four can (*42*). The single expression of THI3 in a quadruple deleted (*pdc1*Δ *pdc5*Δ *pdc6*Δ *aro10*Δ) gene *S. cerevisiae* strain had no α-keto-acids decarboxylase activity (*38, 39*). Further studies involving THI3 suggest that its role in the Ehrlich pathway is rather regulatory than catalytic (*43*).

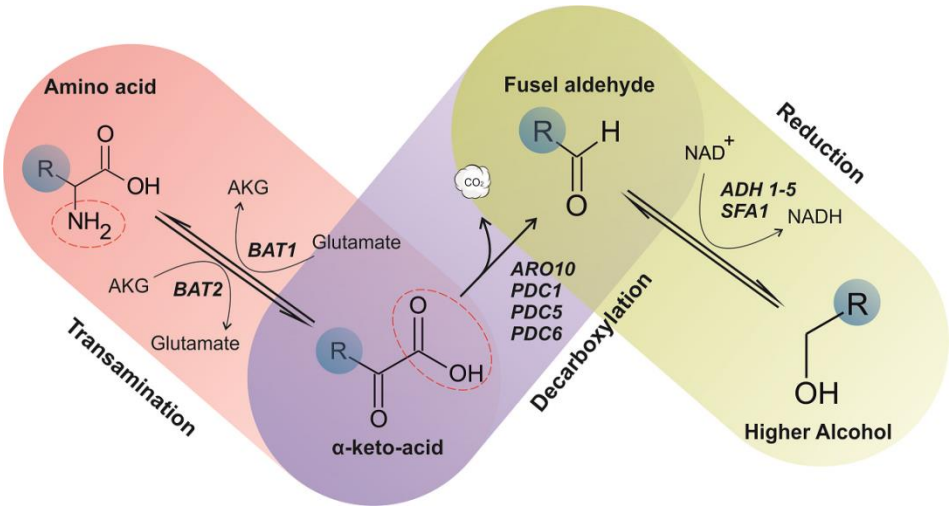


Figure 3.1 - The Ehrlich Pathway and the main genes involved in the synthesis of enzymes catalyzing each reaction. The reversible transamination reaction uses different BAT-encrypted enzymes – while BAT2 catalyze the transfer of the amino group from the amino acid to α-ketoglutarate (AKG), BAT1 is usually required on the reverse transamination for amino acid biosynthesis. 5

Although the lager brewing yeast *S. pastorianus* is long known to be a natural aneuploid hybrid of *S. cerevisiae* with another *Saccharomyces spp.* (*44*), only recently the missing link was proven to be *S. eubayanus* (*45*). This fact has called the attention of Bolat and colleagues (*37*) upon the contribution of ARO10 gene expression from each of the subgenomes on the production of higher alcohols. The authors amplified by PCR both *S. eubayanus*-like and *S. cerevisiae*-like alleles of ARO10 (*LgSeubARO10* and *LgScARO10*, respectively) from genomic DNA of *S. pastorianus*. The alleles showed a sequence identity of 80% at the DNA level and 84% at the protein level. The results have also shown that *S. cerevisiae* alleles of ARO10 are present in a ratio of 3:1 from those present in *S. eubayanus* subgenome. These authors have equally demonstrated that both *S. eubayanus*-like and *S. cerevisiae*-like ARO10-encoded isoenzymes had similar activity for most of the substrates tested with preferred decarboxylation action against phenylpyruvate. However, the activity of *LgSeubARO10* encrypted enzyme towards ketoisovalerate (precursor of isobutanol) was twofold higher than that encoded by *LgScARO10*. Moreover, those authors also suggest that *S. eubayanus*-like and *S. cerevisiae*-like ARO10-derived α-oxo-acid decarboxylases exert different roles during beer fermentation by *S. pastorianus*. Fusel alcohols produced by Ehrlich pathway would involve preferentially the *S. cerevisiae*-ARO10 decarboxylase. Conversely, higher alcohols formed by *de novo* synthesis would rely almost exclusively on the *LgSeubARO10* encrypted isoenzyme.

3.3.1.3 Reduction to higher alcohols

After decarboxylation, the fusel aldehydes enter in the last step of the Ehrlich pathway, where they are converted into their respective alcohols by action of alcohol dehydrogenases. Any one of the *S. cerevisiae* alcohol dehydrogenases encoded by Adh1, Adh2, Adh3, Adh4 and Adh5 or the formaldehyde dehydrogenase encrypted by Sfa1 are able to catalyze the conversion of fusel aldehydes into higher alcohols (*42*). Thus, studies related to these genes often discuss ethanol production rather than fusel alcohols. Adh1-encoded enzyme is the constitutive, cytoplasmic form that reduces acetaldehyde into ethanol during fermentation (*46*). Adh2p is also a cytoplasmic enzyme, but it is directly repressed in the presence of glucose acting in the opposite direction of Adh1p by oxidizing ethanol (*31, 47*). Contrary to cytosolic counterparts, Adh3p is responsible for the reduction of acetaldehyde inside the mitochondria in order to keep the redox balance during anaerobic growth (*48*).

3.3.2 Regulation of higher alcohols

Iraqui and coworkers (*28*) were the first to identify the Aro80 gene as a pathway-specific regulator of the Aro9 transaminase and Aro10 decarboxylase in the presence of the aromatic amino acids tryptophan, phenylalanine and tyrosine. The nitrogen catabolite repression (NCR) on *S. cerevisiae* is mediated by GATA transcription factors consisting of activators (Gat1 and Gln3) and repressors (Gzf3 and Dal80) (*49*). The NCR works as a global nitrogen quality control system, activating GATA genes in the presence of poor nitrogen sources and repressing them when nitrogen-rich materials are available (*50*). Recent findings have shown that Aro9 and Aro10 transcription also requires GATA activators (*51*). In other words, Aro80 not only induces the transcription of Aro9 and Aro10 by directly binding to their promoters in the presence of aromatic amino acids, but is required for the recruitment of Gat1 and Gln3 activators. Lee and coworkers (*52*) assessed whether environmental conditions would also affect Aro9 and Aro10 expression. Among the conditions tested, only heat shock stress was able to activate Aro9 and Aro10 transcription. Furthermore, the authors tested a knocked down *aro80Δ* strain upon the same stress conditions and no Aro9 or Aro10 expression was observed in heat shock growth. These data strongly suggest that the transcription of Aro9 and Aro10 is activated by Aro80 under heat shock stress in *S. cerevisiae*. Back in Bolat and colleagues (*37*) studies with *S. pastorianus*, a deletion of Aro80 from *S. eubayanus*-like allele did not eliminate phenylalanine induction of LgSeubARO10. This

finding suggests that LgScAro80 can also cross-activate LgSeubARO10 compensating the loss of *S.cerevisiae*-type activator.

3.3.3 Anabolic pathway

The brewing wort normally has all proteinogenic amino acids required by the fermenting yeast to grow. However, α-keto acids (intermediates in the Ehrlich pathway) are also formed via biosynthesis de novo of amino acids through carbohydrate metabolism (Fig. 3.2) (*23*). Thus, in order to evaluate the contribution of anabolic pathway in the synthesis of higher alcohols, Eden and coworkers (*53*) have blocked the transamination of amino acids from the growth medium by using a knockout strain (*eca39Δ and eca40Δ*). In addition to these deletions, *ilv2Δ* was also investigated, and thus the activity of acetolactate synthase (ILV2) could be assessed. Without ILV2, the synthesis of isoleucine is hindered, causing an increase of the main precursor (after pyruvate) - α-ketobutyrate. As this α-keto is the precursor of propanol, the authors evidenced a significant increase in this fusel alcohol produced by *eca39Δ eca40Δ ilv2Δ* strain (*53*). This strain was also unable to produce isobutanol as α-acetolactate could not be synthetized from pyruvate due to lack of ILV2. Thus, as no external amino acid could be used in the Ehrlich pathway due to *eca39Δ eca40Δ,* the role of ILV2 gene was confirmed in the anabolic pathway of isobutanol. On the other hand, active amyl alcohol and isoamyl alcohol synthese were reduced, but still unexpectedly present (*53*). ILV2 was recently addressed to be integrated to a protein network module of functional similar proteins involved in BCAA and physically connected to the mitochondria (*31*).

3.4 Esters

Compared to other yeast metabolites, esters are only trace elements. Nevertheless, despite being "a drop in the ocean" of beer´s constituents, esters are the most important aroma elements produced by yeast. That is because esters have a very low odour threshold in beer (*14, 54*) and yet to a large extent may define its final aroma (*3, 9, 11, 12, 17, 54-57*). However, if overproduced, they can negatively affect the beer with a bitter, over fruity taste. Thus, it is crucial for the brewer to keep the optimum conditions to obtain a balanced beer in terms of its ester profile.

Esters are mainly formed during the vigorous phase of primary fermentation by enzymatic chemical condensation of organic acids and alcohols. Volatile esters in beer can be divided in two major groups: the acetate esters, and the medium-chain fatty acids

(MCFA) ethyl esters. The former group comprises esters synthetized from acetic acid (acetate) with ethanol or a higher alcohol. In ethyl esters' family, ethanol will form the alcohol radical and the acid side is a MCFA. Although dozens of different esters can be found in any beer (*14, 17*), six of them are of major importance as aromatic constituents: ethyl acetate (solvent-like aroma); isoamyl acetate (banana aroma); isobutyl acetate (fruity aroma); phenyl ethyl acetate (roses and honey aroma); ethyl hexanoate (sweet apple aroma); and ethyl octanoate (sour apple aroma).

Esters are synthetized in the cytoplasm of the brewing yeast, but readily leave the cell as they are lipophilic. However, while small chain acetate esters easily diffuse through the plasmatic membrane, MCFA ethyl esters may have their passage hindered (*4, 58, 59*).

To be synthetized into esters, organic acids must be linked to a coenzyme A to form an acyl-CoA molecule. Acyl-CoAs are highly energetic entities, which in the presence of oxygen can be β-oxidized ("cut") into smaller units (Acetyl-CoA) in the mitochondria. This will happen unless the organic acid involved is the acetic acid itself, which in this case will be turned into Acetyl-CoA. However, the great majority of Acetyl-CoA produced by the yeast cells comes from the oxidative decarboxylation of pyruvate. Aerobic conditions inside the mitochondria make Acetyl-CoA to enter in the Krebs cycle to form ATP (respiration). In the absence of oxygen, Acetyl-CoA will be enzymatic esterified with an alcohol to form the acetate esters. Moreover, MCFA ethyl esters are formed from longer chains of Acyl-CoA with ethanol. Figure 3.2 drafts the main metabolic routes of the brewing yeast contributing to higher alcohols and ester synthesis.

3.4.1 Biosynthesis of acetate esters

Acetate esters are the major flavour components of beer as they are present in much higher concentrations than other volatile esters. The enzymatic involvement of synthases on ester production dates from the 60´s (60), but the enzyme was only purified and named as Alcohol Acetyltransferase (AAT) back in 1981 by Yoshioka & Hashimoto (61). The most studied and best characterized enzymes responsible for esters synthesis are the AATases I and II (EC 2.3.1.84), encoded by the genes ATF1 and ATF2 (13, 61-68). It was also found that bottom fermenting lager yeasts have an extra ATF1 homologous gene (Lg-ATF1) (64), which encodes an AAT very similar to that encoded from the original ATF1 gene (69). This additional gene expression on lager yeast enhances acetate ester production and ultimately beer´s aroma profile.

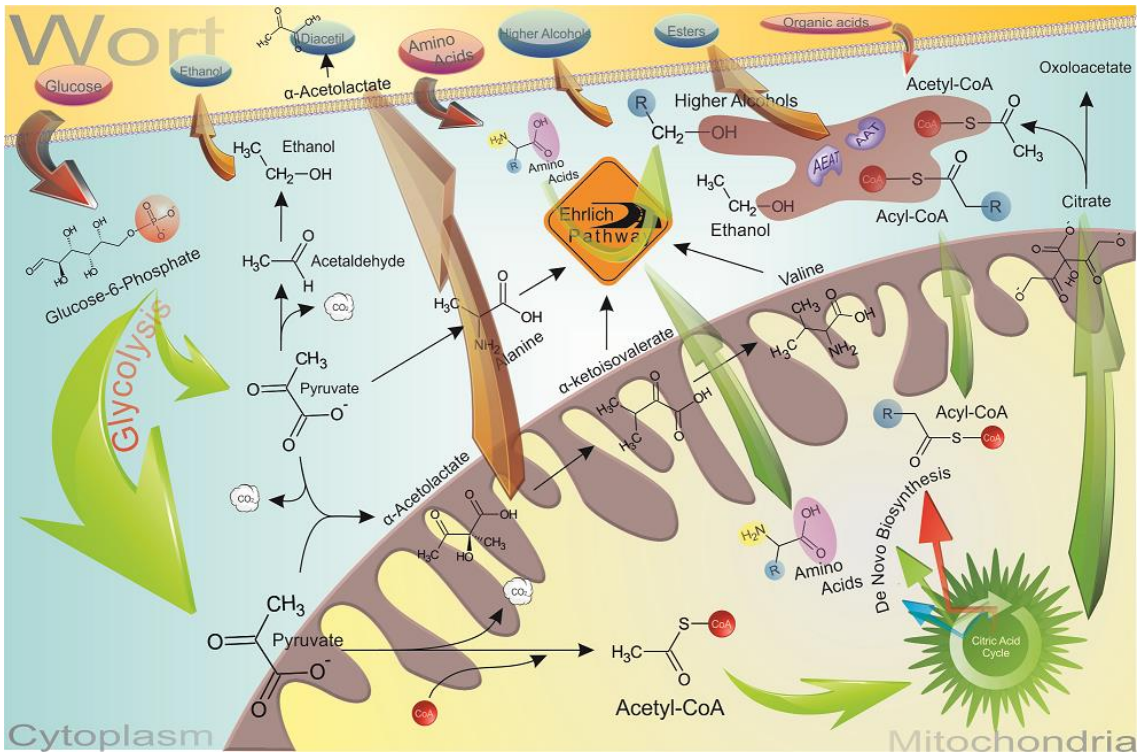


Figure 3.2 – A schematic overview of the main metabolic routes inside brewing yeast cell contributing to higher alcohols and esters synthesis when inserted in the fermenting wort.

The best way to understand the role of a gene's expression is by either overexpressing or deleting it. A heavy body of literature focus on these genetic modifications to better understand role of ATF1, ATF2 and Lg-ATF1 gene expression on the total acetate ester production (*62, 64-67, 69*). Very recently a brewer´s yeast strain was designed to increase ester/higher alcohol ratio by overexpressing ATF1 and knocking down a gene related to higher alcohol synthesis (*65*). Ester production by the genetically modified strains was considerably higher than that of parental cells. Verstrepen and coworkers (*62*) have earlier carried out a more detailed work concerning deletion and overexpression of not only the AFT1, but also of its homologous Lg-ATF1 as well as the ATF2. As others in the past (*64, 66, 69*), those authors clearly demonstrated the strong impact exerted by the expression levels of ATF genes on acetate esters production. For example, they have shown that overexpressing ATF1 strains may have up to 180-fold increased isoamyl acetate production and a 30-fold increased ethyl acetate production when compared to wild type cells. In fact, their analysis also revealed that ATF1-encrypted ATTases seem to be responsible for the great majority of acetate esters production. Through specific deletion of ATF1 and ATF2, no acetate esters originated from alcohols with more than five carbon atoms, like isoamyl acetate and phenyl ethyl acetate, were formed. This means that the most desirable banana

aroma (isoamyl acetate) in beer depends exclusively on ATF1 and ATF2-encoded enzymes. Later on 2008, Saerens and coworkers (*70*) confirmed that the maximum expression levels of ATF1 and ATF2 are directly correlated to the final concentration of acetate esters. However, the knock down (*atf1*Δ*atf*2Δ) executed by Verstrepen and coworkers (*62*), could only reduce in 50% the production of smaller esters such as ethyl acetate. Together with other pieces of evidence (*63, 71*), this result makes clear that there might be more ATTases involved on acetate esters production, but this goes beyond the knowledge present in published data. Figure 3.3A schematizes the chemical reaction for production of the main acetate esters and genes involved in these reactions.

The presence of acetate esters on alcohol-free beers (AFBs) is imperative. AFBs can be produced either from physical removal of ethanol from the finished beer or by controlling the biological process involved in beer fermentation (*72*). AFBs produced by membrane processes have usually less body and a low aromatic profile, thermally dealcoholized AFBs may suffer heat damages, while beers obtained by biological methods have often a sweet and worty off-flavor (*73*). The lack of ethanol itself greatly affects the retention of volatile aroma active compounds (*74*). Very recently, Strejc and colleagues (*75*) isolated a brewing yeast mutant capable of overproducing isoamyl acetate and isoamyl alcohol. The sweet banana odour from isoamyl acetate could then be a solution to overcome the undesirable worty off-flavor of AFB. Sensory analyzes showed that the increased level of isoamyl acetate ester had a positive effect on the fruity (banana) palate fullness and aroma intensity of the AFB produced.

3.4.2 Biosynthesis of ethyl esters

When inserted in all history of volatile ester research, MCFA ethyl esters received less attention from the literature in the past. This happened because they are much less present in beer if compared to their acetate counterparts. Nonetheless, works focused on ethyl esters in brewing fermentations have become much more common in the past decade, most of them carried by Saerens and colleagues (12, 76). Before them, with evidence published long ago (63), Mason and Dufour (77) suggested that apart from ATF1 and ATF2-encoded enzymes, there should be a different enzyme involved on ethyl esters synthesis. This ester-synthesizing enzyme, called ethanol hexanoyl transferase, is responsible for generating ethyl hexanoate from ethanol and hexanoyl-CoA (77). Later in 2006, Saerens and coworkers proved that MCFA ethyl esters are produced by the brewing yeast through a condensation reaction between an acyl-CoA

unity and ethanol (Fig. 3.3B), catalyzed by two acyl-CoA:ethanol O-acyltransferases (AEATases) encoded by Eeb1 and Eht1 genes (76). Moreover, these authors were highly convinced of the role of each of these genes on the final MCFA ethyl esters content. A single deletion on EeB1 reduced the formation of ethyl butanoate, ethyl hexanoate, ethyl octanoate and ethyl decanoate in 36%, 88%, 45% and 40%, respectively. Eht1 knocked out strain on the other hand, only had ethyl hexanoate and ethyl octanoate production affected. Additionally, a double deletion (*eeb1Δ and eht1Δ*) strain produced a similar ethyl ester profile to the *eeb1Δ* single deletion strain. This means that EeB1 is the most relevant gene on MCFA ethyl ester synthesis (76). However, even though double deletion caused a pronounced drop in ethyl esters measured, only ethyl hexanoate production was virtually extinguished. Thus, there must be other, yet unknown, AEATases involved on the MCFA ethyl esters synthesis. Also, overexpression of those genes did not increase MCFA ethyl esters production even when more precursors of these esters were added to the fermenting medium. This fact was explained as a consequence of extra esterase (breakdown) activity exerted by Eeb1- and Eht1-encoded proteins, which was also demonstrated in-vitro by the same authors (76).

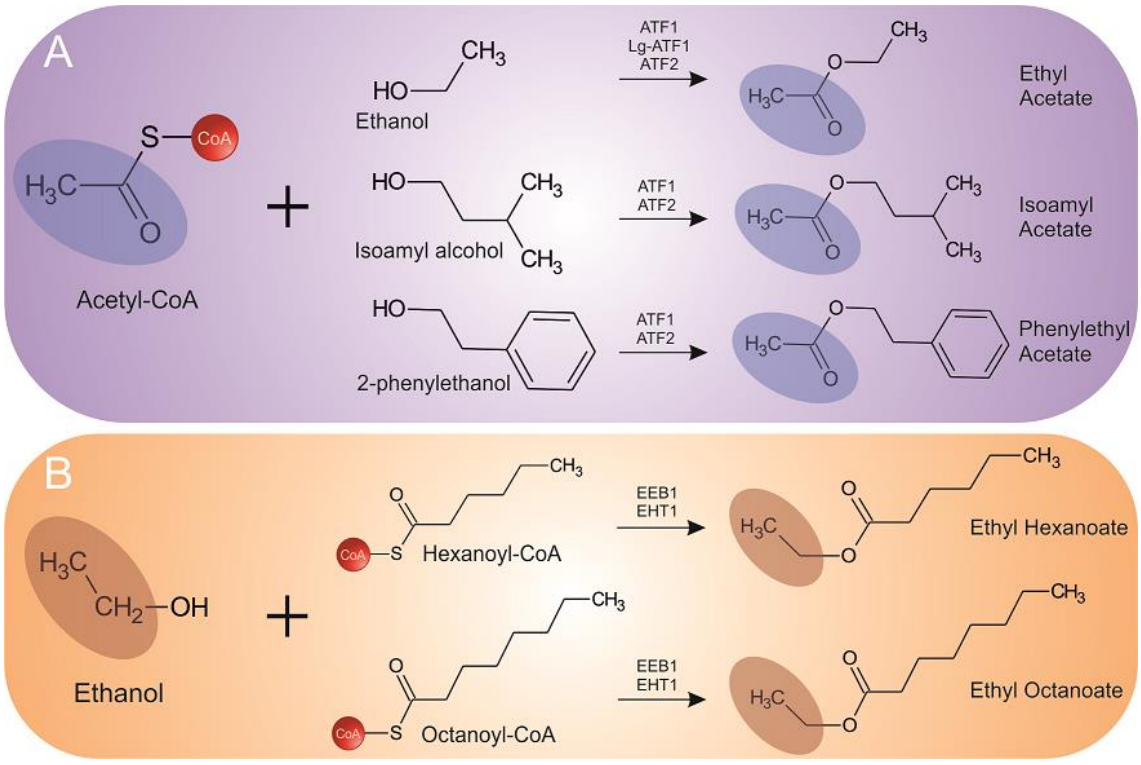


Figure 3.3 – A scheme of the chemical reactions involving acetate esters (A) and medium-chain fatty acid ethyl esters (B) biosynthesis. The main genes involved in each reaction are presented above the reaction arrows. 7

3.4.3 Ester regulation

The net rate of ester production depends not only on the availability of the precursor´s substrates (*9, 76*), but to a significant extent on the enzymatic balance of synthesis (by alcohol acetyltransferases- AATases) (*62, 65, 67, 76, 77*) and breakdown (by esterases) of esters (*78-80*). Esterases are a group of hydrolyzing enzymes that catalyze the cleavage and/or avoid the formation of ester bonds. Fukuda and coworkers (*81*) have chosen another strategy to raise the end net of isoamyl acetate production by a sake strain of *S. cerevisiae*. Instead of enhancing AATases activity, they avoided isoamyl acetate cleavage by deleting the acetate-hydrolyzing esterase gene (IAH1, previous known as EST2) responsible for encoding the carboxylesterase that hydrolyzes that ester (*79*). The mutant deficient strain produced approximately 19 times higher amounts of isoamyl acetate when compared with the parent strain. Fukuda and colleagues (*78*) have further proven the important balance activity between AATases and esterases for the net rate of ester accumulation by *S. cerevisiae*. More evidence of the IAH1-encoded esterase on the breakdown of esters was buildup recently by Lilly and coworkers (*80*). In addition to isoamyl acetate, the authors also reported decreased production of ethyl acetate, phenyl ethyl acetate and hexyl acetate by the overexpressing IAH1 mutant strain. These findings are in agreement with recent published data by Ma and collaborators (*82*) whose work determined the crystalline structure of the enzyme encrypted by IAH1 gene. They have shown that an additional C-terminus was involved on the substrate-binding region. Furthermore, it was also demonstrated that this C-terminus restricts access to the active site of the enzyme, playing a vital role in determining substrate specificity. Non-modified IAH1-encoded esterase had highest hydrolytic activity against shorter acetate esters. Moreover, this activity was greatly reduced against ethyl hexanoate and almost null for ethyl decanoate, which suggests that IAH1-encrypted enzyme is more specific for shorter chain esters. This was confirmed by truncating the additional C-terminus present in the enzyme. This modified variant was now able to hydrolyze longer ethyl esters chains such as decanoate. Those authors conclude that deletion of the C-terminus provides better access to the active site of the enzyme, which allows it to accommodate longer acyl chains (*82*).

3.4.4 Esters on aging beer

The ester profile of a given beer may change drastically during storage either by action of yeast (bottle refermentation) (*83*) or by spontaneous chemical condensation of

organic acids with ethanol (*54*, *84*, *85*). With time, hop derived components are oxidized to form 3-methyl-butyric and 2-methyl-butyric acid, which are spontaneously esterified to their respective ethyl esters (3-methyl-butyrate and 2-methyl-butyrate) (*86*) that impart the aged beer a winy aroma (*87*). On the other hand, some esters as isoamyl acetate are known to be hydrolyzed during beer storage (*88*). Chemical hydrolysis and esterification are acid-catalyzed (*85*), but remaining esterases from yeast autolysis can also play its role in unpasteurized beers (*88*). Other ethyl esters as ethyl nicotinate (medicinal, solvent, anis-like aromas), ethyl pyruvate (peas, freshly cut grass) and ethyl lactate (fruity, buttery) are also formed in aging beers (*54*). For all reasons mentioned in these lines, aging beers tend to lose fresh fruity aromas giving place to sweeter odours.

3.5 Yeast response to fermentation parameters

3.5.1 Yeast strain

As regards the lines written above, it is easy to conclude that the production of most aroma active compounds is strictly dependent on the yeast strain chosen for the fermentation. The genome associated to each strain is unique and will ultimately define the final aroma profile of the product (*2*, *5*). This makes the selection of the right strain the most important task to make good beer. Is yet crucial that the brewer keeps his strain safe not only from "wild" yeast contaminations, but from genetic drifts that may occur in the course of serial repitching (*89-91*). While the repeated repitching processes will not cause prominent loss of physiological characteristics of the brewing yeast (*90*, *92*, *93*), the accumulation of variants may eventually cause certain characteristics to linger on subsequent generations. That is why brewers must keep frozen stocks of their yeast strains for periodical restart of fresh pitching cultures.

3.5.2 Temperature

It has been reported that rising fermentation temperatures increase BAP2 expression in the brewing yeast *S. cerevisiae* (*94*). This gene is responsible for encoding a broad-substrate specificity permease that promotes the transport of the BCAAs valine, leucine and isoleucine into the yeast cell (*95*). The higher availability of amino acids within the cell favors the catalytic Ehrlich pathway, increasing higher alcohol production (*94*). Saerens and coworkers (*70*) obtained increasing levels of propanol, isobutanol, isoamyl alcohol and phenyl ethanol by rising the fermentation temperature using two different brewing yeast strains. Conversely, those authors have shown that despite increasing temperatures promotes the expression of all BAT1, BAT2 or BAP2, only BAT1 could

be strongly correlated with the final concentration of higher alcohols, in particular of propanol (*70*).

As higher alcohols formation is temperature-dependent (*96*), changes in temperature may cause changes in the availability of fusel alcohols, which are necessary for ester formation (*97*). Indeed, a slight change in temperature from 10 to 12 ºC can increase ester production of up to 75% (*98*). Saerens and coworkers (*70*) have shown that the AATases encrypting genes ATF1 and ATF2 are more expressed with increasing temperatures during beer fermentation. Furthermore, the maximum expression of these genes clearly correlated with the end concentration of ethyl acetate, isoamyl acetate and phenyl ethyl acetate.

3.5.3 Hydrostatic pressure

With increasing market demands, breweries are continuously increasing reactor sizes for beer production. The incredibly high fermenters naturally generate a huge hydrostatic pressure that generally increases the concentration of carbon dioxide dissolved in beer. The excess in dissolved CO_2 inhibits yeast growth by unbalancing decarboxylation reactions (*96, 99-102*). As said before, decarboxylation is a fundamental step in either higher alcohol or acetyl-CoA synthesis. As acetyl-CoA is the main precursor of acetate esters, hydrostatic pressure unbalances beer flavor most probably by limiting the substrate availability for ester formation (*96*).

3.5.4 Wort composition

It is not hard to understand that wort´s composition will greatly influence the final beer aroma. After all, fermenting wort is nothing more than a growth medium from which the brewing yeast absorbs nutrients for living and to where it lays its metabolic by-products. Thus, changes in the nutrient content will trigger different yeast responses.

3.5.4.1 Sugars

High gravity brewing (HGB) or even very high gravity (VHG) became a common practice for commercial breweries as it can bring great economic benefits (103, 104). The use of HGB can not only increase the brewery capacity in up to 20-30% without any investment in equipment, but also improve the haze and smoothness of the beer (105). However, HGB often brings an unbalanced flavour profile to the finished beer, being the most common perturbation the overproduction of acetate esters, impairing the beer with over-fruity and solvent-like aromas (3, 70, 106). Anderson and Kirsop (106)

observed up to eightfold increase in acetate esters production when the specific gravity of wort was doubled. Saerens and coworkers (70) have tested ale and lager strains upon increasing specific wort gravity. Although all higher alcohols measured showed an increased accumulation, after dilution to reach the normal ethanol content (5.1% v/v), only the fermentations performed by the ale strain remained with unbalanced high levels of fusel alcohols. Additionally, all acetate esters were overproduced by both lager and ale strains when increasing the initial specific gravity of the fermenting wort. Yet, not only the amount, but also the type of sugars may influence the changes in the aromatic profile of the final beer. Easily assimilable glucose- and fructose-rich worts normally generate beers with higher contents of esters than those rich in maltose (107-110). Both 21 and 24 °P wort enriched with maltose syrup fermentations performed by Piddocke and colleagues (110) produced less acetate esters compared to fermentations carried out with glucose syrup-enriched worts. The reason why an individual assimilable sugar has different impact on ester production is still unknown. Younis and Stewart (108) suggested that higher levels of glucose increase acetyl-CoA, which is the main substrate for acetate esters synthesis. In the same way, maltose-rich worts may weakly induce acetyl-CoA formation for acetate ester production (111). Moreover, while glucose rapidly enhances ester synthase activity in carbon-starved cells by directly inducing ATF1 transcription through Ras/cAMP/PKA nutrient pathway, maltose relies on the slow "fermentable growth medium-induced" (FGM) pathway to do so (112). Furthermore, a nitrogen source is needed to maintain the transcription of ATF1 and Lg-ATF1 in the course of the fermentation (112). Increasing levels of maltose as sole carbon source in synthetic medium showed an increasing tendency to accumulate acetate esters (12). Conversely, Dekoninck and coworkers (13) have shown that although sucrose had higher impact on ATF1 expression when compared to maltose, a remarkable decrease in acetate esters was observed during HGB. The high amount of sucrose stimulated yeast´s metabolism and growth, which ultimately increased the uptake of amino acids. This discussion leads to another important factor on HGB altering aroma profile of the beer - the carbon-to-nitrogen (C|N) ratio. The addition of sugary syrups is a common practice to increase the specific gravity of the wort in HGB. However, these syrups generally lack on nitrogen, which normally reduces the total free amino nitrogen (FAN) content of the wort. Therefore, adjuncts usually increase the C|N ratio, which in turn may lead nitrogen to be a growth-limiting factor (12, 112-114). Any alteration in sugar or FAN levels affects acetate ester accumulation, but not ethyl esters

(12). Additionally, diluted FAN content observed in HGB leads to abnormal yeast physiology and unbalanced beer flavor (113).

3.5.4.2 FANs

Although a wide range of nitrogen-containing compounds are dissolved in the wort, the brewing yeast can only assimilate the smaller molecules, generally called FANs. The discussion of FANs interfering with beer aroma will inevitably lead to the absorption of amino acids to form higher alcohols through the Ehrlich pathway. The type and amount of amino acids will also lead the yeast to different responses and ultimately to final beer aromatic profile (*114, 115*). In fact, treating the wort with proteases increases the final FAN and ultimately increases higher alcohols and esters production by the brewing yeast in either HGB or normal gravity brewing (*116*). The addition of BCAAs like valine, leucine and isoleucine to the fermenting wort increases the formation of their respective fusel alcohols – isobutanol, isoamyl alcohol and amyl alcohol (*115, 117, 118*). Recently, Procopio and coworkers (*118*) have shown that not only the addition of valine, leucine and isoleucine increased the formation of fusel alcohols, but intriguingly also did proline. Since proline cannot be converted into a higher alcohol via Ehrlich pathway, its role on fusel alcohol formation induction was attributed to the synthesis of glutamate from this amino acid. A recent study showed that the supplementation of wort with lysine and histidine improved the performance of a lager brewing yeast in HGB (*114*). Compared to lysine, histidine greatly affected the aromatic profile by increasing higher alcohols and ester formation. Moreover, recent reports confirmed that FAN content of wort can affect the transcription of both ATF1 and BAT1 genes (*70, 113*).

3.5.5 Oxygen and unsaturated fatty acids (UFAs)

Dissolved oxygen and UFAs in wort are remarkably known as negative regulators of esters synthesis by brewing yeast (*71, 106, 119-124*). Oxygen was once thought to indirectly reduce ester formation by decreasing acetyl-CoA availability (*106*). However, when genetic studies came into fashion, oxygen and UFAs were proven to directly inhibit the expression of ATF1 and ATF2 (*119*). Fujiwara and colleagues (*122*) have further complemented that oxygen and UFAs repress the expression of ATF1 by different regulatory pathways. While the repression of ATF1 by oxygen is mediated by the Rox1–Tup1–Ssn6 hypoxic repressor complex (*125*), UFAs intermediates through the low-oxygen response element (*126*). In addition to acetate esters, it has been also

shown that increasing levels of UFAs in the fermenting medium reduces the production of ethyl esters by the brewing yeast (*12*).

Considering what is written above, Moonjai and coworkers (*127*) assessed the potential of rich UFAs lipid supplements to decrease the need of wort aeration. The results have shown that the treated yeast with UFAs can be pitched into poor-oxygenated worts without losing fermentation potency or organoleptic quality. A reduced amount of oxygen supplied to the wort may increase flavour stability of the final beer and will limit potential oxidative stress upon the brewing yeast (*128*). Inspired by this potential, Hull (*129*) assessed the replacement of wort oxygenation by treatment of the pitching yeast with olive oil rich in UFAs. The industrial scale test succeeded without major effects on the acceptability of the beer produced. Therefore, UFA-treated yeast may be of particular help on HGB, once worts with specific high gravity have limited oxygen solubility (*130*).

3.6 Conclusions

Higher alcohols and esters formation by brewing yeast involves complexes enzymatic and regulatory pathways. Nonetheless, much progress has been made in elucidating not only the genes involved in the transcription of key enzymes during the biosynthesis of these aroma-active substances but also on the importance of the subcellular location of these enzymes inside the yeast cell. Also important steps have been taken towards substrate specificity as regards the analysis of the crystalline structure of IAH1-encoded esterase for small acetate esters.

Much progress has been also done in proteomics describing the B1B2 module of functionally-related proteins. The core of this module is composed by Bat1p and Bat2p – two paralogous enzymes involved in the metabolism of the BCAA catalyzing the same metabolic reaction in opposite directions. It was also interesting to know the contribution of subgenomes of *S. pastorianus* to the final concentration of higher alcohols. Fusel alcohols originated from the catabolic pathway involve preferentially the *S. cerevisiae*-ARO10 decarboxylase. Conversely, higher alcohols formed by *de novo* synthesis rely almost exclusively on the *LgSeubARO10* encrypted isoenzyme.

While acetate esters production is largely dependent on ATF1 and ATF2 encoding enzymes, substrate concentration seems to be the major limiting factor for ethyl esters synthesis.

The negative aspects involving HGB affecting beer aroma are still a reality. However, each day breweries and scientists head forward to overcome these problems. Not only genetically-modified brewing yeasts are being developed, but also interesting techniques like pre-treatment of pitching yeast with oil-rich UFAs to substitute wort oxygenation are being applied. In addition to those well-known BCAAs valine, leucine and isoleucine increasing the formation of their respective fusel alcohols (isobutanol, isoamyl alcohol and amyl alcohol), proline interestingly can also raise the end net of fusel alcohols through the formation of glutamate – a key compound in yeast metabolism that may be used as amine donor in amino acid synthesis.

Despite the clear progress describing fusel alcohols and ester synthesis by brewing yeast, there is still much to be found in this field.

3.7 Acknowledgements

The financial contributions of the EU FP7 project EcoBioCAP - Ecoefficient Biodegradable Composite Advanced Packaging, Grant agreement no: 265669 as well as of the Grant Agency of the Czech Republic (project GAČR P503/12/1424) are also gratefully acknowledged. The authors thank the Ministry of Education, Youth and Sports of the Czech Republic (MSM 6046137305) for their financial support.

3.8 References

1. Meilgaard, M. C. (1975) Flavor chemistry of beer: Part I: Flavor interaction between principal volatiles, *MBAA, Techn. Quart. 12*, 107-117.
2. Ramos-Jeunehomme, C., Laub, R., and Masschelein, C. A. (1991) Why is ester formation in brewery fermentations yeast strain dependent?, In *23rd European Brewery Convention Congress*, pp 257-264, Oxford University Press, Lisbon.
3. Peddie, H. A. B. (1990) Ester formation in brewery fermentations, *J. Inst. Brew. Dig. 96*, 327-331.
4. Nykanen, L., and Nykanen, I. (1977) Production of esters by different yeast strains in sugar fermentations., *J. Inst. Brew. 83*, 30-31.
5. Rossouw, D., Naes, T., and Bauer, F. F. (2008) Linking gene regulation and the exo-metabolome: a comparative transcriptomics approach to identify genes that impact on the production of volatile aroma compounds in yeast, *BMC genomics 9*, 530.
6. Berner, T. S., and Arneborg, N. (2012) The role of lager beer yeast in oxidative stability of model beer, *Letters in applied microbiology 54*, 225-232.
7. Blasco, L., Vinas, M., and Villa, T. G. (2011) Proteins influencing foam formation in wine and beer: the role of yeast, *International microbiology : the official journal of the Spanish Society for Microbiology 14*, 61-71.
8. Bravi, E., Perretti, G., Buzzini, P., Della Sera, R., and Fantozzi, P. (2009) Technological steps and yeast biomass as factors affecting the lipid content of beer during the brewing process, *Journal of agricultural and food chemistry 57*, 6279-6284.
9. Hiralal, L., Olaniran, A. O., and Pillay, B. (2013) Aroma-active ester profile of ale beer produced under different fermentation and nutritional conditions, *Journal of bioscience and bioengineering*.

10. Lodolo, E. J., Kock, J. L., Axcell, B. C., and Brooks, M. (2008) The yeast *Saccharomyces cerevisiae*- the main character in beer brewing, *FEMS yeast research 8*, 1018-1036.

11. Verbelen, P. J., Dekoninck, T. M., Saerens, S. M., Van Mulders, S. E., Thevelein, J. M., and Delvaux, F. R. (2009) Impact of pitching rate on yeast fermentation performance and beer flavour, *Applied microbiology and biotechnology 82*, 155-167.

12. Saerens, S. M., Delvaux, F., Verstrepen, K. J., Van Dijck, P., Thevelein, J. M., and Delvaux, F. R. (2008) Parameters affecting ethyl ester production by *Saccharomyces cerevisiae* during fermentation, *Applied and environmental microbiology 74*, 454-461.

13. Dekoninck, T., Verbelen, P. J., Delvaux, F., Van Mulders, S. E., and Delvaux, F. (2012) The Importance of Wort Composition for Yeast Metabolism During Accelerated Brewery Fermentations, *J. Am. Soc. Brew. Chem. 70*, 195-204.

14. Meilgaard, M. C. (1975) Flavour chemistry of beer. Part II: Flavor and threshold of 239 aroma volatiles, *MBAA, Techn. Quart. 12*, 151-168.

15. Alvarez, P., Malcorps, P., Almeida, A. S., Ferreira, A., Meyer, A. M., and Dufour, J. P. (1994) Analysis of Free Fatty-Acids, Fusel Alcohols, and Esters in Beer - an Alternative to Cs2 Extraction, *J. Am. Soc. Brew. Chem. 52*, 127-134.

16. Krogerus, K. a., and Gibson, B. R. (2013) Diacetyl and its control during brewery fermentation, *J. Inst. Brew. 119*, 86-97.

17. Engan, S. (1974) Esters in Beer, *J. Inst. Brew. Dig. 49*, 40-48.

18. Engan, S. (1981) Beer composition: Volatile substances, In *Brewing Science* (J. R. A. Pollock, e., Ed.), pp 93-165, Academic Press, London.

19. Ehrlich, F. (1907) Über die Bedingungen der Fuselölbildung und über ihren Zusammenhang mit dem Eiweissaufbau der Hefe, *Ber. Dtsch. Chem. Ges. 40*, 1027-1047.

20. Neubauer, O., and Fromherz, K. (1911) Über den Abbau der Aminosäuren bei der Hefegärung. , *Hoppe-Seyler's Z. Physiol. Chem. 70*, 326-350.

21. Sentheshanuganathan, S. (1960) The mechanism of the formation of higher alcohols from amino acids by *Saccharomyces cerevisiae*, *The Biochemical journal 74*, 568-576.

22. Sentheshanmuganathan, S., and Elsden, S. R. (1958) The mechanism of the formation of tyrosol by *Saccharomyces cerevisiae*, *The Biochemical journal 69*, 210-218.

23. Chen, E.-H. (1978) Relative contribution of Ehrlich and biosynthetic pathways to the formation of fusel alcohols, *J. Am. Soc. Brew. Chem. 36*, 39-43.

24. Oshita, K., Kubota, M., Uchida, M. a., and Ono, M. (1995) Clarification of the relationship between fusel alcohol formation and amino acid assimilation by brewing yeast using 13C-labeled amino acid, In *25th European Brewery Convention Congress*, pp 387-402, Brussels.

25. Dickinson, J. R., and Norte, V. (1993) A study of branched-chain amino acid aminotransferase and isolation of mutations affecting the catabolism of branched-chain amino acids in *Saccharomyces cerevisiae*, *FEBS letters 326*, 29-32.

26. Kispal, G., Steiner, H., Court, D. A., Rolinski, B., and Lill, R. (1996) Mitochondrial and cytosolic branched-chain amino acid transaminases from yeast, homologs of the myc oncogene-regulated Eca39 protein, *The Journal of biological chemistry 271*, 24458-24464.

27. Eden, A., Simchen, G., and Benvenisty, N. (1996) Two yeast homologs of ECA39, a target for c-Myc regulation, code for cytosolic and mitochondrial branched-chain amino acid aminotransferases, *The Journal of biological chemistry 271*, 20242-20245.

28. Iraqui, I., Vissers, S., Andre, B., and Urrestarazu, A. (1999) Transcriptional induction by aromatic amino acids in *Saccharomyces cerevisiae*, *Molecular and cellular biology 19*, 3360-3371.

29. Urrestarazu, A., Vissers, S., Iraqui, I., and Grenson, M. (1998) Phenylalanine- and tyrosine-auxotrophic mutants of *Saccharomyces cerevisiae* impaired in transamination, *Molecular & general genetics : MGG 257*, 230-237.

30. Boer, V. M., Tai, S. L., Vuralhan, Z., Arifin, Y., Walsh, M. C., Piper, M. D., de Winde, J. H., Pronk, J. T., and Daran, J. M. (2007) Transcriptional responses of *Saccharomyces cerevisiae* to preferred and nonpreferred nitrogen sources in glucose-limited chemostat cultures, *FEMS yeast research 7*, 604-620.

31. Picotti, P., Clement-Ziza, M., Lam, H., Campbell, D. S., Schmidt, A., Deutsch, E. W., Rost, H., Sun, Z., Rinner, O., Reiter, L., Shen, Q., Michaelson, J. J., Frei, A., Alberti, S., Kusebauch, U., Wollscheid, B., Moritz, R. L., Beyer, A., and Aebersold, R. (2013) A complete mass-spectrometric map of the yeast proteome applied to quantitative trait analysis, *Nature 494*, 266-270.

32. Schoondermark-Stolk, S. A., Tabernero, M., Chapman, J., Ter Schure, E. G., Verrips, C. T., Verkleij, A. J., and Boonstra, J. (2005) Bat2p is essential in *Saccharomyces cerevisiae* for fusel alcohol production on the non-fermentable carbon source ethanol, *FEMS yeast research 5*, 757-766.

33. Avalos, J. L., Fink, G. R., and Stephanopoulos, G. (2013) Compartmentalization of metabolic pathways in yeast mitochondria improves the production of branched-chain alcohols, *Nature biotechnology 31*, 335-341.

34. Kohlhaw, G. B. (2003) Leucine biosynthesis in fungi: entering metabolism through the back door, *Microbiology and molecular biology reviews : MMBR 67*, 1-15, table of contents.

35. Dickinson, J. R., Lanterman, M. M., Danner, D. J., Pearson, B. M., Sanz, P., Harrison, S. J., and Hewlins, M. J. (1997) A 13C nuclear magnetic resonance investigation of the metabolism of leucine to isoamyl alcohol in *Saccharomyces cerevisiae*, *The Journal of biological chemistry 272*, 26871-26878.

36. Romagnoli, G., Luttik, M. A., Kotter, P., Pronk, J. T., and Daran, J. M. (2012) Substrate specificity of thiamine pyrophosphate-dependent 2-oxo-acid decarboxylases in *Saccharomyces cerevisiae*, *Applied and environmental microbiology 78*, 7538-7548.

37. Bolat, I., Romagnoli, G., Zhu, F., Pronk, J. T., and Daran, J. M. (2013) Functional analysis and transcriptional regulation of two orthologs of ARO10, encoding broad-substrate-specificity 2-oxo-acid decarboxylases, in the brewing yeast *Saccharomyces pastorianus* CBS1483, *FEMS yeast research 13*, 505-517.

38. Vuralhan, Z., Luttik, M. A., Tai, S. L., Boer, V. M., Morais, M. A., Schipper, D., Almering, M. J., Kotter, P., Dickinson, J. R., Daran, J. M., and Pronk, J. T. (2005) Physiological characterization of the ARO10-dependent, broad-substrate-specificity 2-oxo acid decarboxylase activity of *Saccharomyces cerevisiae*, *Applied and environmental microbiology 71*, 3276-3284.

39. Vuralhan, Z., Morais, M. A., Tai, S. L., Piper, M. D., and Pronk, J. T. (2003) Identification and characterization of phenylpyruvate decarboxylase genes in *Saccharomyces cerevisiae*, *Applied and environmental microbiology 69*, 4534-4541.

40. Dickinson, J. R., Harrison, S. J., and Hewlins, M. J. (1998) An investigation of the metabolism of valine to isobutyl alcohol in *Saccharomyces cerevisiae*, *The Journal of biological chemistry 273*, 25751-25756.

41. Dickinson, J. R., Harrison, S. J., Dickinson, J. A., and Hewlins, M. J. (2000) An investigation of the metabolism of isoleucine to active Amyl alcohol in *Saccharomyces cerevisiae*, *The Journal of biological chemistry 275*, 10937-10942.

42. Dickinson, J. R., Salgado, L. E., and Hewlins, M. J. (2003) The catabolism of amino acids to long chain and complex alcohols in *Saccharomyces cerevisiae*, *The Journal of biological chemistry 278*, 8028-8034.

43. Mojzita, D., and Hohmann, S. (2006) Pdc2 coordinates expression of the THI regulon in the yeast *Saccharomyces cerevisiae*, *Molecular genetics and genomics : MGG 276*, 147-161.

44. Vaughan, M. A., and Kurtzman, C. P. (1985) Deoxyribonucleic acid relatedness among species of the genus *Saccharomyces sensu stricto*, *Int J Syst Bacteriol 35*, 508-511.

45. Libkind, D., Hittinger, C. T., Valerio, E., Goncalves, C., Dover, J., Johnston, M., Goncalves, P., and Sampaio, J. P. (2011) Microbe domestication and the identification of the wild genetic

stock of lager-brewing yeast, *Proceedings of the National Academy of Sciences of the United States of America 108*, 14539-14544.

46. Bennetzen, J. L., and Hall, B. D. (1982) The primary structure of the *Saccharomyces cerevisiae* gene for alcohol dehydrogenase, *The Journal of biological chemistry 257*, 3018-3025.

47. Russell, D. W., Smith, M., Williamson, V. M., and Young, E. T. (1983) Nucleotide sequence of the yeast alcohol dehydrogenase II gene, *The Journal of biological chemistry 258*, 2674-2682.

48. Bakker, B. M., Bro, C., Kotter, P., Luttik, M. A., van Dijken, J. P., and Pronk, J. T. (2000) The mitochondrial alcohol dehydrogenase Adh3p is involved in a redox shuttle in *Saccharomyces cerevisiae*, *Journal of bacteriology 182*, 4730-4737.

49. Hofman-Bang, J. (1999) Nitrogen catabolite repression in *Saccharomyces cerevisiae*, *Molecular biotechnology 12*, 35-73.

50. Cooper, T. G. (2002) Transmitting the signal of excess nitrogen in *Saccharomyces cerevisiae* from the Tor proteins to the GATA factors: connecting the dots, *FEMS microbiology reviews 26*, 223-238.

51. Lee, K., and Hahn, J. S. (2013) Interplay of Aro80 and GATA activators in regulation of genes for catabolism of aromatic amino acids in *Saccharomyces cerevisiae*, *Molecular microbiology 88*, 1120-1134.

52. Lee, K., Sung, C., Kim, B. G., and Hahn, J. S. (2013) Activation of Aro80 transcription factor by heat-induced aromatic amino acid influx in *Saccharomyces cerevisiae*, *Biochemical and biophysical research communications 438*, 43-47.

53. Eden, A., Van Nedervelde, L., Drukker, M., Benvenisty, N., and Debourg, A. (2001) Involvement of branched-chain amino acid aminotransferases in the production of fusel alcohols during fermentation in yeast, *Applied microbiology and biotechnology 55*, 296-300.

54. Saison, D., De Schutter, D. P., Uyttenhove, B., Delvaux, F., and Delvaux, F. R. (2009) Contribution of staling compounds to the aged flavour of lager beer by studying their flavour thresholds, *Food Chem 114*, 1206–1215.

55. Meilgaard, M. C. (1991) The flavor of beer, *MBAA Tech. Q 28*, 132-141.

56. Nykanen, I., and Suomalainen, H. (1983) Formation of aroma compounds by yeast, In *Aroma of beer, wine and distilled beverages.* (Suomalainen, N. a. H., Ed.), pp 3-16, Reidel Publishing Company, Netherlands.

57. Suomalainen, H. (1981) Yeast esterases and aroma esters in alcoholic beverages, *J. Inst. Brew. 87*, 296-300.

58. Dufour, J. P. (1994) Higher alcohols, acids and ester secretion during yeast growth, In *6th Jean De Clerck Chair*, pp 1-40, Academic Press, Leuven, Belgium

59. Nykiinen, L., Nykiinen, I., and Suomalainen, H. (1977) Distribution of esters produced during sugar fermentation between the yeast cell and the medium. , *J. Inst. Brew. 83*, 32-34.

60. Nordström, K. (1962) Formation of ethyl acetate in fermentation with brewer's yeast III. Participation of coenzyme A., *J. Inst. Brew. 68*, 398-407.

61. Yoshioka, K., and Hashimoto, N. (1981) Ester formation by alcohol acetyltransferase from brewers yeast. , *Agr. Biol. Chem. 45*, 2183-2190.

62. Verstrepen, K. J., Van Laere, S. D., Vanderhaegen, B. M., Derdelinckx, G., Dufour, J. P., Pretorius, I. S., Winderickx, J., Thevelein, J. M., and Delvaux, F. R. (2003) Expression levels of the yeast alcohol acetyltransferase genes ATF1, Lg-ATF1, and ATF2 control the formation of a broad range of volatile esters, *Applied and environmental microbiology 69*, 5228-5237.

63. Malcorps, P., and Dufour, J. P. (1992) Short-chain and medium-chain aliphatic-ester synthesis in *Saccharomyces cerevisiae*, *European journal of biochemistry / FEBS 210*, 1015-1022.

64. Fujii, T., Nagasawa, N., Iwamatsu, A., Bogaki, T., Tamai, Y., and Hamachi, M. (1994) Molecular cloning, sequence analysis, and expression of the yeast alcohol acetyltransferase gene, *Applied and environmental microbiology 60*, 2786-2792.

65. Zhang, C., Liu, Y., Qi, Y., Zhang , J., Dai, L., Lin, X., and Xiao, D. (2013) Increased esters and decreased higher alcohols production by engineered brewer's yeast strains, *Eur. Food. Res. Technol. 236*, 1009-1014.

66. Nagasawa, N., Bogaki, T., Iwamatsu, A., Hamachi, M., and Kumagai, C. (1998) Cloning and nucleotide sequence of the alcohol acetyltransferase II gene (ATF2) from *Saccharomyces cerevisiae* Kyokai No. 7, *Bioscience, biotechnology, and biochemistry 62*, 1852-1857.

67. Yoshimoto, H., Fujiwara, D., Momma, T., Ito, C., Sone, H., Kaneko, Y., and Tamai, Y. (1998) Characterization of the ATF1 and Lg-ATF1 genes encoding alcohol acetyltransferases in the bottom fermenting yeast *Saccharomyces pastorianus.* , *J Ferment Bioeng 86*, 15-20.

68. Molina, A. M., Swiegers, J. H., Varela, C., Pretorius, I. S., and Agosin, E. (2007) Influence of wine fermentation temperature on the synthesis of yeast-derived volatile aroma compounds, *Applied microbiology and biotechnology 77*, 675-687.

69. Fujii, T., Yoshimoto, H., Nagasawa, N., Bogaki, T., Tamai, Y., and Hamachi, M. (1996) Nucleotide sequences of alcohol acetyltransferase genes from lager brewing yeast, *Saccharomyces carlsbergensis*, *Yeast 12*, 593-598.

70. Saerens, S. M., Verbelen, P. J., Vanbeneden, N., Thevelein, J. M., and Delvaux, F. R. (2008) Monitoring the influence of high-gravity brewing and fermentation temperature on flavour formation by analysis of gene expression levels in brewing yeast, *Applied microbiology and biotechnology 80*, 1039-1051.

71. Malcorps, P., Cheval, J. M., JamilS., and Dufour, J.-P. (1991) A new model for the regulation of ester synthesis by alcohol acetyltransferase in *Saccharomyces cerevisiae*, *J. Am. Soc. Brew. Chem. 49*, 47-53.

72. Branyik, T., Silva, D. P., Baszczynski, M., Lehnert, R., and Silva, J. (2012) A review of methods of low alcohol and alcohol-free beer production, *J. Food Eng.*, 493-506.

73. Montanari, L., Marconi, O., Mayer, H., and Fantozzi, P. (2009) Production of alcohol-free beer, In *Beer in Health and Disease Prevention*, pp 61-75, Elsevier Inc., Burlington, MA.

74. Perpete, P., and Collin, S. (2000) Influence of beer ethanol content on the wort flavour perception, *Food Chem. 71*, 379-385.

75. Strejc, J., Siříšťová, L., Karabín, M., Silva, J., and Brányik, T. (2013) Production of alcohol-free beer with elevated amounts of flavouring compounds using lager yeast mutants, *J. Inst. Brew.*

76. Saerens, S. M., Verstrepen, K. J., Van Laere, S. D., Voet, A. R., Van Dijck, P., Delvaux, F. R., and Thevelein, J. M. (2006) The *Saccharomyces cerevisiae* EHT1 and EEB1 genes encode novel enzymes with medium-chain fatty acid ethyl ester synthesis and hydrolysis capacity, *The Journal of biological chemistry 281*, 4446-4456.

77. Mason, A. B., and Dufour, J. P. (2000) Alcohol acetyltransferases and the significance of ester synthesis in yeast, *Yeast 16*, 1287-1298.

78. Fukuda, K., Yamamoto, N., Kiyokawa, Y., Yanagiuchi, T., Wakai, Y., Kitamoto, K., Inoue, Y., and Kimura, A. (1998) Balance of Activities of Alcohol Acetyltransferase and Esterase in *Saccharomyces cerevisiae* Is Important for Production of Isoamyl Acetate, *Applied and environmental microbiology 64*, 4076-4078.

79. Fukuda, K., Kuwahata, O., Kiyokawa, Y., Yanagiuchi, T., Wakai, Y., Kitamoto, K., Inoue, Y., and Kimura, A. (1996) Molecular cloning and nucleotide sequence of the isoamyl acetate-hydrolyzing esterase gene (EST2) from *Saccharomyces cerevisiae J. Ferment. Bioeng. 82*, 8-15.

80. Lilly, M., Bauer, F. F., Lambrechts, M. G., Swiegers, J. H., Cozzolino, D., and Pretorius, I. S. (2006) The effect of increased yeast alcohol acetyltransferase and esterase activity on the flavour profiles of wine and distillates, *Yeast 23*, 641-659.

81. Fukuda, K., Yamamoto, N., Kiyokawa, Y., Yanagiuchi, T., Wakai, Y., Kitamoto, K., Inoue, Y., and Kimura, A. (1998) Brewing properties of sake yeast whose EST2 gene encoding isoamyl acetate-hydrolyzing esterase was disrupted *J. Ferment. Bioeng. 85*, 101-106.

82. Ma, J., Lu, Q., Yuan, Y., Ge, H., Li, K., Zhao, W., Gao, Y., Niu, L., and Teng, M. (2011) Crystal structure of isoamyl acetate-hydrolyzing esterase from *Saccharomyces cerevisiae* reveals a novel active site architecture and the basis of substrate specificity, *Proteins 79*, 662-668.

83. Vanderhaegen, B., Neven, H., Coghe, S., Verstrepen, K. J., Derdelinckx, G., and Verachtert, H. (2003) Bioflavoring and beer refermentation, *Applied microbiology and biotechnology 62*, 140-150.

84. Rodrigues, J. A., Barros, A. S., Carvalho, B., Brandao, T., and Gil, A. M. (2011) Probing beer aging chemistry by nuclear magnetic resonance and multivariate analysis, *Analytica chimica acta 702*, 178-187.

85. Vanderhaegen, B., Neven, H., Verachtert, H., and Derdelinckx, G. (2006) The chemistry of beer aging – a critical review, *Food Chem 95*, 357–381.

86. Williams, R. S., and Wagner, H. P. (1979) Contribution of hop bitter substances to beer staling mechanisms, *J. Am. Soc. Brew. Chem. 37*, 13-19.

87. Williams, R. S., and Wagner, H. P. (1978) The isolation and identification of new staling related compounds form beer, *J. Am. Soc. Brew. Chem. 36*, 27-31.

88. Neven, H., Delvaux, F., and Derdelinckx, G. (1997) Flavor evolution of top fermented beers, *MBAA Tech. Q 34*, 115-118.

89. Jenkins, C. L., Kennedy, A. I., Hodgson, J. A., Thurston, P. a., and Smart, K. A. (2003) Impact of serial repitching on lager brewing yeast quality, *J. Am. Soc. Brew. Chem. 61*, 1-9.

90. Powell, C. D., and Diacetis, A. N. (2007) Long Term Serial Repitching and the Genetic and Phenotypic Stability of Brewer's Yeast *J. Inst. Brew. 113*, 67-74.

91. Sato, M., Watari, J., Sahara, H. a., and Koshino, S. (1994) Instability in electrophoretic karyotype of brewing yeasts., *J. Am. Soc. Brew. Chem. 52*, 148-151

92. Buhligen, F., Rudinger, P., Fetzer, I., Stahl, F., Scheper, T., Harms, H., and Muller, S. (2013) Sustainability of industrial yeast serial repitching practice studied by gene expression and correlation analysis, *Journal of biotechnology*.

93. Vieira, E., Brandao, T., and Ferreira, I. M. (2013) Evaluation of Brewer's Spent Yeast To Produce Flavor Enhancer Nucleotides: Influence of Serial Repitching, *Journal of agricultural and food chemistry 61*, 8724-8729.

94. Yukiko, K., Fumihiko, O., Keiji, M., and Toshihiko, A. (2001) Control of higher alcohol production by manipulation of the BAP2 gene in brewing yeast, *J. Am. Soc. Brew. Chem. 59*, 157-162.

95. Didion, T., Grauslund, M., Kielland-Brandt, M. C., and Andersen, H. A. (1996) Amino acids induce expression of BAP2, a branched-chain amino acid permease gene in *Saccharomyces cerevisiae*, *Journal of bacteriology 178*, 2025-2029.

96. Landaud, S., Latrille, E., and Corrieu, G. (2001) Top Pressure and Temperature Control the Fusel Alcohol/Ester Ratio through Yeast Growth in Beer Fermentation, *J. Inst. Brew. 107*, 107–117.

97. Calderbank, J., and Hammond, J. R. M. (1994) Influence of higher alcohol availability on ester formation by yeast, *J. Am. Soc. Brew. Chem. 52*, 84-90.

98. Engan, S., and Aubert, O. (1977) Relations between fermentation temperature and the formation of some flavour components,, In *16th European Brewery Convention Congress*, pp 591-607, Netherlands, Amsterdam.

99. Rice, J. F., Chicoye, E., and and Helbert, J. R. (1977) Inhibition ofbeer volatiles formation by carbon dioxide pressure, *J. Am. Soc. Brew. Chem. 35*, 35-40.

100. Knatchbull, F. B., and Slaughter, J. C. (1987) The effect of low CO2 pressure on the absorption of amino acids and production of flavour-active volatiles by yeast, *J. Inst. Brew. 93*, 420-424.

101. Renger, R. S., Van Hateren, S. H., and , and Luyben, K. C. A. M. (1992) The formation of esters and higher alcohols during brewery fermentation -the effect of carbon dioxide pressure, *J. Inst. Brew. 98*, 509-513.

102. Shanta Kumara, H. M. C., Fukui, N., Kojima, K., and, and Nakatani, K. (1995) Regulation mechanism of ester formation by dissolved carbon dioxide during beer fermentation., *MBAA Tech. Q 32*, 159-162.

103. Yu, Z., Zhao, H., Li, H., Zhang, Q., Lei, H., and Zhao, M. (2012) Selection of Saccharomyces pastorianus variants with improved fermentation performance under very high gravity wort conditions, *Biotechnology letters 34*, 365-370.

104. Lei, H., Zhao, H., and Zhao, M. (2013) Proteases supplementation to high gravity worts enhances fermentation performance of brewer's yeast, *Biochem Eng J 77*, 1-6.

105. Stewart, G. (2007) High gravity brewing- the pros and cons., *New Food 1*, 42-46.

106. Anderson, R. G., and Kirsop, B. H. (1974) The control of volatile ester synthesis during the fermentation of wort of high specific gravity, *J. Inst. Brew. 80*, 48-55.

107. Younis, O. S., and Stewart, G. G. (2000) The effect of wort maltose content on volatile production and fermentation performance in brewing yeast, In *Brewing yeast fermentation performance* (Smart, K., Ed.) 1st ed., pp 170-176, Blackwell Science, Oxford, UK.

108. Younis, O. S., and Stewart, G. G. (1998) Sugar uptake and subsequent ester and higher alcohol production by *Saccharomyces cerevisiae*, *J. Inst. Brew. 104*, 255-264.

109. Younis, O. S., and Stewart, G. G. (1999) The effect of malt wort, very high gravity malt wart and very high gravity adjunct wort on volatile production in *Saccharomyces cerevisiae*, *J. Am. Soc. Brew. Chem. 57*, 39-45.

110. Piddocke, M. P., Kreisz, S., Heldt-Hansen, H. P., Nielsen, K. F., and Olsson, L. (2009) Physiological characterization of brewer's yeast in high-gravity beer fermentations with glucose or maltose syrups as adjuncts, *Applied microbiology and biotechnology 84*, 453-464.

111. Shindo, S., and Murakani, J., and Koshino, S. (1992) Control of acetate ester formation during alcohol fermentation with immobilized yeast., *J Ferment Bioeng 73*, 370-374.

112. Verstrepen, K. J., Derdelinckx, G., Dufour, J. P., Winderickx, J., Pretorius, I. S., Thevelein, J. M., and Delvaux, F. R. (2003) The *Saccharomyces cerevisiae* alcohol acetyl transferase gene ATF1 is a target of the cAMP/PKA and FGM nutrient-signalling pathways, *FEMS yeast research 4*, 285-296.

113. Lei, H., Zhao, H., Yu, Z., and Zhao, M. (2012) Effects of wort gravity and nitrogen level on fermentation performance of brewer's yeast and the formation of flavor volatiles, *Applied biochemistry and biotechnology 166*, 1562-1574.

114. Lei, H., Li, H., Mo, F., Zheng, L., Zhao, H., and Zhao, M. (2013) Effects of Lys and His supplementations on the regulation of nitrogen metabolism in lager yeast, *Applied microbiology and biotechnology 97*, 8913-8921.

115. Äyräptää, T. (1971) Biosynthetic formation of higher alcohols by yeast. Dependence on the nitrogen nutrient level of the medium, *J. Inst. Brew. 77*, 266-276.

116. Lei, H., Zheng, L., Wang, C., Zhao, H., and Zhao, M. (2013) Effects of worts treated with proteases on the assimilation of free amino acids and fermentation performance of lager yeast, *International journal of food microbiology 161*, 76-83.

117. Engan, S. (1970) Wort composition and beer flavour I: the influence of some amino acids on the formation of higher aliphatic alcohols and esters, *J. Inst. Brew. 76*, 254-261.

118. Procopio, S., Krausea, D., Hofmannb, T., and, and Beckera, T. (2013) Significant amino acids in aroma compound profiling during yeast fermentation analyzed by PLS regression, *LWT - Food Science and Technology 51*, 423–432.

119. Fujii, T., Kobayashi, O., Yoshimoto, H., Furukawa, S., and Tamai, Y. (1997) Effect of aeration and unsaturated fatty acids on expression of the *Saccharomyces cerevisiae* alcohol acetyltransferase gene, *Applied and environmental microbiology 63*, 910-915.

120. Thurston, P. A., Quain, D. E., and , and Tuhh, R. S. (1982) Lipid metabolism and the regulation of volatile synthesis in Srrccharomyces cerevisiae, *J. Inst. Brew. 88*, 90-94.

121. Taylor, G. T., Tburston, P. A., and , and Kirsop, B. H. (1979) lnfluence of lipids derived from malt spent grains on yeast metabolism and fermentation, *J. Inst. Brew. 85*, 219-227.

122. Fujiwara, D., Yoshimoto, H., Sone, H., Harashima, S., and Tamai, Y. (1998) Transcriptional co-regulation of *Saccharomyces cerevisiae* alcohol acetyltransferase gene, ATF1 and delta-9 fatty acid desaturase gene, OLE1 by unsaturated fatty acids, *Yeast 14*, 711-721.

123. Anderson, R. G., and Kirsop, B. H. (1975) Oxygen as a regulator of ester accumulation during the fermentation of worts of high specific gravity, *J. Inst. Brew. 81*, 111-115.

124. Anderson, R. G., and Kirsop, B. H. (1975) Quantitative aspects of the control by oxygenation of acetate ester formation of worts of high specific gravity, *J. Inst. Brew. 81*, 269-301.

125. Fujiwara, D., Kobayashi, O., Yoshimoto, H., Harashima, S., and , and Tamai, Y. (1999) Molecular mechanism of the multiple regulation of the *Saccharomyces cerevisiae* ATF1 gene encoding alcohol acetyltransferase, *Yeast 15*, 1183-1197.

126. Vasconcelles, M. J., Jiang, Y., McDaid, K., Gilooly, L., Wretzel, S., Porter, D. L., Martin, C. E., and Goldberg, M. A. (2001) Identification and characterization of a low oxygen response element involved in the hypoxic induction of a family of *Saccharomyces cerevisiae* genes. Implications for the conservation of oxygen sensing in eukaryotes, *The Journal of biological chemistry 276*, 14374-14384.

127. Moonjai, N., Verstrepen, K. J., Delvaux, F. R., Derdelinckx, G., and and Verachtert, H. (2002) The effects if linoleic acid supplementation of cropped yeast on its subsequent fermentation performance and acetate ester synthesis, *J. Inst. Brew. 108*, 227-235.

128. Gibson, B. R., Lawrence, S. J., Boulton, C. A., Box, W. G., Graham, N. S., Linforth, R. S., and Smart, K. A. (2008) The oxidative stress response of a lager brewing yeast strain during industrial propagation and fermentation, *FEMS yeast research 8*, 574-585.

129. Hull, G. (2008) Olive oil addition to yeast as an alternative to wort aeration, *Tech. Q. Master Brew. Assoc. Am. 45*, 17-23.

130. Baker, C. a., and Morton, S. (1977) Oxygen levels in air-saturated worts, *J. Inst. Brew. 83*, 348-349.

Chapter 4

A new approach on brewers´ spent grains treatment and potential use as lignocellulosic yeast cells carriers

In this chapter a wide range of chemical treatments were suggested in order to obtain lignocellulosic yeast carriers from the brewer´s spent grains.

4.1 Abstract

The major objective of this work is to improve the pre-treatments of Brewers´ Spent Grains (BSG) aiming at their use as a source for lignocellulosic yeast carriers' (LCYC) production. Therefore, several pre-treatments of BSG have been designed aiming at obtaining various yeast carriers, differing on their physicochemical composition. Cellulose, hemicellulose, lignin, fat, protein and ash content were determined for crude BSG and the LCYCs. The long chain fatty acids profile for the crude BSG was also analyzed. Chemical treatments' successfully produced several different LCYC based on BSG. The highest cellulose content in LCYC was achieved upon application of caustic (NaOH) treatment during 40 min. Either caustic or combined acid/caustic treatments predominately generated hydrophobic, negatively charged LCYC. The feasibility of using BSG for LCYC production is strengthened by the fact that added-value by-products can be extracted before the chemical treatments are applied.

Keywords: Brewers´ Spent Grains, lignocellulosic yeast carriers', cellulose, lignin, hemicellulose and treatments.

4.2 Introduction

BSG is the major by-product produced by the beer industry. Nowadays, in accordance with the new tendency to find solutions for waste utilization, mainly for industry residues *(1)*, BSG proved to be useful in applications such as human and animal nutrition, energy production, paper manufacture, as absorbent material, etc. *(2,3)*. However, in Europe, BSG is mainly used for cattle feeding and its real value as a source of marketable product seems to be underestimated.

BSG are solid remains from wort preparation process and consist of a complex mixture of barley grain husk, pericarp and endosperms' fragments *(4)*. For cell immobilization itself, the husks´ grains are used once they are composed mainly of a rigid matrix of cellulose (30%), hemicellulose (34.9%) and lignin (17.7%) *(5)*.

Continuous beer fermentation is based on a high-density yeast population, often achieved by attachment to solids substrates (carriers) and subsequent biofilm formation. Besides being food grade, the carrier chosen for this use should meet some requirements like enhanced adhesion capacity, physical resistance, low cost of production as well as broad availability *(6)*. Although several types of materials have been suggested for this purpose until now, including polyvinyl alcohol particles *(7)*, k-carrageenan beads *(8)*, alginate microbeads *(9)* and ceramic foam *(10)*, they are relatively expensive if compared to BSG. The carrier cost is extremely important and restrictive if it is to be applied on scale-up processes. Since BSG is a residue for the beer company itself, it can clearly be integrated in the continuous beer fermentation technology.

Pre-treated BSG was earlier evaluated as a lignocellulosic yeast carrier (LCYC) for continuous beer fermentation *(11–16)* and it possesses a high yeast loading capacity triggered by both carrier and cells´ biochemical and physicochemical properties.

Both caustic (NaOH) and acid-caustic (HCl + NaOH) treatments have previously been proposed to deal with BSG during LCYC preparation *(11, 17-19)* and they are mainly time consuming. Base-treated carriers are more hydrophobic if compared to acid-base treated, enhancing adhesion in one hand, but more floatable and easily washed out from the reactor in the other hand *(13)*. Thus, a balance between hydrophilic (cellulose) and hydrophobic (lignin) composition of carriers obtained from BSG must be idealized. A simplified caustic-based treatment could also be attractive from an economic point of view *(13)*.

The major aim of this work was to provide a fast and simple treatment for LCYC preparation from BSG. In addition, a deeper study was carried out on BSG as well as on the LCYC obtained from the treatments proposed in this work, directed to strengthen the feasibility of using this material for continuous beer production.

4.3 Materials and methods

4.3.1 Raw material

Fresh BSG was kindly provided by UNICER Bebidas de Portugal, S.A. (S. Mamede de Infesta, Portugal) from its main line of beer production. The company uses the CH-9240 Uzwil (Buhler, Germany) grain mill to make the grist.

Previous to chemical treatments the crude BSG was sieved under water (500 μm) aiming at removing the majority of residues thus obtaining a cleaner material, with higher barley husk content. After this operation, the material was placed in an oven at 50 °C until complete drying and stored at room temperature for further use.

4.3.2 Lignocellulosic yeast carrier (LCYC) preparation

In order to be used as yeast carrier, the washed BSG received a series of different chemical treatments (Table 4.1 summarizes the conditions used), as well as the treatments proposed in the literature *(11)*. Aiming at comparing the final yield of treated material, crude BSG (non-washed) received the same treatments. For all circumstances, 50 g of dried BSG have been treated using several combinations of NaOH and/or HCl (Panreac, Spain) concentrations, always in a 1:15 (w/v) ratio. All treatments were done in triplicate and treated material was dried and stored at room temperature for subsequent chemical analysis.

4.3.3 Chemical treatments

The treatments proposed in the literature to deal with BSG are mainly time consuming (3-26 hours) *(11,17–19)* and a faster and equally efficient treatment is needed. Pulping industry uses modified Kraft pulping processes for cellulose purification from wood. However, the Kraft method and its enhanced varieties use high temperatures and very strong caustic solutions composed mainly of NaOH and Na_2S (20), which would probably destroy BSG completely. Thus, double caustic treatments have heuristically been proposed to fit a simpler material like BSG.

Table 4.1 – Chemical treatments applied on Brewers´ Spent Grains (BSG) for Ligno-cellulosic yeast carrier (LCYC) preparation

Treatment	Description
Caustic [a]	NaOH 2%; 120 rpm; 30 °C; 24 h
Acid-Caustic [a]	(HCl 3%; 60 °C; 2.5 h) + (NaOH 2%; 120 rpm; 30 °C; 24 h)
Fast Caustic	NaOH 3%; 70 °C; 20 min
Double Caustic 3% & 6%	(NaOH 3%; 70 °C; 20 min) + (NaOH 6%; 90 °C; 20 min)
Double Caustic 3% & 3%	(NaOH 3%; 70 °C; 20 min) + (NaOH 3%; 90 °C; 20 min)
Double Caustic 3% & 1%	(NaOH 3%; 70 °C; 20 min) + (NaOH 1%; 90 °C; 20 min)
Double Caustic 0.5%	(NaOH 0.5%; 70 °C; 20 min) + (NaOH 0.5%; 90 °C; 20 min)
Fast Caustic and Acid	(NaOH 1%; 70 °C; 20 min) + (HCl 1%; 70 °C; 20 min)

[a] Methods performed according to Bránik and coworkers *(11)*

4.3.4 Physico-chemical characterization of LCYC

4.3.4.1 Cellulose and hemicellulose characterization

All carriers and washed BSG were assessed for sugars' polymers content through high performance liquid chromatography (HPLC) using a Metacarb 67H Column (300 x 6.5 mm, Varian, USA) pre-heated to 60 °C by a thermostated column compartment (Chrompack Instruments AG, Neuheim, Switzerland). The mobile phase (H_2SO_4 0.005 $mol.L^{-1}$) was pumped in a flow rate of 0.7 $mL.min^{-1}$ through the Jasco 880 PU pump (Jasco, Japan). Samples were automatically injected (Jasco Intelligent Sampler AS 2057 Plus, Jasco, Japan). The methods and data analyses were carried out according to Ruiz and coworkers (2011) *(21)*. Briefly, approximately 0.5 g of each dried carrier was treated with H_2SO_4 (Sigma-aldrich, UK) 72% for one hour, at 30 °C, under agitation. The treated material was then settled in 500 mL autoclavable glass flasks (Simax, Czech Republic) containing 148.67 g of pure water and autoclaved for 1 h at 121 °C. The flasks contents were then filtered through a 20 µm polyester mesh. Solids were held in reserve for lignin content determination, while the liquid was placed in vials for sugars' assessment.

4.3.4.2 Klason lignin determination

The solids obtained after the treatment with sulfuric acid, as described above, are composed mainly by lignin and ash. Thus, these solids were dried in an oven at 105 °C for 24 h. After that, dry weight was determined followed by combustion of the material in a muffle at 600 °C for 8 h for ash weight determination. The Klason lignin fraction is

the difference weight between the dry weight and ash divided by the carrier dry weight before digestion.

4.3.4.3 Contact angle

Carriers and non-treated barley husks from washed BSG were clamped one by one to glass slides on a double-sided glue tape, helped by a stereoscopic microscope. Contact angles of three different pure liquids (water, formamide and α-bromo-naphtalene) were assessed by the sessile drop technique with a drop volume varying from 0.1 to 0.3 µl, using a contact angle apparatus (OCA 20, Dataphysics, Germany). At least 30 readings were carried out for each sample and liquid, at 25 °C. All data were processed and calculated according to van Oss and coworkers (1988) *(22)*.

4.3.4.4 Zeta potential

Carriers and washed BSG were milled to powder with an Agate Ball Mill (Retsch, Germany) and sieved through a 90 µm mesh sieve. Approximately 0.1 g of sieved powder was suspended in 50 mL of KNO_3 0.01 $mol.L^{-1}$ solution and filtered through a Whatman filter paper grade Nº 1 (Whatman, UK) with 11 µm of particle retention size. The pH was adjusted to 5 and the suspension was filled in the electrophoresis cells for zeta potential determination with a Zetasizer Nano ZS (Malvern Instruments, UK). Five repetitions of 50 readings for each sample were carried out and the average zeta potential was calculated for each one of them.

4.3.4.5 Total fat analysis

The Soxtec method was performed for total fat content determination from carriers, crude and washed BSG accordingly to the manufacturer's protocol (Tecator, Sweden). Soxtec System HT2 1045 extraction unit helped by the Soxtec System HT Service Unit (Tecator, Sweden) was used in the process. Approximately 1 g of each material was used in the extraction and diethyl ether (Sigma-aldrich, UK) was used as solvent. All extractions were carried out in triplicate for each material.

4.3.4.6 Protein content

The Kjeldahl technique was implemented for total nitrogen determination on carriers, crude and washed BSG. Digestion was carried out in a Digestion System 20 1015 Digester (Tecator, Sweden) while the distillation was held in a Kjeltec system 1026 Distilling Unit (Tecator, Sweden). Briefly, approximately 0.1 g of each material was digested with 10 mL of sulfuric acid 96% (Sigma-aldrich, UK), using selenium

(Kejeltabs, Thompson & Capper LTD, UK) as catalyst at 350 °C. Distillation was further performed and a 40 $g.L^{-1}$ boric acid (Fisher Scientific, UK) solution containing the pH indicators Methyl red (Acrós Organics, USA) and Bromocresol green (Acrós Organics, USA) was used as receiving solution. Back titration was then executed using a 0.025 $mol.L^{-1}$ sulfuric acid solution. Experiments were done in triplicate for all materials.

4.3.4.7 Long chain fatty acids (LCFA)

Lipids were extracted by Soxtec (Tecator, Sweden) method from approximately 5 g of dried crude BSG. LCFA composition was analyzed by a pre-established method *(23)*. Dichloromethane (DCM) standard solutions were used to make a calibration curve for each of the following LCFA: Lauric (C12:0), myristic (C14:0), palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1) and linoleic (C18:2). The synthetic fatty acid pentadecanoic (C15:0) was used as internal standard. Both DCM and all LCFA mentioned were acquired from Fluka (Buchs, Switzerland). The LCFA analysis was done by gas chromatography (GC) (CP-9001, Chrompack, Germany), separated by an eq. CP-Sil 52CB 30 m x 0.32 mm x 0.25 µm Tr-wax column (Teknokroma, Spain). Helium was used as carrier gas at 1.0 $mL.min^{-1}$ flow rate.

4.3.5 Statistical analyses

Statistical analyses were performed using analysis of variance (ANOVA). The multiple comparison test (α = 0.05) was used to determine any significance of differences between specific means (Matlab, version 7.6.0, R2008a software, USA).

4.4 Results and Discussion

4.4.1 Carriers' yield

The bulk yields for the chemical treatments are displayed on Table 4.2. Treatments applied on washed BSG were expressively ($p < 0.05$) more efficient if compared to crude ones. The treatments proposed in the literature *(11)* were successfully replicated in this work and strongly support the yield presented on Table 4.2. Undoubtedly the washing step played an important role on treatments´ performance, possibly because the washed BSG has a higher ratio of barley husks (w/w). Thus, effectively alkali is more available to act in the husks if no other residue is present to compete with it. Hence, a simple washing step applied to BSG could reflect directly in a final lower cost of carriers' production by savings in the total amount of reagents needed in the subsequent

Table 4.2 - Bulk yield of the chemical treatments

Treatment	Yield (%) [a]	
	Crude	Washed
Caustic [b]	10	29.6
Acid-Caustic [b]	8.7	15.1
Fast Caustic	9.1	30
Double Caustic 3% & 6%	5.9	20.1
Double Caustic 3% & 3%	6.8	22.7
Double Caustic 3% & 1%	8	25.2
Double Caustic 0.5%	15.9	36.4
Fast Caustic and Acid	8.7	30.6

[a] Weight of carrier obtained from 100 grams of BSG.
[b] Methods performed by Brányik and coworkers *(11)*

processes. In addition, the residual washed material (approximately 80% of total material), which was taken apart from the treatment could be used for other purposes such as animal nutrition (as stated, this is the major destination of BSG); energy production; and on biotechnological processes as substrate for microorganisms and enzyme production *(2,3)*.

4.4.2 BSG and LCYCs´ chemical analyses

Composition of BSG may vary with barley variety, time of harvest, characteristics of hops and other adjuncts added during the brewing process; changes may also occur due to brewery technology, even within the same beer industry *(24)*. The average chemical compositions for the crude, washed (non-treated) BSG and LCYCs are shown on Table 4.3.

Considering the concentrations ($g.L^{-1}$) of monosaccharides (mainly glucose, xylose and arabinose) and considering correction factors like sugar degradation, density, water evaporation and stoichiometry, it is possible to calculate the amount of polymer that has been hydrolyzed to monomers for the given sample *(21)*. Cellulose, hemicellulose and lignin composition for the crude BSG from UNICER, strongly agrees with data present in the literature *(2,3,25)*. The present work is pioneer on LCYCs´ chemical analysis and this feature will allow a better understanding of yeast adhesion to lignocellulosic matrices in further studies. Caustic and acid-caustic treatments *(11)*, for example, greatly differed in lignocellulosic composition. The former treatment resulted in almost half of the cellulose content (in %) than the last one. As already reported for the washing step, it is possible that the acid treatment removes most of the non-lignocellulosic residues that would compete directly with the effective alkali, which

could then more efficiently remove lignin from the husks. In the first stage of Kraft pulping for example, only a small amount of lignin is removed from the wood because more than 40% of effective alkali is consumed by other residues, while in the second stage more than 65% of lignin is extracted (20,26). To reinforce this fact, the cellulosic content of carriers obtained by the acid-caustic treatment (11) from crude (non-washed) BSG was also measured (46.1% ± 0.2). This clearly demonstrates that a direct chemical treatment on crude residue-rich BSG greatly reduces the treatments´ efficiency, once the same method applied to washed BSG achieved 61.1% ± 2.27 of cellulose content. Still, either acid-caustic or caustic treatments (11) take more than 24 hours to obtain the final carrier; such a long treatment time would be unacceptable for industrial production. Double caustic (3 & 6%) treatment on the other hand allowed achieving a similar (55.6 ± 0.6%) cellulose content in just 40 minutes.

The average fat, protein and ash percentages of crude BSG, agree with previous reports *(2,3,24,27)*. All treatments almost completely removed fat and protein residues from BSG. Therefore, the remaining material was nearly a pure lignocellulosic rigid substance that can be further used as yeast carrier. The effective removal of these

Table 4.3 – Average content percentage from crude, washed BSG and carriers obtained from washed BSG 5

Material (%)	*Fat*	*Protein*	*Lignin*	*Cellulose*	*Hemicell*	*Ash*
Crude	10.5^{a}	39.08^{a}	25.62^{a}	20.97^{a}	15.18^{a}	$2.21^{a,b,c,e,f}$
Washed BSG	3.85^{b}	30.06^{b}	31.01^{b}	19.21^{a}	8.26^{b}	$2.51^{a,b,c,e,f}$
Caustic [a]	$0.74^{c,g,h,i,j}$	$3.76^{c,e}$	$22.65^{a,c,d}$	35.26^{b}	11.45^{c}	$2.97^{a,b,c,e,j}$
DC 0.5%	$1.81^{d,e,i}$	$1.43^{d,f,g,h,i,j}$	$22.99^{a,c,d}$	35.82^{b}	12.89^{d}	$1.02^{d,f,g,h,i}$
FCA	$1.84^{d,e,i}$	$3.31^{c,e,j}$	$27.79^{a,b}$	40.17^{c}	7.12^{e}	$2.49^{a,b,c,e,f}$
Fast Caustic	$0.47^{c,f,g,h,j}$	$1.76^{d,f,g,h,i,j}$	$19.39^{c,e}$	$41.38^{c,d}$	10.17^{f}	$1.56^{a,b,d,e,f,g,h,i}$
DC 3 & 1%	$0.6^{c,f,g,h,i,j}$	$0.91^{d,f,g,h,i,j}$	$20.14^{c,d,e}$	45.14^{d}	8.15^{b}	$0.98^{d,f,g,h,i}$
DC 3 & 3%	$0.69^{c,f,g,h,i,j}$	$0.97^{d,f,g,h,i,j}$	$28.39^{c,d,e}$	50.03^{e}	$7.87^{b,e}$	$0.77^{d,f,g,h,i}$
DC 3 & 6%	$1.18^{c,d,e,g,h,i}$	$0.68^{d,f,g,h,i,j}$	$16.25^{c,e}$	55.64^{f}	6.29^{e}	$0.75^{d,f,g,h,i}$
Acid-Caustic [a]	$0.25^{c,f,g,h,j}$	$2.01^{d,e,f,g,h,i,j}$	$21.19^{c,d,e}$	61.09^{g}	0^{g}	$3.74^{c,j}$

The letters a-j represent the statistical similarity among data from the same column.
[a] Methods by Brányik and coworkers (11); DC- Double Caustic; FCA- Fast caustic and Acid

substances is essential if this material is to be applied on continuous beer production once the residual fat or protein contents could easily interfere with beer flavor and/or turbidity *(28–30)*.

Both saturated and unsaturated LCFA are present in the lipid profile of BSG from UNICER. The prevailing LCFA on BSG are linoleic acid (55.5%) and palmitic acid (32.5%) (Figure 4.1). Not surprisingly, these LCFA are predominant in the pre-boiled wort *(28,31,32)*. Linoleic acid has earlier been reported to play a major role in detrimental raise of wort turbidity thus affecting the end product quality *(28–30)*. Bad for brewing on the one hand, yet good as an added-value substance on the other hand, these oils could be extracted from BSG previous to the chemical treatment, purified and sold as such; this is, however, out of the scope of the present work.

4.4.3 The LCYC

The use of lignocellulosic materials as yeast carriers is far from being a modern science as several sources of this material for yeast immobilization have been evaluated since long ago *(11,17,33)*. The use of BSG as source of LCYC was first implemented on continuous beer making *(12,16)* and more recently on wine production *(19)*.

The contact angle is the angle at which the liquid-vapor meets the solid-liquid interface. Since the tendency for a drop of liquid to spread over a plane solid surface increases as the contact angle θ decreases, the contact angle provides a useful, inverse measure of

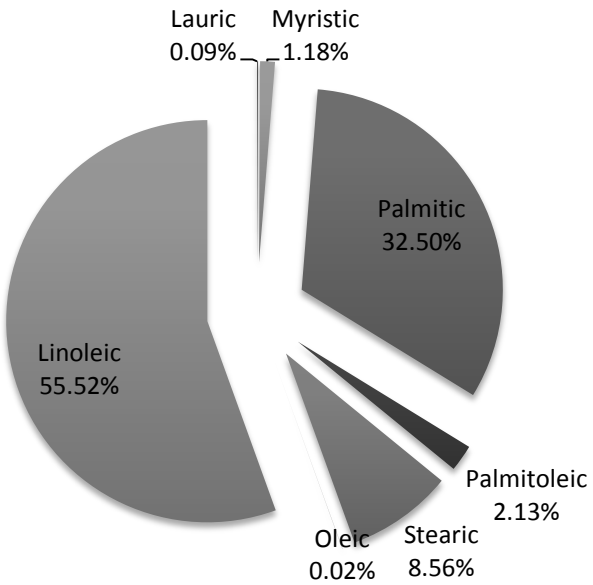


Figure 4.1 - Long chain fatty acids profile from Brewers´ Spent Grains. The LCFAs´ concentrations in grams per liter of liquid oil extracted are: lauric acid 0.06 $g.L^{-1}$, myristic acid 0.77 $g.L^{-1}$, palmitic acid 20.99 $g.L^{-1}$, palmitoleic acid 1.38 $g.L^{-1}$, stearic acid 5.53 $g.L^{-1}$, oleic acid 0.01 $g.L^{-1}$ and linoleic acid 35.86 $g.L^{-1}$.

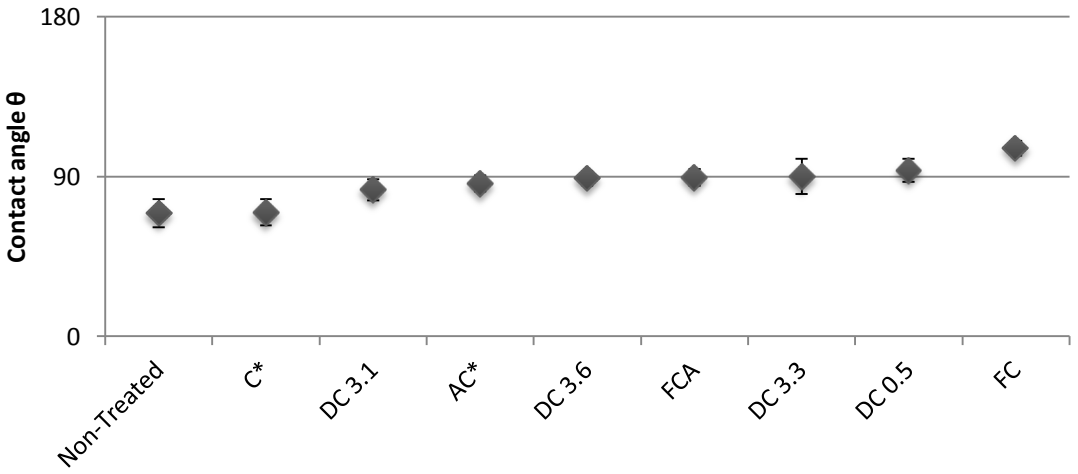


Figure 4.2 – Average contact angle between pure water, non-treated barley husks from washed BSG and carriers. From the left to the right: Non-treated barley husks from washed BSG; Caustic *(11)* treatment applied on washed BSG (C*); Double Caustic 3 & 1% (DC 3.1); Acid-Caustic *(11)* treatment applied on washed BSG; Double Caustic 3 & 6% (DC 3.6); Fast Caustic and Acid (FCA); Double Caustic 3 & 3% (DC 3.3); Double Caustic 0.5% (DC 0.5); and Fast Caustic (FC).

wettability *(34)*. In general, if the contact angle is lower than 90°, the surface is known to be wettable or hydrophilic, while if the contact angle is higher than 90°, the surface is called hydrophobic and is less prone to wet. For practical purposes Figure 4.2 outlines a 90° line, which didactically suggests the wettability limit for the described treatments. Each value represents the average contact angle from 30 readings of pure water droplets on the carrier surface. The same problem reported by Brányik and coworkers *(13)* regarding contact angle measurements at the carriers' surface was experienced in this work. The sessile drop method usually needs a large flat surface where the liquid drop can rest and the contact angle may be measured. Lignin works as a cement matrix that holds cellulose fibers together. The caustic treatment removes the lignin present in the barley husks, greatly increasing the surface roughness by exposing the cellulose fibers. The rough surface now tends to spread the drop by capillarity and this may cause a reading bias. To smooth this bias the discrepant five highest and five lowest contact angle values were discarded. The Fast caustic treated carrier was the most ($p < 0.05$) hydrophobic, while the non-treated barley husks seem to be slightly hydrophilic. Caustic *(11)* treatment applied on washed BSG was also somewhat hydrophilic. All other treatments showed no significant ($p > 0.05$) differences and are grouped around the hydrophobic/hydrophilic line. Hydrophobicity is known to enhance yeast adhesion and biofilm formation *(35–37)*, but this parameter may be tricky for continuous beer production, once unbalanced (in terms of lignin/cellulose content) hydrophobic carriers tend to float and may easily be washed out from the reactor *(13)*.

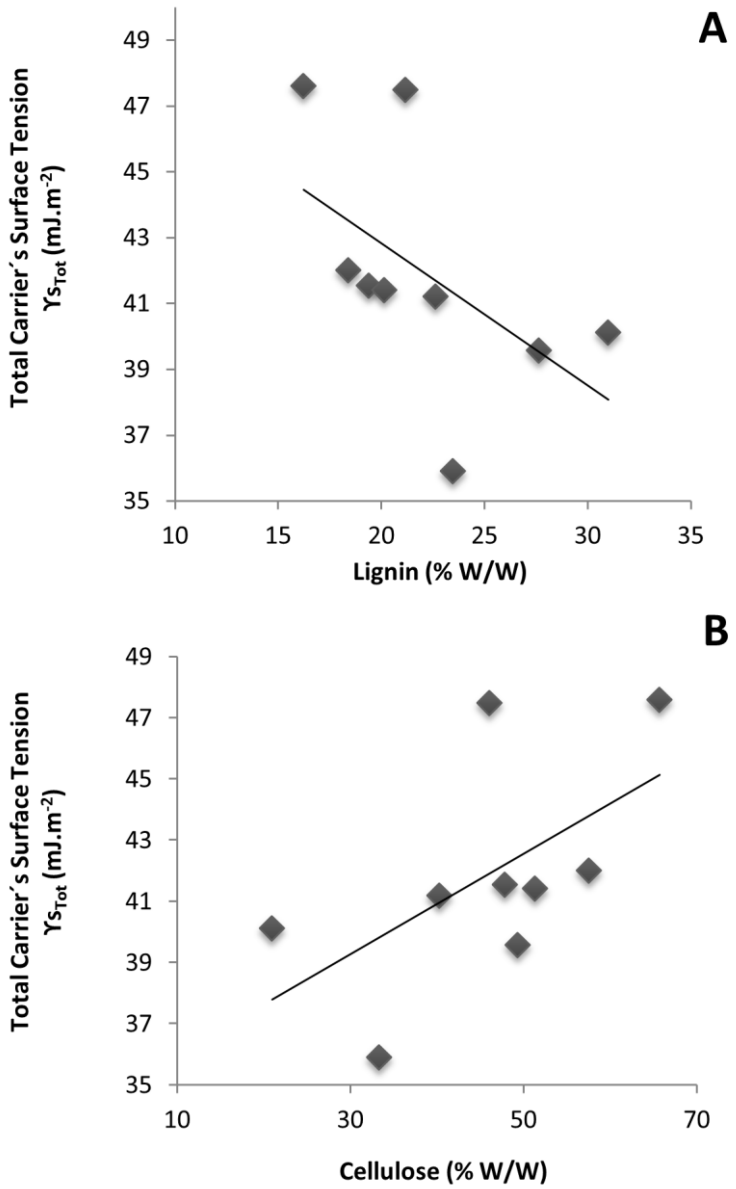


Figure 4.3 – Changes of the total surface tension of carriers as a function of their content in (A) lignin and (B) cellulose.

Figure 4.3 shows the influence of lignocellulosic composition on carriers´ surface tension. Although no strong correlation has been found between lignin or cellulose contents on carriers´ surface tension, possibly due to the lack of homogeneity of the materials used (from industrial origin), it is clear that the presence of cellulose and lignin do contribute to changes in surface properties of the carrier, which is fundamental when considering yeast cells adhesion to surfaces *(13,34)*.

The average zeta potential for the non-treated barleys´ husks from washed BSG is very close to zero (-0.07 ± 0.12 mV). All treatments have slightly reduced the zeta potential of carriers´ surface to negative values (Figure 4.4). The lower zeta potential values ($p <$

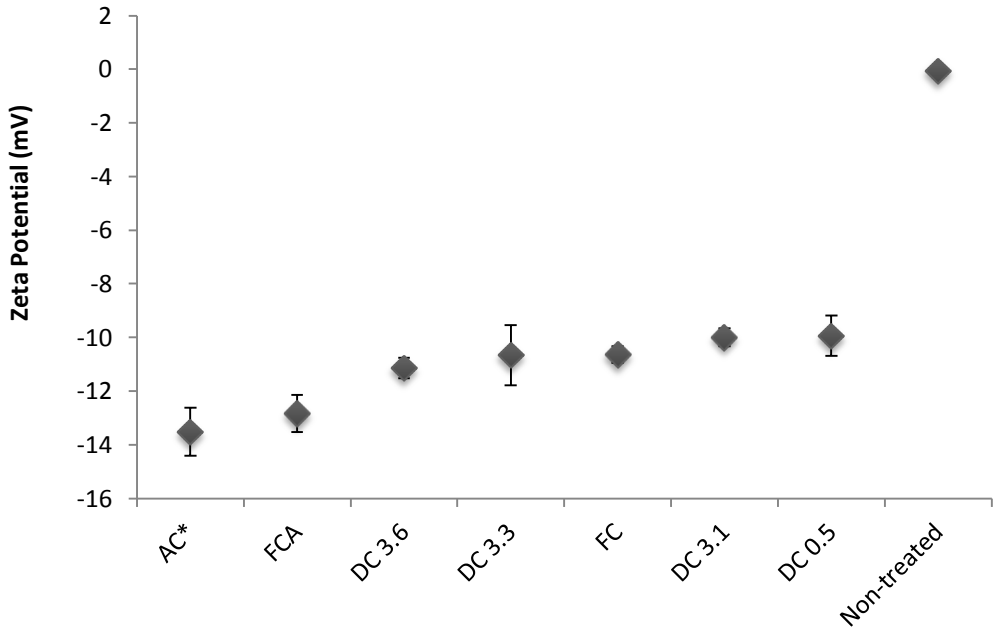


Figure 4.4 – Zeta potential of carriers and non-treated barley husks. From left to right: Acid-Caustic (AC) *(11)*; Fast Caustic and Acid (FCA); Double Caustic 3 and 6% (DC 3.6); Double Caustic 3 and 3% (DC 3.3); Fast Caustic (FC); Double Caustic 3 and 1% (DC 3.1); Double Caustic 0.5% (DC 0.5); Non-treated barley husks from washed BSG.

0.05) were observed for acid treated carriers with both Acid-Caustic (11) (-13.52 ± 0.9 mV) and Fast Caustic and Acid (-12.84 ± 0.69 mV), but they are statistically similar. This suggests that acid treatments may not be a good choice if this material is to be applied on cell immobilization. Still, post-treatments with positively charged substances are known to help on this matter *(11)*. All other treatments had no significant ($p > 0.05$) influence on the values of the carriers' zeta potential, which varied between -9.94 and -11.14 mV.

Back in 2004, Branyik and coworkers *(13)* already emphasized the need for a simpler caustic treatment – "… the equilibrium between the carrier's hydrophobic (cell adhesion sites) and hydrophilic (wettable) properties should be maintained by shortening the acidic hydrolysis of the spent grains. The resulting simplified preparation procedure, consisting only of the base treatment, will have also an attractive economic feature". Thus, the current work successfully established several simpler and faster treatments for LCYC preparation. Although the highest percentage of cellulose was achieved with the acid-caustic treatment *(11)* applied on washed BSG, Double Caustic 3 & 6% must be highlighted for faster LCYC achievement at similar yields of cellulose content.

4.5 Conclusions

The present work evaluated several chemical treatments for fast production of LCYCs in view of their use in scaled-up processes and further implementation in continuous beer production. The key element for using BSG in LCYC production goes beyond the contents of lignocellulosic material in barley husks, once this by-product is rich in other components that would be wasted by direct treatment of the raw BSG. Thus, an extraction of fat and protein contents from BSG before the chemical treatment for LCYC production is highly recommended as it could greatly add value to the process as a whole. This added-value would lay both on the improvement of the yield of the LCYC production process and on the extraction of high-value compounds from the rejected protein/lipid fraction.

4.6 Acknowledgements

The material supplied by UNICER Bebidas de Portugal, S.A. (S. Mamede de Infesta, Portugal) is gratefully acknowledged. The financial contribution of the EU FP7 project EcoBioCAP - Ecoefficient Biodegradable Composite Advanced Packaging, Grant agreement no: 265669 is also gratefully acknowledged.

4.7 References

1. Orzua, M. C.; Mussatto, S.; Contreras-Esquivel, J. C.; Rodriguez, R.; de la Garza, H.; Teixeira, J.; Aguilar, C. N. (2009) Exploitation of agro industrial wastes as immobilization carrier for solid-state fermentation. *Ind Crop Prod*, *30*, 24-27.
2. Mussatto, S.; Dragone, G.; Roberto, I. (2006) Brewers' spent grain: generation, characteristics and potential applications. *J Cereal Sci*, *43*, 1-14.
3. Mussatto, S.; Fernandes, M.; Rocha, G. J. M.; Orfão, J. J. M.; Teixeira, J.; Roberto, I. (2010) Production, characterization and application of activated carbon from brewer's spent grain lignin. *Bioresource technol*, *101*, 2450-7.
4. Forssell, P.; Kontkanen, H.; Schols, H. A.; Hinz, S.; Eijsink, V. G. H.; Treimo, J.; Robertson, J. A.; Waldron, K. W.; Faulds, C. B.; Buchert, J. (2008) Hydrolysis of Brewers' Spent Grain by Carbohydrate Degrading Enzymes. *J Inst Brew*, *114*, 306–314.
5. Palmarola-adrados, B.; Galbe, M.; Zacchi, G. (2005) Pretreatment of barley husk for bioethanol production. *J Chem Technol Biotechnol, 91*, 85-91.
6. Cheng, K.-C.; Demirci, A.; Catchmark, J. M. (2010) Advances in biofilm reactors for production of value-added products. *Appl Microbiol Biot, 87*, 445-56.
7. Bezbradica, D.; Obradovic, B.; Leskosek-cukalovic, I.; Bugarski, B.; Nedovic, V. (2007) Immobilization of yeast cells in PVA particles for beer fermentation. *Process Biochem, 42*, 1348-1351.
8. Decamps, C.; Norton, S.; Nationale, E.; Cedex, N.; Neufeld, R. J. (2004) Continuous Pilot Plant – Scale Immobilization of Yeast in K-Carrageenan Gel Beads. *AIChE Journal, 50*, 1599-1605.
9. Nedovic, V. A.; Obradovic, B.; Leskos, I. (2001) Electrostatic generation of alginate microbeads loaded with brewing yeast. *Process Biochem*, *37*, 17-22.

10. CHENG, J.; SU, Z.; LIANG, C.; YE, Q. (2009) Immobilization technology of *Saccharomyces cerevisiae* in Foam Ceramic. *China Brewing*.

11. Brányik, T.; Vicente, A.; Cruz, J. M.; Teixeira, J. (2001) Spent grains – a new support for brewing yeast immobilisation. *Biotechnol Lett*, *23*, 1073-1078.

12. Brányik, T.; Vicente, A.; Cruz, J. M.; Teixeira, J. (2002) Continuous Primary Beer Fermentation with Brewing Yeast Immobilized on Spent Grains. *Scan Electron Micros*, *108*, 410-415.

13. Brányik, T.; Vicente, A.; Oliveira, R.; Teixeira, J. Physicochemical surface properties of brewing yeast influencing their immobilization onto spent grains in a continuous reactor. *Biotechnol Bioeng* **2004**, *88*, 84-93.

14. Brányik, T.; Vicente, A.; Kuncová, G.; Podrazký, O.; Dostálek, P.; Teixeira, J. (2004) Growth model and metabolic activity of brewing yeast biofilm on the surface of spent grains: a biocatalyst for continuous beer fermentation. *Biotechnol Progr*, *20*, 1733-40.

15. Brányik, T.; Vicente, A.; Dosta, P.; Teixeira, A. (2005) Continuous Beer Fermentation Using Immobilized Yeast Cell Bioreactor Systems. *Biotechnol Progr*, *21*, 653-663.

16. Brányik, T.; Silva, D. P.; Vicente, A.; Lehnert, R.; e Silva, J. B. A.; Dostálek, P.; Teixeira, J. (2006) Continuous immobilized yeast reactor system for complete beer fermentation using spent grains and corncobs as carrier materials. *J Ind Microbiol Biot*, *33*, 1010-8.

17. Bardi, E. P.; Koutinas, A. A. (1994) Immobilization of Yeast on Delignified Cellulosic Material for Room Temperature and Low-Temperature Wine Making. *J. Agric. Food Chem.*, *42*, 221-226.

18. Kopsahelis, N.; Kanellaki, M.; Bekatorou, A. (2007) Food Chemistry Low temperature brewing using cells immobilized on brewer ' s spent grains. *Food Chem*, *104*, 480-488.

19. Tsaousi, K.; Velli, A.; Akarepis, F.; Bosnea, L.; Drouza, C.; Koutinas, A. A.; Bekatorou, A. (2011) Low-Temperature Winemaking by Thermally Dried Immobilized Yeast on Delignified Brewer ' s Spent Grains. *Food Technol Biotechnol*, *49*, 379-384.

20. Santiago, A. S.; Neto, C. P.; Vilela, C. (2008) Impact of effective alkali and sulfide profiling on Eucalyptus globulus kraft pulping . Selectivity of the impregnation phase and its effect on final pulping results. *J Chem Technol Biotechnol*, *251*, 242-251.

21. Ruiz, H. ; Ruzene, D. S.; Silva, D. P.; da Silva, F. F. M.; Vicente, A.; Teixeira, J. (2011) Development and characterization of an environmentally friendly process sequence (autohydrolysis and organosolv) for wheat straw delignification. *Appl Biochem Microbiol*, *164*, 629-41.

22. van Oss, C. J. V. A. N.; Good, R. J. (1988) Interfacial Lifshitz-van der Waals and Polar Interactions in Macroscopic Systems. *Chem Rev*, *88*, 927-941.

23. Neves, L.; Pereira, M. A.; Mota, M.; Alves, M. M. (2009) Bioresource Technology Detection and quantification of long chain fatty acids in liquid and solid samples and its relevance to understand anaerobic digestion of lipids. *Bioresource technol*, *100*, 91-96.

24. Santos, M.; Jiménez, J. J.; Bartolomé, B.; Gómez-Cordovés, C.; Nozala, M. J. (2003) Detection and quantification of long chain fatty acids in liquid and solid samples and its relevance to understand anaerobic digestion of lipids. *Food Chem*, *80*, 17-21.

25. Mandalari, G.; Faulds, C. B.; Sancho, A. I.; Saija, A.; Bisignano, G.; Locurto, R.; Waldron, K. W. (2005) Fractionation and characterisation of arabinoxylans from brewers ' spent grain and wheat bran. *J Cereal Sci*, *42*, 205-212.

26. Aurell, R.; Hartler, N. (1965) Kraft pulping of pine: part 1 - the changes in the composition of the wood residue during the cooking process. *Sven Papperstidn*, *68*, 59-68.

27. Kanauchi, O.; Mitsuyama, K.; Araki, Y. (2001) Development of a functional germinated barley foodstuff from brewers' spent grain for the treatment of ulcerative colitis. *J Am Soc Brew Chem*, *59*, 59-62.

28. Kühbeck, F.; Back, W.; Krottenthaler, M.; Brew, J. I. (2006) Influence of Lauter Turbidity on Wort Composition , Fermentation Performance and Beer Quality – A Review. *J Inst Brew*, *112*, 215-221.

29. Dufour, J. P.; Alvarez, P.; Devreux, A.; Gerardi, W. (1986) Influence of the filtration procedure on the relationship between wort turbidity and its lipid content. *Mschr. Brauwiss*, *39*, 115-121.

30. Klopper, W. J.; Tuning, B.; Vermeire, H. A. (1975) Free fatty acids in wort and beer. In *Proceedings of the European Brewery Convention Congress*; Scientific, E., Ed.; Amsterdam,; pp. 659–667.

31. Anness, B. J.; Reed, R. J. R. (1985) Lipids in the brewery – a material balance. *J Inst Brew*, *91*, 82-87.

32. Stewart, G. G.; Martin, S. A. Wort clarity: Effects on fermentation. In *Convention of the Master Brewers Association of the Americas*; 2004; pp. 18-26.

33. Moo-Young, M.; Lamptey, J.; Robinson, C. W. (1980) Immobilization of yeast cells on various supports for ethanol production. *Biotechnol Lett*, *2*, 541-548.

34. Shafrin, E. G.; Zisman, W. A. (1960) Constitutive relations in the wetting of low energy surfaces and the theory of the reaction method of preparing monolayers. *J Phys Chem*, *64*, 519-524.

35. Karagöz, P.; Erhan, E.; Keskinler, B.; Ozkan, M. (2009) The use of microporous divinyl benzene copolymer for yeast cell immobilization and ethanol production in packed-bed reactor. *Appl Biochem Microbiol*, *152*, 66-73.

36. Schmolke, H.; Demming, S.; Edlich, A.; Magdanz, V.; Büttgenbach, S.; Franco-Lara, E.; Krull, R.; Klages, C.-P. (2010) Polyelectrolyte multilayer surface functionalization of poly(dimethylsiloxane) (PDMS) for reduction of yeast cell adhesion in microfluidic devices. *Biomicrofluidics*, *4*, 044113.

37. White, J. S.; Walker, G. M. (2011) Influence of cell surface characteristics on adhesion of Saccharomyces cerevisiae to the biomaterial hydroxylapatite. *A van Leeuw*, *99*, 201-9.

Chapter 5

Maintaining yeast viability in continuous primary beer fermentation

This chapter evaluated the applicability of one type of the lignocellulosic yeast carriers suggested in the previous chapter for continuous beer production. It also approaches the feasibility of biomass skimming through foaming.

5.1 Abstract

Continuous fermentation is a long known and vastly studied process. The use of Immobilized Cell Technology (ICT) is exploited in a significant number of works due to the associated high volumetric productivity, time-savings and low capital demand. This work was aimed at solving one of the most relevant obstacles to implement ICT on a large scale in beer fermentations, namely the control of biomass and the maintenance of cell viability in a gas-lift bioreactor. For this purpose, foam fractionation by skimming was proposed as a tool for control of continuous biomass concentration. The consequences of foaming on lignocellulosic yeast carriers (LCYCs) losses were assessed and discussed. A steady consumption of sugars from wort, as well as consistent ethanol production were achieved. The viability of the suspended cells in the reactor was compared to that of the cell population in the foam using flow cytometry. Results suggest that foam might be used as a promising tool to skim non-viable biomass out of the gas-lift reactor, thus ensuring the maintenance of a cell culture with optimum viability.

Keywords: Continuous fermentation, lignocellulosic yeast carrier (LCYC), excess of biomass, foam fractionation.

5.2 Introduction

Without question, the vast majority of fermentation processes are performed in batch mode. However, it is also well known that batch fermentations are time consuming and their replacement by continuous fermentation processes could lead to important improvements in process productivity and, possibly, to economic gains.

Beer production is one of the processes that have been addressed by several authors concerning the application of continuous fermentation systems (*1-7*). In traditional batch processes, primary beer fermentation, along with maturation, can take more than a month. Continuous fermentation with immobilized biomass has been proposed as an attractive alternative that may provide the final product in less than a week (*1, 2, 8, 9*). This high volumetric productivity may lead to savings in capital and operational costs (*10*). Among the technical issues reported to date as needing improvement (*11, 12*), the maintenance of viable and metabolically active biomass inside the reactor for a prolonged period of time is, undoubtedly, one of the most important. This objective should be achieved while preventing biomass clogging inside the bioreactor (*1, 13*). Moreover, the immobilized biomass must be capable of producing beer that meets the required quality standards (*14*).

Individual yeast cells have a limited lifespan (*15-17*) and a restricted time of metabolic activity (*18, 19*). For these reasons, in a continuous reactor, dead and injured cells are prone to accumulate with negative consequences on the quality of the final product (*20*). In the batch process, after primary fermentation, dead cells are removed from the vessel by preferential sedimentation of the older cell population (*21-24*). In continuous systems where mixing is applied, such as the gas-lift reactor, biomass separation through preferential sedimentation is not feasible. As a consequence, it is fundamental to develop strategies to match the challenge of removing the excess of dead and injured cells without stopping the process in a continuous gas-lift bioreactor.

On the one hand, biofilm formation onto lignocellulosic yeast carrier's (LCYC) is a dynamic process, involving cell deposition, growth of immobilized biomass, and detachment (*25*). On the other hand, aging in brewing yeast cells is followed by changes in the surface properties of the cell wall, with older yeast cells being more hydrophobic then their younger counterparts (*24*). Hydrophobic particles can be removed from a system by adhering to bubbles and rising to the reactor´s top where they are removed by skimming. This process is called foam fractionation (*26-28*). The hydrophobic character

of older cells can thus be used as a strategy for foam fractionation and as a method for the constant removal of aged biomass from the bioreactor.

This work evaluated the effect of foaming on biomass and on the LCYC removal in a continuous beer primary fermentation process in an airlift reactor aiming at an effective control of biomass skimming and at the possibility of differentially skimming non-viable biomass.

5.3 Material and Methods

5.3.1 Brewing Yeast

The brewing yeast *Saccharomyces carlsbergensis* was generously been supplied by UNICER Bebidas, S.A. (S. Mamede de Infesta, Portugal) from its production line. The yeast was taken from the storage tank for further fermentations, previous to acid treatment. The sample was inoculated on Yeast-extract Peptone Dextrose (YPD) broth (Sigma, UK) with 2% agar (Fluka, Switzerland) for yeast colony isolation. A flocculent *Saccharomyces carlsbergensis* strain nº 96 from the culture collection of brewing yeast (Research Institute of Brewing and Malting, Plc., Prague, Czech Republic) was used in a second experiment. Strain 96 was inoculated in Petri dishes as described above. For both strains, one colony was pitched into 400 mL of wort and incubated at 20 ºC, at 120 rpm, for 48 h, previous to being used as the inoculum for the gas-lift reactor.

5.3.2 Beer wort

Two continuous fermentation trials were performed. In the first trial, wort with original specific gravity of 15º Plato (ºP), supplied by UNICER, was used. It was boiled, filled into 50 L autoclavable polypropylene carboys (Nalgene, USA) and autoclaved for 1 h. In the second trial, concentrated wort (Research Institute of Brewing and Malting – RIBM, Prague) was diluted to the desired final concentration, which varied from 5-11 ºP. Thereafter, it was filled into 20 L autoclavable polypropylene carboys (Nalgene, USA) and autoclaved for 3.5 h.

5.3.3 Brewers´ Spent Grains (BSG)

BSG were kindly provided by UNICER Bebidas, S.A. (S. Mamede de Infesta, Portugal) from its beer production process. It was double caustic (3 and 6%) treated as previously described (*29, Chapter 4*) for LCYC production. After drying, the LCYC was used in both fermentations at 10 $g.L^{-1}$ (ratio of 1% dried LCYC/reactor working volume).

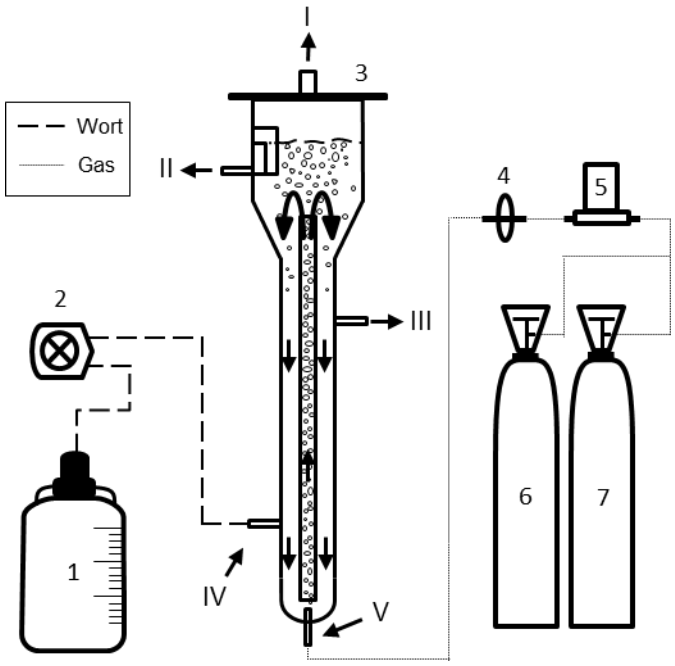


Figure 5.1 – Immobilized yeast reactor system used in the current work: 1 wort barrel; 2 peristaltic pump; 3 gas-lift reactor; 4 air filter; 5 flow mass controller; 6 pressurized air; 7 CO_2 bottle; I gas outflow and foam sampling point; II green beer outflow/sampling point; III yeast (biocatalyst, flocculated and free biomass) sampling point; IV wort inlet; V gas sparger.

5.3.4 Continuous fermentation

All experiments were performed in a Perspex gas-lift reactor with 4 L of total working volume. Gas flow was kept constant (500 $mL.min^{-1}$) by a mass flow controller (GFC17, AALBORG, USA). The temperature was held at 15 ºC using a JULABO F32 Refrigerated/Heating Circulator (JULABO, Germany) for both fermentations. The wort was fed to the reactor with a peristaltic pump at a constant dilution rate of 0.043 h^{-1}.

The reactor was sterilized using a 3% (v/v) solution of commercial sodium hypochlorite with 1.5% of active chlorine, 48 h before use. After this time, the solution was discarded and 50 L of sterile water was used to wash the reactor.

After washing, the reactor was filled with wort and inoculated. It remained under batch operation for 48 h previous to the addition of 40 g of LCYC and the start of the continuous phase. During the batch phase, the supplied gas was pressurized air that was changed to pure CO_2 at the beginning of the continuous phase. In both cases, the gas was sterilized by passing through a 0.2 µm sterile filter (Whatman, UK). Figure 5.1 displays a schematic view of the experimental setup.

The first fermentation was carried out without the addition of antifoam and the yeast strain used was from UNICER. In the second fermentation, foaming was inhibited by the direct addition into the reactor of a sterile Antifoam B emulsion, containing 10% of active silicone (Sigma, UK) and yeast strain 96 was used.

5.3.5 Biomass Measurements

Immobilized biomass was assessed as previously reported (*2*). Measurements of free biomass were carried out. Three samples of 10 mL from the bulk liquid of the reactor, free of biocatalysts, were collected into pre-weighted 15 mL Falcon tubes and centrifuged at 4000 x *g* for 5 min. The liquid phase was discarded and the tubes were dried at 105 ^{o}C for 24 h and weighed for biomass determination. Controls were samples using the inlet wort to correct for the presence of trub-like proteins that could interfere at weighting.

5.3.6 Flow Cytometric Assays

Stock solutions were prepared for fluorescein diacetate (FDA) and propidium iodide (PI). The FDA (Sigma, UK) was diluted with dimethyl sulfoxide (DMSO) (Sigma, UK) to a final concentration of 500 µg.mL^{-1}. The PI (Sigma, UK) solution was diluted in ultra-pure water to a final concentration of 1 mg.mL^{-1}. The stock solutions were kept at -20 ^{o}C until used. For the double staining with FDA and PI, 100 µL of ≈1x10^{6} cell suspension were added to 400 µL of phosphate buffered saline - PBS (Sigma, UK) containing 1 µL of PI stock solution and 4 µL of FDA. Incubation was carried out at room temperature for 20 min.

From the continuous reactor, three types of yeast populations were chosen for flow cytometric analyses: free (not attached to support) flocculated cells, immobilized cells on the LCYC support and cells collected from the foam. Immobilized cells population was prepared by successive washing of a small amount of biocatalyst (LCYC plus cells) in PBS followed by a strong agitation for cell release. The cells in the supernatant were used for further characterization. As the biocatalysts had higher density than flocculated cells, it was easy to separate them by sedimentation. Therefore, after sedimentation, yeast flocks were harvested with the help of a pipette and centrifuged at 4000 x g for 5 min. Liquid phase was discarded and the cells were washed two times with PSB previous to staining. Samples were double stained with FDA and PI as described above, and analyzed using a Partec Pas III (Partec GmbH, Münster, Germany) analyzer equipped with an argon ion laser (15 mW laser power with excitation wavelength 488 nm). The FDA green fluorescence was detected by the FL1 (530 nm) channel, while the PI red fluorescence was captured by the FL3 (590-610 nm). More than 20000 cell readings were carried out for each sample in triplicate. Data were analyzed in the

Flowing Software version 2.2.0 (Freeware provided by Perttu Terho - Turku Centre for Biotechnology, University of Turku, Finland).

5.3.7 Epifluorescence Microscopy

Samples were periodically analyzed by epifluorescence microscopy, using an Olympus BX51 (OLYMPUS, Japan) microscope for staining control (previous to flow cytometry) and biofilm visualization. The FDA and PI signals were obtained by long pass filters: one in the green wavelength range with an excitation bandpass of 470–490 nm and emission at 516 nm, and a second filter in the red wavelength range with an excitation bandpass of 530–550 nm and emission at 591 nm.

5.3.8 Wort sugars and ethanol determination

All sugars and ethanol levels were assessed by high-performance liquid chromatography (HPLC) in a Jasco chromatograph equipped with refraction-index detector (Jasco, Tokyo, Japan) using a Metacarb 67H column (300 × 6.5 mm, Varian) preheated to 80 °C by a thermostated column compartment (Chrompack Instruments AG, Neuheim, Switzerland). The mobile phase (H_2SO_4 0.005 mol·L^{-1}) was pumped at a flow rate of 0.3 mL·min^{-1} through a JASCO 880 PU pump. Samples were automatically injected (JASCO Intelligent Sampler AS 2057 Plus, USA). Standard calibration curves were obtained using different concentrations of pure sugars (2.5-20 g.L^{-1}) and ethanol (1.25-10% v/v) (D-glucose (Sigma, UK), D-fructose (Acros Organics, USA), D-maltose monohydrate (Himedia, India), maltotriose (Sigma, USA) and ethanol (Sigma, UK)) previous to wort and green beer samples analyses.

5.4 Results and Discussion

5.4.1 Biomass growth and biofilm formation

In order to evaluate the consequences of foam formation on biomass losses (biocatalysts and free biomass), the effect of the antifoam agent addition was assessed. The use of the antifoam agent caused a massive accumulation of free biomass (flocculated plus suspended cells) inside the gas-lift reactor (Fig. 5.2b). This accumulation is the first proof of the negative impact caused by the inhibition of a “foaming mode of operation”, which stops biomass skimming. Additionally, the inhibition of foam formation caused an increase in the size of cell clusters (flocs), which could also contribute to the retention of biomass inside the reactor, as the dilution rate used would not cause its wash-out. Moreover, bearing in mind that the lack of fermentable sugars promotes yeast

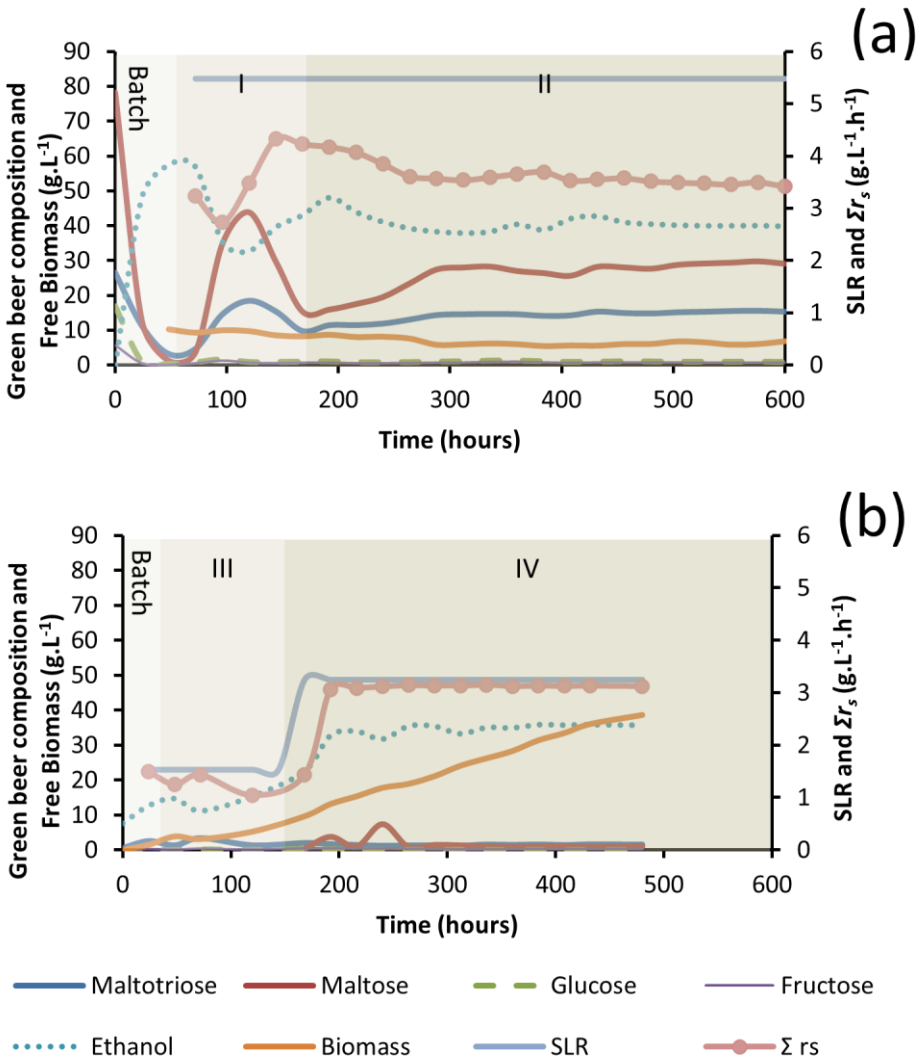


Figure 5.2 – Composition of the green beer and total free biomass concentration in the bioreactor for both foaming (a) and non-foaming (b) experiments. **(a)** The initial phase (I) of foaming experiment, marked by a great loss of yeast carriers, was followed by a stabilization phase (II) associated with a steady-state consumption of sugars and ethanol production from 15 ºP wort. **(b)** The start-up phase of non-foaming experiment (III) carried out with 5 ºP wort was associated with a slight increase in biomass and followed by a high increase in ethanol and biomass (IV) after 11 ºP wort supply. (SLR) Saccharide loading rate; (Σr_s) total saccharide consumption rate.

flocculation (*30-32*), the lower amount of sugars supplied in this non-foaming experiment (5-11 ºP) compared to that fed in the foaming experiment (15 ºP) may also have caused higher flocculation, and hence lower biomass losses. Although calcium concentration (*33*) and pH (*34*) may also interfere with flocculation, the possible impact of these two parameters was not evaluated in this work.

The foaming experiment exhibited a completely different behavior regarding the free biomass profile (Fig. 5.2a). Due the continuous biomass removal through the foam, the free biomass initially decreased (from 10 to 6 $g.L^{-1}$) and then remained almost constant

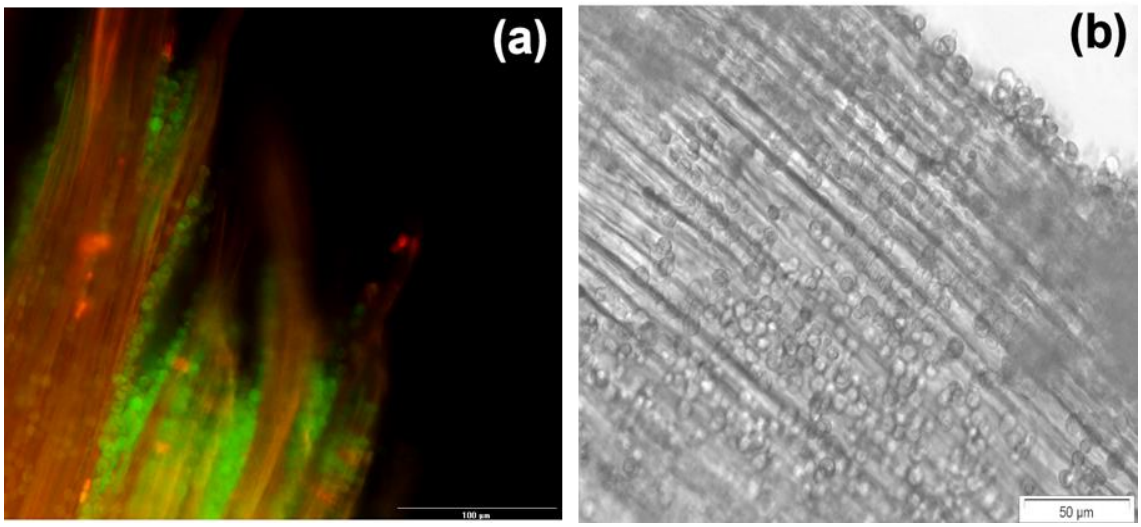


Figure 5.3 – Photomicrographs of yeast biofilm on the lignocellulosic yeast carriers. (a) Double staining with FDA (green cells with esterase activity) and PI (red cells with compromised plasma membrane) of a biocatalyst at the end of foaming experiment. (b) Biofilm visualization using an optical microscope.

until the end of the continuous culture. Considering that both yeast strains tested were flocculent in batch cultures, the differences observed in biomass accumulation could be ascribed to the different fermentation conditions and wort composition, rather than to differences in their flocculation capability (*32*).

Whether or not an antifoaming agent is used, the maximum cell load achieved in the bioreactor was 0.51±0.086 g of immobilized yeast per g of LCYC. This was consistent with previous work, which had shown that yeast adhesion to LCYC in a gas-lift reactor reached its maximum loading after few days and remained quite stable during the continuous fermentation (*1, 25*). Figure 5.3 shows the biofilm formation on LCYC used in this work.

5.4.2 Sugars consumption and ethanol production

During foaming experiments, it was observed that at the same time as glucose and fructose were almost completely consumed, approximately 65% of the maltose and 46% of the maltotriose from the original wort sugars were still present in the young beer (Fig. 5.2 – a, II). This happens because monosaccharides in wort (glucose and fructose) are preferentially consumed over maltose and maltotriose (*35-39*) due to direct repression by glucose over gene expression involved in maltose utilization (*40, 41*). In fact, the time required for wort fermentation could be greatly reduced if the brewing yeast could ferment maltose at the same time as glucose and fructose (*36-38*).

Specific saccharide consumption rate (r_s) and volumetric ethanol productivity (r_p) can be useful tools for comparing different reactor systems (*8, 42*). The former considers the

difference of sugar concentrations between inlet and outlet of the reactor, thus referring to the amount of sugar consumption per liter of bioreactor volume per hour. The latter considers the difference of ethanol concentrations between the same points, being that at the inlet the ethanol concentration is considered to be null. Additionally, the ratio between the values of the r_s and the inlet saccharide loading rate (SLR) provides valuable information about the capacity of sugar attenuation of the setup in a given moment. Yet, this ratio should be kept within the desired real attenuation, which is normally around 70% for a regular lager primary fermentation (*35, 43*). As dilution rate was constant, during foaming experiment the SLR was 5.48 $g.L^{-1}.h^{-1}$, while for the non-foaming experiment it varied from 1.53 (5 ºP phase) to 3.25 $g.L^{-1}.h^{-1}$ (11 ºP phase) (Fig. 5.2).

The average r_s measured in the current work can be found in Table 5.1**.** Through the steady phase of foaming experiment a stable average total r_s (considering all fermentable sugars present in wort) of 3.62 ± 0.33 $g.L^{-1}.h^{-1}$, was measured for several days. This means that an average of 66 ± 6% of all fermentable sugars was being used through primary continuous fermentation in the foaming setup. Thus, real attenuation in that phase oscillated within the desired 70%, but further fine-tuning of the dilution rate would be recommended. Conversely, during the non-foaming experiment, the yeast overpopulation led to sugar exhaustion (96 ± 1%) (Fig. 5.2b). Considering that the dilution rate was kept constant at 0.043 h^{-1}, it was predictable that the supply of sugars would be consumed completely by the growing yeast population. In order to ensure a desirable residual sugar concentration in green beer, increasing free biomass concentration in the continuous reactor should be accompanied by a gradual increase in the dilution rate. Besides the difficult on-line process control of such a non-equilibrium system, the increasing biomass load would lead to both engineering (high viscosity, mixing and mass transfer limitations) and yeast physiological (accumulation of dead cells, autolysis and ethanol stress) problems. In addition, the use of antifoaming agent would also certainly affect negatively the foam stability of the final product. The maximum r_s obtained by Šmogrovičová and co-workers (*44*), using yeast entrapment on calcium pectate and calcium alginate in a gas-lift reactor, varied from 5.64 to 6.08 $g.L^{-1}.h^{-1}$, respectively, although these authors have also reported up to 29 $g.L^{-1}$ of residual sugars present in the outflow of the reactor. Such high values of r_s can be ascribed to mono and disaccharide rich worts, which may have a faster consumption rate than of

Table 5.1 – Average volumetric saccharide consumption (r_s) and average volumetric ethanol productivity (r_p) measured during the steady-state phase of both the foaming and non-foaming experiments

	Foaming	Non-foaming
r_s Maltotriose ($g.L^{-1}h^{-1}$)	0.52 ± 0.08	0.43 ± 0.07
r_s Maltose ($g.L^{-1}h^{-1}$)	2.2 ± 0.26	1.87 ± 0.21
r_s Glucose ($g.L^{-1}h^{-1}$)	0.68 ± 0.01	0.47 ± 0.02
r_s Fructose ($g.L^{-1}h^{-1}$)	0.21 ± 0.01	0.18 ± 0.01
Σ r_s ($g.L^{-1}h^{-1}$)	3.62 ± 0.33	2.95 ± 0.30
r_p *Ethanol* ($g.L^{-1}h^{-1}$)	1.73 ± 0.12	1.33 ± 0.21

maltotriose. If in the one hand cell entrapment technology provides a higher concentration of immobilized biomass inside the reactor, on the other hand it can also be restrictive in terms of carrier cost and cell viability maintenance. Tata and coworkers (*8*) proposed a couple of different reactor setups: a two stage fluidized bed reactor; and another composed of a two-stage reactor containing a silicon carbide cartridge. The average r_s for both reactor systems used by these authors varied from1.9-2 $g.L^{-1}.h^{-1}$. Using a similar setup as in the current work, Brányik and coworkers (45) obtained relatively stable r_s values (3-4 $g.L^{-1}.h^{-1}$) during almost 2 months of continuous primary beer fermentation. The authors highlighted that the maximum r_s measured (4.2 $g.L^{-1}.h^{-1}$) during the experiment was considerably higher than the average r_s (0.8 $g.L^{-1}.h^{-1}$) observed in a batch fermentations with similar wort attenuations.

During the steady state phase of the foaming experiment, an average of 40 $g.L^{-1}$ of ethanol was continuously produced, thus for this phase of the foaming experiment, the average volumetric ethanol productivity was 1.73 $g.L^{-1}.h^{-1}$. However for the retention time used in this experiment (23.25 h), there were sugars still present in the outflow current; suggesting that r_p can be further improved for higher values of the retention time. The average r_p measured during the non-foaming experiment was lower (1.35 $g.L^{-1}.h^{-1}$) than that observed in the course of the foaming experiment. In 2002, Brányik and coworkers (*2*) used a similar setup for the continuous primary beer fermentation of a 13 ºP wort and obtained an average r_p of 1.6 $g.L^{-1}.h^{-1}$. Later in 2004, Brányik and coworkers (*45*) kept r_p near 2 $g.L^{-1}.h^{-1}$ for almost two months using a 14 ºP wort. Šmogrovičová and coworkers (*44*) used entrapped yeast on calcium alginate and calcium pectate in a gas-lift reactor and obtained r_p values from 1.69 to 2.4 $g.L^{-1}.h^{-1}$.

5.4.3 LCYC losses and yeast viability

Dead cells will naturally accumulate in the gas-lift reactor if only free suspended cells leave the reactor from the outflow, while the flocculated and immobilized yeast would be retained behind the sedimentation barrier of the reactor's outflow (*15, 17, 18*). To avoid this situation, regular replacement of LCYC is required, which allows for the formation of new biocatalysts and for the preservation of yeast activity during the wort fermentation inside the gas-lift reactor (*1-3, 25, 46*). In the present work however, there was no replacement of biocatalyst by fresh LCYC in the reactor, since the experimentation was aimed at evaluating the losses of carriers during either foaming or non-foaming fermentations. Regarding these losses, 21 g of LCYC was washed away from the reactor during the foaming experiment i.e. 52.5% from the initial carrier load. Most of these losses were observed during the first week of continuous operation (data not shown). On the other hand, during the non-foaming experiment, only 4.5 g (11.25%) of LCYC was retrieved from the outflow. A detailed distribution of LCYC at the end of both fermentations can be seen in Figure 5.4. The massive loss of carrier during the foaming experiment can be in large part explained due to the hydrophobic character of the LCYC (29) with its tendency to adhere to CO_2 bubbles, and therefore to be dragged out of the reactor.

The concentration of LCYC used was 1% (w/v) and considering the maximum yeast load onto carriers, the maximum concentration of immobilized biomass was approximately 5.1 $g.L^{-1}$ for the non-foaming experiment and 4.38 $g.L^{-1}$ for the foaming

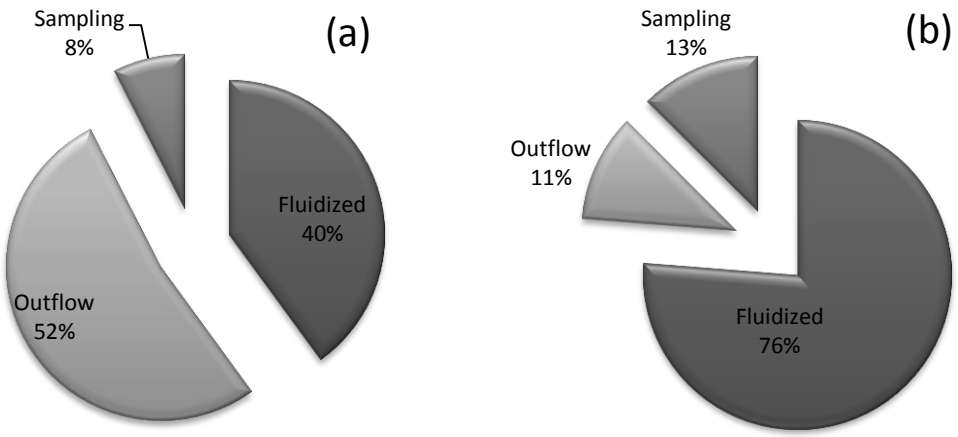


Figure 5.4 – The lignocellulosic yeast carrier distribution and loss during both foaming (a) and non-foaming (b) experiments. The fluidized population represents the amount of carriers still suspended in the reactor at the end of the fermentation. Outflow and sampling, represents the losses of carriers by these means.

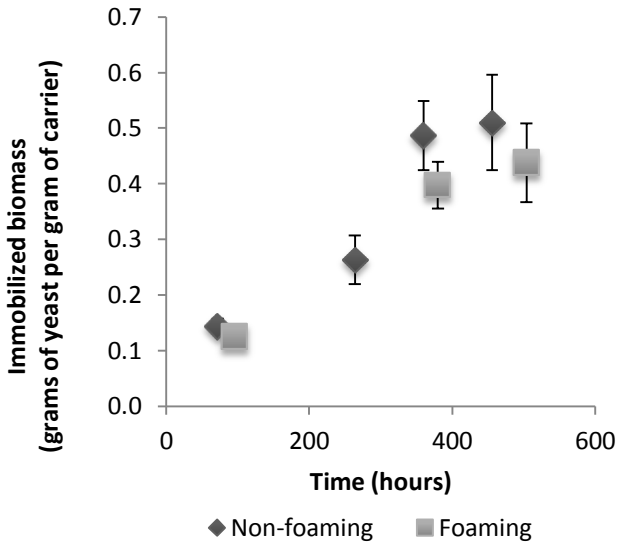


Figure 5.5 – Immobilized biomass concentration measured during experiments.

experiment. However, considering carrier losses, the concentration of immobilized biomass in the non-foaming experiment was, at the end, approximately 4.5 $g.L^{-1}$, while for foaming experiment it was approximately 2.1 $g.L^{-1}$. The immobilized biomass load measured during both experiments is shown in Figure 5.5. Although 4.50 $g.L^{-1}$ may sound irrelevant when compared to 47.63 $g.L^{-1}$ of the free biomass present at the end of the non-foaming experiment, 2.1 $g.L^{-1}$ is about one third of the total biomass (around 6 $g.L^{-1}$) in the foaming experiment.

Cell viability assessed by esterase activity and preservation of plasma membrane integrity was monitored by a FDA/PI double staining protocol on the last day of fermentation for both the foaming and the non-foaming experiments (Table 5.2). Whilst foam production resulted in great losses of carrier, it helped to ensure that the free biomass had a high content of viable cells until the late stages of fermentation. At 24 days of continuous fermentation there was still a 98.74% FDA positive PI negative (FDA+PI-) stained cell population among the flocculated free biomass. The immobilized cell population in this experiment also exhibited 97.42% FDA+PI- cells, while the foam´s cell sub-population displayed 90.9% FDA+PI- cells and 8.49% FDA negative PI positive staining cells (FDA-PI+). These data suggest that a cell sub-population, with loss of plasma membrane integrity, was being washed out through the reactor´s outflow at a considerably higher concentration compared to their presence in the free biomass inside of the reactor (0.98%). This result, which presumably indicates a "positive discrimination" of the viable cells to stay within the bioreactor, supports the

initial hypothesis that biomass removal through foaming could serve to keep the biomass load of a continuously operating fermenter in a viable state for a longer period of time.

For the non-foaming experiment, after 24 days of continuous fermentation, 73.1% of flocculated cells and 62.6% of immobilized cells were FDA+PI-, whereas 15.4% and 17.6% of cells were FDA-PI+, respectively. These data support the concept that without their periodical removal, accumulation of injured and dead cells occurs in the continuous immobilized bioreactor. However, these numbers may be overestimated due to possible cell starvation caused by the lack of sugars during biomass growth over the constant dilution rate. Yeast cells, which preserve their plasma membrane integrity, are not permeable to PI (*47*). Some other cells however, may have lost esterase activity and become metabolically affected or inactive, but still have intact membranes, and thus are not permeable to PI. The percentage of these cells (FDA-PI-) was 11.6% and 19.9% in flocculated and immobilized cells fractions in the non-foaming fermentation, respectively. For comparison, this sub-population was lesser than 0.1% among cells in the foaming experiment.

The accumulation of yeast cells with compromised plasma membrane integrity must be avoided in continuous beer fermentation, otherwise the sensorial quality of the final product could be compromised.

Table 5.2 - Yeast viability assessed by esterase activity and preservation of plasma membrane integrity of different cells sub-populations as determined by double staining with FDA and PI

Experiment	Cell Sub-population	Stained yeast (%)*		
		FDA(+)PI(-)	FDA(-)PI(+)	FDA(-)PI(-)
Foaming	Flocculated	98.7±0.2	1.0±0.09	-
	Immobilized	97.4±0.2	2.0±0.1	-
	Foam	91.0±0.8	8.5±0.3	-
Non-foaming	Flocculated	73.1±0.5	15.4±0.4	11.6±0.6
	Immobilized	62.6±0.7	17.5±0.9	19.9±0.3

FDA – Fluorescein Diacetate
PI – Propidium Iodide
**Numbers obtained from an average of 20000 cell counts, performed in triplicate.*

5.5 Conclusions

The production of green beer with a constant composition can only be achieved by carefully controlling the amount and viability of the biomass inside a reactor. For this purpose, foam fractionation should be encouraged as a natural solution to control biomass in a gas-lift reactor.

Despite the great losses of LCYC through foam formation during fermentation, this experimental set-up showed steady performance (within desired values) of sugar consumption rate and ethanol productivity. Additionally, it was demonstrated that the flocculated biomass represents the large majority of the total biomass present inside the reactor.

Saccharide consumption and ethanol productivity observed in this work were comparable with other primary continuous beer fermentation setups.

Even though small in number, the PI positive cell sub-population was higher in the foam when compared to the free and immobilized cell sub-populations in the foaming experiment. These data strengthen the hypothesis that foam fractionation could be of help for the constant removal of dead or injured biomass from a gas-lift reactor.

5.6 Acknowledgements

Cristina Ribeiro, from the Centre of Molecular and Environmental Biology/Department of Biology of the University of Minho is gratefully acknowledged for her technical support. This work was also supported by FEDER through POFC – COMPETE and by national funds from FCT through the project PEst-C/BIA/UI4050/2011. The materials supplied by UNICER Bebidas, S.A. (S. Mamede de Infesta, Portugal) are gratefully acknowledged. The financial contributions of the EU FP7 project EcoBioCAP - Ecoefficient Biodegradable Composite Advanced Packaging, Grant agreement no: 265669 as well as of the Grant Agency of the Czech Republic (project GAČR P503/12/1424) are also gratefully acknowledged.

5.7 References

1. Branyik, T., Silva, D. P., Vicente, A. A., Lehnert, R., e Silva, J. B., Dostalek, P., and Teixeira, J. A. (2006) Continuous immobilized yeast reactor system for complete beer fermentation using spent grains and corncobs as carrier materials, *J. Ind. Microbiol. Biotechnol. 33*, 1010-1018.

2. Branyik, T., Vicente, A. A., Cruz, J. M., and Teixeira, J. A. (2002) Continuous primary beer fermentation with brewing yeast immobilized on spent grains, *J. Inst. Brew. 108*, 410-415.

3. Branyik, T., Vicente, A. A., Dostalek, P., and Teixeira, J. A. (2005) Continuous beer fermentation using immobilized yeast cell bioreactor systems, *Biotechnol. Prog. 21*, 653-663.

4. Decamps, C., Norton, S., Poncelet, D., and Neufeld, R. J. (2004) Continuous pilot plant–scale immobilization of yeast in κ-carrageenan gel beads, *AIChE 50*, 1599–1605.

5. Smogrovicová, D., and Dömény, Z. (1999) Beer volatile by-product formation at different fermentation temperature using immobilised yeasts., *Process Biochem. 34*, 785–794.

6. Nedovic, V. A., Cukalovic, I. L., Bezbradica, D., Obradovic, B., and Bugarski, B. (2005) New porous matrices and procedures for yeast cell immobilisation for primary beer fermentation., In *Proc. Eur. Brew. Conv. Congr.*, pp 401–413, Fachverlag Hans Carl: Nürnberg., Prague.

7. Bardi, E. P., Koutinas, A. A., and Kanellaki, M. (1997) Room and low temperature brewing with yeast immobilized on gluten pellets, *Proc. Biochem. 32*, 691–696.

8. Tata, M., Bower, P., Bromberg, S., Duncombe, D., Fehring, J., Lau, V. V., Ryder, D., and Stassi, P. (1999) Immobilized yeast bioreactor systems for continuous beer fermentation, *Biotechnol. Prog. 15*, 105-113.

9. Dömény, Z., Smogrovicová, D., Gemeiner, P., Sturdík, E., Pátková, J., and Malovíková, A. (1998) Continuous secondary fermentation using immobilised yeast, *Biotechnol Lett 20*, 1041-1045.

10. Linko, M., Haikara, A., Ritala, A., and Penttila, M. (1998) Recent advances in the malting and brewing industry, *J. Biotechnol. 65*, 85-98.

11. Mensour, N. A., Margaritis, A., Briens, C. L., Pilkington, H., and Russell, I. (1997) New developments in the brewing industry using immobilised yeast cell bioreactor systems, *J. Inst. Brew. 103*, 363-370.

12. Verbelen, P. J., De Schutter, D. P., Delvaux, F., Verstrepen, K. J., and Delvaux, F. R. (2006) Immobilized yeast cell systems for continuous fermentation applications, *Biotechnol. Lett. 28*, 1515-1525.

13. Willaert, R., and Nedovic, V. (2006) Primary beer fermentation by immobilised yeast – a review on flavour formation and control strategies, *J. Chem. Technol. Biotechnol. 81*, 1353-1367.

14. Masschelein, C. A. (1997) A realistic view on the role of research in the brewing industry today, *J. Inst. Brew. 103*, 103-113.

15. Mortimer, R. K., and Johnston, J. R. (1959) Life span of individual yeast cells, *Nature 183*, 1751–1752.

16. Koc, A., Gasch, A. P., Rutherford, J. C., Kim, H., and Gladyshev, V. N. (2004) Methionine sulfoxide reductase regulation of yeast lifespan reveals reactive oxygen species-dependent and -independent components of aging, *Proc. Natl. Acad. Sci. U.S.A. 101*, 7999–8004.

17. Kaeberlein, M. (2010) Lessons on longevity from budding yeast., *Nature 464*, 513–519.

18. Muller, I., Zimmermann, M., Becker, D., and Flomer, M. (1980) Calendar life span versus budding life span of Saccharomyces cerevisiae, *Mech. Ageing. Dev. 12*, 47-52.

19. Österlund, T., Nookaew, I., Bordel, S., and Nielsen, J. (2013) Mapping condition-dependent regulation of metabolism in yeast through genome-scale modeling, *BMC Chem. Biol. 7*, 36.

20. Kuřec, M., Baszczyňski, M., Lehnert, R., Mota, A., Teixeira, J. A., and Brányik, T. (2009) Flow Cytometry for Age Assessment of a Yeast Population and its Application in Beer Fermentations, *J. Inst. Brew. 115*, 253–258.

21. Powell, C. D., Van Zandycke, S. M., Quain, D. E., and Smart, K. A. (2000) Replicative ageing and senescence in Saccharomyces cerevisiae and the impact on brewing fermentations, *Microbiol. 146*, 1023–1034.

22. Deans, K., Pinder, A., Catley, B. J., and Hodgson, J. A. (1997) Effects of cone cropping and serial repitch on the distribution of cell ages in brewery yeast., *Proc. Eur. Brew. Conv. Congr. 26*, 469-476.

23. Barker, M. G., and Smart, K. A. (1996) Morphological changes associated with the cellular ageing of a brewing yeast strain, *J. Am. Soc. Brew. Chem. 54*, 121-126.

24. Powell, C. D., Quain, D. E., and Smart, K. A. (2003) The impact of brewing yeast cell age on fermentation performance, attenuation and flocculation, *FEMS yeast research 3*, 149-157.

25. Branyik, T., Vicente, A. A., Kuncova, G., Podrazky, O., Dostalek, P., and Teixeira, J. A. (2004) Growth model and metabolic activity of brewing yeast biofilm on the surface of spent grains: a biocatalyst for continuous beer fermentation, *Biotechnol. Prog. 20*, 1733-1740.

26. Perry, E. S. (1968) *Progress in Separation and Purification*, Vol. 1, Wiley-interscience, New York.

27. Lemlich, R., and Lavi, E. (1961) Foam fractionation with reflux, *Science 134*, 191.

28. Hutzler, S., Tobin, S. T., Meagher, A. J., Marguerite, A., and Weaire, D. (2013) A model system for foam fractionation, *Proc. R. Soc. A 469*, 1471-2946

29. Pires, E. J., Ruiz, H. A., Teixeira, J. A., and Vicente, A. A. (2012) A New Approach on Brewer's Spent Grains Treatment and Potential Use as Lignocellulosic Yeast Cells Carriers, *J. Agr. Food. Chem. 60*, 5994-5999.

30. Eddy, A. A. (1955) Flocculation characteristics of yeasts II. Sugars as dispersing agents., *J. Inst. Brew. 61*, 313-317.

31. Bokulicha, N. A., and Bamforth, C. W. (2013) The Microbiology of Malting and Brewing, *Microbiol. Mol. Biol. Rev. 77*, 157-172.

32. Soares, E. V. (2011) Flocculation in *Saccharomyces cerevisiae*: a review, *J. App. Microbiol. 110*, 1-18.

33. Stratford, M. (1989) Yeast flocculation: Calcium specificity, *Yeast 5*, 487-496.

34. Stratford, M. (1996) Induction of flocculation in brewing yeasts by change in pH value, *FEMS Microbiol. Lett. 136*, 13-18.

35. Kunze, W. (2010) Technology Brewing and Malting, In *4th International Edition*, p 368, VLB Berlin.

36. Michels, C. A., and Romanowski, A. (1980) Pleiotropic glucose repression-resistant mutation in Saccharomyces carlsbergensis., *J. Bacteriol. 143*, 674-679.

37. Novak, S., D'Amore, T., and Stewart, G. G. (1990) 2-Deoxy-D-glucose resistant yeast with altered sugar transport activity., *FEBS Lett. 269*, 202-204.

38. Kodama, Y., Fukui, N., Ashikari, T., Shibano, Y., Morioka-Fujimoto, K., Hiraki, Y., and Nakatani, K. (1995) Improvement of maltose fermentation efficiency: constitutive expression of MAL genes in brewing yeast., *J. Am. Soc. Brew. Chem. 563*, 24-29.

39. Rautio, J., and Londesborough, J. (2003) Maltose transport by brewer´s yeast in brewer´s wort. , *J. Inst. Brew. 109*, 251-261.

40. Entian, K. D., and Shüller, H. J. (1997) Glucose repression (Carbon Catabolite Repression) in Yeast, In *Yeast Sugar Metabolism* (Zimmermann, F., and Entian, K., Eds.), pp 410-434, Technomic Publishing Company, Pennsylvania.

41. Govender, P., Domingo, J. L., Bester, M. C., Pretorius, I. S., and Bauer, F. F. (2008) Controlled expression of the dominant flocculation genes FLO1, FLO5, and FLO11 in Saccharomyces cerevisiae, *Appl. Environ. Microbiol. 74*, 6041-6052.

42. Kumar, S., Singh, S. P., Mishra, I. M., and Adhikari, D. K. (2011) Continuous ethanol production by Kluyveromyces sp. IIPE453 immobilized on bagasse chips in packed bed reactor, *J. Petroleum Technol. Altern. Fuels 2*, 1-6.

43. Patel, G. B., and Ingledew, W. M. (1973) Trends in wort carbohydrate utilization, *Appl. Microbiol. 26*, 349-353.

44. Smogrovicová, D., Dömény, Z., Gemeiner, P., Malovíková, A., and Sturdík, E. (1997) Reactors for continuous primary beer fermentation using immobilised yeast, *Biotechnol. Tech. 11*, 261–264.

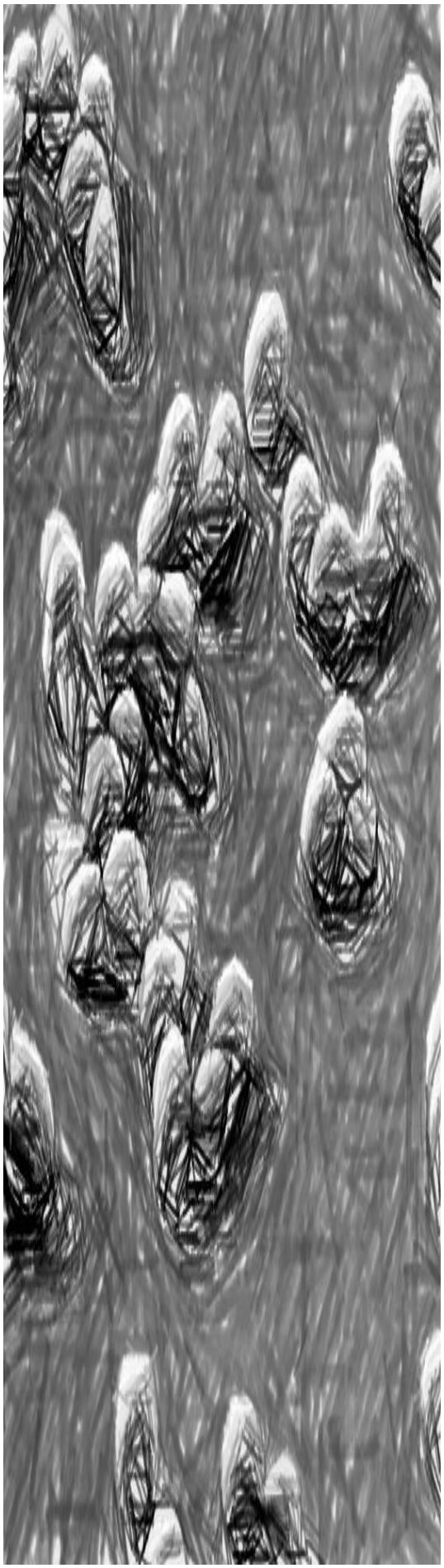
45. Branyik, T., Vicente, A., Cruz, J. M., and Teixeira, J. (2004) Continuous Primary Fermentation of Beer with Yeast Immobilized on Spent Grains—The Effect of Operational Conditions, *J. Am. Soc. Brew. Chem. 62*, 29-34.

46. Branyik, T., Vicente, A. A., Oliveira, R., and Teixeira, J. A. (2004) Physicochemical surface properties of brewing yeast influencing their immobilization onto spent grains in a continuous reactor, *Biotechnol. Bioeng. 88*, 84-93.

47. Paravicini, G., Cooper, M., Friedli, L., Smith, D. J., Carpentier, J. L., Klig, L. S., and Payton, M. A. (1992) The osmotic integrity of the yeast cell requires a functional PKC1 gene product, *Mol. Cell. Biol. 12*, 4896-4905.

Chapter 6

Carrier-free, continuous primary beer fermentation



This chapter evaluated the feasibility of accumulation of immobilized biomass solely by yeast flocculation using an airlift bioreactor.

6.1 Abstract

Developing a sustainable continuous fermentation reactor is one of the most ambitious tasks in brewing science as it could bring great benefits regarding volumetric productivity to modern breweries. Immobilized Cell technology (ICT) is often applied to reach large densities of yeast needed in continuous fermentations. Yeast carrier´s price is however one of the most limiting parts of the process. Thus, this work proposed yeast flocculation as a way of biomass immobilization not requiring carrier in a gas-lift reactor. Almost 25 $g.L^{-1}$ of yeast was flocculated in the reactor before interrupting the fermentation. Stable sugar consumption and ethanol production (4.5% ABV) from 11°P wort was evidenced for several days. The key esters and higher alcohols measured in the young beer met the standards of finished primary beer fermentation.

Key words: Continuous beer fermentation; yeast flocculation; gas-lift reactor.

6.2 Introduction

The advantages of continuous beer fermentation are based mainly on high volumetric densities of yeast cells inside the bioreactor. The considerable amount of yeast allows a short residence time of wort, which is continuously supplied for beer production. This feature is reflected in high productivity, reduced space and time needed to reach the final product (*1-3*). Despite being very attractive, the continuous fermentation of beer is still marginalized to lab benches, pilot plants and very few courageous companies like DB Breweries (New-Zealand), which has been continuously brewing since 1959 (*1*).

Immobilized cell technology (ICT) uses solid carriers for cell adsorption and biofilm formation (*4-11*) or forced physical entrapment of yeast cells to solid matrixes (*12-15*) to increase cell density inside the reactor. The carrier cost is a key component for the financial viability of ICT (*16, 17*) and the composition of solid matrix may also interfere in the final beer quality and flavor profile(*1, 18*). Additionally, the relatively short lifetime of a single yeast cell (*19*) results in accumulation of dead biomass into biocatalyst demanding constant replacement (*20*).

Some brewing yeast strains are remarkably flocculent and this feature is widely used to harvest yeast at the end of primary fermentation (*21*). Flocculation is a multifactorial inheritance triggered by both genetic (*22-24*) and environmental factors (*25-28*). This self-aggregation characteristic is a free-of-charge way of immobilization and seems to be poorly exploited as ICT for continuous fermentation.

Gas-lift bioreactors are remarkably efficient on mass transfer for either liquid-liquid or solid-liquid phases triggered by rapid mixing, low shear stress, simple design and low energy consumption (*29, 30*). The low shear environment provides excellent conditions for ICT implementation (*31*) and has previously been adopted for continuous beer fermentation (*5, 15*).

This work is exploiting the feasibility of self-aggregation as single biomass immobilization method for young beer production in a gas-lift reactor.

6.3 Material and Methods

6.3.1 Brewing Yeast

The flocculent *Saccharomyces carlsbegensis* strain nº 96 from the culture collection of brewing yeast (Research Institute of Brewing and Malting, Plc., Prague, Czech Republic) was used. It was inoculated in a complex media with the following

composition ($g.L^{-1}$): glucose, 30; KH_2PO_4, 5; $MgSO_4.7H_2O$, 0.4; $(NH_4)_2SO_4$, 2; Yeast extract, 2; Agar, 20. The media was disposed in Petri dishes for isolation of yeast colonies. A colony was then inoculated in 400 mL of 5º Plato wort and incubated at 20 ºC, 120 rpm, for 48 h previous to be used as inoculum for the gas-lift reactor.

6.3.2 Beer wort

Pale wort concentrate was acquired from the Research Institute of Brewing and Malting (RIBM, Prague). It was diluted to final desired concentration – 5 and 11º Plato. Final wort was filled in 20 L polyethylene carboys (Nalgene, USA) and autoclaved for 3.5 hours.

6.3.3 Continuous fermentation

The experiment was carried out in a Perspex gas-lift reactor with 4 L of total work volume. Gas flow was kept constant at 0.5 $L.min^{-1}$ by the GFC17 mass controller (AALBORG, USA). The temperature was hold at 15 ºC using a JULABO F32 Refrigerated/Heating Circulator (JULABO, Germany). The dilution rate was kept at 0.043 h^{-1} by a peristaltic pump PDC 83 (Kouřil, Czech Republic). Figure 6.1 shows the scheme of the entire setup used in the current work.

The reactor was sterilized by bleaching, using a 3% (v/v) solution of commercial sodium hypochlorite with 1.5% of active chlorine, 48 h previous to use. After this time, the solution was discarded and 50 L of sterile water was used to wash the reactor. It was then filled with 5º Plato wort and inoculated.

Batch growth was kept in the first 48 hours using pressurized air as gas supply at 500 $mL.min^{-1}$. After that, gas was changed to CO_2 in the same flow rate and continuous

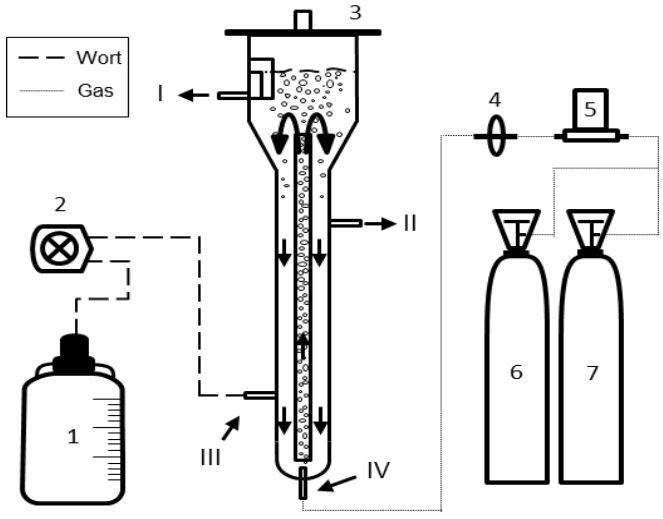


Figure 6.1 - Immobilized yeast bioreactor system used in the current work: 1 wort barrel; 2 peristaltic pump; 3 gas-lift reactor; 4 air filter; 5 flow mass controller; 6 pressurized air; 7 CO_2 bottle; I green beer outflow/sampling point; II biomass sampling point; III wort inlet; IV gas sparger.

phase was started with 5° P supply at 0.043 h^{-1} dilution rate. Four days later, wort supply was changed to 11 °P keeping dilution rate unchanged.

6.3.4 Biomass measurements

The flocculated biomass was evaluated daily. Three 15 mL plastic Falcon curvets were dried at 105 °C for 12 h and weighted. Then 10 mL of cell suspension from the reactor was added to each one of them and centrifuged at 4000 x ***g*** for 5 min. Liquid phase was discarded and the falcons were dried (105 °C) for 24 h previous to weighting. Blank experiments were carried out using the inlet wort to correct for the presence of trub-like proteins that could interfere at weighting.

6.3.5 Sugars and ethanol measurements

A daily sample was taken from the reactor´s outflow for green beer analysis. Sugars and ethanol were evaluated through high-performance liquid chromatography (HPLC) in an Agilent 1100 series equipped with Agilent G 1362A RID detector (Agilent, USA). The column used was the Rezex^{TM}RSO-Oligosaccharide 200x10 mm (Phenomenex, USA) and the eluent was deionized degassed water pumped in a flow of 0.4 $mL.min^{-1}$. Sugar and ethanol standards were calibrated previous to wort and young beer measurements using the following reagents: D-Fructose (Chemapol, Praha, CZ); D-Maltose monohydrate (Fluka, Japan); *D*-Glucose (Fluka, Japan); Maltotriose (Sigma, USA); Ethanol (Sigma, USA).

6.3.6 Flavor-active compounds measurements

Higher alcohols and esters were analyzed by gas chromatography using an Agilent HP-6890N GC–MS system (Agilent Technologies, USA) coupled to a mass detector Agilent 5975B Inert MSD (Agilent Technologies, USA). Compounds were separated on a InnoWax (30 m x 0.25 mm x 0.25 μm) column (Agilent Technologies, USA). Helium has been used as carrier gas at a flow rate of 6 $mL.min^{-1}$. The oven temperature was programmed to start temperature of 30 °C for 10 min, then it raised at 2 $°C.min^{-1}$ up to 52 °C (2 min), plus 2 $°C.min^{-1}$ up to 65 °C and finally up to 250 °C at 5 $°C.min^{-1}$ (3 min). Samples were injected at 260 °C. Standards were analyzed previous to samples using: 2-Methyl-1-butanol (> 98%), 3-methyl-1-butanol (> 98.5%), Isobutanol (> 99%), Isoamyl acetate (~ 99%), Ethyl acetate (99.7%), Ethyl butyrate (> 98%), Ethyl hexanoate (> 99%), ethyl octanoate (> 98%), ethyl decanoate (> 99%), 2-phenyl ethyl

acetate (> 99%) (Fluka, Germany). Internal standards were used: 3-octanol (99%) (Aldrich, USA) and ethyl heptanoate (99%) (Aldrich, Germany)

6.4 Results and Discussion

6.4.1 Biomass buildup

The initial phase of continuous fermentation (5 °P) was marked by low biomass accumulation. In the course of 11° P wort fermentation, the flocculation strengthened and the buildup of biomass was achieved (Fig. 6.2). One of the main conditions triggering yeast flocculation is the reduced amount of sugars present at the end of primary fermentation (*25, 28*). Considering that dilution rate and other parameters were kept constant during either 5 or 11 °P, nutrients may have been restrictive for biomass growth in the course of the former stage, otherwise biomass buildup in this phase should have been more expressive. As there were still sugars present in the outflow (Table 6.1), there might have been a lack of assimilable nitrogen in the diluted 5 °P wort. In fact, although flocculation could be visualized through the Perspex reactor, a nitrogen starvation might have caused reduced cell division and biomass growth (*32*). Apart from being very diluted (5 °P), concentrated worts as used in this work may have insufficient levels of free amino nitrogen (FAN) (*33*).

As no solid carriers were used in this study, immobilization of yeast cells inside the gas-lift reactor depended exclusively on flocculation. At the end of the reactor operation, the biomass suspended inside the gas-lift reactor was nearly 25 $g.L^{-1}$ in dry matter. Most of this biomass was flocculated (data not shown). The separation of free cells from flocs is virtually impossible due the small size of flocs and dynamics of cell aggregation (*34, 35*). Hence, biomass expressed in the figure 6.2 denotes the total biomass (X_{tot} = flocs + free cells) suspended in the system.

Comparing X_{tot} in different setups used by other authors is a tough task. Whereas cell adsorption is very dynamic, entrapment based studies often does not discuss biomass growth on biocatalysts. For example, Tata and coworkers (*10*) compared a couple of two-stage systems for continuous beer production. Each of these systems was composed of two reactors connected in series: two fluidized bed reactors with porous glass beads for cell immobilization; and two loop reactors with silicon carbide cartridge for yeast load. The maximum X_{tot} reported by the authors for each of these systems was 29.7 and 18.2 $g_{cell\ dry\ wt}.L^{-1}$, respectively. In a further work involving continuous primary beer

Table 6.1 - Wort and green beer composition measured in the course of both 5 and 11 degree Plato (°P) wort supply through the continuous primary fermentation.

Sample Type	Time	Sugars [g.L^{-1}]				ABV*	Ethanol	X_{tot}
	(h)	Maltotriose	Maltose	Glucose	Fructose	%	[g.L^{-1}]	[g.L^{-1}]
5 °P Wort	-	6.45	23.49	5.71	2.46	-	-	-
GB5	48	2.34	1.76	0.14	0.32	1.96	15.50	3.9
GB5	72	2.29	1.69	0.17	0.33	1.96	15.48	4.3
GB5	96	2.61	1.93	0.22	0.36	1.93	15.26	4.5
GB5	120	3.24	2.85	0.30	0.48	1.90	14.99	4.0
GB5	144	3.63	4.11	0.34	0.55	1.97	15.57	3.4
11 °P Wort	-	13.69	50.96	12.91	6.69	-	-	-
GB11	168	7.59	18.61	1.57	1.70	2.04	16.09	4.1
GB11	192	8.36	20.18	1.22	1.48	2.63	20.81	5.8
GB11	216	7.68	14.38	1.17	1.45	3.26	25.72	7.5
GB11	240	6.24	8.31	0.68	1.02	3.90	30.78	10.6
GB11	264	4.78	3.90	0.39	0.71	4.21	33.29	14.0
GB11	288	3.78	2.71	0	0.53	4.47	35.28	16.8
GB11	312	3.20	2.20	0	0	4.48	35.41	20.0
GB11	336	3.03	1.97	0	0	4.52	35.68	22.0
GB11	360	2.75	1.92	0	0	4.50	35.58	24.2
GB11	384	2.56	1.31	0	0	4.62	36.54	24.8

* ABV = Alcohol by Volume
GB5 – Green Beer from 5 °P wort
GB11 – Green Beer from 11 °P wort

fermentation carried by Brányik and coworkers (*36*) the X_{tot} reported using a gas-lift reactor with lignocellulosic carriers obtained from brewer´s spent grains (BSG) varied from 9.3 to 10.5 $g_{cell\ dry\ wt}.L^{-1}$. However, the yeast load of a specific system does not necessarily reflect on good results. Therefore, the performance of different ICT setups must be compared in terms of specific saccharide consumption (r_s) and volumetric ethanol productivity (r_p) (*10, 37*) as further discussed.

6.4.2 Sugars consumption and ethanol yield

The efficacy of the gas-lift reactor for primary beer fermentation has long been studied, using several types of yeast immobilization methods (*15, 36, 38*). The present work focused on self-aggregation to attain the biomass needed for the continuous primary fermentation of the wort provided. As temperature and dilution rate were kept constant during the whole experiment, sugar consumption and ethanol production were directly

dependent on X_{tot} and wort gravity. Table 6.1 shows in detail the amount of each fermentable sugars present at the inlet wort for both 5 and 11º P stages. It also shows the residual content of these sugars and the ethanol present in the green beer in the course of the continuous fermentation. By changing the composition of wort during continuous phase, an adaption phase was experienced between 144 h (start of inlet supply of 11º P barrel) and 388 h (beginning of steady phase) period of the continuous fermentation (Fig. 6.2). Environmental changes alter yeast metabolism, which takes time to adapt to the new conditions (*39*). This adaptation phase is not desirable in large-scale production plants. Thus, instead of changing wort gravity, dilution rate should have gradually been raised, using a steady wort gravity, to avoid delays. After that adaption stage, the consumption of sugars was satisfactory and stable until the end of the fermentation trial. Extract attenuation during this stage should have been very close to the limit, once very few reducing sugars were present in the young beer. The residual sugars in young beer during this stage were maltose and maltotriose in an average of 2 and 3 $g.L^{-1}$, respectively. Not surprisingly, glucose and fructose were not present in the young beer at this phase (Table 6.1) due to preferential sugar consumption of monosaccharides by yeast (*39-42*). Furthermore, a reduced amount of fermentable sugars is required in the green beer for further carbon dioxide development in the maturation stage.

The r_s value designates the consumption rate of all fermentable sugars ($g.L^{-1}.h^{-1}$) in the course of the retention time. Through the initial 5º P stage, the average r_s measured was 0.66 $g.L^{-1}.h^{-1}$ and this value increased during the steady phase of 11º P to 3.4 $g.L^{-1}.h^{-1}$ (Fig. 6.2). This consumption rate is consistent with r_s values reported by other studies regarding continuous primary beer fermentation (*5, 10, 15, 36, 38, 43*).

Ethanol yield obtained during the steady phase 11º P was very satisfactory and an average of 4.5% (v/v) alcohol by volume (ABV) has been measured during this period (Table 6.1). As RT_{tot} = 23.25 h, the average r_p measured in the course of steady phase of 11º P wort was 1.54 $g.L^{-1}.h^{-1}$ (Fig. 6.2). In the studies involving entrapped yeast carried out by Šmogrovičová and co-workers (*38*), they reported r_p values from 1.7 to 2.4 $g.L^{-1}.h^{-1}$. However, the higher yields of ethanol reported by these authors were followed by higher amounts of residual sugars in the young beer. Short RT_{tot} may ultimately increase r_s and r_p, but without enough biomass, the result will be an unfinished primary fermentation. The r_p values observed in the current study are also in accordance with

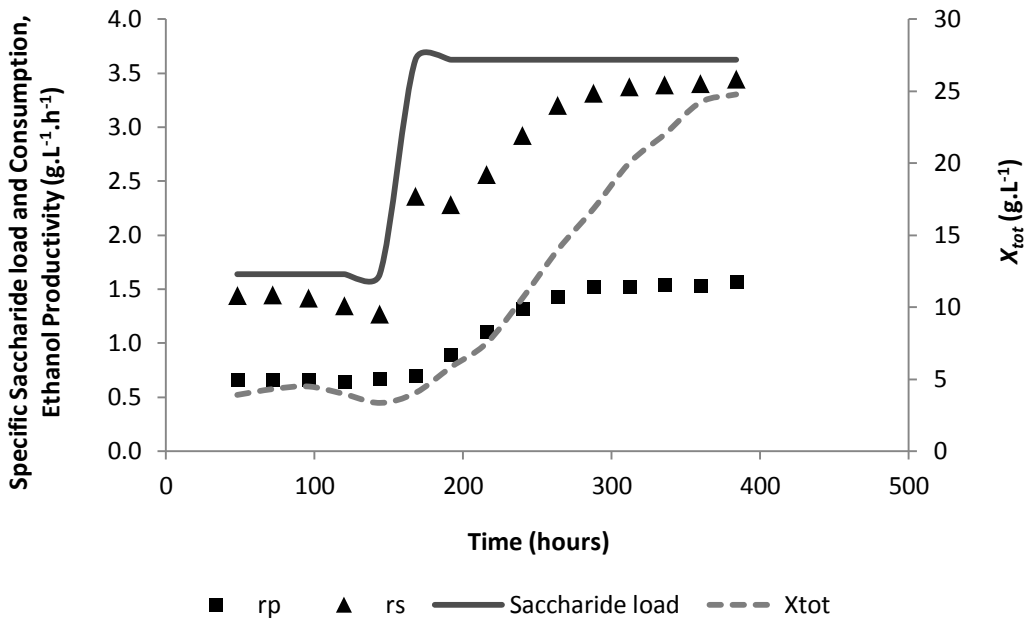


Figure 6.2 - Specific saccharide loading and consumption rates (r_s), Ethanol productivity (r_p) and total biomass (X_{tot}) inside the fermenting reactor through the primary continuous fermentation.

previous reports involving continuous primary beer fermentation: 1.6 $g.L^{-1}.h^{-1}$ (36); 2 $g.L^{-1}.h^{-1}$ (43).

6.4.3 Volatile profile

The consequences of continuously operated systems using ICT over the aroma profile produced during primary continuous beer fermentation has previous been reviewed by Willaert & Nedovic (*1*).

The great majority (nearly 80%) of higher alcohols present in the final beer are produced by yeast during the primary fermentation (*39*) either from catabolism (*44*, *45*) or synthesis of amino acids (*46*). Higher alcohols not only contribute directly to final beer´s aroma, but they also are precursors of the most desirable esters. Data involving the production of higher alcohols using ICT are rather inconsistent as some works have reported low production of higher alcohols using ICT (*47-49*), while others have shown even better performance when compared to batch fermentation (*50*). In the current work, isoamyl alcohol (3-methyl-1-butanol) and the active amyl alcohol (2-methyl-1-butanol) were measured together and are henceforth mention as amyl alcohols. The threshold for these alcohols when considered together is 50 $mg.L^{-1}$ (*51*). Higher alcohols measured in the green beer produced in current work are presented in Table 6.2. When fermentation reached steady-state upon 11º P wort supply, the threshold of amyl alcohols was achieved. At this time, X_{tot} was nearly 14 $g.L^{-1}$ and the amyl alcohols

concentration was 52 mg.L^{-1}. Levels of these alcohols have slightly changed (55 mg.L^{-1}) up to the interruption of fermentation when the total biomass was close to 25 g.L^{-1}. Using polyvinyl alcohol for yeast entrapment, Smogrovicová and co-workers (*52*) obtained 57.8 mg.L^{-1} of amyl alcohols in the young beer. Amyl alcohols yield measured in Brányik and coworkers (*43*) setup using brewer´s spent grains as yeast carriers was somewhat higher and 60.1 mg.L^{-1} was measured. In an early study, Smogrovicová and Dömény used calcium-pectate beads for yeast immobilization and observed a similar amyl alcohols content of 56.8 mg.L^{-1} (*48*). In this particular last mentioned work the authors used the same yeast strain, temperature (15 ^{o}C) and similar wort gravity (11.5^{o} P) as used in the current work, which may also support the similarity in amyl alcohols content. Higher alcohols production is totally dependent on the fermentation conditions adopted and usually enhanced by conditions that favor yeast growth (*39, 53*). The isobutanol levels measured in the current work are also in agreement with published data for green beer produced by ICT (*43, 48*). Surprisingly, 2-phenylethanol was considerably high (Table 6.2) in the green beer produced from 11^{o} P wort, which could ultimately contribute to a pleasant floral aroma to the finished beer.

Esters are pleasant aroma compounds present in beer contributing with positive smells like flowers, honey and fruity. They are mainly produced at the initial phase of primary fermentation by action of yeast acyltransferase activities catalyzing the condensation reaction between either acetyl/acyl-CoA and higher alcohols or ethanol. Accordingly, successful primary beer fermentation must produce enough esters, which will be present in the final product. The most present ester in beer is the fruit/solvent-like ethyl acetate, which has a flavor threshold around 25-30 mg.L^{-1} (*51, 54*). For the current work, this threshold was reached at the beginning of steady phase of continuous experiment (11^{o} P). From this time forth, ethyl acetate values slightly changed up to 36.7 mg.L^{-1} at the end of the continuous fermentation. This number is higher than any other data found in the literature concerning ethyl acetate in young beer produced by an ICT system (*5, 48, 52, 55-57*). Ester production is greatly influenced by yeast strain, pitching rate, temperature, top pressure, aeration and agitation (*39*). An increased production of acetate esters by immobilized yeast was evidenced during beer fermentation due to higher *ATF1* gene expression in immobilized cell population when compared to free cells (*58*). Alcohol acetyltransferases' (ATF1 and ATF2) gene expression is the most important aspect determining acetate ester levels during fermentation (*59*). Isoamyl

Table 6.2 - Flavour-active compounds present in the young beer at different fermentation times

Fermentation Time (h)	120	216	264	384
Esters (mg.L^{-1})				
Ethyl acetate	3.328	15.38	28.863	36.709
Isoamyl acetate	0.088	0.655	1.242	1.985
Ethyl butyrate	-	0.015	0.033	0.067
Phenyl ethyl acetate	0.301	0.328	0.488	0.522
Ethyl hexanoate (Caproate)	0.008	0.042	0.048	0.139
Ethyl octanoate (Caprylate)	0.005	0.015	0.067	0.144
Ethyl decanoate (Caprate)	0.001	0.004	0.042	0.039
Total	3.731	16.439	30.783	39.605
Higher Alcohols (mg.L^{-1})				
Amyl alcohols (3-methyl-1-butanol, 2-methyl-1-butanol)	24.551	28.241	52.371	55.496
Isobutanol	-	4.761	11.052	11.551
2-Phenylethanol	40.277	40.423	61.490	67.547
Total	64.828	73.425	124.913	134.594
A/E	17.38	4.47	4.06	3.40

acetate is also an important constituent of final beer with threshold values around 1.2 mg.L^{-1} (*51*). The performance of the current setup on the production of isoamyl acetate was also superior to other data involving primary beer fermentation (*5, 47, 55*).

Medium-chain fatty acids (MCFA) ethyl esters as ethyl hexanoate (caproate), ethyl octanoate (caprylate) and ethyl decanoate (caprate) are produced in much lower levels than the acetate esters in beer (*54*). Thus, ICT studies often focus more attention on the acetate family rather than the ethyl ester group. MCFA ethyl esters measured in this study are consistent with past records involving immobilized yeast during primary beer fermentation (*48, 52, 55-57*). Table 6.2 shows the ester profile of the green beer at different fermentation times for the present work.

Both higher alcohols and esters present in the green beer from the outflow of the gas-lift reactor could be correlated with X_{tot} (Fig. 6.3) for a specific sampling time. This data can give crucial insights for planning new continuous fermentations. The use of higher pitching yeast rates on the startup of the reactor may also quicken the expected results.

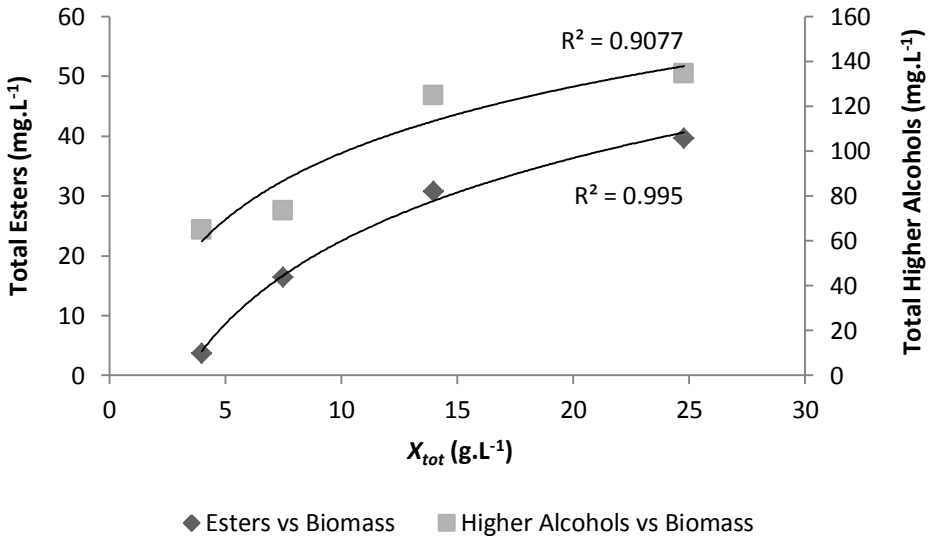


Figure 6.3 – Correlations between X_{tot} present in the gas-lift reactor and the outlet contents of total esters and higher alcohols.

6.5 Conclusions

The current work has shown that it is totally feasible to use flocculation as single way of yeast immobilization in a gas-lift bioreactor for continuous primary beer fermentation. This fact is supported not only by the successful tendency for biomass accumulation relying only on self-aggregation of yeast cells, but also by the good performance on specific saccharide consumption and ethanol volumetric productivity demonstrated on this work. The data obtained was comparable to other ICT systems using several types of yeast carriers. Keeping the composition of wort as constant as possible is highly recommended as it may interfere with biomass buildup during the startup of the reactor. A carrier-free setup shall provide advantages concerning financial cost and management of the reactor when scaled-up.

6.6 Acknowledgements

Marcel Karabín from the Department of Biotechnology of the Institute of Chemical Technology (Prague) is gratefully acknowledged for his technical support. The financial contributions of the EU FP7 project EcoBioCAP - Ecoefficient Biodegradable Composite Advanced Packaging, Grant agreement no: 265669 as well as of the Grant Agency of the Czech Republic (project GAČR P503/12/1424) are also gratefully acknowledged.

6.7 References

1. Willaert, R., and Nedovic, V. A. (2006) Primary beer fermentation by immobilised yeast – a review on flavour formation and control strategies, *J Chem Technol Biotechnol 81*, 1353-1367.
2. Linko, M., Haikara, A., Ritala, A., and Penttila, M. (1998) Recent advances in the malting and brewing industry, *Journal of Biotechnology 65*, 85-98.
3. Pilkington, P., Margaritis, A., Mensour, N., and Russell, I. (1998) Fundamentals of immobilized yeast cells for continuous beer fermentation: A Review, *J. Insi. Brew. 104*, 19-31.
4. Berlowska, J., Kregiel, D., and Ambroziak, W. (2013) Enhancing adhesion of yeast brewery strains to chamotte carriers through aminosilane surface modification, *World Journal of Microbiology and Biotechnology*.
5. Branyik, T., Silva, D. P., Vicente, A. A., Lehnert, R., e Silva, J. B., Dostalek, P., and Teixeira, J. A. (2006) Continuous immobilized yeast reactor system for complete beer fermentation using spent grains and corncobs as carrier materials, *Journal of industrial microbiology & biotechnology 33*, 1010-1018.
6. Branyik, T., Vicente, A., Oliveira, R., and Teixeira, J. (2004) Physicochemical surface properties of brewing yeast influencing their immobilization onto spent grains in a continuous reactor, *Biotechnology and bioengineering 88*, 84-93.
7. Brányik, T., Vicente, A. A., Cruz, J. M. M., and Teixeira, J. A. (2001) Spent grains – a new support for brewing yeast immobilisation, *Biotechnology Letters 23*, 1073–1078.
8. VA, N., IL, C., D, B., B, O., and B, B. (2005) New porous matrices and procedures for yeast cell immobilisation for primary beer fermentation., In *30th European Brewery Convention.*, pp 401–413, Prague.
9. Verbelen, P. J., De Schutter, D. P., Delvaux, F., Verstrepen, K. J., and Delvaux, F. R. (2006) Immobilized yeast cell systems for continuous fermentation applications, *Biotechnol Lett 28*, 1515-1525.
10. Tata, M., Bower, P., Bromberg, S., Duncombe, D., Fehring, J., Lau, V. V., Ryder, D., and Stassi, P. (1999) Immobilized yeast bioreactor systems for continuous beer fermentation, *Biotechnology progress 15*, 105-113.
11. Bezbradica, D., Obradovic, B., Leskosek-Cukalovic, I., Bugarski, B., and Nedovic, V. (2007) Immobilization of yeast cells in PVA particles for beer fermentation, *Process Biochemistry 42*, 1348–1351.
12. Pilkington, H., Margaritis, A., Mensour, N., Sobczak, J., Hancock, I., and Russell, I. (1999) Kappa-Carrageenan Gel Immobilisation of Lager Brewing Yeast, *J. Insi. Brew. 105*, 398-404.
13. Almonacid, S. F., Nájera, A. L., Young, M. E., Simpson, R. J., and Acevedo, C. A. (2012) A Comparative Study of Stout Beer Batch Fermentation Using Free and Microencapsulated Yeasts, *Food and Bioprocess Technology 5*, 750-758.
14. Smogrovicova, D., Domény, Z., Gemeiner, P., Malovikova, A., and Sturdik, E. (1997) Reactors for continuous primary beer fermentation using immobilised yeast, *Biotechnology Techniques 11*, 261-264.
15. Dömény, Z., Smogrovicová, D., Gemeiner, P., Sturdík, E., Pátková, J., and Malovíková, A. (1998) Continuous secondary fermentation using immobilised yeast, *Biotechnol Lett 20*, 1041-1045.
16. Virkajärvi, I. (2001) Feasibility of Continuous Main Fermentation of Beer Using Immobilized Yeast, In *Technical Research Centre of Finland*, VTT Publications 430, Espoo.
17. Virkajärvi, I. (1999) Profiting from Immobilized Fermentation, In *Fifth Aviemore Conference on Malting, Brewing and Distilling*, pp 290-293, Institute of Brewing: London.
18. Virkajarvi, I., and Pohjala, N. (2000) Primary fermentation with immobilized yeast: Some effects of Carrier Materials on the Flavour of the Beer *J. Insi. Brew. 106*, 311-318.

19. RK, M., and JR, J. (1959) Life span of individual yeast cells, *Nature 183*, 1751–1752.

20. Kuřec, M., Baszczyński, M., Lehnert, R., Mota, A., Teixeira, J. A., and Brányik, T. (2009) Flow Cytometry for Age Assessment of a Yeast Population and its Application in Beer Fermentations, *J. Insi. Brew. 115*, 253-258.

21. Powell, C. D., Quain, D. E., and Smart, K. A. (2003) The impact of brewing yeast cell age on fermentation performance, attenuation and flocculation, *FEMS yeast research 3*, 149-157.

22. R, G. (1951) The Flocculation characteristics of brewing yeast during fermentation., In *Eur. Brew. Congr.*, pp 35-57.

23. Kurec, M., and Brányik, T. (2011) The role of physicochemical interactions and FLO genes expression in the immobilization of industrially important yeasts by adhesion, *Colloids and surfaces. B, Biointerfaces 84*, 491-497.

24. Bester, M. C., Jacobson, D., and Bauer, F. F. (2012) Many Saccharomyces cerevisiae Cell Wall Protein Encoding Genes Are Coregulated by Mss11, but Cellular Adhesion Phenotypes Appear Only Flo Protein Dependent, *G3 (Bethesda) 2*, 131-141.

25. Eddy, A. A. (1955) Flocculation characteristics of yeasts II. Sugars as dispersing agents., *J. Inst. Brew. 61*, 313-317.

26. Stratford, M. (1989) Yeast flocculation: Calcium specificity*Yeast 5*, 487-496.

27. Stratford, M. (1996) Induction of flocculation in brewing yeasts by change in pH value, *FEMS Microbiol. Lett. 136*, 13-18.

28. Stratford, M., and Carter, A. T. (1993) Yeast flocculation: lectin synthesis and activation, *Yeast 9*, 371-378.

29. Merchuk, J. C. (1991) Shear effects on suspended cells, *Advances in biochemical engineering/biotechnology 44*, 65-95.

30. Chisti, Y., and Moo-Young, M. (1989) On the calculation of shear rate and apparent viscosity in airlift and bubble column bioreactors, *Biotechnology and bioengineering 34*, 1391-1392.

31. Chun-Min Chang, W. J. L., Ku Song Own, Shyh-Jye Hwang. (1993) Operation conditions affecting the performance of airlift reactors for immobilized enzyme reactions, *Biotechnology Techniques 7*, 317-322.

32. Smit, G., Straver, M. H., Lugtenberg, B. J., and Kijne, J. W. (1992) Flocculence of Saccharomyces cerevisiae cells is induced by nutrient limitation, with cell surface hydrophobicity as a major determinant, *Applied and environmental microbiology 58*, 3709-3714.

33. Lei, H., Zhao, H., Yu, Z., and Zhao, M. (2012) Effects of wort gravity and nitrogen level on fermentation performance of brewer's yeast and the formation of flavor volatiles, *Applied biochemistry and biotechnology 166*, 1562-1574.

34. Stratford, M., Coleman, H. P., and Keenan, M. (1988) Yeast flocculaiton: A dynamic equilibrium, *Yeast 4*, 199–208.

35. Panteloglou, A. G., Smart, K. A., and Cook, D. J. (2012) Malt-induced premature yeast flocculation: current perspectives, *Journal of industrial microbiology & biotechnology 39*, 813-822.

36. Branyik, T., Vicente, A., Cruz, J. M., and Teixeira, J. (2002) Continuous primary beer fermentation with brewing yeast immobilized on Spent Grains, *J. Inst. Brew. 108*, 410–415.

37. Kumar, S., Singh, S., Mishra, I., and Adhikari, D. (2011) Continuous ethanol production by Kluyveromyces sp. IIPE453 immobilized on bagasse chips in packed bed reactor, *J. Petroleum Technol. Altern. Fuels 2*, 1-6.

38. Smogrovicová, D., Dömény, Z., Gemeiner, P., Malovikova, A., and Sturdik, E. (1997) Reactors for continuous primary beer fermentation using immobilised yeast, *Biotechnol Tech 11*, 261-264.

39. Kunze, W. (2010) Beer Production, In *Technology Brewing and Malting*, pp 367-525.

40. Michels, C. A., and Romanowski, A. (1980) Pleiotropic glucose repression-resistant mutation in Saccharomyces carlsbergensis., *J Bacteriol 143*, 674-679.

41. Novak, S., D'Amore, T., and Stewart, G. G. (1990) 2-Deoxy-D-glucose resistant yeast with altered sugar transport activity., *FEBS Lett 269*, 202-204.

42. Kodama, Y., Fukui, N., Ashikari, T., Shibano, Y., Morioka-Fujimoto, K., Hiraki, Y., and Nakatani, K. (1995) Improvement of maltose fermentation efficiency: constitutive expression of MAL genes in brewing yeast., *J Am Soc Brew Chem 563*, 24-29.

43. Brányik, T., Vicente, A., Cruz, J., and Teixeira, J. (2004) Continuous primary fermentation of beer with yeast immobilized on spent grains - the effect of operational conditions., *J Am Soc Brew Chem 62*, 29-34.

44. Hazelwood, L. A., Daran, J. M., van Maris, A. J., Pronk, J. T., and Dickinson, J. R. (2008) The Ehrlich pathway for fusel alcohol production: a century of research on Saccharomyces cerevisiae metabolism, *Applied and environmental microbiology 74*, 2259-2266.

45. Sentheshanuganathan, S. (1960) The mechanism of the formation of higher alcohols from amino acids by Saccharomyces cerevisiae, *The Biochemical journal 74*, 568-576.

46. Oshita, K., Kubota, M., Uchida, M., and Ono, M. (1995) Clarification of the relationship between fusel alcohol formation and amino acid assimilation by brewing yeast using 13C-labeled amino acid, In *25th European Brewery Convention Congress*, pp 387–402.

47. Ryder, D., and Masschelein , C. (1985) The growth process of brewing yeast and the biotechnological challenge., *J Am Soc Brew Chem 43*, 66-75.

48. Smogrovicová, D., and Dömény, Z. (1999) Beer volatile by-product formation at different fermentation temperature using immobilized yeasts., *Process Biochem 34*, 785–794.

49. Mensour, N., Margaritis, A., Briens, C., Pilkington, H., and Russell, I. (1997) New developments in the brewing industry using immobilised yeast cell bioreactor systems., *J Inst Brew 103*, 363-370.

50. Pajunen, E., Tapani, K., Berg, H., Ranta, B., Bergin, J., Lommi, H., and Viljava, T. (2001) Controlled beer fermentation with continuous on-stage immobilized yeast reactor., In *28th EBC Congress*, pp Chap. 49, 41-12.

51. Engan, S. (1972) Organoleptic threshold values of some alcohols and esters in beer., *J Inst Brew 78*, 33-36.

52. Smogrovicová, D., Dömény, Z., Navrátil, M., and Dvorák, P. (2001) Continuous beer fermentation using polyvinyl alcohol entrapped yeast, In *28th EBC Congress*, pp Chapter 50, pp. 51-59

53. MacDonald, J., Reeve, P., Ruddlesden, J., and White, F. (1984) Current approaches to brewery fermentations., *Prog Ind Microbiol 19*, 47-198.

54. Meilgaard, M. C. (1975) Flavour chemistry of beer. part i: flavour interaction between principal volatiles, *Tech Q Master Brew Assoc Am 12*, 107-117.

55. Virkajärvi, I., and Kronlöf, J. (1998) Long-term stability of immobilized yeast columns in primary fermentation., *J Am Soc Brew Chem 56*, 70-75.

56. Andersen, K., Bergin, J., Ranta, B., and Viljava, T. (1999) New process for the continuous fermentation of beer, In *27th European Brewery Convention Congress EBC*, pp 771-778.

57. Kronlöf, J., and Virkajärvi, I. (1999) Primary fermentation with immobilized yeast, In *27th EBC Congress*, pp 761-770.

58. Shen, H., Moonjai, N., Verstrepen, K., Delvaux, F., and Delvaux, F. (2003) Immobilization of Saccharomyces cerevisiae induces changes in the gene expression levels of HSP12, SSA3 and ATF1 during beer fermentation., *J Am Soc Brew Chem 61*, 175-181.

59. Verstrepen, K. J., Van Laere, S. D., Vanderhaegen, B. M., Derdelinckx, G., Dufour, J. P., Pretorius, I. S., Winderickx, J., Thevelein, J. M., and Delvaux, F. R. (2003) Expression levels of the yeast alcohol acetyltransferase genes ATF1, Lg-ATF1, and ATF2 control the formation of a broad range of volatile esters, *Applied and environmental microbiology 69*, 5228-5237.

Chapter 7

High-gravity primary continuous beer fermentation by flocculated biomass

This chapter approaches a long-term, high gravity continuous beer fermentation attained through flocculated biomass in an airlift reactor.

7.1 Abstract

The present work regards a long-term (54 days) continuous primary fermentation of lager beer. For this purpose, it was suggested an airlift bioreactor setup using flocculation as single way of yeast immobilization. Self-aggregation was able to keep up to nearly 53 $g_{cell\ dry\ wt}.L^{-1}$ immobilized in the system. The flexibility of this setup was tested and two types of beer were produced without interrupting the reactor, based in two types of wort: the Pilsener type had a high specific gravity of 15.6±0.3 °P, while Dark Lager wort was less concentrated with around 14.4 °P. Even when using high gravity wort, the desired attenuation was achieved without either recirculation or auxiliary second stage bioreactor. Specific saccharide consumption rate (r_s) was kept around 7.9 ± 0.4 $g.L^{-1}.h^{-1}$ and ethanol productivity (r_p) oscillated within 3.36 ± 0.2 $g.L^{-1}.h^{-1}$ for nearly a month. During this period the volumetric productivity of the current setup reached 1.6 $L_{green\ beer}.L_{bioreactor}^{-1}.day^{-1}$. The green beer produced met the standards of regular finished primary lager beer fermentation for both Pilsener and Dark Lager. Despite the high yeast load, diacetyl productivity seemed to be correlated with free amino nitrogen (FAN) consumption rate.

Keywords: Yeast flocculation, continuous beer fermentation, volumetric productivity, diacetyl accumulation.

7.2 Introduction

High-gravity brewing (HGB) and continuous fermentation are two well-known options for increasing the productivity of a brewery. However, while HGB is a fully implemented and reliable technology, continuous brewing is still avoided by most breweries. One of the main reasons is that the former requires very low additional investments in equipment, which would not be the case for continuous fermentation. Additionally, continuous beer fermentation still needs fine-tunings concerning technical (*1, 2*) and sensorial (*3, 4*) problems affecting the final product. Nonetheless, considering that continuous fermentation can greatly increase volumetric productivity, the efforts and investment cost could probably be justified.

Continuous fermentation relies on high pitching rates of yeast attained by immobilized cell technology (ICT). Regarding ICT, there are two main methods for yeast immobilization: attachment to solid carriers and biofilm formation (*5-8*); and forced physical entrapment of yeast cells into semi-rigid matrixes (*9-12*). For both of these methods the yeast carrier price often limits the technology for further pilot or industrial scale application. Flocculation on the other hand is a particular natural (free of charge) way of immobilization as yeast cells attach to each other to form cell aggregates – flocs. Flocculation is a multifactorial inheritance triggered by both genetic (*13, 14*) and environmental factors (*15-17*) and is widely used to harvest yeast at the end of primary beer fermentation (*18, 19*).

Airlift bioreactors are remarkably efficient on mass transfer for either liquid-liquid or solid-liquid phases triggered by rapid mixing, low shear stress, simple design and low energy consumption (*20*). Airlifts' low shear environment provides excellent conditions for ICT implementation and has previously been used in continuous mode for beer production (*4, 6, 11, 12*).

For a long-term continuous fermentation, relying only on yeast flocculation as immobilization method can be challenging as self-aggregation is hard to predict (*15, 16, 19*). This task becomes even harder when the input wort has a high specific gravity (SG) as sugars inhibit yeast flocculation (*16, 17, 19*). Additionally, an extended continuous fermentation may also compromise yeast viability due to limited lifespan (*21, 22*) and restricted time of metabolic activity (*23, 24*). When mechanical agitation is absent like in batch beer fermentations dead cells are removed from the cylindroconical fermenters by preferential sedimentation of the older cells (*18, 25, 26*), which is not compatible

with the constant agitation of an airlift reactor. Yet ambitious, the possible synergy offered by placing together HGB and continuous fermentation could greatly increase productivity numbers.

Therefore, this work assessed the feasibility of a long-term continuous HGB using flocculation as sole way of biomass immobilization using an airlift bioreactor. Key parameters such as yeast viability and chemical composition of green beer were evaluated in detail in the course of the entire fermentation. Also, maturation was performed in bottles to allow access the final quality of the beer.

7.3 Material and Methods

7.3.1 Brewing Yeast

The flocculent *Saccharomyces carlsbergensis* strain nº 96 from the culture collection of brewing yeast (Research Institute of Brewing and Malting, Plc., Prague, Czech Republic) was used. Yeast was isolated in a Petri dish containing yeast extract peptone dextrose (YPD) broth (Sigma-Aldrich, UK) with 2% of Agar (Sigma-Aldrich, UK). Few colonies were then inoculated in 400 mL of 15º Plato wort and incubated at 30 ºC, 120 rpm, for 48 h previous to be used as inoculum for the gas-lift reactor.

7.3.2 Beer wort

All the wort used in this experiment was supplied by UNICER Bebidas, S.A. (S. Mamede de Infesta, Portugal). The wort was collected from the main production line after cooling stage and previous to aeration. It was stored in 50 L barrels (Nalgene, USA), taken to the laboratory, heated to 100 ºC and autoclaved for 1.5 h at 121 ºC.

Table 7.1 – Composition of inlet wort from the barrels

(Barrel nº) Wort type	Period in days (from-to)	RT (h)	SG (°P)	pH	Color (EBC)	EBU	FAN ($mg.L^{-1}$)
(1) Pilsener	$0\text{-}11^{th}$	59-27	15.95	4.91	26.5	25	203
(2) Pilsener	$11\text{-}20^{th}$	20	15.67	4.79	26.0	27	178
(3) Pilsener	$20\text{-}27^{th}$	17	14.98	4.82	20.6	26	176
(4) Pilsener	$27\text{-}34^{th}$	15	15.76	4.74	31.6	34	209
(5) Pilsener	$34\text{-}40^{th}$	15	15.50	4.76	28.1	33	207
(6) Dark Lager	$40\text{-}48^{th}$	15	14.43	4.77	224	19	183
(7) Dark Lager	$48\text{-}54^{th}$	15	14.40	4.75	219	20	191

SG – Specific gravity; RT – Retention time; °P – Plato degree; EBC – European Brewery Convention; EBU - European Bitterness Units; FAN – Free-amino nitrogen

Considering the high volume of the barrels, heat treatment is mandatory for keeping the wort free of contaminations during long-term experiments. The composition of each barrel of wort supplied to the reactor is presented in Table 7.1.

7.3.3 Continuous fermentation

The continuous fermentation was carried out in a Perspex airlift bioreactor with 4 L of total work volume. Gas flow was regulated by two independent mass controllers (Alicat Scientific, USA) using CO_2 (100 mL.min^{-1}) and pressurized air (10 mL.min^{-1}). This mixture and flow were kept constant in the course of the entire experiment. The temperature was hold at 15 ^{o}C using a JULABO F32 Refrigerating/Heating Circulator (JULABO, Germany). The dilution rate was controlled by a peristaltic pump 101U/R (Watson-Marlow, UK). Figure 7.1 shows an overview of the setup used in the current work.

The reactor was sterilized using a 3% (v/v) solution of commercial sodium hypochlorite with 1.5% of active chlorine, 48 h before use. After this time, the solution was discarded and 50 L of sterile water were used to wash the reactor. It was then filled with 15^{o} Plato wort and inoculated.

Batch reactor operation was kept in the first 48 h at 20 ^{o}C, using only pressurized air as gas supply at 200 mL.min^{-1}. After that, air-flow was reduced to 10 mL.min^{-1} at the same time that CO_2 injection started at 100 mL.min^{-1}. Afterwards, a continuous reactor operation phase was started with the first wort barrel at 0.019 h^{-1} dilution rate, which was raised gradually (up to 0.066 h^{-1}) depending on the attenuation level of the green

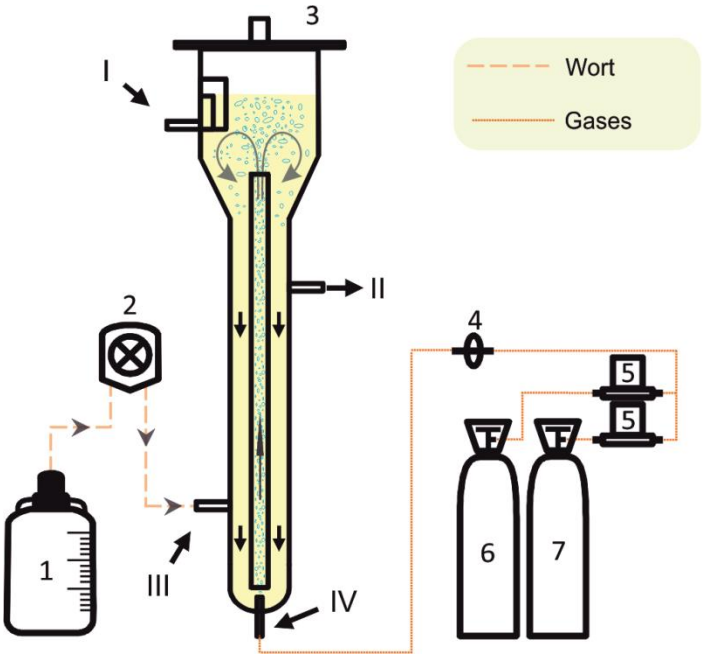


Figure 7.1 - Immobilized yeast bioreactor system used in the current work: 1 - wort barrel; 2 - peristaltic pump; 3 - gas-lift reactor; 4 - air filter; 5 - flow mass controllers; 6 - pressurized air; 7 - CO_2 bottle; I - green beer outflow/sampling point; II - biomass sampling point; III - wort inlet; IV - gas sparger.

beer.

7.3.4 Biomass measurements

Flocculated biomass was evaluated daily. Three 15 mL plastic Falcon tubes were dried at 105 ºC for 12 h and weighted. Then 10 mL of X_{tot} (corresponding to flocculated plus free cells suspended inside the reactor) taken from the reactor sampling point II (Fig. 7.1) were added to each Falcon and centrifuged at 4000 x ***g*** for 5 min. Liquid phase was discarded and the Falcons were dried (105 °C) for 24 h previous to weighting. The same procedure was also carried out with samples of green beer directly from the outflow (Fig. 7.1,I) in order to evaluate the loss of biomass. Blank experiments were carried out using the inlet wort to correct for the presence of trub-like compounds that could interfere with weighting.

7.3.5 Yeast viability

Yeast viability from either X_{tot} or cells from the outflow was assessed by flow cytometric measurements. Stock solutions were prepared for fluorescein diacetate (FDA) and propidium iodide (PI). FDA (Sigma, UK) was diluted with dimethyl sulfoxide (DMSO) (Sigma, UK) to a final concentration of 500 µg.mL^{-1}. PI (Sigma, UK) solution was diluted in ultra-pure water to a final concentration of 1 mg.mL^{-1}. The stock solutions were kept at -20 ºC until use. For the double staining with FDA and PI, 100 µL of ≈1x10^{6} cell suspension was added to 400 µL of phosphate buffered saline (PBS) (Sigma, UK) containing 1 µL of PI stock solution and 4 µL of FDA. The incubation was carried out at room temperature for 20 min.

Samples were double stained with FDA and PI as described above, and analyzed using a Beckman-Coulter EPICS XL flow cytometer (Beckman Coulter, Inc., USA) equipped with an Argon ion air cooled laser (emission at 488 nm/15 mW power). The FDA green fluorescence was detected by the FL1 (530 nm) channel, while the PI red fluorescence was captured by the FL3 (590-610 nm). More than 20.000 cell readings were carried out for each sample in triplicate. Data were analyzed in the Flowing Software version 2.5.1 (Freeware provided by Perttu Terho - Turku Centre for Biotechnology, University of Turku, Finland).

7.3.6 Beer Maturation

Green beer collected at the outflow was stored in a closed plastic recipient, immersed in ice. After 12 h storage, the green beer was filled into 1.5 L PET bottles containing 50 mL of the respective original wort for carbonation and maturated for 30 days at 6 °C.

7.3.7 Beer and wort analysis

Characterization of wort, green and maturated beer was done as suggested in the present Analytica EBC methods. All reagents were purchased from Merck (Germany).

Periodically, the green beer coming directly from the outflow was filtered using a vacuum filtration apparatus (New Star Environmental, USA) coupled to a filtering flask immersed in ice. Cellulose nitrate membranes with 0.2 µm pore size (Whatman, USA) were used for filtration. Samples were then placed into 50 mL Falcon tubes and frozen (-20 °C) up to analysis.

Alcohol, specific gravity, original and real extract, pH, colour and degree of fermentation were performed by the automatic beer analyzer Anton Paar Beer Analysis System DMA 4500 (Anton Paar GmbH, Austria).

Total diacetyl was determined by gas chromatographic analysis of the static headspace using a Varian CP-3800 gas chromatograph (GC) (Varian, USA). Separation was carried by a Wall Coated Open Tubular (WCOT) fused silica capillary column (60 m x 0.25 mm) CP-Sil 8 CB low bleed/ms (Varian, Netherlands) using nitrogen as carrier gas. Samples were automatically injected by a Combi PAL headspace autosampler (CTC Analytics, Switzerland).

Acetaldehyde, higher alcohols and esters were measured by Varian star 3400 CX GC (Varian, USA) with a flame ionization detector (FID) and a thermoconductivity detector (TCD). The volatiles were separated by a WCOT fused silica capillary column (60 m x 0.53 mm) CP-Wax 52 CB (Varian, Netherlands) using a pure air and hydrogen as carrier. Samples were automatically injected by a Combi PAL headspace autosampler (CTC Analytics, Switzerland).

The free-amino nitrogen (FAN) and bitterness results were measured in a Varian Spectrophotometer Cary 50 Bio UV-Visible (Varian, USA).

7.4 Results and Discussion

7.4.1 Yeast growth and viability

As Figure 7.2 shows, X_{tot} had a consistent tendency to accumulate in the airlift reactor in the course of all continuous fermentations. It is important to emphasize that other works involving ICT normally distinguish between immobilized and free cells, where the sum of these two fractions is equal to the X_{tot} used in this work.

In order to build the needed X_{tot} to achieve high productivities, the wort fed to the airlift started at a very slow flow rate (Q = 0.074 $L.h^{-1}$). As a consequence, the total retention time (RT_{tot}) was 59 h, but before completing the first RT_{tot} cycle (48 h) the X_{tot} was already 14.7 $g_{cell\ dry\ wt}.L^{-1}$. As the current system relied on flocculation, the tendency for

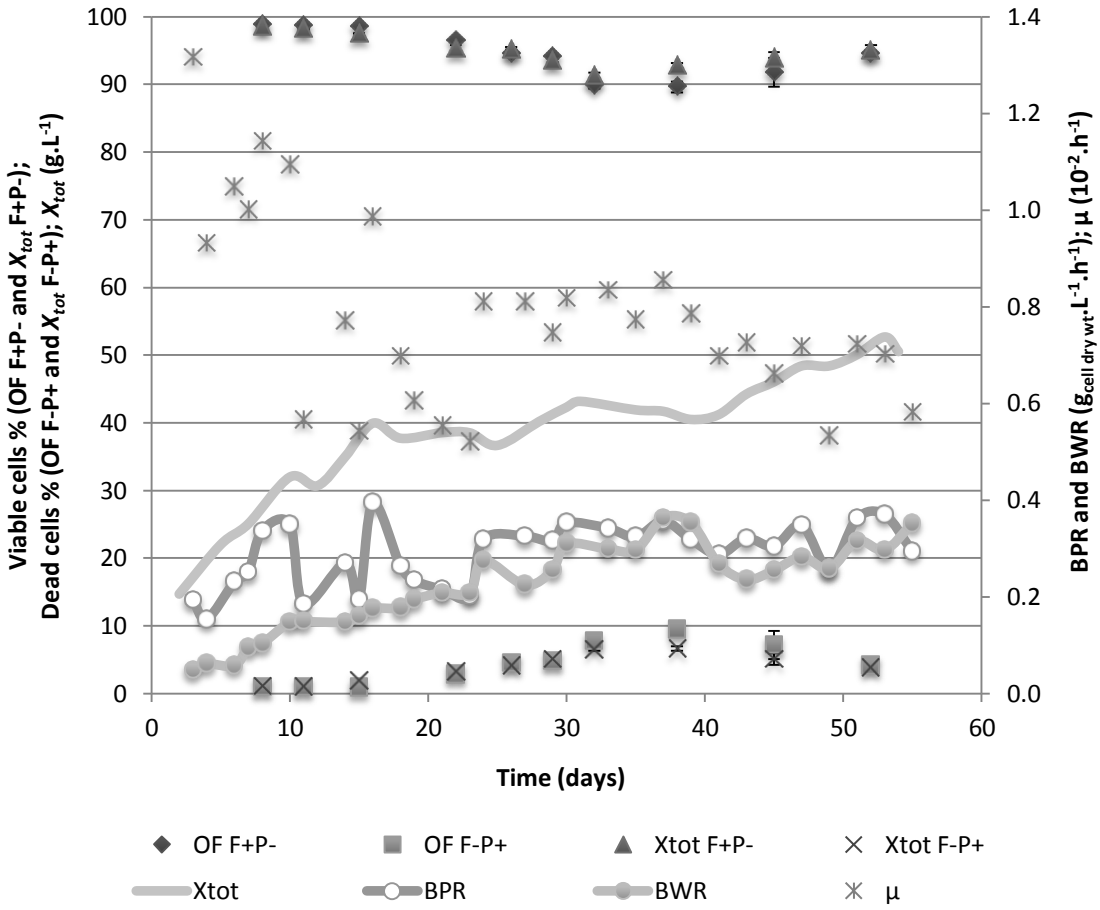


Figure 7.2 - Biomass growth rate (μ), biomass productivity rate (*BPR*), biomass washout rate (*BWR*), and yeast viability assessed by flow cytometry during the continuous fermentation. *OF* F+P-: Viable cells from outflow (Fig. 7.1,I) with positive staining for fluorescein diacetate and negative staining for propidium iodide; X_{tot} F+P-: Viable cells from sampling point II (Fig. 7.1) representing the X_{tot} with positive staining for fluorescein diacetate and negative staining for propidium iodide; *OF* F-P+: Dead cells from outflow (Fig. 7.1,I) with positive staining for propidium iodide and negatively stained by fluorescein diacetate; X_{tot} F-P+: Dead cells from sampling point II (Fig. 7.1) representing the X_{tot} with positive staining for propidium iodide and negatively stained by fluorescein diacetate. The viability values presented in this figure are the average of 20.000 cells readings, carried out in triplicate.

X_{tot} accumulation inside the airlift depended on the biomass productivity rate (*BPR*) and biomass washout rate (*BWR*). *BPR* has been estimated to be the capacity of biomass productivity by X_{tot} in given time, calculated as follows:

$$BPR = X_{tot} * \mu \qquad \textbf{eq. 1}$$

BWR has been evaluated experimentally by constant measurements of biomass concentration in the outflow (X_{out}) of the airlift reactor and can be expressed as:

$$BWR = X_{out} * D \qquad \textbf{eq. 2}$$

Theoretically, if the values of *BPR* and *BWR* would have attained equilibrium, X_{tot} would have been kept constant. Specific growth rate (μ) has been calculated based in experimental data acquired in this experiment through different stages of the continuous fermentation, calculated as follows:

$$\mu = \frac{\Delta X}{\Delta t_{(1-0)}} * \frac{1}{X_{tot_{t0}}} \qquad \textbf{eq. 3}$$

However, considering that biomass was constantly being removed from the reactor through the outflow, ΔX in the current system is represented by:

$$\Delta X = \left(X_{tot_{t1}} - X_{tot_{t0}}\right) + X_w \qquad \textbf{eq. 4}$$

Where *Xw* is the total biomass concentration removed from the system during Δt. Thus:

$$\mu = \frac{\left(X_{tot_{t1}} - X_{tot_{t0}}\right) + X_w}{\Delta t_{(1-0)}} * \frac{1}{X_{tot_{t0}}} \qquad \textbf{eq. 5}$$

The maximum specific growth rate, μ_{max}, was observed in the first days of continuous operation and was around 0.013 h^{-1}, being the average value through the whole experiment around 0.008 h^{-1}. Moreover, it is possible to see (Fig. 2) that *BWR* oscillated below *BPR* in a great part of the continuous operation experiment, marking a positive tendency on X_{tot} retention by flocculation. This was true except in the period comprised between the 27th to the 40th day of continuous fermentation, when *BWR* began to rise

reaching its peak above *BPR* in the 39th day, which reflected in a small period of X_{tot} decrease. This period matches with the increase of saccharide loading rate (*SLR*) and free amino nitrogen loading rate (*FLR*) caused by changing wort barrels and raising *Q* (Fig. 3 A,B). Flocculation is affected by a combination of many variables being one of the most important nutrients' availability (e.g. sugars and FANs) (*19*).

Intriguingly, the same change in *SLR* and *FLR* reported in the last lines has also decreased X_{tot} viability (Fig. 7.2), which showed signs of recovery when *SLR* and *FLR* were reduced. Yeast viability was assessed by esterase activity and preservation of plasma membrane integrity monitored by FDA and PI, respectively.

The maximum value of X_{tot} measured for the current work was 52.7 $g_{cell\ dry\ wt}.L^{-1}$. Tata and coworkers (*8*) compared a couple of two-stage systems for continuous beer production. Each of these systems comprised two reactors connected in series: two fluidized bed reactors with porous glass beads for cell immobilization; and two loop reactors with silicon carbide cartridge for yeast load. The X_{tot} reported by the authors for each of these systems was 29.7 and 18.2 $g_{cell\ dry\ wt}.L^{-1}$, respectively. In the continuous primary beer fermentation carried by Brányik and coworkers (*7*) the X_{tot} reported using an airlift reactor with lignocellulosic carriers obtained from brewer´s spent grains (BSG) varied from 9.3 to 10.5 $g_{cell\ dry\ wt}.L^{-1}$. In a further work, using a similar setup with BSG, Brányik and coworkers (*4*) measured a X_{tot} of nearly 15 $g.L^{-1}$. However, the amount of yeast associated to a specific system does not necessarily reflect on good results. Therefore, the performance of different ICT setups should be compared in terms of specific saccharide consumption (r_s) and volumetric ethanol productivity (r_p) as discussed in the next section of this manuscript.

7.4.2 SLR, FLR, nutrient consumption and productivities

The performance of the bioreactor not only depends on its ability to assimilate what is being fed, but also on the yield of the desired product. Figure 7.3 drafts the entire course of *SLR* and *FLR* fed to the airlift reactor, their respective consumption rate and the productivity values of most important components of the green beer - ethanol, higher alcohols and esters.

Coupling continuous fermentation with HGB, associated to reduced RT_{tot} resulted in r_s values as high as 8.7 $g.L^{-1}.h^{-1}$ and r_p of ethanol reaching the 3.7 $g.L^{-1}.h^{-1}$. Moreover, none of these values were obtained with loss of attenuation performance (Table 7.2). In

fact, the values observed in the current work are far greater than any other published data for continuous beer fermentation (*4, 6, 8, 11, 12*). Additionally, as long as RT_{tot} was fixed at 15 h, r_s and ethanol r_p remained steady within 7.9 ± 0.4 and 3.36 ± 0.2 respectively, for nearly a month until interruption of the fermentation (Fig. 7.3A). In other words, during all stages (including the highest productivities RT_{tot} = 15 h) the desired attenuation was reached, which means a complete primary beer fermentation. Throughout all this period, the volumetric productivity of the current system was 1.6 $L_{green\ beer}.L_{bioreactor}^{-1}.day^{-1}$. This means that a 4 L reactor was producing 6.4 L of green beer per day.

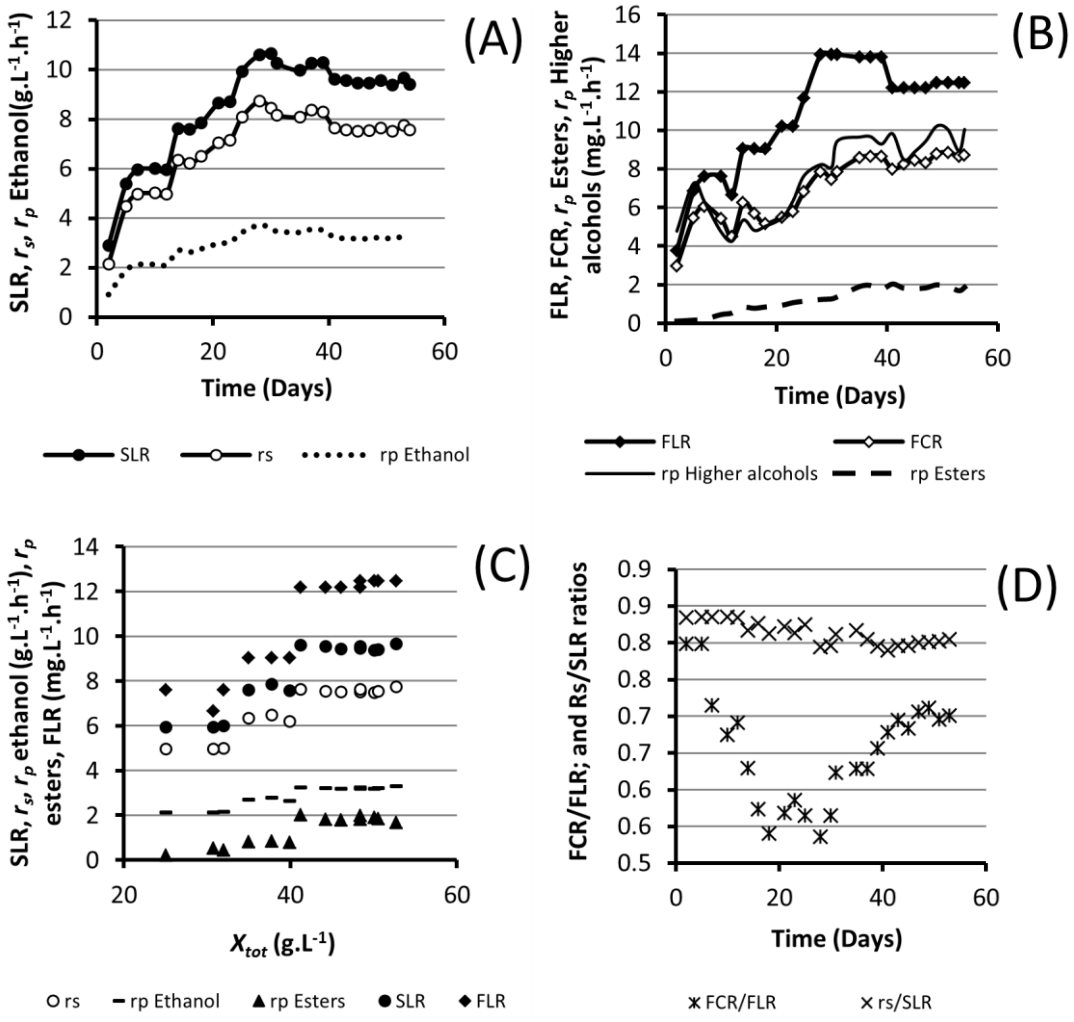


Figure 7.3 – Organic loading/consumption rate of nutrients present in the feed wort and productivity rates of main components measured in the green beer from the outflow during the continuous fermentation. (A) - Saccharide loading rate (*SLR*); Saccharide consumption rate (r_s); and Ethanol productivity (r_p Ethanol), in the course of continuous fermentation time. (B) – Free amino nitrogen loading rate (FLR); Free amino nitrogen consumption rate (FCR); Total higher alcohols productivity (r_p Higher alcohols); and Total acetate esters productivity (r_p Esters), through fermentation time. (C) – Consumption and productivity rates during steady inlet phases of SLR/FLR and the influence of total biomass (X_{tot}). (D) – Ratios of consumption and loading rates of saccharide and free amino nitrogen.

Table 7.2 – Average composition (± standard deviation) of the green beer (recalculated to 5.2% ethanol) taken from the outflow (Fig. 7.1,I) of the airlift reactor

Beer Type	Pilsener	Pilsener	Pilsener	Pilsener	Pilsener	Dark Lager	Dark Lager
Days (from-to)	0-11th	11-20th	20-27th	27-34th	34-40th	40-48th	48-54th
Profile							
Real Att. (%)	67±3.0	68.8±0.4	67.7±0.4	67.0±1.4	67.3±0.5	66.0±0.0	66.5±0.5
FAN (mg.L^{-1})	33.8±4.8	48.2±6.5	60.3±2.8	70.1±2.8	60.0±1.3	50.1±1.8	46.8±0.6
EBU	15±2	15±1	16±0	20±1	20±1	12±0	12±0
Color (EBC)	17±2	15±0	14±1	18±0	16±0	157±11	159±7
pH	3.8±0.1	4.0±0.0	4.2±0	4.2±0	4.1±0	4.1±0	4.1±0
Volatiles (mg.L^{-1})							
Diacetyl	0.44±0.05	0.25±0.03	0.30±0.03	0.31±0.04	0.45±0.03	1.02±0.22	1.20±0.07
Acetaldehyde	17.3±2.8	12.3±2.1	11.5±1.4	11.0±0.3	9.0±0.4	12.5±1.1	11.0±1.1
n-Propanol	37.1±11.4	19.2±0.8	22.4±2.7	26.3±2.9	27.8±1.3	21.4±3.7	17.9±1.4
Isobutanol	23.1±10	8.2±1.1	7.9±0.8	9.4±1.0	12.7±0.1	16.7±2.7	18.6±1.9
Amyl alcohols	84.6±24.3	50.5±1.8	53.5±3.5	61.6±5.7	70.5±1.5	78.7±4.6	87.7±5.2
Ethyl acetate	5.2±1.9	11.5±0.9	13.1±0.6	13.9±1.0	20.7±1.0	22.6±1.1	22.1±1.5
Isoamyl acetate	0.20±0.11	0.40±0.02	0.48±0.04	0.70±0.06	1.14±0.05	1.32±0.1	1.56±0.11
A/E	31.2±13.3	6.6±0.8	6.1±0.4	6.6±0.2	5.1±0.1	4.9±0.2	5.3±0.1

EBC – European Brewery Convention; EBU - European Bitterness Units; FAN – Free-amino nitrogen;

Although high osmolarity has been targeted as being one of the main stress factors during HGB (*27, 28*), ethanol tolerance developed by lager yeast strains was recently observed to increase fermentation rates (*29*). As fermentation in this work ran in continuous mode, sugar levels inside the airlift reactor were low due to fast metabolism, but on the other hand ethanol was always around 52±3 g.L^{-1}. Although (together with other stress factors) the ethanol stress might have discouraged biomass growth (μ_{max}=0.013 h^{-1}), the evidence reported by Hasegawa and coworkers (*29*) may support the good performance of fermentation rates for the current setup.

In contrast with what is observed in the ratio of SLR/r_s, which remained quite stable during all experiment, the free amino nitrogen consumption rate (*FCR*) did not follow linearly the *FLR* (Fig. 7.3D). This could be explained by the uptake rate of amino acids, which takes some time and energy to be accomplished (*30*). Moreover, the initial fall of *FCR*/*FLR* observed in the early stages of continuous fermentation (Fig. 7.3D) could be ascribed to the increased yeast metabolism and growth rate that may have triggered the

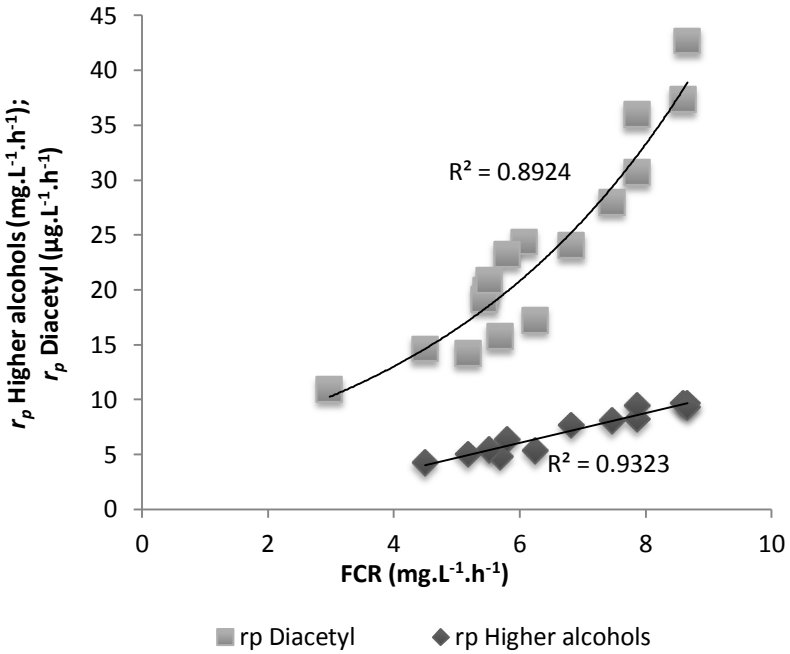


Figure 7.4 – Correlation of free amino nitrogen consumption rate (FCR) influencing the productivities of higher alcohols and diacetyl.

synthesis de novo of amino acids, decreasing the need of external intake of FANs. But not surprisingly, *FCR* exerted a strong impact (Fig. 7.4) on the productivity of higher alcohols (r_p higher alcohols) as the main route for their synthesis is by the catabolism of amino acids through the Ehrlich pathway (*31*).

Despite the vast presence of precursors as higher alcohols (Table 7.2), the main factor influencing the synthesis of acetate esters is not substrate availability, but the expression of genes (ATF1, ATF2, Lg-ATF1) encoding alcohol acetyltransferases (AATases) (*32-34*). However, the expression of these genes is directly inhibited in the presence of oxygen (*35-39*). Yet, in order to keep a positive tendency for biomass accumulation, oxygen is crucial for the synthesis of plasmatic membrane structures (*40*). It is in this context that the core paradox for the feasibility of long-term continuous fermentation emerges: how to build biomass without inhibiting ester synthesis? In batch fermentations the dissolved oxygen present in wort is rapidly exhausted by the pitched yeast to form new cells. Breweries rely on this initial yeast growth for maturation and crop for re-pitching. Thus, continuous bioreactors as suggested in the current work must provide not only a sensorially balanced green beer, but also the needed yeast to keep the fermenter working and also yeast to proceed with carbonation and diacetyl removal in the maturation step. So, despite using direct aeration to the airlift reactor, which could

impair ester synthesis, the current setup presented consistent acetate esters productivity (Fig. 7.3).

From the analysis of Figure 7.3C, it is possible to observe that in the course of constant inlet of nutrients (stable *SLR* and *FLR*) the growing X_{tot} did not influence consumption and productivity rates. This data suggests that the X_{tot} present during these stages was potentially prepared to increase even more the productivities by raising *Q*. However, this was not performed due to logistic difficulties of transporting a higher number of wort barrels from the brewing industry to the laboratory in order to support the increased demand of *SLR* and *FLR*.

7.4.3 Beer analysis

The high initial RT_{tot} needed for building the X_{tot} also delayed the washout of the green beer produced during the initial batch phase of the reactor operation. As a result, the green beer from the outflow in the first days of continuous fermentation was completely unbalanced especially for the values of acetaldehyde and higher alcohols (Table 7.2). As long as enough X_{tot} was immobilized inside the airlift reactor and altered green beer was washed-out (≈ 10^{th} day of continuous experiment), the higher alcohol to ester ratio (*A/E*) was kept within acceptable values (5.7 ± 0.83) until interruption of fermentation. This ratio was attained both by decreased higher alcohols measured in the green beer after the washout period and also by increased rate of ester synthesis when there was sufficient X_{tot} (> 30 $g.L^{-1}$) in the airlift reactor (Table 7.2).

The vast majority (≈ 80%) of higher alcohols present in the final beer are produced by yeast during the primary fermentation either from catabolism (*41, 42*) or biosynthesis of amino acids (*43*). These fusel alcohols not only contribute directly to the final beer´s aroma and taste, but also indirectly as they are precursors of the most desirable esters. Data involving the production of higher alcohols using ICT is rather inconsistent as some works have reported low production of higher alcohols by immobilized cells (*44-46*), while others have shown increased biosynthesis when compared to batch fermentations (*47*). To a large extent, the average total higher alcohols measured in the green beer produced by the current setup were within the expected concentration range (*48, 49*) for both Pilsener (119 ± 18 $mg.L^{-1}$) and Dark Lager (142 ± 9 $mg.L^{-1}$). In the present work, isoamyl alcohol (3-methyl-1-butanol) and the active amyl alcohol (2-methyl-1-butanol) were measured together and are henceforth mentioned as amyl alcohols (Table 7.2). In the course of the continuous fermentation, these alcohols

remained always above the flavour threshold in the green beer (*50*). The average content of amyl alcohols in the green beer during the inlet of Pilsener wort was 84.6±21.82 $mg.L^{-1}$ and Dark Lager 98.26±7.6 $mg.L^{-1}$. Polyvinyl alcohol was used for lager yeast entrapment producing a green beer with 57.8 $mg.L^{-1}$ of amyl alcohols (*51*). Brányik and coworkers (*4*) used treated BSG as yeast carriers, also for lager beer continuous fermentation with yields of amyl alcohols around 60 $mg.L^{-1}$. In the calcium-pectate beads system proposed by Smogrovicová and Dömény (*45*), the reported value for amyl alcohols in the green beer was 56.8 $mg.L^{-1}$. Bearing in mind that the current work used high gravity wort for continuous beer fermentation (which means that beer produced should still be diluted) not only amyl alcohols, but all other higher alcohols reported in Tables 7.2 and 7.3 are in the desired concentration range for lager beers for either Pilsener or Dark Lager (*48, 49*).

Esters are the less present, but the most important of beer constituents produced by the brewing yeast. As esters are also mainly produced during initial vigorous fermentation, the quality of final beer will ultimately rely on a successful primary fermentation. Only acetate esters were measured in the current work as they are much more present in beer than medium-chain fatty acids ethyl esters. Ethyl acetate is synthetized by the brewing yeast through the action of alcohol acetyltransferase catalyzing the condensation reaction between acetyl-CoA with ethanol and is the most abundant ester in beer. After approximately 10 days of continuous fermentation (when there was enough $X_{tot} > 30$ $g.L^{-1}$) the average ethyl acetate content in the green beer for Pilsener type was 18.4±0.4 $mg.L^{-1}$ and 26.35±0.18 for Dark Lager. These numbers not only agree with other reported setups for primary continuous beer fermentation (*45, 51-54*), but are also within the desired concentration range (*48, 49*). Isoamyl acetate has a much lower flavour threshold (1.2 $mg.L^{-1}$) than its counterpart ethyl acetate (20-30 $mg.L^{-1}$) (*48, 49*). In the current work, isoamyl acetate was present below the flavour threshold in the green beer up to late stages of Pilsener wort inlet. It is possible that the increased cell activity driven by increasing *SLR*/*FLR* and biomass itself have progressively exhausted dissolved oxygen, leading to gradually higher expressions of ATF genes (*35*), and hence higher levels of isoamyl acetate (Fig. 7.3B).

As regards past literature involving ICT (*4, 6, 45, 55, 56*), one of the main problems impairing beer produced under such dense pitching rates is the overproduction of diacetyl. This vicinal diketone (VDK) has a very low odour threshold (around 0.1 $mg.L^{-}$

[1] (*49*)), affecting lager beers with unpleasant sweetish buttery off-flavour. Diacetyl is spontaneously formed outside yeast cells by non-enzymatic oxidative decarboxylation of α-acetolactate, which in turn is also intermediate for the *de novo* synthesis of valine. Additionally, the accumulation of α-acetolactate inside yeast cells is rate-limiting for the biosynthesis of that amino acid and therefore excreted (*57*). As recommended by the EBC, the measurements of total diacetyl performed in the current work also consider α-acetolactate content. Therefore, all values mentioned for diacetyl should be henceforth understood as total diacetyl. As shown in Figure 7.4, diacetyl productivity was influenced by *FCR*. Very recently, Krogerus and Gibson (*58*) demonstrated that worts with less FAN produced less diacetyl during fermentation. Before them, Pugh and colleagues (*59*) not only evidenced the same correlation, but also have shown that levels lower than 122 $mg.L^{-1}$ started to increase diacetyl production. It was clear that depletion of FAN stimulated the *de novo* synthesis of amino acids increasing the pool of α-acetolactate. Still recently, Lei and coworkers (*30*) observed that the intake of valine decreased with increasing FAN content. These authors attributed the rise of available FAN to induce yeast in the absorption of preferred amino acids rather than valine. Valine acts as feedback inhibitor of ILV2-encrypting acetohydroxy acid synthase (AHAS), which catalyzes the irreversible conversion of pyruvate to α-acetolactate (*60*, *61*). Thus, it is possible that the increased *FLR* from the 27^{th} day onwards, associated with *FCR* (of preferred amino acids) has decreased valine uptake in the current setup stimulating α-acetolactate formation, which ultimately led to increased levels of diacetyl (Fig. 7.4). Regardless of the increased diacetyl productivity, after maturation the total diacetyl was reduced well below the threshold value for the Pilsener beer (0.04 $mg.L^{-1}$) and slightly above for the dark lager (0.12-0.17 $mg.L^{-1}$) produced in the current work (Table 7.3).

Table 7.3 – Profile of three continuously produced green beers and their corresponding maturated beers. Each shaded row represents a single beer, being the number above the values of green beer and the bolded numbers below the respective final value after maturation.

SG (°P)	ABV (%)	Real Att. (%)	pH	Color (EBC)	EBU	Diacetyl ($mg.L^{-1}$)	Acetaldehyde ($mg.L^{-1}$)	Propanol ($mg.L^{-1}$)	Isobutanol ($mg.L^{-1}$)	Amyl alcohols ($mg.L^{-1}$)	Total Higher Alcohols	Ethyl acetate ($mg.L^{-1}$)	Isoamyl Acetate ($mg.L^{-1}$)	Total Acetate Esters	A/E
15.4	6.8	67	4.07	20.8	25	0.64	11.14	34.24	16.21	88.96	139.4	25	1.39	26.45	5.3
15.9	**7.5**	**71.92**	**4.15**	**21.3**	**25**	**0.04**	**8.28**	**35.3**	**16.4**	**90.5**	**142.2**	**36.4**	**2.01**	**38.4**	**3.7**
14.1	6.1	67	4.11	194	14	1.55	12.3	20.7	25.5	104.6	150.8	26.14	1.85	28	5.39
14.8	**6.5**	**67.4**	**4.13**	**213**	**15**	**0.17**	**7.2**	**21**	**19.9**	**99**	**139.9**	**31.9**	**2.31**	**34.2**	**4.1**
14.5	6.3	67	4.10	197	14	1.44	11.53	19	19.6	95.3	133.9	23.7	1.66	25.35	5.28
14.7	**6.5**	**67.39**	**4.12**	**202**	**14**	**0.12**	**6.66**	**23.3**	**17**	**89.4**	**129.7**	**29.9**	**1.88**	**31.8**	**4.1**

SG – Specific gravity; °P – Plato degree; EBC – European Brewery Convention; EBU - European Bitterness Units; and A/E – alcohol to ester ratio.

As maturation time was somehow extended (30 days), it is possible that some yeast present in maturating bottles started autolysis, slightly increasing the pH in the maturated beer (Table 7.3). As more original extract was added to the maturating bottles the final beer has certainly changed (Table 7.3). The addition of sugars during maturation step is a common practice to improve beer quality in some Belgian Ales (*62*). As it is expected, the dissolved oxygen in the green beer should be null. Thus, yeast metabolism during re-fermentation is completely reductive. As a result, yeast will accumulate the reduced coenzymes NADH and NADPH. In order to keep the redox balance the brewing yeast exposed to this situation uses this "reduction power" to reduce aldehydes to alcohols through the action of aldoketoreductases (*63-65*). The reduction of aldehydes like diacetyl and 2-trans-nonenal into their respective alcohols improves beer flavour as these alcohols have much higher flavour threshold than the original aldehydes. In the present work, this was true for acetaldehyde as values were below the threshold (*49*) in the finished beer (Table 7.3).

7.5 Conclusions

Concerning the results observed in the present work, the volumetric productivity of the airlift for primary continuous beer fermentation was greatly enhanced by HGB. The current setup has shown not only an extraordinary performance at the level of real attenuation and ethanol productivity, but has also proven to be flexible as two types of beer were produced without interrupting the airlift reactor operation.

It should not be forgotten that yeast immobilization was carried out by flocculation, which could bring great benefits in terms of the financial costs of further scale-up. Additionally, this simple self-aggregation method was able to keep an amount of immobilized yeast suspended in the system greater than any other ICT setup reported. Moreover, irrespective of the wort fed, the desired concentration range of main flavour-active compounds was achieved in the green beer at the outflow throughout the continuous experiment. A fine-tuning in the amount of dissolved oxygen provided to the airlift reactor can further increment ester productivity.

Bearing in mind that current work was a laboratory-based study, faster startups of the current setup could be achieved by increasing the initial pitching rate (e.g. using industrial yeast slurry).

The current work also inspires new studies of diacetyl formation in ICT, which could be triggered by increasing *FLR* and *FCR*.

7.6 Acknowledgements

All material supplied by UNICER Bebidas de Portugal, S.A. (S. Mamede de Infesta, Portugal) as well as the technical support of company members Sonia Meireles and Helena Cunha is forever acknowledged. The financial contributions of the EU FP7 project EcoBioCAP - Ecoefficient Biodegradable Composite Advanced Packaging, Grant agreement no: 265669 as well as of the Grant Agency of the Czech Republic (project GAČR P503/12/1424) are also gratefully acknowledged. The authors thank the Ministry of Education, Youth and Sports of the Czech Republic (MSM 6046137305) for their financial support.

7.7 References

1. Verbelen, P. J., De Schutter, D. P., Delvaux, F., Verstrepen, K. J., and Delvaux, F. R. (2006) Immobilized yeast cell systems for continuous fermentation applications, *Biotechnology letters 28*, 1515-1525.
2. Mensour, N. A., Margaritis, A., Briens, C. L., Pilkington, H., and Russell, I. (1997) New developments in the brewing industry using immobilised yeast cell bioreactor systems, *J Inst Brew 103*, 363-370.
3. Willaert, R., and Nedovic, V. A. (2006) Primary beer fermentation by immobilised yeast – a review on flavour formation and control strategies, *J Chem Technol Biotechnol 81*, 1353-1367.
4. Brányik, T., Vicente, A., Cruz, J., and Teixeira, J. (2004) Continuous primary fermentation of beer with yeast immobilized on spent grains - the effect of operational conditions., *J Am Soc Brew Chem 62*, 29-34.
5. Berlowska, J., Kregiel, D., and Ambroziak, W. (2013) Enhancing adhesion of yeast brewery strains to chamotte carriers through aminosilane surface modification, *World J Microbiol Biotechnol 29*, 1307-1316.
6. Branyik, T., Silva, D. P., Vicente, A. A., Lehnert, R., e Silva, J. B., Dostalek, P., and Teixeira, J. A. (2006) Continuous immobilized yeast reactor system for complete beer fermentation using spent grains and corncobs as carrier materials, *Journal of industrial microbiology & biotechnology 33*, 1010-1018.
7. Branyik, T., Vicente, A., Cruz, J. M., and Teixeira, J. (2002) Continuous primary beer fermentation with brewing yeast immobilized on Spent Grains, *J. Inst. Brew. 108*, 410–415.
8. Tata, M., Bower, P., Bromberg, S., Duncombe, D., Fehring, J., Lau, V. V., Ryder, D., and Stassi, P. (1999) Immobilized yeast bioreactor systems for continuous beer fermentation, *Biotechnology progress 15*, 105-113.
9. Pilkington, H., Margaritis, A., Mensour, N., Sobczak, J., Hancock, I., and Russell, I. (1999) Kappa-Carrageenan Gel Immobilisation of Lager Brewing Yeast, *J. Insi. Brew. 105*, 398-404.
10. Almonacid, S. F., Nájera, A. L., Young, M. E., Simpson, R. J., and Acevedo, C. A. (2012) A Comparative Study of Stout Beer Batch Fermentation Using Free and Microencapsulated Yeasts, *Food and Bioprocess Technology 5*, 750-758.

11. Smogrovicová, D., Dömény, Z., Gemeiner, P., Malovikova, A., and Sturdik, E. (1997) Reactors for continuous primary beer fermentation using immobilised yeast, *Biotechnol Tech 11*, 261-264.

12. Dömény, Z., Smogrovicová, D., Gemeiner, P., Sturdík, E., Pátková, J., and Malovíková, A. (1998) Continuous secondary fermentation using immobilised yeast, *Biotechnology letters 20*, 1041-1045.

13. Bester, M. C., Jacobson, D., and Bauer, F. F. (2012) Many Saccharomyces cerevisiae Cell Wall Protein Encoding Genes Are Coregulated by Mss11, but Cellular Adhesion Phenotypes Appear Only Flo Protein Dependent, *G3 (Bethesda) 2*, 131-141.

14. Kurec, M., and Brányik, T. (2011) The role of physicochemical interactions and FLO genes expression in the immobilization of industrially important yeasts by adhesion, *Colloids and surfaces. B, Biointerfaces 84*, 491-497.

15. Stratford, M. (1996) Induction of flocculation in brewing yeasts by change in pH value, *FEMS Microbiol. Lett. 136*, 13-18.

16. Stratford, M., and Carter, A. T. (1993) Yeast flocculation: lectin synthesis and activation, *Yeast 9*, 371-378.

17. Eddy, A. A. (1955) Flocculation characteristics of yeasts II. Sugars as dispersing agents., *J. Inst. Brew. 61*, 313-317.

18. Powell, C. D., Quain, D. E., and Smart, K. A. (2003) The impact of brewing yeast cell age on fermentation performance, attenuation and flocculation, *FEMS yeast research 3*, 149-157.

19. Panteloglou, A. G., Smart, K. A., and Cook, D. J. (2012) Malt-induced premature yeast flocculation: current perspectives, *Journal of industrial microbiology & biotechnology 39*, 813-822.

20. Chisti, Y., and Moo-Young, M. (1989) On the calculation of shear rate and apparent viscosity in airlift and bubble column bioreactors, *Biotechnology and bioengineering 34*, 1391-1392.

21. Kaeberlein, M. (2010) Lessons on longevity from budding yeast., *Nature 464*, 513–519.

22. Mortimer, R. K., and Johnston, J. R. (1959) Life span of individual yeast cells, *Nature 183*, 1751–1752.

23. Österlund, T., Nookaew, I., Bordel, S., and Nielsen, J. (2013) Mapping condition-dependent regulation of metabolism in yeast through genome-scale modeling, *BMC Systems Biology*, 36.

24. Muller, I., Zimmermann, M., Becker, D., and Flomer, M. (1980) Calendar life span versus budding life span of Saccharomyces cerevisiae, *Mechanisms of ageing and development 12*, 47-52.

25. Powell, C. D., Van Zandycke, S. M., Quain, D. E., and Smart, K. A. (2000) Replicative ageing and senescence in Saccharomyces cerevisiae and the impact on brewing fermentations, *Microbiology 146*, 1023–1034.

26. Deans, K., Pinder, A., Catley, B. J., and Hodgson, J. A. (1997) Effects of cone cropping and serial repitch on the distribution of cell ages in brewery yeast., *Proc Eur Brew Con 26*, 469-476.

27. Gibson, B. R., Lawrence, S. J., Leclaire, J. P., Powell, C. D., and Smart, K. A. (2007) Yeast responses to stresses associated with industrial brewery handling, *FEMS microbiology reviews 31*, 535-569.

28. Puligundla, P., Smogrovicova, D., Obulam, V. S., and Ko, S. (2011) Very high gravity (VHG) ethanolic brewing and fermentation: a research update, *Journal of industrial microbiology & biotechnology 38*, 1133-1144.

29. Hasegawa, S., Ogata, T., Tanaka, K., Ando, A., Takagi, H., and Shima, J. (2012) Overexpression of vacuolar H+-ATPase-related genes in bottom-fermenting yeast enhances ethanol tolerance and fermentation rates during high-gravity fermentation, *J. Inst. Brew. 118*, 179–185.

30. Lei, H., Zheng, L., Wang, C., Zhao, H., and Zhao, M. (2013) Effects of worts treated with proteases on the assimilation of free amino acids and fermentation performance of lager yeast, *Int J Food Microbiol 161*, 76-83.

31. Ehrlich, F. (1907) Über die Bedingungen der Fuselölbildung und über ihren Zusammenhang mit dem Eiweissaufbau der Hefe, *Ber. Dtsch. Chem. Ges. 40*, 1027-1047.

32. Zhang, C., Liu, Y., Qi, Y., Zhang , J., Dai, L., Lin, X., and Xiao, D. (2013) Increased esters and decreased higher alcohols production by engineered brewer's yeast strains, *Eur. Food. Res. Technol. 236*, 1009-1014.

33. Saerens, S. M., Verbelen, P. J., Vanbeneden, N., Thevelein, J. M., and Delvaux, F. R. (2008) Monitoring the influence of high-gravity brewing and fermentation temperature on flavour formation by analysis of gene expression levels in brewing yeast, *Applied microbiology and biotechnology 80*, 1039-1051.

34. Verstrepen, K. J., Van Laere, S. D., Vanderhaegen, B. M., Derdelinckx, G., Dufour, J. P., Pretorius, I. S., Winderickx, J., Thevelein, J. M., and Delvaux, F. R. (2003) Expression levels of the yeast alcohol acetyltransferase genes ATF1, Lg-ATF1, and ATF2 control the formation of a broad range of volatile esters, *Applied and environmental microbiology 69*, 5228-5237.

35. Fujii, T., Kobayashi, O., Yoshimoto, H., Furukawa, S., and Tamai, Y. (1997) Effect of aeration and unsaturated fatty acids on expression of the Saccharomyces cerevisiae alcohol acetyltransferase gene, *Applied and environmental microbiology 63*, 910-915.

36. Malcorps, P., Cheval, J. M., JamilS., and Dufour, J.-P. (1991) A new model for the regulation of ester synthesis by alcohol acetyltransferase in Saccharomyces cerevisiae, *J. Am. Soc. Brew. Chem. 49*, 47-53.

37. Anderson, R. G. a. K., B.H. (1975) Quantitative aspects of the control by oxygenation of acetate ester formation of worts of high specific gravity, *J. Inst. Brew. 81*, 269-301.

38. Anderson, R. G. a. K., B.H. (1975) Oxygen as a regulator of ester accumulation during the fermentation of worts of high specific gravity, *J. Inst. Brew. 81*, 111-115.

39. Anderson, R. G. a. K., B.H. (1974) The control of volatile ester synthesis during the fermentation of wort of high specific gravity, *J. Inst. Brew. 80*, 48-55.

40. van Meer, G., Voelker, D. R., and Feigenson, G. W. (2008) Membrane lipids: where they are and how they behave, *Nature reviews. Molecular cell biology 9*, 112-124.

41. Hazelwood, L. A., Daran, J. M., van Maris, A. J., Pronk, J. T., and Dickinson, J. R. (2008) The Ehrlich pathway for fusel alcohol production: a century of research on Saccharomyces cerevisiae metabolism, *Applied and environmental microbiology 74*, 2259-2266.

42. Sentheshanuganathan, S. (1960) The mechanism of the formation of higher alcohols from amino acids by Saccharomyces cerevisiae, *Biochem J 74*, 568-576.

43. Oshita, K., Kubota, M., Uchida, M., and Ono, M. (1995) Clarification of the relationship between fusel alcohol formation and amino acid assimilation by brewing yeast using 13C-labeled amino acid, In *25th European Brewery Convention Congress*, pp 387–402.

44. Ryder, D., and Masschelein , C. (1985) The growth process of brewing yeast and the biotechnological challenge., *J Am Soc Brew Chem 43*, 66-75.

45. Smogrovicová, D., and Dömény, Z. (1999) Beer volatile by-product formation at different fermentation temperature using immobilized yeasts., *Process Biochem 34*, 785–794.

46. Mensour, N., Margaritis, A., Briens, C., Pilkington, H., and Russell, I. (1997) New developments in the brewing industry using immobilised yeast cell bioreactor systems., *J Inst Brew 103*, 363-370.

47. Pajunen, E., Tapani, K., Berg, H., Ranta, B., Bergin, J., Lommi, H., and Viljava, T. (2001) Controlled beer fermentation with continuous on-stage immobilized yeast reactor., In *28th EBC Congress*, pp Chap. 49, 41-12.

48. Engan, S. (1981) Beer composition: Volatile substances, In *Brewing Science* (J. R. A. Pollock, e., Ed.), pp 93-165, Academic Press, London.

49. Meilgaard, M. C. (1975) Flavour chemistry of beer. Part II: Flavor and threshold of 239 aroma volatiles, *MBAA, Techn. Quart. 12*, 151-168.

50. Engan, S. (1972) Organoleptic threshold values of some alcohols and esters in beer., *J Inst Brew 78*, 33-36.

51. Smogrovicová, D., Dömény, Z., Navrátil, M., and Dvorák, P. (2001) Continuous beer fermentation using polyvinyl alcohol entrapped yeast, In *28th EBC Congress*, pp Chapter 50, pp. 51-59

52. Virkajärvi, I., and Kronlöf, J. (1998) Long-term stability of immobilized yeast columns in primary fermentation., *J Am Soc Brew Chem 56*, 70-75.

53. Andersen, K., Bergin, J., Ranta, B., and Viljava, T. (1999) New process for the continuous fermentation of beer, In *27th European Brewery Convention Congress EBC*, pp 771-778.

54. Kronlöf, J., and Virkajärvi, I. (1999) Primary fermentation with immobilized yeast, In *27th EBC Congress*, pp 761-770.

55. Virkajarvi, I., and Pohjala, N. (2000) Primary fermentation with immobilized yeast: Some effects of Carrier Materials on the Flavour of the Beer *J. Insi. Brew. 106*, 311-318.

56. Kronlöf, J., Härkönen, T., Hartwall, P., Home, S., and Linko, M. (1989) Main fermentation with immobilized yeast, In *European Brewery Convention Congress*, pp 355–362.

57. Dillemans, M., Goossens, E., Goffin, O., and Masschelein, C. (1987) The amplification effect of the ILV5 gene on the production of vicinal diketones in Saccharomyces cerevisiae., *J Am Soc Brew Chem 45*, 81-84.

58. Krogerus, K. a., and Gibson, B. R. (2013) Diacetyl and its control during brewery fermentation, *J. Inst. Brew. 119*, 86-97.

59. Pugh, T., Maurer, J., and Pringle, A. (1997) The impact of wort nitrogen limitation on yeast fermentation performance and diacetyl., *Technol Quart MBAA 34*, 185-189.

60. Barton, S., and Slaughter, J. (1992) Amino acids and vicinal diketone concentrations during fermentation., *Technol Quart MBAA 29*, 60-63.

61. Magee, P. T., and Robichon-Szulmajster, H. (1968) The regulation of isoleucine-valine biosynthesis in Saccharomyces cerevisiae. 3. Properties and regulation of the activity of acetohydroxyacid synthetase, *European journal of biochemistry / FEBS 3*, 507-511.

62. Vanderhaegen, B., Neven, H., Coghe, S., Verstrepen, K. J., Derdelinckx, G., and Verachtert, H. (2003) Bioflavoring and beer refermentation, *Applied microbiology and biotechnology 62*, 140-150.

63. Debourg, A., Laurent, M., Dupire, S., and Masschelein, C. (1993) The specific role and interaction of yeast enzymatic systems in the removal of flavour-potent wort carbonyls during fermentation., In *Eur Brew Conv*, pp 437-444.

64. van Iersel, M. F., Eppink, M. H., van Berkel, W. J., Rombouts, F. M., and Abee, T. (1997) Purification and characterization of a novel NADP-dependent branched-chain alcohol dehydrogenase from Saccharomyces cerevisiae, *Applied and environmental microbiology 63*, 4079-4082.

65. Van Nedervelde, L., Verlinden, V., Philipp, D., and Debourg, A. (1997) Purification and characterization of yeast 3-methyl butanal reductases involved in the removal of wort carbonyls during fermentation., *Process Biochem 32*, 447-454.

Chapter 8

Continuous beer fermentation – what is missing?

A primary continuous beer fermentation was again performed, but this time with improved conditions to controlling key parameters as it was carried at the laboratories of UNICER. The aim of this chapter was to show that good quality beer could be produced in the setup used in Chapter 7.

8.1 Abstract

This work tested the viability of producing beer of good quality after maturation of green beer obtained by primary continuous fermentation of high gravity wort using an airlift bioreactor with flocculated biomass. Fermentation performance of the tested setup was unique as it reached a maximum saccharide consumption rate of 9.43 $g.L^{-1}.h^{-1}$ and ethanol productivity of 3.75 $g.L^{-1}.h^{-1}$. Despite the high levels of diacetyl present in the green beer, a regular maturation was able to reduce it below threshold values in up to 15 days. It was observed that diacetyl production was deeply correlated with wort composition injected into the system, rather than with the large amount of biomass immobilized in the bioreactor (up to 727 x 10^6 $cell.mL^{-1}$). Organoleptic tests showed that the maturated beer had no major defects.

Keywords: Continuous beer fermentation, beer maturation, diacetyl.

8.2 Introduction

Large breweries are constantly investing in new technologies to increase productivity, being high-gravity brewing (HGB) the most widespread. However, continuous beer fermentation can be far more productive than HGB. Despite a few disadvantages concerning HGB have been identified, such as yeast stress affecting cell physiology (*1*) and unbalanced aromatic profile (*2, 3*), the cost-benefit associated with this technology is certainly favorable. Moreover, for today's large breweries, accelerating processes by using higher fermentation temperatures and yeast selection is an established reality that may provide the final product in less than 20 days. Nevertheless, continuous fermentation can provide a finished beer in a period as short as 3 days (*4*). Although continuous fermentation shares some common disadvantages with HGB, the radical change in fermentation process and the associated costs has hindered the decision to step forward with this technology. Thus, to counterbalance the drawbacks, continuous fermentation setup should offer enough advantages that would justify its application.

The first decision to make concerns the bioreactor type. Airlift reactors are remarkably efficient on mass transfer for either liquid-liquid or solid-liquid phases triggered by rapid mixing, low shear stress, simple design and low energy consumption (*5*). These qualities have elected airlifts as being the reactor of choice for primary continuous beer fermentation (*6-8*). Another decisive parameter to choose regards to the yeast immobilization method. Despite several yeast carriers have been suggested so far (*7-12*), just few were considered for scale-up application due to either their cost or problems affecting the final beer (*4, 13*). Yet, the only free-of-charge way of immobilizing yeast in a bioreactor relies on self-aggregation of cells – flocculation.

As continuous fermentations are characterized by constant supply of amino acids, sugars, oxygen and other nutrients into the bioreactor, the course of metabolic routes of the yeast cells is naturally changed. For example, the large availability of amino acids can increase higher alcohols production through the Ehrlich pathway (*14*). Accordingly, that incessant supply may trigger a preferential uptake of some amino acids in detriment of others with lower uptake rates like valine or isoleucine (*15, 16*). The affected uptake of valine will ultimately divert metabolism to its synthesis *de novo*, increasing the bulk of α-acetolactate - diacetyl precursor (*15*). Also, a deliberate feed of oxygen may inhibit directly the expression of genes encoding crucial alcohol acetyltransferases responsible for catalyzing the synthesis of precious acetate esters (*17, 18*).

This work evaluated the development of aromatic profile of beers obtained after maturation of green beer produced by primary continuous fermentation using flocculated yeast in a high cell density airlift bioreactor. A key element in the current work was to assess the time required for diacetyl reduction (through regular maturation) in beer produced under continuous operation.

8.3 Material and Methods

8.3.1 Brewing Yeast

The industrial strain of *Saccharomyces pastorianus* was provided by UNICER Bebidas, S.A. (S. Mamede de Infesta, Portugal). Approximately 1.5 L of yeast slurry was harvested from the storage tank, suspended in 3 L of Pilsner wort (≈ 16º Plato), and filled into the airlift reactor.

8.3.2 Wort

The all grain wort used in the current experiment was supplied by UNICER Bebidas, S.A. (S. Mamede de Infesta, Portugal). The wort was collected from the main production line after cooling stage and previous to aeration. It was filled into pre-autoclaved 20 L barrels (Nalgene, USA) and stored at 2 ºC during continuous injection to the bioreactor. Two Pilsner wort recipes were used and a detailed composition of each is presented in Table 8.1.

8.3.3 Continuous fermentation

Continuous fermentation was carried out in a Perspex airlift bioreactor with 4 L of total work volume. Gas flow was regulated by two independent mass controllers (Alicat Scientific, USA) using CO_2 (200 mL.min^{-1}) and pressurized air (10 mL.min^{-1}). This mixture and flow were kept constant in the course of the entire experiment. Temperature was held at 15 ºC using a JULABO F32 Refrigerated/Heating Circulator (JULABO, Germany). The dilution rate was controlled by a peristaltic pump 101U/R (Watson-

Table 8.1 - Composition of feeding wort[13]

Wort	SG (°P)	pH	Color (EBC)	EBU	FAN (mg.L^{-1})
Pilsner 1 (P1)	15.92	4.93	11.9	28	206
Pilsner 2 (P2)	13.41	5.07	14.5	29	242

SG – Specific gravity; °P – Plato degree; EBC – European Brewery Convention; EBU - European Bitterness Units; FAN – Free-amino nitrogen

Marlow, UK). Figure 8.1 shows an overview of the setup used.

The reactor was sterilized by bleaching, using a 3% (v/v) solution of commercial sodium hypochlorite with 1.5% of active chlorine, 48 h previous to use. After this time, the solution was discarded and sterile water was used for washing the reactor. Thereafter, it was filled with the mixture of yeast slurry and 16 °P P1 wort as reported above.

Batch growth was kept until complete real attenuation (> 67%) was achieved (≈ 24 h). Afterwards, continuous phase has been started with a total retention time (RT_{tot}) of 23.67 h and yeast cell count of 3.8 x 10^8 cell.mL^{-1}. Beer produced in the first 24 h of continuous fermentation was discarded as it regarded to the beer produced over batch mode.

8.3.4 Beer maturation

Green beer was stored in a ball lock keg of 20 L capacity. It was placed at the same refrigerator (2 °C) as the wort barrel was, receiving the green beer through the silicon tubing right from the airlift outflow (Fig. 8.1). From the keg, green beer was filled into 0.33 L glass bottles helped by a counter pressure bottle filler, which ensures little/no oxygen makes contact with the beer. This process was carried out in two lots of bottles for P1 (P1 and P1'), while P2 was maturated in the own keg. After bottling/locking the keg beer was settled in a cooling chamber (6 °C) for maturation.

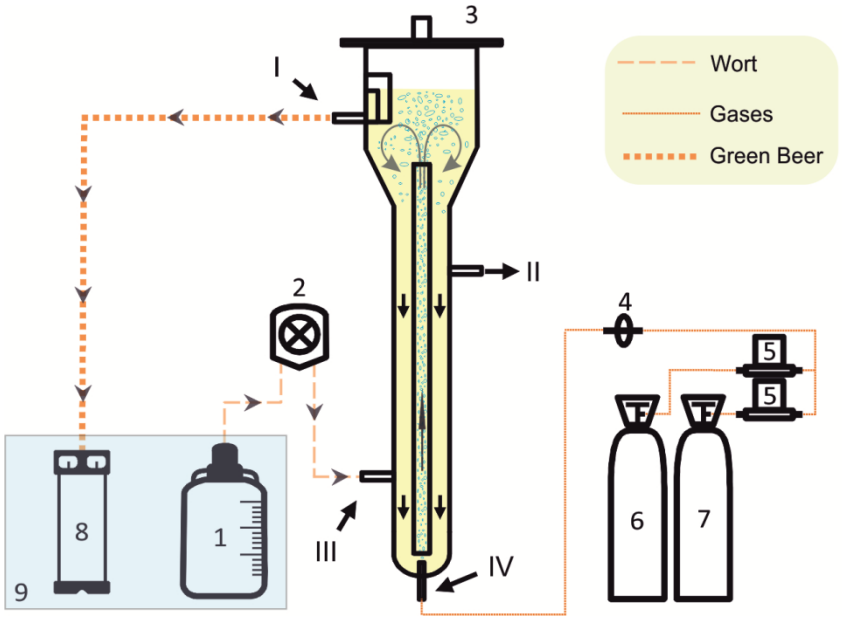


Figure 8.1 - Immobilized yeast bioreactor system used in the current work: 1 - wort barrel; 2 - peristaltic pump; 3 - airlift reactor; 4 - air filter; 5 - flow mass controllers; 6 - pressurized air; 7 - CO_2 bottle; 8 - ball lock keg as green beer storage; 9 - refrigerator at 2 °C; I - green beer outflow/sampling point; II - biomass sampling point; III - wort inlet; IV - gas sparger.

8.3.5 Cell count

Yeast immobilized in the airlift reactor was measured daily by an automated cell counter - Z2 (Beckman Coulter, Inc., USA). Samples from the reactor were treated as yeast slurry (due to the high cell count) and thus serially diluted following the manufacturer's instructions. Briefly, 1 g of yeast suspension from the airlift was placed into a 100 mL volumetric flask, plus 1 mL of sulfuric acid 2 $mol.L^{-1}$ (to separate budding yeasts/flocs) and diluted with ISOTON II diluent (Beckman Coulter, Germany). This dilution was followed by two other dilutions (5/50 and 4/200 mL) before cell counting. Yeast suspended in maturating bottles was directly diluted (400 µl/200 mL) and analyzed.

8.3.6 Beer and wort analysis

Characterization of wort, green and maturated beer was done as suggested in the present Analytica EBC methods. All reagents were purchased from Merck (Germany).

Alcohol, specific gravity, original and real extract, pH, colour and degree of fermentation were performed by the automatic beer analyzer Anton Paar Beer Analysis System DMA 4500 (Anton Paar GmbH, Austria).

Total diacetyl was determined by gas chromatographic analyses of the static headspace using a Varian CP-3800 gas chromatograph (GC) (Varian, USA). Separation was carried by a Wall Coated Open Tubular (WCOT) fused silica capillary column (60 m x 0.25 mm) CP-Sil 8 CB low bleed/ms (Varian, Netherlands) using nitrogen as carrier gas. Samples were automatically injected by a Combi PAL headspace autosampler (CTC Analytics, Switzerland).

Acetaldehyde, higher alcohols and esters were measured by Varian star 3400 CX GC (Varian, USA) with a flame ionization detector (FID) and a thermoconductivity detector (TCD). The volatiles were separated by a WCOT fused silica capillary column (60 m x 0.53 mm) CP-Wax 52 CB (Varian, Netherlands) using pure air and hydrogen as carrier. Samples were automatically injected by a Combi PAL headspace autosampler (CTC Analytics, Switzerland).

The free-amino nitrogen (FAN) and bitterness results were measured in a Varian Spectrophotometer Cary 50 Bio UV-visible (Varian, USA).

8.4 Results and Discussion

8.4.1 High pitching rates – is it really a threat to beer quality?

As a general rule, all immobilized cell technology (ICT) based systems for continuous fermentation are characterized by high concentration of yeast in the bioreactor. This abnormal amount of yeast is then ready to incessantly process the inlet wort into beer. In the current work for example, the maximum immobilized biomass obtained through flocculation was 727 x 10^6 cells.mL^{-1}. Flocculation has been avoided as a way of biomass immobilization on ICT systems as it can be affected by many parameters, such as nutrient availability, agitation, calcium levels, pH, temperature, yeast handling and storage conditions (*19-21*). However, as Table 8.2 shows, biomass accumulation by flocculation presented an increasing trend throughout the whole experiment, unaffected by altering process conditions. This tendency was also observed in a previously performed long-term (56 days) experiment (unpublished data). Traditional batch fermentations on the other hand use around 10-20 x 10^6 cells.mL^{-1} as initial pitching rate that will reach nearly 70 x 10^6 cells.mL^{-1} after exponential growth. As ICT systems use far greater concentrations of yeast, they often express the biomass concentration in dry weight of yeast per volume. Nonetheless, the estimate (10^9 cells.mL^{-1} is equivalent to 50 $g_{dry\ wt}$.L^{-1}) made by Šmogrovičová and coworkers (*8*) can be used for comparison. Thus, each 1 $g_{dry\ wt}$.L^{-1} would correspond approximately to 20 x 10^6 cells.mL^{-1}. Yeast entrapment methods have been highlighted as being the best techniques for attaining extremely high cell loadings (*4*). These methods normally use between 10^7 to 10^9 cells.$mL_{gel\ matrix}^{-1}$ (*8, 10, 22*). Yet, when the beads (biocatalysts) are introduced in the bioreactor, the total immobilized biomass concentration will drop to the same the extent of the solid loading capacity of the system. Tata and coworkers (*10*) compared a couple

Table 8.2 – Continuous fermentation parameters[14]

Time* (h)	RT_{tot} (h)	Yeast Cells (10^8 cells.mL^{-1})	Real Att. (%)	SLR	r_s	r_p	FLR	FCR
				(g.L^{-1}.h^{-1})			(mg.L^{-1}.h^{-1})	
48	23.67	3.53	70	7.15	5.94	2.36	8.70	6.00
72	19.70	4.04	71	8.59	7.22	2.87	10.46	7.46
96	17.24	5.22	71	9.81	8.64	3.43	11.95	8.24
120	15.10	5.70	70	11.21	9.43	3.75	13.64	9.34
192	15.10	7.27	70	9.35	7.93	3.14	16.03	9.21

* Continuous fermentation time including the 24 h washout needed to get rid of the green beer produced during batch fermentation. (RT_{tot}) total retention time; (*SLR*) saccharide loading rate; (r_s) saccharide consumption rate; (r_p) ethanol productivity; (*FLR*) free-amino nitrogen loading rate; (*FCR*) free-amino nitrogen consumption rate.

of two-stage systems for continuous beer production. Each system consisted of two reactors connected in series: two fluidized bed reactors with porous glass beads for cell immobilization; and two loop reactors with a silicon carbide cartridge for yeast load. The maximum loading capacity of yeast immobilization reported by the authors for each of these systems was around 594 and 364 x 10^6 cells.mL^{-1}, respectively. The most promising carrier for cell adhesion was suggested by Brányik and coworkers (*23*). The carrier obtained from brewer´s spent grains (BSG) was used in many experiments with promising results (*6, 7, 24*). However, despite being simpler and cheaper, the maximum cell loading capacity of systems using BSG (≈300 x 10^6 cells.mL^{-1}) is lower than that of entrapment-based systems (*23*, Chapter 5). The current flocculation-based system proved to be extremely efficient not only in cell loading capacity but also in production rates. The absence of gel matrices of carrier particles may be responsible for the increased productivity, triggered by better mass and heat transfers.

The increased productivity rate resulting from higher amounts of yeast either for batch or continuous setups is unquestionable (*6, 8, 20, 25, 26*). Accordingly, the present study obtained uniquely high values of saccharide consumption rate (r_s = 9.43 g.L^{-1}.h^{-1}) and ethanol productivity (r_p = 3.75 g.L^{-1}.h^{-1}), higher than any other setup for continuous primary beer fermentation. Additionally, the productivity of the current setup was potentially greater as real attenuation was always above 70% even when increasing rates of saccharide loading (*SLR*) were used (Table 8.2). Even under such extreme conditions, the final beer produced had many parameters around the expected values for lager beers and no major defects affecting the aroma profile (*27*). Although increased pitching rates have been identified as detrimental for beer quality (*4, 20*), the demand for higher productivities led to efforts to demystify this (*25, 26*). For example, not long ago, Verbelen and coworkers (*28*) proved that the negative impact impaired by increasing pitching rates in batch fermentations was yeast strain-dependent. Thus it would be possible to take advantage of higher production rates by selecting the right yeast strain. In a further study, Verbelen and coworkers (*26*) also concluded that increasing pitching rates only cause minor changes in beer´s flavour-active constituents, with exception of diacetyl. As in these batch experiments, the only real problem observed in the current work that required closer attention was diacetyl levels. Yet, there is evidence that the problem does not necessarily regard to the amount of immobilized yeast, as further discussed.

As the storage keg in this study (Fig. 8.1) also worked as sedimentation tank for the excess yeast that came through the outflow, the yeast present in the P1 bottles for maturation was in acceptable amount (≈ $6x10^6$ cells.mL^{-1}). However, as P2 maturation occurred inside the own keg, some of the excess yeast could have started autolysis releasing esterases in the maturating beer. This fact could explain the decrease in isoamyl acetate values observed during maturation (Fig. 8.2). Horsted and coworkers (*29*) and Neven and coworkers (*30*) showed that the excretion of esterases leads to a considerable change of the ester profile of beer and the most affected esters are isoamyl acetate, ethyl hexanoate and ethyl octanoate. Taking these facts into account, continuous beer produced in this experiment was of excellent quality. Indeed, beer was blind tasted by a small panel (four people) of professionals at UNICER. The tasting was carried out with the concentrated version (not diluted to match 5.2% ethanol) of beers shown in Table 8.3. Continuous P1 beer was considered of very good quality and was (overall) considered as similar to the batch produced ones. On the other hand, as diacetyl level of concentrated P2 was 0.09 mg.mL^{-1}, it could be perceived by all professionals, causing a negative impression. However, few days later and without the interference of diacetyl, P2 presented a very pleasant (flower predominant) aroma, good taste and aspect.

If there is a key parameter affecting continuous beer, this would be the FAN

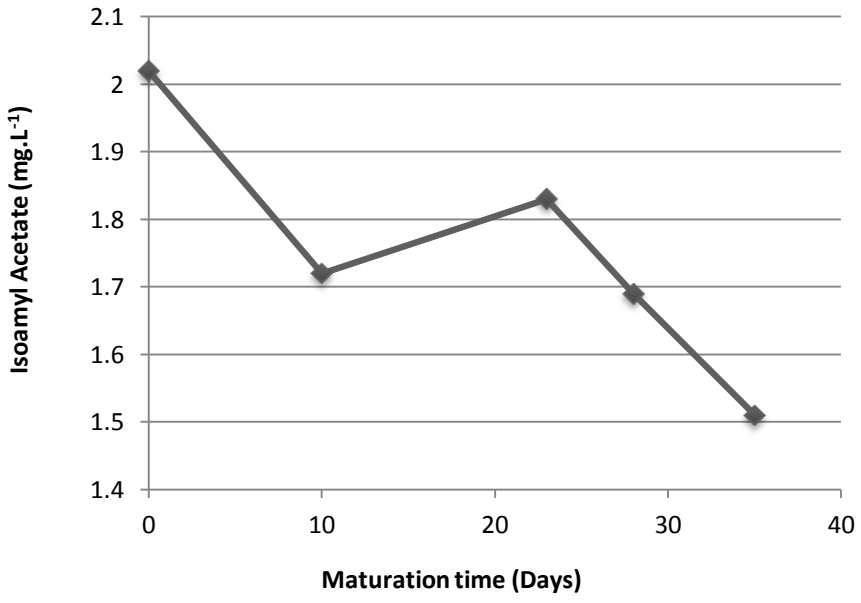


Figure 8.2 – Decrease observed in isoamyl acetate concentrations during Pilsner 2 maturation inside the keg.

Table 8.3 – Main parameters of finished beer (recalculated for 5.2% of ethanol) produced under primary continuous fermentation, compared to batch fermented beers of the same type.

Compound (mg.L^{-1})	P2 Keg	P2 CC1	P2 CC2	P1	P1'	P1 CC1	P1 CC2
FAN	78.08	83.31	78.94	49.03	43.04	89.18	76.38
Diacetyl	0.07	0.03	0.03	0.03	0.02	0.05	0.04
Acetaldehyde	3.79	2.84	1.85	3.43	3.61	8.70	8.94
n-Propanol	29.47	44.56	33.45	32.01	34.01	21.49	20.31
Isobutanol	14.49	15.04	17.58	20.61	17.60	12.19	14.59
Amyl alcohols	89.42	92.45	92.54	82.90	86.03	75.32	79.60
Total Higher Alcohols	133.39	152.05	143.57	135.53	137.64	109.00	114.51
Ethyl Acetate	27.74	15.77	16.82	13.63	17.15	18.37	20.17
Isoamyl Acetate	1.32	1.89	1.89	0.55	0.82	1.48	1.62
Total Esters	29.06	17.65	18.71	14.18	17.97	19.86	21.79
A/E	3.58	7.55	6.59	6.60	5.32	3.60	3.35

(P2) Pilsner 2; (P1) Pilsner 1; (CC) Cylindroconical industrial reactors; Shaded columns refer to beer produced by continuous fermentation; (A/E) Higher alcohols to esters ratio.

consumption rate (FCR). The environment provided by the current setup (strong agitation, oxygen and high organic loading rate) was very favorable for increased yeast metabolism. This fact results in increased *FCR* and consequently increased synthesis of higher alcohols through the Ehrlich pathway (14). As a consequence, the final beer has normally lower FAN levels and increased amounts of fusel alcohols (Table 8.3). This fact was evident for P1 but not for P2, which could be explained by different FAN composition triggered by the difference between the two recipes. This data also suggests that FCR in continuous fermentation can be controlled by altering the amino acid composition of the fermenting wort. In fact the uptake rate of amino acids is controlled by the presence of preferred amino acids through nitrogen catabolite repression (NCR) (31). In other words, some amino acids will impair the intake of others until they are completely exhausted from the wort. Recently, Lei and coworkers (32) have shown that FCR increased with increasing specific gravity of the wort. This is also in agreement with the fact that the brewing yeast needs more assimilable nitrogen to handle intense stress conditions (*33*). These data also support the increased ratio (0.68) between *FCR* and FAN loading rate (*FLR*) experienced during P1 wort (≈ 16° Plato) inlet, when compared to *FCR*/*FLR* ratio (0.57) of P2 (≈ 13.4° Plato). Yet, while FAN values for P1 (49 and 43 mg.L^{-1}) could for instance affect foam stability, higher alcohols were in the

expected range of concentrations. On the other hand, yeast entrapment techniques often limit cell proliferation and activity, due to low mass transfer rates within the matrices. This condition results in deficient FAN consumption and also impairs unbalanced flavour profile to the finished beer (*4*). Additionally, recent findings have shown the crucial role of FAN consumption rate in diacetyl formation (*15, 16*).

8.4.2 Diacetyl – Still a villain!

The main problem affecting beers produced by (either batch or continuous) accelerated fermentations obtained through increased pitching rates is still diacetyl (*6-8, 12, 13, 15, 16*). Taking the current work as a clear example, the time saved by primary continuous fermentation would not be justified by 10-20 days to get rid of diacetyl (Fig. 8.3). It is good to remember that commercial breweries already have enough technology and know-how to produce a finished product (from the grain to the bottle) in less than 20 days. Whilst continuous maturation systems have been suggested (*12, 34-36*), the main challenging task is to put together both continuous primary fermentation and continuous maturation (*37*). As a matter of fact, only continuous maturation and alcohol-free beer production are fully implemented in industrial scale (*4*). That is because primary fermentation is far more complex as it is responsible in a large extent for producing the great majority of the final beer constituents like ethanol, higher alcohols and esters; as well as to the synthesis of all diacetyl that must be further reduced through maturation.

Although both 2,3-butanedione (diacetyl) and 2,3-pentanedione are formed during fermentations carried out by *Saccharomyces spp.*, it is the former that is regarded as being the key tracer for beer maturation. This happens because diacetyl has approximately ten times lower flavour threshold than 2,3-pentanedione and if present in concentrations higher than 0.1 $mg.L^{-1}$ (*27*), it will affect lager beers with an unpleasant sweetish buttery off-flavour. Accordingly, the current work used diacetyl as maturation

Table 8.4 – Profile of green beer sampled from the airlift outflow[16]

Time* (h)	SG (°P)	ABV (%)	Real Att. (%)	pH	Color (EBC)	EBU	FAN ($mg.L^{-1}$)	Diacetyl ($mg.L^{-1}$)
48	15.61	7.13	70	3.97	12.9	19	64	1.18
72	15.53	7.22	71	3.81	10.7	14	59	1.23
96	16.26	7.55	71	3.84	10.9	16	64	0.93
120	15.84	7.22	70	3.94	10.4	24	65	1.10
192	13.39	6.04	70	4.19	11.8	21	103	1.70

* Continuous fermentation time including the 24 h washout needed to get rid of the green beer produced during batch fermentation. SG – Specific gravity; ABV – Alcohol by volume; °P – Plato degree; EBC – European Brewery Convention; EBU - European Bitterness Units; FAN – Free-amino nitrogen

tracer for the beer produced by primary continuous fermentation through the regular maturation in bottles or keg. As diacetyl is also a by-product of intense cell division (favored by the conditions offered by the current setup) the green beer from the airlift outflow had also the highest peaks of diacetyl concentration (Table 8.4). This scenario drastically changes inside the storage keg and bottles where growth is arrested and anaerobiosis prevails. While P1 beers reached maturation in 15 days after bottling, it took P2 28 days to reach diacetyl values below the threshold (Fig. 8.3).

Diacetyl is formed outside yeast cells by spontaneous non-enzymatic oxidative decarboxylation of α-acetolactate, which in turn is also intermediate for the *de novo* synthesis of valine. The accumulation of α-acetolactate inside the yeast cell is rate-limiting for the biosynthesis of that amino acid and therefore it is excreted (*38*). It has been demonstrated that worts with lower FAN contents produce less diacetyl during batch fermentations (*15, 16, 39*). However, FAN values below 122 $mg.L^{-1}$ trigger the opposite response (*39*). Furthermore, Lei and coworkers (*32*) have shown that the intake of valine decreased with increasing FAN content. Those authors attributed the rise of available FAN to induce yeast in the absorption of preferred amino acids rather than valine. Bearing in mind that valine acts as feedback inhibitor of ILV2-encrypting acetohydroxy acid synthase (AHAS - that catalyzes the irreversible conversion of

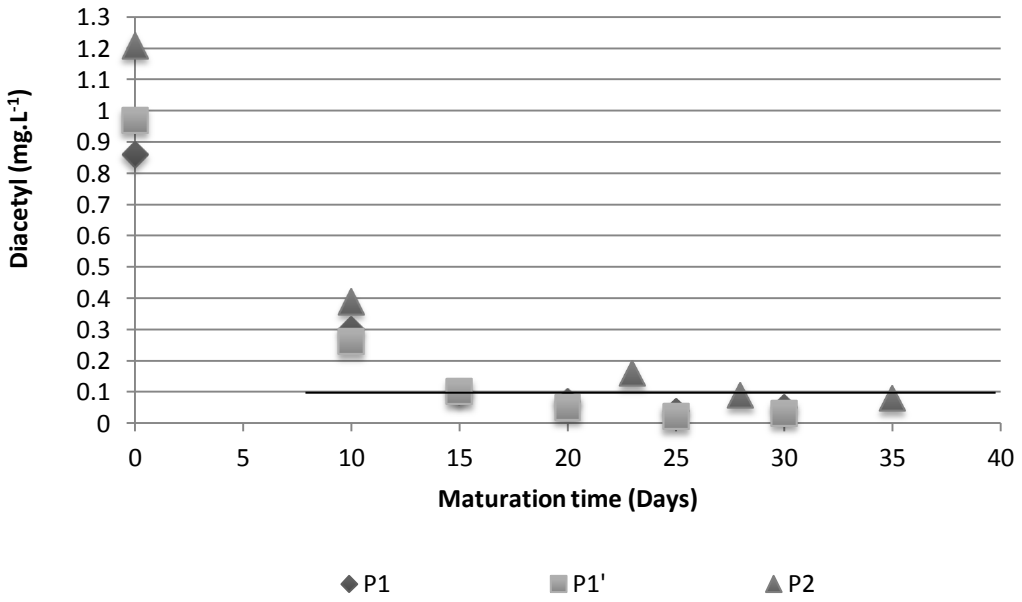


Figure 8.3 – Dynamics of diacetyl reduction from the beers produced under continuous primary fermentation during regular maturation. The line at 0.1 $mg.L^{-1}$ marks the threshold of diacetyl for lager beers (*27*). (P1) Pilsner 1; (P2) Pilsner 2.

pyruvate to α-acetolactate), the lack of this amino acid inside the yeast will deliberately stimulate α-acetolactate synthesis and hence diacetyl formation (*40, 41*). Therefore, the increased diacetyl production during the inlet of P2 wort, could be explained by impaired valine intake triggered by increased availability of preferred amino acids. These pieces of evidence may provide valuable insights for future works regarding continuous fermentation with reduced production of diacetyl. Another parameter that also deserves attention regards to dissolved oxygen, as diacetyl production in ICT can be reduced by lowering the inlet of oxygen into the system (*22*).

8.5 Conclusions

The current work emphasized the feasibility of flocculation as a very promising way of yeast immobilization using an airlift bioreactor. The simple design and good performance offered in the present work may benefit new research on implementing continuous fermentations. It is worth mentioning that flocculation is not only a free of charge immobilization method, but also provides the best conditions for mass/heat transfers as well as for yeast recovery.

This work also brought evidence that the key parameter affecting quality of continuously produced beers might be *FCR*. New insights from recent literature with regard to the type of amino acids present in wort as being the main factor altering *FCR*. Thus, the implementation of an appropriate control of *FCR* in continuous fermentations would provide not only a continuous beer with a balanced aroma profile and good foam stability, but also solve the bigger and only real problem – diacetyl. Yet further research in continuous fermentations should be carried out to confirm this.

8.6 Acknowledgements

All material supplied by UNICER Bebidas de Portugal, S.A. (S. Mamede de Infesta, Portugal) as well as the technical support of company members Sonia Meireles and Helena Cunha is forever acknowledged. The financial contributions of the EU FP7 project EcoBioCAP - Ecoefficient Biodegradable Composite Advanced Packaging, Grant agreement no: 265669 as well as of the Ministry of Education, Youth and Sports of the Czech Republic (MSM 6046137305) are also gratefully acknowledged.

References

1. Gibson, B. R., Lawrence, S. J., Leclaire, J. P., Powell, C. D., and Smart, K. A. (2007) Yeast responses to stresses associated with industrial brewery handling, *FEMS microbiology reviews 31*, 535-569.
2. Saerens, S. M., Verbelen, P. J., Vanbeneden, N., Thevelein, J. M., and Delvaux, F. R. (2008) Monitoring the influence of high-gravity brewing and fermentation temperature on flavour formation by analysis of gene expression levels in brewing yeast, *Applied microbiology and biotechnology 80*, 1039-1051.
3. Lei, H., Zhao, H., Yu, Z., and Zhao, M. (2012) Effects of wort gravity and nitrogen level on fermentation performance of brewer's yeast and the formation of flavor volatiles, *Applied biochemistry and biotechnology 166*, 1562-1574.
4. Willaert, R., and Nedovic, V. A. (2006) Primary beer fermentation by immobilised yeast – a review on flavour formation and control strategies, *J Chem Technol Biotechnol 81*, 1353-1367.
5. Chisti, Y., and Moo-Young, M. (1989) On the calculation of shear rate and apparent viscosity in airlift and bubble column bioreactors, *Biotechnology and bioengineering 34*, 1391-1392.
6. Brányik, T., Vicente, A., Cruz, J., and Teixeira, J. (2004) Continuous primary fermentation of beer with yeast immobilized on spent grains - the effect of operational conditions., *J Am Soc Brew Chem 62*, 29-34.
7. Branyik, T., Silva, D. P., Vicente, A. A., Lehnert, R., e Silva, J. B., Dostalek, P., and Teixeira, J. A. (2006) Continuous immobilized yeast reactor system for complete beer fermentation using spent grains and corncobs as carrier materials, *Journal of industrial microbiology & biotechnology 33*, 1010-1018.
8. Smogrovicová, D., Dömény, Z., Gemeiner, P., Malovikova, A., and Sturdik, E. (1997) Reactors for continuous primary beer fermentation using immobilised yeast, *Biotechnol Tech 11*, 261-264.
9. Berlowska, J., Kregiel, D., and Ambroziak, W. (2013) Enhancing adhesion of yeast brewery strains to chamotte carriers through aminosilane surface modification, *World J Microbiol Biotechnol 29*, 1307-1316.
10. Tata, M., Bower, P., Bromberg, S., Duncombe, D., Fehring, J., Lau, V. V., Ryder, D., and Stassi, P. (1999) Immobilized yeast bioreactor systems for continuous beer fermentation, *Biotechnology progress 15*, 105-113.
11. Pilkington, H., Margaritis, A., Mensour, N., Sobczak, J., Hancock, I., and Russell, I. (1999) Kappa-Carrageenan Gel Immobilisation of Lager Brewing Yeast, *J. Insi. Brew. 105*, 398-404.
12. Dömény, Z., Smogrovicová, D., Gemeiner, P., Sturdík, E., Pátková, J., and Malovíková, A. (1998) Continuous secondary fermentation using immobilised yeast, *Biotechnol Lett 20*, 1041-1045.
13. Virkajarvi, I., and Pohjala, N. (2000) Primary fermentation with immobilized yeast: Some effects of Carrier Materials on the Flavour of the Beer *J. Insi. Brew. 106*, 311-318.
14. Ehrlich, F. (1907) Über die Bedingungen der Fuselölbildung und über ihren Zusammenhang mit dem Eiweissaufbau der Hefe, *Ber. Dtsch. Chem. Ges. 40*, 1027-1047.
15. Krogerus, K., and Gibson, B. R. (2013) Influence of valine and other amino acids on total diacetyl and 2,3-pentanedione levels during fermentation of brewer's wort, *Applied microbiology and biotechnology 97*, 6919-6930.
16. Krogerus, K. a., and Gibson, B. R. (2013) Diacetyl and its control during brewery fermentation, *J. Inst. Brew. 119*, 86-97.
17. Fujii, T., Kobayashi, O., Yoshimoto, H., Furukawa, S., and Tamai, Y. (1997) Effect of aeration and unsaturated fatty acids on expression of the Saccharomyces cerevisiae alcohol acetyltransferase gene, *Applied and environmental microbiology 63*, 910-915.

18. Anderson, R. G., and Kirsop, B. H. (1975) Oxygen as a regulator of ester accumulation during the fermentation of worts of high specific gravity, *J. Inst. Brew. 81*, 111-115.

19. Verstrepen, K. J., Derdelinckx, G., Verachtert, H., and Delvaux, F. R. (2003) Yeast flocculation: what brewers should know, *Applied microbiology and biotechnology 61*, 197-205.

20. Verbelen, P. J., De Schutter, D. P., Delvaux, F., Verstrepen, K. J., and Delvaux, F. R. (2006) Immobilized yeast cell systems for continuous fermentation applications, *Biotechnol Lett 28*, 1515-1525.

21. Sampermans, S., Mortier, J., and Soares, E. V. (2005) Flocculation onset in Saccharomyces cerevisiae: the role of nutrients, *Journal of applied microbiology 98*, 525-531.

22. Onaka, T., Nakanishi, K., Inoue, T., and Kubo, S. (1985) Beer brewing with immobilized yeast, *Bio/Technol. 3*, 467-470.

23. Brányik, T., Vicente, A. A., Machado Cruz, J. M., and Teixeira, J. A. (2001) Spent grains – a new support for brewing yeast immobilisation, *Biotechnol Lett 23*, 1073–1078.

24. Branyik, T., Vicente, A. A., Kuncova, G., Podrazky, O., Dostalek, P., and Teixeira, J. A. (2004) Growth model and metabolic activity of brewing yeast biofilm on the surface of spent grains: a biocatalyst for continuous beer fermentation, *Biotechnology progress 20*, 1733-1740.

25. Erten, H., Tanguler, H., and Cakıroz, H. (2007) The Effect of Pitching Rate on Fermentation and Flavour Compounds in High Gravity Brewing, *J. Inst. Brew. 113*, 75–79.

26. Verbelen, P. J., Dekoninck, T. M., Saerens, S. M., Van Mulders, S. E., Thevelein, J. M., and Delvaux, F. R. (2009) Impact of pitching rate on yeast fermentation performance and beer flavour, *Applied microbiology and biotechnology 82*, 155-167.

27. Meilgaard, M. C. (1975) Flavour chemistry of beer. Part II: Flavor and threshold of 239 aroma volatiles, *MBAA, Techn. Quart. 12*, 151-168.

28. Verbelen, P. J., Mulders, S. V., Saison, D., Laere, S. V., Delvaux, F., and Delvaux, F. R. (2008) Characteristics of High Cell Density Fermentations with Different Lager Yeast Strains, *J. Inst. Brew. 114*, 127–133.

29. Horsted, M. W., Dey, E. S., Holmberg, S., and Kielland-Brandt, M. C. (1998) A novel esterase from Saccharomyces carlsbergensis, a possible function for the yeast TIP1 gene, *Yeast 14*, 793-803.

30. Neven, H., Delvaux, F., and Derdelinckx, G. (1997) Flavor evaluation of top fermented beers, *Tech Q Master Brew Assoc Am 34*, 115-118.

31. Beltran, G., Novo, M., Rozes, N., Mas, A., and Guillamon, J. M. (2004) Nitrogen catabolite repression in Saccharomyces cerevisiae during wine fermentations, *FEMS yeast research 4*, 625-632.

32. Lei, H., Zheng, L., Wang, C., Zhao, H., and Zhao, M. (2013) Effects of worts treated with proteases on the assimilation of free amino acids and fermentation performance of lager yeast, *International journal of food microbiology 161*, 76-83.

33. Piddocke, M. P., Fazio, A., Vongsangnak, W., Wong, M. L., Heldt-Hansen, H. P., Workman, C., Nielsen, J., and Olsson, L. (2011) Revealing the beneficial effect of protease supplementation to high gravity beer fermentations using "-omics" techniques, *Microbial cell factories 10*, 27.

34. Yamauchi, Y., Okamoto, T., Murayama, H., Kajino, K., Amikura, T., Hiratsu, H., Nagara, A., Kamiya, T., and Takashi, I. (1995) Rapid maturation of beer using an immobilized yeast bioreactor. 1. Heat conversion of α-acetolactate, *J Biotechnol 38*, 101–108.

35. Pajunen, E., Gronqvist, A., and Lommi, H. (1989) Continuous secondary fermentation and maturation of beer in an immobilized yeast reactor, *Tech Q Master Brew Assoc Am 26*, 147-151.

36. Yamauchi, Y., Okamoto, T., Murayama, H., Kajino, K., Nagara, A., and Noguchi, K. (1995) Rapid maturation of beer using an immobilized yeast bioreactor. 2. Balance of total diacetyl reduction and regeneration, *J Biotechnol 38*, 109-116.

37. Branyik, T., Vicente, A. A., Dostalek, P., and Teixeira, J. A. (2005) Continuous beer fermentation using immobilized yeast cell bioreactor systems, *Biotechnology progress 21*, 653-663.

38. Dillemans, M., Goossens, E., Goffin, O., and Masschelein, C. (1987) The amplification effect of the ILV5 gene on the production of vicinal diketones in Saccharomyces cerevisiae., *J Am Soc Brew Chem 45*, 81-84.

39. Pugh, T., Maurer, J., and Pringle, A. (1997) The impact of wort nitrogen limitation on yeast fermentation performance and diacetyl., *Technol Quart MBAA 34*, 185-189.

40. Barton, S., and Slaughter, J. (1992) Amino acids and vicinal diketone concentrations during fermentation., *Technol Quart MBAA 29*, 60-63.

41. Magee, P. T., and Robichon-Szulmajster, H. (1968) The regulation of isoleucine-valine biosynthesis in Saccharomyces cerevisiae. 3. Properties and regulation of the activity of acetohydroxyacid synthetase, *European journal of biochemistry / FEBS 3*, 507-511.

Chapter 9

Conclusions and perspectives

A general conclusion of the whole thesis is presented in the next pages. This chapter also briefly discusses the future perspectives on continuous beer fermentation.

9.1 Conclusions

This thesis fulfilled all steps suggested in the original project to study continuous beer fermentation either by using LCYC from BSG or by an alternative and simpler way of yeast immobilization in the airlift bioreactor – flocculation.

If on the one hand it is clear that the setup of choice for future studies involving continuous beer fermentation (based on flocculated biomass) would abandon the LCYC obtained from the suggested chemical treatments, on the other hand it has also been discussed that BSG are rich in added-value components that deserve further exploitation. Furthermore, the suggested treatments efficiently and quickly obtained several cellulose-rich materials that could be used in a wide range of industrial applications. These materials were also successfully used as LCYC, with biomass loadings comparable to other biofilm-based carriers suggested by existing literature.

In continuous fermentations, the interruption of bioreactor would be significantly less frequent than that needed in batch fermentations. This means that it is crucial to maintain the viability of yeast throughout the extended time taken by a continuous fermentation. For this purpose, and taking advantage of the constant formation of foam observed under the airlift mode of operation, foam fractionation has shown to be an efficient tool.

The key innovative character of this thesis is clearly the use of flocculation as a biomass immobilization strategy in a gas-lift reactor. The setup used in the present work has shown great performance on biomass retention, higher than those observed in other configurations. Additionally, as there were no matrices enclosing the yeast in these configurations, a clear improvement of fermentation rates was observed. This resulted in higher productivity rates, most probably triggered by improved mass and heat transfer rates between yeast and wort. The final beer produced by this setup was considered to be of excellent quality when tasted by professionals.

Unlike past studies involving ICT, which attributed directly or indirectly the unbalanced beer flavour to the higher pitching rates used, the current work has shown that this association can be wrong. Conversely, changing the composition of wort fed to the bioreactor possibly diverted yeast metabolism to other paths, thus affecting beer flavour in different manners. The principal factor influencing the quality of beer was *FCR*.

9.2 Perspectives

- ✓ Future works involving ICT should use flocculation as biomass immobilization strategy because it is not only free-of-charge, but also provides better mass and heat transfer rates, thus increasing productivity rates. Additionally, as yeast is not attached to or entrapped in any type of material, it can easily be recovered by traditional sedimentation methods.
- ✓ The correct FAN composition should be determined by experimental trials in order to reduce diacetyl concentration and obtain the desired sensorial profile.
- ✓ The configuration based in yeast flocculation could be linked to known methods to accelerate diacetyl removal, thus achieving complete continuous beer fermentation.