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Physiology of Yeasts in Alcoholic Fermentation Processes

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Physiology of Yeasts in Alcoholic Fermentation Processes

Tese de Doutoramento em Engenharia Química e Biológica

Trabalho efectuado sob a orientação da
Doutora Lucília Domingues e do
Professor Doutor José A. Teixeira

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I dedicate this thesis

to my parents,

and to Ana.

ABSTRACT

Physiology of Yeasts in Alcoholic Fermentation Processes

This thesis is focused on physiological aspects of the yeasts used in two alcoholic fermentation processes: primary brewing fermentation and fermentation of lactose (particularly lactose derived from cheese whey) to ethanol by recombinant *Saccharomyces cerevisiae* flocculent strains.

The brewing fermentation is probably the most extensively studied alcoholic fermentation process. Nevertheless, developments in brewing technology demand deeper understanding of yeast physiology under process conditions. The studies with brewer's yeast reported in this thesis addressed two specific questions that had not yet been effectively investigated. First, it is here directly demonstrated for the first time that the brewer's yeast lipid composition, particularly the amounts of sterols and unsaturated fatty acids, affects the activity of maltose transporters. The maltose uptake rates were correlated with the amount of ergosterol in yeast, showing that proper function of the maltose permeases requires adequate amounts of ergosterol in the plasma membrane. This effect may partly explain the low maltose (and maltotriose) transport rates at the beginning and during the second half of brewery fermentations, when the sterol content of the yeast is low. Second, the energetic state of the yeast was studied under the specific environments of high- and very high-gravity brewing fermentations. The adenylate energy charge (EC) of the yeast was high (>0.8) throughout fermentation until residual sugar concentrations became low and specific rates of ethanol production became less than 5% of the maximum values observed in early fermentation. At that point, the EC fell to around 0.5 – 0.6. The results suggest that the ethanol tolerance of brewer's yeast is high so long as fermentation continues. However, when residual α-glucoside concentrations no longer support adequate rates of fermentation both the EC and the yeast viability collapse.

The development of microorganisms that efficiently ferment lactose has a high biotechnological value for the design of processes for the bioremediation of cheese whey with simultaneous production of bio-ethanol. A new lactose-fermenting flocculent *S. cerevisiae* recombinant strain is described here. This strain (T1-E) was obtained by evolutionary engineering of an original recombinant (T1, constructed in previous work) that had shown rather poor lactose fermentation and flocculation performances. The new strain T1-E consumed lactose 2-fold faster producing 30% more ethanol than T1. Its flocculation performance was also significantly better than that of T1. A series of physiological and genetic

studies were done to compare T1 and T1-E. The contribution of the identified molecular differences to the improved lactose fermentation phenotype of strain T1-E is discussed. In batch fermentations with mineral medium, the new strain T1-E consumed rapidly and completely lactose at initial concentrations up to 150 $g \cdot L^{-1}$. The maximum ethanol titre reached was 8% (v/v) and the highest ethanol productivity was 1.5 – 2 $g \cdot L^{-1} \cdot h^{-1}$. T1-E was also able to ferment 3-fold concentrated cheese whey (about 150 $g \cdot L^{-1}$ of lactose), producing 7% (v/v) of ethanol. These results demonstrate that T1-E is the most efficient lactose-fermenting *S. cerevisiae* recombinant strain reported in the literature. Being highly flocculent, this strain is suitable for developing high cell density fermentation systems that, when operated in continuous with flocculated biomass retention, can reach very high productivities.

Sugar transport is a key factor determining fermentation efficiency in both processes studied in this thesis. Studies of lactose transport by the recombinant *S. cerevisiae* strains (T1 and T1-E) and by *Kluyveromyces lactis* revealed that zero-*trans* uptake rates of lactose measured by standard methodology (i.e. using suspensions of yeast harvested from fermentation, washed and stored in nutrient-free buffer at low temperature before they are assayed using radiolabeled sugar) were too small (by factors of 3 to 8) to account for the lactose consumption rates observed during fermentations. A short incubation (1 – 7 min) with glucose (10 – 30 mM) increased the low intracellular ATP and EC of cells in the starved yeast suspensions to the levels found in actively fermenting yeast cells, and simultaneously increased the activity (V_{max}) of the lactose transporters by factors of 1.5 to 5. Similar observations were made for maltose transport in brewer's yeasts. These results suggest that the electrochemical proton potential that drives transport through sugar/H^+ symports is significantly smaller in the starved yeast suspensions used for the zero-*trans* assays than in actively metabolising cells. Short exposure of the starved cells to glucose is suggested as a quick method to approach more closely the sugar/H^+ symport capacity of the actively fermenting cells.

Keywords: brewer's yeast, maltose transport, ergosterol, adenylate energy charge, cheese whey, lactose fermentation, bio-ethanol, *Kluyveromyces lactis*, recombinant *Saccharomyces cerevisiae*, evolutionary engineering, sugar uptake assays.

RESUMO

Fisiologia de Leveduras em Processos de Fermentação Alcoólica

Esta tese foca-se em aspectos relacionados com a fisiologia de leveduras utilizadas em dois processos de fermentação alcoólica: a fermentação primária da cerveja e a fermentação de lactose (em particular a lactose derivada do soro do queijo) para produção de etanol por estirpes floculantes de *Saccharomyces cerevisiae* geneticamente modificadas.

A fermentação da cerveja é provavelmente o processo de fermentação alcoólica mais extensivamente estudado. Todavia, o desenvolvimento da tecnologia para o fabrico de cerveja requer uma compreensão mais aprofundada da fisiologia da levedura nas condições específicas deste processo. Os estudos com levedura de cerveja descritos nesta tese abordaram duas questões específicas que não haviam ainda sido efectivamente investigadas. Em primeiro lugar, é aqui demonstrado pela primeira vez que a composição lipídica da levedura de cerveja, em particular o seu conteúdo em esteróis e ácidos gordos insaturados, afecta a actividade dos transportadores de maltose. Verificou-se uma relação entre as taxas de transporte de maltose e a quantidade de ergosterol na levedura, demonstrando que o funcionamento adequado das permeases da maltose requer quantidades adequadas de ergosterol na membrana plasmática. Este efeito poderá explicar parcialmente as baixas taxas de transporte de maltose (e maltotriose) no início e durante a segunda metade das fermentações de cerveja, fases em que o conteúdo de esteróis da levedura é baixo. Em segundo lugar, o estado energético da levedura foi estudado nas condições específicas de fermentações de cerveja com mostos de alta e muito alta gravidade. A carga energética (EC) da levedura foi elevada (>0.8) durante a fermentação até a concentração residual de açúcar se tornar baixa e as taxas específicas de produção de etanol baixarem para menos de 5% dos valores máximos observados na fase inicial da fermentação. Nesse ponto, a EC decaiu para cerca de 0.5 – 0.6. Os resultados sugerem que a tolerência da levedura de cerveja ao etanol é elevada enquanto a fermentação prosseguir. No entanto, quando as concentrações residuais de α-glucosídeos não mais sustentam taxas de fermentação adequadas tanto a EC como a viabilidade da levedura colapsam.

O desenvolvimento de microrganismos que fermentem de forma eficiente a lactose tem um elevado valor em termos biotecnológicos para o desenho de processos para a biorremediação do soro do queijo com produção de bio-etanol em simultâneo. Uma nova estirpe recombinante de *S. cerevisiae* capaz de fermentar a lactose e floculante é descrita nesta tese. Esta estirpe (T1-E) foi obtida por evolução dirigida a partir de uma estirpe recombinante original (T1, construída em trabalho prévio) que havia demonstrado um fraco desempenho em termos de fermentação de lactose e de floculação. A nova estirpe, T1-E, foi

capaz de consumir a lactose duas vezes mais rapidamente e de produzir 30% mais etanol do que a estirpe T1. Foram efectuados vários estudos fisiológicos e genéticos para comparar as estirpes T1 e T1-E. A contribuição das diferenças identificadas ao nível molecular para o fenótipo melhorado da estirpe T1-E em termos de fermentação de lactose é aqui discutida. Em fermentações descontínuas com meio mineral, a nova estirpe T1-E consumiu rapidamente e por completo lactose em concentrações iniciais até 150 $g \cdot L^{-1}$. A concentração máxima de etanol atingida foi 8% (v/v) e a produtividade máxima foi 1.5 – 2 $g \cdot L^{-1} \cdot h^{-1}$. A estirpe T1-E foi também capaz de fermentar soro do queijo três vezes concentrado (cerca de 150 $g \cdot L^{-1}$ de lactose), produzindo 7% (v/v) de etanol. Estes resultados demonstram que a estirpe T1-E é a estirpe recombinante de *S. cerevisiae* mais eficiente para fermentação de lactose descrita na literatura. Sendo floculante, esta estirpe é adequada para desenvolver sistemas de fermentação de alta densidade celular que, quando operados em continuo com retenção da biomassa floculada, permitem atingir produtividades muito elevadas.

O transporte de açúcares é um factor chave na determinação da eficiência de fermentação em ambos os processos estudados nesta tese. Estudos do transporte de lactose nas estirpes recombinantes de *S. cerevisiae* (T1 e T1-E) e em *Kluyveromyces lactis* revelaram que as taxas iniciais de transporte de lactose medidas pela metodologia padrão (i.e. usando suspensões de leveduras colhidas da fermentação, lavadas e guardadas em tampão sem nutrientes a baixa temperatura antes de serem utilizadas para ensaios com açúcar marcado radioactivamente) eram demasiado baixas (por factores de 3 a 8) para explicar as taxas de consumo de lactose observadas durante as fermentações. Uma breve incubação (1 – 7 min) com glucose (10 – 30 mM) aumentou a baixa EC e os baixos níveis de ATP intracelulares encontrados nas suspensões de leveduras guardadas sem nutrientes para os níveis encontrados em células de leveduras a fermentar activamente. Simultaneamente, essa incubação aumentou a actividade (V_{max}) dos transportadores de lactose por factores de 1.5 a 5. Foram feitas observações semelhantes para o transporte de maltose em leveduras de cerveja. Estes resultados sugerem que o potencial electroquímico de protões responsável pelo transporte por mecanismos de simporte açúcar-protão é significativamente mais reduzido nas células guardadas em tampão sem nutrientes, utilizadas para os ensaios de transporte, do que nas células a fermentar activamente. Um breve tratamento das células guardadas sem nutrientes com glucose é sugerido como um método rápido para abordar de forma mais aproximada a capacidade dos sistemas de simporte açúcar-protão das leveduras a fermentar activamente.

Palavras-chave: levedura de cerveja, transporte de maltose, ergosterol, carga energética, soro do queijo, fermentação de lactose, bio-etanol, *Kluyveromyces lactis*, *Saccharomyces cerevisiae* recombinante, evolução dirigida, ensaios de transporte de açúcar.

LIST OF PUBLICATIONS

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CHAPTER 5

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CHAPTER 6

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Figure 6.10 – Intracellular adenine nucleotides levels during incubation of a buffered and starved

suspension of brewer's yeast (strain A15) with glucose at 20 °C. Portions of the suspension were pre-equilibrated for 5 min at 20 °C, after which glucose was added (at 0 min) to 27 mM. Intracellular adenine nucleotides (AXP; ATP, ADP, AMP) and total adenine nucleotides (Total AXP = ATP + ADP + AMP) were measured and the EC calculated after incubation with glucose for the times indicated and (plotted at -2.5 min) in samples taken from the yeast culture immediately before harvesting. For the incubation with glucose, error bars indicate the ranges between duplicate assays. For the culture, error bars correspond to the ranges between two samples. EC data (EC II) from a replicate experiment using independently grown yeast are also shown.

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LIST OF ABBREVIATIONS

A	Absorbance
Acetyl-coA	Acetyl coenzyme A
ADP	Adenosine 5'-diphosphate
AK	Adenylate kinase
AMP	Adenosine 5'-monophosphate
App. Att.	Apparent attenuation
ATP	Adenosine 5'-triphosphate
AXP	ATP, ADP or AMP
BLAST	Basic local alignment search tool
BOD	Biochemical oxygen demand
bp	base pairs
BSA	Bovine serum albumine
CBS	Centraalbureau voor Schimmelcultures *(The Netherlands)*
cDNA	complementary DNA
CEB-UM	Centre of Biological Engineering of the University of Minho
COD	Chemical oxygen demand
CTP	Cytidine triphosphate
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
CWPS	Cheese whey powder solution
DW	Dry weight
DY	Dry yeast
EBC	European Brewery Convention
EC	Energy charge
EDTA	Ethylenediaminetetraacetic acid
FAN	Free amino nitrogen
GO	Gene ontology
GRAS	Generally regarded as safe
G6PHD	Glucose-6-phosphate dehydrogenase

HEPES	N-(2-hydroxyethyl)-piperazine-2'-(2-ethanesulfonic acid)
HG	High gravity
HPLC	High-performance liquid chromatography
ITS	Internal transcribed spacer
kb	kilobase pairs
LACIR	Intergenic region between the genes *LAC4* and *LAC12* of *K. lactis*
LB	Luria-Bertani
L-L	Luciferin-luciferase
mRNA	messenger RNA
NAD	Nicotinamide adenine dinucleotide
NCBI	National Center for Biotechnology Information *(USA)*
NCYC	National Collection of Yeast Cultures *(UK)*
OD	Optical density
ORF	Open reading frame
PCA	Perchloric acid
PCR	Polymerase chain reaction
PGI	Phosphoglucoisomerase
PK	Pyruvate kinase
PMSF	Phenylmethanesulfonyl fluoride
pNPG	p-nitrophenyl-β-D-galactopyranoside
rDNA	ribosomal DNA
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RT-PCR	Reverse transcription-PCR
SC	Synthetic complete
SCC	Sodium chloride citrate
SCP	Single cell protein
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SGD	*Saccharomyces* genome database
SS	Semi-synthetic

TCA cycle	Trycarboxylic acid cycle
U	Unit
UAS	Upstream activating sequences
UFA	Unsaturated fatty acids
UV	Ultraviolet
VHG	Very-high gravity
VTT	Valtion Teknillinen Tutkimuskeskus *(Technical Research Centre of Finland)*
vvm	Volume of gas per volume of liquid per minute
WPC	Whey protein concentrate
Xgal	5-bromo-4-chloro-3-indolyl- β-D-galactopyranoside
YNB	Yeast nitrogen base
YP	Yeast extract Peptone
YPD	Yeast extract Peptone Dextrose

BACKGROUND AND OUTLINE OF THE THESIS

Yeasts stand out as beneficial microorganisms, traditionally represented by the species *Saccharomyces cerevisiae* that is particularly famous for the ability to convert sugars to ethanol, i.e. to carry out the alcoholic fermentation. Back in the nineteenth century, Louis Pasteur, one of the most distinguished microbiologists of all time, carried out extensive physiological studies of sugar fermentation by yeast cells, thus launching yeasts as central organisms in microbiology and more broadly in life science research. However, long before the foundations of microbiology and the unravelling of the physiological nature of fermentation, yeasts had already been used by mankind to make e.g. bread, beer and wine, what we now refer to as the traditional yeast biotechnology.

Alcoholic fermentation has been traditionally one of the main driving forces for the research on yeasts, with an important financial support from the industry. With the recent interest in bio-ethanol for fuel, the subject of alcoholic fermentation by yeasts continues to be highly attractive both for industry and for research. For obvious reasons, brewers are very interested in yeast and have enthusiastically contributed to their study. Beer fermentation is a very well established industrial process. Despite all the empirical knowledge accumulated over the centuries by brewers, extensive research has been carried out in fermentation technology. Thereafter, new fermentation systems are emerging, challenging the 21st century brewers. The improvement of traditional fermentation processes and the development of innovative solutions requests a deeper understanding of yeast physiology, biochemistry and genetics, thus brewers certainly continue to have high expectations from the knowledge being generated by scientists working with yeast. On the other hand, scientists and engineers must face the challenging questions raised by novel brewing technologies.

In addition to the main role in traditional fermentation processes, yeasts play other essential roles in modern biotechnology. The already mentioned bio-ethanol production to be used as fuel is only one example that has recently focused much attention, with the production of other molecules, such as glycerol or lactic acid, being also attractive. More recently, yeasts have been considered as good hosts for the production of recombinant proteins and other high-added value compounds, such as prebiotics. From the environmental technology perspective, yeasts may be applied in processes of bioremediation.

The cheese industry generates a highly pollutant by-product rich in lactose, the cheese whey, which is produced in large amounts (around 9 L per Kg of cheese) and therefore must be valorised and not just regarded as an effluent. The cheese whey has been the subject of research in the Centre of Biological Engineering of the University of Minho (CEB-UM) for many years now. In this perspective, a process for cheese whey valorisation/bioremediation has been proposed, consisting of a first step to separate the nutritionally valuable proteins and a second step involving the alcoholic fermentation of the lactose fraction (permeate). This

second step reduces the pollutant charge of the whey, by converting the lactose into potable ethanol, which can be used as food additive or as fuel. In order to make this latter process viable a microorganism that efficiently ferments lactose to ethanol is needed. The yeast traditionally used in alcoholic fermentation processes, *S. cerevisiae*, cannot metabolise lactose. However, this problem can be overcome by genetic engineering.

Besides their biotechnological interest, yeasts constitute simple model organisms to study the eukaryotic cell. Studies of cellular physiology, biochemistry, genetics and also evolution in yeasts provide valuable knowledge for investigations in higher eukaryotic organisms.

This dissertation focuses on physiological aspects of yeasts used in two alcoholic fermentation processes: (1) the primary brewing fermentation and (2) lactose fermentation by recombinant *S. cerevisiae* strains. The first chapter corresponds to a general literature review of the main subjects of this thesis. Chapters 2 to 6 describe the experimental work and the main results attained, and finally, chapter 7 is a summary of the main conclusions and some future perspectives. A short summary of the contents of each chapter follows.

Chapter 1 starts with an introduction to yeast biotechnology and alcoholic fermentation, providing some basic topics on these two subjects that constitute the main theme of the thesis. There follows a section about brewing, in which the brewing process is outlined with emphasis on brewer's yeast and fermentation. High- and very high-gravity fermentations are discussed, given that part of the experimental work dealt with these types of fermentations. The following section introduces the cheese whey problem and lactose fermentation as part of a sustainable solution for it. Lactose metabolism and the *GAL*/*LAC* regulon of *Kluyveromyces lactis* are discussed, particularly in comparison with the closely related *GAL*/*MEL* regulon of *S. cerevisiae.* Finally, ethanol production from lactose-based media by yeasts and the principal strategies for the construction of lactose-consuming *S. cerevisiae* recombinant strains are reviewed.

Chapter 2 describes experiments in which the sterol and fatty acid compositions of brewer's yeast were manipulated by growing it, with maltose as the sole carbon source, under strictly anaerobic conditions in the presence or absence of lipid supplements. The maltose uptake rates in yeast with varied sterol and unsaturated fatty acid compositions were then determined. The results obtained provide the first direct evidence that proper function of maltose transporters requires adequate amounts of ergosterol in the yeast, and this effect is discussed as to its meaning during different stages of brewery fermentations.

Chapter 3 deals with the energy status of yeast during high- and very high-gravity brewing fermentations. Intracellular and extracellular ATP, ADP and AMP concentrations were measured and the energy charge of yeast was calculated throughout the various phases of

these fermentations. The energy charge was high throughout fermentation, and only dropped when residual α-glucoside concentrations no longer supported adequate rates of fermentation. The high ethanol concentrations reached in the fermentations did not decrease the energy charge below values that permitted synthesis of new proteins. The results suggest that, during wort fermentations, the ethanol tolerance of brewer's yeast is high so long as fermentation continues.

Chapter 4 describes an evolutionary engineering experiment with a flocculent lactose-consuming *S. cerevisiae* recombinant strain constructed during previous work at CEB-UM. An evolved recombinant that consumed lactose 2-fold faster and produced 30% more ethanol was obtained. Comparative physiological and genetic studies of the original recombinant and the evolved strain are presented and the mechanisms underlying the evolutionary adaptation of the recombinant to lactose are discussed. A comparative transcriptome analysis between the original and the evolved recombinants growing in lactose, using *S. cerevisiae* cDNA microarrays, is also discussed.

Chapter 5 describes batch fermentations with high concentrations of lactose using the evolved recombinant strain characterised in chapter 4, showing that this strain is the best lactose-fermenting *S. cerevisiae* recombinant described in the literature and that it is suitable for the fermentation of concentrated cheese whey.

In chapter 6, lactose transport by *K. lactis* and by the recombinant strains described in chapter 4 is examined. The lactose uptake rates measured by standard methodology, i.e. adding radiolabeled lactose to a washed yeast suspension, could not account for the actual lactose consumption rates during fermentation. A short treatment of the cells with glucose before assay increased their ATP levels and lactose uptake rates. The methodology for sugar proton symport measurements in yeast is thus discussed in relation to the energetic state of the cells used for the assays. In addition to lactose transport, this same problem is also evaluated with maltose transport in brewer's yeast.

The work with brewer's yeast was developed in the perspective of increasing the knowledge of the physiology of yeast under the specific environment of brewery fermentations. This increased knowledge is a demand of new technological solutions, such as very high-gravity brewing. To my point of view, the work presented here provides valuable contributions to current discussions among brewers and scientists dedicated to brewing fermentation. The studies on lactose fermentation appear as a deeper exploitation of previous research in CEB-UM, specifically dealing with the development of *S. cerevisiae* recombinant strains for efficient lactose fermentation processes. A new strain with great potential was obtained using a simple evolutionary engineering approach. The work reported here is centred on the characterization

of that strain and of the evolutionary process that originated it, illustrating the usefulness of evolutionary engineering as part of strain development strategies.

There are many interesting connections between the two fermentation systems studied in this thesis, and the results presented demonstrate that the studies done in one of the systems may achieve conclusions that are relevant to the other system as well. For instance, sugar transport is a key factor determining fermentation efficiency in both systems. Nevertheless, there is still limited knowledge about the factors affecting the sugar transport rates. The results described in this thesis emphasise two of those factors: the lipid composition of the plasma membrane, particularly its sterol content, and the yeast adenylate energy charge.

CHAPTER 1

INTRODUCTION TO YEAST BIOTECHNOLOGY AND ALCOHOLIC FERMENTATION PROCESSES

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1.1 Introduction

Traditionally and historically yeasts are associated with alcoholic fermentation. This can be well understood in face of the role of yeasts in the production of alcoholic beverages, like beer and wine. From a scientific standpoint, this straight association dates back to the earlier steps of microbiology in the middle of the nineteenth century. By that time, yeasts became widely recognized as living organisms and yeasts and fermentation began to be studied from the biological point of view, in parallel with studies by chemists. Scientific work on alcoholic fermentation by yeasts therefore played a central role in the foundation of both microbiology and biochemistry, with contributions of brilliant scientists like Theodor Schwann, Louis Pasteur or Eduard Buchner. Nowadays, yeast biotechnology does not mean exclusively alcoholic fermentation. A wide range of new biotechnological applications for yeasts emerged, driven by the development of recombinant DNA technology and, more recently, by the publication of the *Saccharomyces cerevisiae* genome.

1.2 Historical notes on yeast and fermentation research[1]

The first observations of yeast cells were reported in the seventeenth century by Antonie van Leeuwenhoek (1632-1723). For a long time, yeasts were called “ferments” (a term also used by some nineteenth century scientists for what we know now as enzymes), a designation still often used in the common language, and their biological nature was unknown. The improvements in microscopes early in the nineteenth century led to the first descriptions of yeasts as living organisms, namely by Theodor Schwann (1810-1882). The view of yeasts as living organisms was however contested by influential chemists, represented by Justus von Liebig (1803-1873), which claimed that fermentation occurred by means of catalysis. By the middle of the nineteen century, the idea that yeasts were living organisms started to get acceptance both from scientists and from brewers. Between 1855 and 1875, Louis Pasteur (1822-1895), which started as a prominent chemist himself, established unequivocally fermentation as a physiological phenomenon, the role of yeast in alcoholic fermentation, and the differences between the aerobic and anaerobic utilization of sugars by yeasts. During this period of controversy, the chemists with their catalytic view helped to found enzymology, while the biologists made advances in microbiology, especially microbial physiology. An important

[1] The references for these notes on how yeast and fermentation research contributed to the establishment of microbiology and biochemistry were a number of articles by James A. Barnett about the history of research on yeasts (Barnett, 1997; Barnett, 1998; Barnett, 2000; Barnett, 2003; Barnett and Lichtenthaler, 2001).

consequence of establishing that yeasts caused fermentation was that, since fermentation had already been compared with putrefaction and disease, this finding encouraged the search for other kinds of microbes, responsible for different kinds of fermentation, as well as different diseases.

Antoine Lavoisier (1743-1794) and Joseph Gay-Lussac (1778-1850) made the first studies on alcoholic fermentation. Lavoisier was able to publish the first clear account of the chemical changes occurring during fermentation, describing quantitatively how sugar is converted into carbonic acid gas and alcohol. Few years later, Gay-Lussac revised Lavoisier's figures, reaching figures astonishingly close to present-day estimates. Pasteur tried to establish an equation for alcoholic fermentation but the ignorance at that time of the empirical formulae of some of the compounds involved and his attempt to include glycerol and succinic acid (by-products of fermentation) in the equation ultimately led him to decide that "Science is too little advanced to hope to put into a rigorous equation a chemical act correlative to a vital [biological] phenomenon".

The acceptance of yeasts as living organisms that cause alcoholic fermentation started a new debate, mainly between Pasteur and another French experimental scientist, Pierre Berthelot (1827-1907), to find whether fermentation should be attributed to intracellular enzymes of microorganisms or to the action of extracellular enzymes. Berthelot studied the break down of sucrose into glucose and fructose by brewer's yeast and was able to obtain invertase by macerating yeast and precipitating the enzyme with ethanol. The controversy arose because Pasteur thought solely in terms of intracellular activities, while Berthelot considered only extracellular phenomena. By that time, the specificity of the enzymatic action was unclear and there were difficulties in distinguishing between microbial action and enzymatic action, even amongst the most important scientists. The confusion produced by the double meaning of the word "ferment" led Wilhelm Friedrich Kühne (1837-1900) to coin the term "enzyme" for the soluble ferments, with the aim of distinguishing between "ferments" meaning microbes, and "ferments" meaning chemical substances that have catalytic properties.

It was Edward Buchner (1860-1917) who solved the question whether living cells are necessary for fermentation or fermentation is a process of chemical catalysis with a physiological basis. Buchner achieved fermentation by cell-free extracts, demonstrating that fermentation can occur outside living cells. His findings opened the way to study the biochemistry of fermentation *in vitro* and allowed to elucidate the main reactions of glycolysis in yeasts. In 1908, Arthur Harden (1865-1940) and William Young (1878-1942) published the equation for overall alcoholic fermentation. Between 1910 and 1940, studies on alcoholic fermentation by yeast extracts and lactic acid fermentation by muscle extracts revealed the glycolytic pathway. The discovery that the reactions were the same in yeast and muscle cells

was important, as it revealed an underlying unity in biochemistry.

Throughout the twentieth century, yeast research continued to contribute to the development of science in areas like physiology, cytology, genetics, taxonomy or evolution. Yeasts continue nowadays in the forefront of both fundamental and applied research in life sciences. Their roles as cell factories and as eukaryotic model organisms has placed yeasts at the very heart of modern biology revolutions like recombinant DNA technology or, more recently, the advent of genomics and systems biology.

1.3 Yeast biotechnology

Yeasts are unicellular fungi. Fungi are lower eukaryotes classified by modern biologists into their own kingdom, sometimes called “The Fifth Kingdom” based on their absorptive mode of nutrition. Fungi range from microscopic molds and yeasts to macroscopic mushrooms and truffles (Bennett, 1998; Gawel and Kosikowski, 1978). Molds or filamentous fungi, as well as yeasts, are used as cell factories in fine-chemical, pharmaceutical or food industries to produce molecules of interests such as enzymes, vitamins, antibiotics, amino acids, polysaccharides, alcohols, pigments, lipids and glycolipids (Adrio and Demain, 2003; Bennett, 1998).

The traditional or conventional yeast species is *Saccharomyces cerevisiae* (*Saccharomyces* meaning sugar moulds and *cerevisiae* meaning beer), which has been used by mankind to make bread, beer or wine since the prebiblical times. The earliest evidence of wine making, which was probably man’s first experience with yeast, has been dated to approximately 7000 years ago (Mortimer, 2000). Though Pasteur and his contemporaries were already fully aware of different sorts of yeast, which they described from many sources such as beer, wine, cheese, rotten wood, trees and truffles or even human urine, intestines, mouth, skin and hair (Barnett, 2000), “yeast” and “*S. cerevisiae*” have been used as synonyms, even often in some scientific literature, for a long time. Hence, to most people yeasts are still exemplified by the species *S. cerevisiae* and with the production of bread or alcoholic beverages, in spite of the fact that this domesticated microorganism represents only a fragment of the vast biodiversity and broad biotechnological potential of the yeast world (Buzzini and Vaughan-Martini, 2006). It is yet noteworthy that, in many physiological aspects, *S. cerevisiae* is an exceptional yeast. It is one of the few yeasts capable of grow under strictly anaerobic conditions. Moreover, the regulation of pyruvate metabolism in *S. cerevisiae*, with its strong tendency towards alcoholic fermentation, is also clearly different from many other yeasts (Pronk *et al.*, 1996).

The classification of yeasts within the *Saccharomyces* genus at the species level has always been problematic. A number of classifications have been proposed over the years, and as a

consequence, names of individual strains and species have undergone several changes that created confusion among yeast scientists and fermentation technologists. The *Saccharomyces* genus includes two groups of species: the *Saccharomyces sensu stricto*, which includes most of the strains relevant in the fermentation industry as well as in basic science, and the *Saccharomyces sensu lato*, which comprises species that are more distantly related to *S. cerevisiae*. The *Saccharomyces sensu stricto* group is composed of four species: *S. bayanus*, *S. cerevisiae*, *S. paradoxus* and *S. pastorianus*. More recently, three new species have been introduced: *S. cariocanus*, *S. kudriavzevii* and *S. mikatae*. The polyploid nature, the capability of exchanging genetic material, the high genetic variability, and the complexity of evolution in *Saccharomyces sensu stricto* yeasts, make species definition very troublesome. *S. bayanus* and *S. pastorianus* species contain strains of high relevance for the fermentation industry. *S. bayanus* includes wine and cider strains as well as grape must contaminants. *S. pastorianus* includes all lager brewing strains. Both these species are extremely heterogenous and have over the years been associated with several synonyms. Many strains that are isolated for industrial purposes are selected for their unique characteristics, and are therefore usually not representative of typical strains naturally available (Rainieri *et al.*, 2003).

The usefulness of *S. cerevisiae* and closely related species in a wide variety of scientific, commercial and medical applications is unquestionable, but the other 800-plus yeast species (Buzzini and Vaughan-Martini, 2006) constitute a precious source of genetic and biotechnological biodiversity. In recent decades, the impact of the so-called non-conventional (non-*Saccharomyces*) yeasts, such as *Kluyveromyces* spp., *Pichia* spp. or *Yarrowia lipolytica*, just to mention some examples, has been rapidly growing, with the biotechnological interest in potential industrial, medical and environmental applications driving fundamental research on their metabolic diversity. If *S. cerevisiae* stands in an exceptional position among yeasts because of its fermentation-oriented physiology, *Kluyveromyces lactis* appears to be a good model of the large number of more aerobic species that are currently used in yeast biotechnology. Furthermore, the fermentation-less *Yarrowia lipolytica* may be a model for highly aerobic species, with its well-established genetic system (Fukuhara, 2006).

Recently, Buzzini and Vaughan-Martini (2006) reviewed yeast biodiversity and their biotechnological application on alcoholic fermentation processes, on the production of single-cell protein and industrially relevant molecules (namely enzymes, lipids, carotenoids, flavour compounds, vitamins, organic acids, extracellular polysaccharides and amino acids), as probiotic microorganisms, as agents of biological control and as bioremediation agents (e.g. for the degradation of oil spills on land or water surfaces as consequence of ecological disasters or as biosorbent agents of heavy or radioactive metals).

The impact of yeasts in food and beverages, beyond the traditional fermentations, has also been the subject of recent surveys (Fleet, 2006; Fleet, 2007), which consider beneficial as well as detrimental aspects. In a positive context, yeasts contribute to the fermentation and maturation of a broad range of food products (cheese, kefir, koumiss, meat sausages, hams, cereal products, coffee and cocoa beans, soy sauce). Non-*Saccharomyces* yeasts can make also positive contributions to wine, cider and rum fermentations as well as in some types of beer. Furthermore, yeasts may be useful as sources of ingredients and additives for food processing, such as enzymes, antioxidants, flavourants, colorants, vitamins or polysaccharides which may be produced by controlled bioprocesses or obtained from yeast extracts, autolysates or dried yeast preparations. Yeasts are also potential probiotics, already in use in the livestock and aquaculture industries and, in the case of *S. cerevisiae* var. *boulardii*, in humans. Antagonistic interactions between yeasts and other microorganisms (bacteria, fungi or even other yeasts) prompt their use as agents in the biocontrol of food spoilage. On the negative side, yeasts can cause spoilage of many products, a problem with commercial and economic consequences. On the other hand, even though unlike many bacteria and viruses yeast are not known as aggressive infectious pathogens, there is some concern about species that can be considered as opportunistic pathogens, specially because of the increased frequency of individuals with weakened health and immune function.

As part of daily food consumption, humans, in most cases unknowingly and inadvertently, ingest large populations of a diversity of yeast species without adverse impact on their health (e.g. yeasts in many cheeses, fermented and cured meats, fruits, home-brewed beer and wine). Moreover, as already mentioned, yeast products, principally derived from *S. cerevisiae*, have been used for many years as ingredients and additives in food processing (Fleet, 2006; Fleet, 2007). Yeasts, as sources of single-cell protein, have also been used as an animal feed additive or even for human consumption (Buzzini and Vaughan-Martini, 2006), substituting meat and fish as protein suppliers. For all these reasons, yeasts are unquestionably part of our alimentary chain, but, unlike bacteria, viruses and some filamentous fungi, yeasts are rarely associated with outbreaks of foodborne gastroenteritis or other foodborne infections or intoxications (Fleet, 2006). The safe association between yeasts and foods created a positive image for these microbes amongst consumers. Moreover, many yeasts, starting with *S. cerevisiae*, have attained the GRAS (Generally Regarded As Safe) status given by the USA Food and Drug Administration. The recognition as GRAS is important for virtually all biotechnological applications, but especially in respect to food, biomedical and pharmaceutical uses.

The development of the recombinant DNA technology made possible to express heterologous genes in yeasts, providing new ways to use yeasts as cell factories, particularly in the

production of heterologous proteins and enzymes as well as other high-added value metabolites (Hadfield *et al.*, 1993; Romanos *et al.*, 1992). Amongst the advantages of yeasts as hosts for foreign protein expression are the ease of cultivation and availability of advanced fermentation technology, the accumulated genetic knowledge and the established molecular biology tools (including yeast-based expression kits currently available from major research tool companies), the eukaryotic environment and the consequent ability to perform many eukaryote-specific post-translational modifications, the GRAS status of some species (such as *S. cerevisiae* and *K. lactis*), their capacity to secrete certain recombinant proteins, as well as the high yields that can be obtained. Several species have been proposed as hosts, but *S. cerevisiae* and *Pichia pastoris* (which has several advantages but also the main drawback of being non-GRAS) have been the most exploited so far (Adrio and Demain, 2003; Cereghino and Cregg, 1999; Hadfield *et al.*, 1993; Romanos *et al.*, 1992). All yeast-based therapeutic proteins for use in humans are currently produced in *S. cerevisiae*, but therapeutic proteins expressed in *P. pastoris* have entered clinical trials (Gerngross, 2004). The replication of human glycosylation pathways in engineered yeasts allowing these hosts to produce human-like glycoproteins, may prompt a switch from mammalian cell culture to yeast-based platforms for the production of this type of therapeutic proteins (Gerngross, 2004; Wildt and Gerngross, 2005).

S. cerevisiae is the best characterized of all eukaryotes and therefore acts as a model for eukaryotes in general, since the basic functions of eukaryotic cells appear to be highly evolutionarily conserved (Hadfield *et al.*, 1993). It is even a helpful model to study a number of cellular processes occurring in human cells (Thuret and Blondel, 2006). *S. cerevisiae* is a perfect example of a living biotechnology tool (Thuret and Blondel, 2006): it is a simple unicellular eukaryote with a complete set of basic eukaryotic genes, it has GRAS status, it is simple to cultivate and inexpensive to work with compared to any other eukaryotic organism, there is a wealth of knowledge on its physiology and genetics as well as a large set of molecular biology tools and genome-scale technologies for the modification at will at the nucleotide level and the global study of the cell.

The first eukaryote genome completely sequenced was that of *S. cerevisiae* (Goffeau *et al.*, 1996). The genome consists of 16 chromosomes that sum approximately 12 million base pairs. The current estimate for the number of yeast ORFs that actually encode proteins is 5794, of which 1129 are listed as “uncharacterized” (SGD, 2007). The genome sequence deeply transformed yeast research. The transformation began with technical improvements that greatly accelerated research, allowing for instance the identification of pieces of cloned DNA by simple sequence runs. The development of genome-scale methods endowed the yeast research community with tools unimaginable before, such as DNA microarrays that

made possible to study expression of all the yeast genes at once (Dolinski and Botstein, 2005). With the advances in global approaches – transcriptome, proteome, metabolome and fluxome analyses – new areas of research are emerging, changing the perspective from focus on individual genes and functionalities to a more global view of how the cellular networks and systems interact and function together to produce the properties of the organism (Dolinski and Botstein, 2005).

Meanwhile, other yeast genome projects followed. There are currently at least seven complete yeast genomes available (*Candida glabrata*, *Debaryomyces hansenii*, *Kluyveromyces lactis*, *Pichia stipitis*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Yarrowia lipolytica*) and many more ongoing sequencing projects at the assembly stage or in progress (NCBI, 2007). With the availability of a large number of genome sequences (not only of yeasts, but already covering a large variety of organisms) the comparative genomics era is launched. Yeasts, with their small and compact genomes and broad range of phylogenetic distances covered, are once more at the forefront (Liti and Louis, 2005).

The advances in fundamental research often endorse developments in aspects related to the industrial applications of *S. cerevisiae*. Areas of yeast biology related with the traditional applications, including the production of alcoholic beverages and baker's yeast, continue to merit research. This is partly due to the need for improved process control, which requires a quantitative description of the metabolic fluxes within the cells. Furthermore, in spite of the long history of *S. cerevisiae* as an industrial microorganism, some inherent problems are still not completely understood or solved (Pronk *et al.*, 1996). For example, in industrial processes requiring optimized biomass production (such as baker's yeast production) the tendency of *S. cerevisiae* to perform alcoholic fermentation represents a problem, since fermentative sugar metabolism leads to a much lower biomass yield than respiratory metabolism. Recently, Otterstedt *et al.* (2004) reported an engineered *S. cerevisiae* strain showing a fully respiratory metabolism even at high external glucose concentrations, switching to fermentation only when oxygen is lacking. This fully respiratory strain is presented both as a unique research tool to study metabolic control in the yeast model system, and as a potential host for increased heterologous protein production in simple batch cultures, due to its higher biomass yield (Otterstedt *et al.*, 2004).

1.4 Alcoholic fermentation

Fermentation can be defined as an ATP-generating oxido-reduction biochemical process that can occur in the absence of oxygen and in which organic compounds act as both donors and

acceptors of electrons (Sols *et al.*, 1971; Stryer, 1995).

Alcoholic fermentation is the conversion of sugars into ethanol and carbon dioxide. Yeasts have the ability to metabolise a wide range of carbohydrates – mono-, di- and trissacharides, higher dextrins and starches, pentoses – depending on the species and cultivation conditions (for general reviews see e.g. Barnett, 1997; Dickinson and Kruckeberg, 2006). Yeasts predominantly use the Embden-Meyerhof-Parnas pathway (glycolytic pathway) for conversion of hexose phosphates to pyruvate. However, important differences occur in the initial steps leading from extracellular sugar to intracellular hexose phosphates (that ultimately enter the glycolytic pathway), depending on the yeast species and on the kind of sugar. The first step in the metabolism of a sugar is either its passage into the cell through the plasma membrane or its hydrolysis by extracellular enzymes. Disaccharides may be hydrolysed by extracellular enzymes or transported by permeases and hydrolysed intracellularly. Disaccharide transport appears to occur exclusively by proton symport, while monosaccharide transport may involve either facilitated diffusion or proton symport (Figure 1.1).

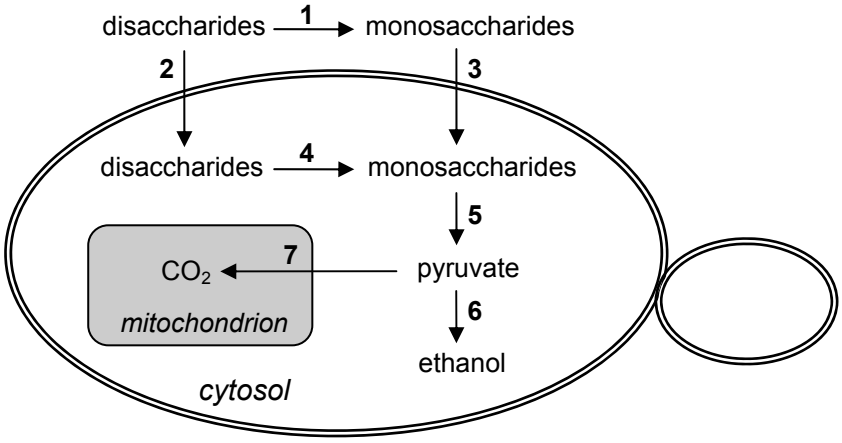

Figure 1.1 – Schematic representation of carbohydrate dissimilation by yeasts. Hydrolysis of disaccharides may occur by extracellular enzymes with a low pH optimum (1) or intracellularly (4) by enzymes with a near-neutral pH optimum. Disaccharide transport (2) appears to occur exclusively via proton symport, whereas monosaccharide transport (3) may involve either facilitated diffusion or proton symport, depending on yeast species and environmental conditions. In the case of hexoses, the Embden-Meyerhoff-Parnas pathway is the main route of sugar dissimilation (5) in yeasts. Pyruvate is either converted to ethanol and carbon dioxide (6) or respired to carbon dioxide and water (7) in the mitochondria (after Pronk *et al.*, 1996).

Glycolysis (glycolytic pathway or Ebden-Meyerhoff-Parnas pathway) is the sequence of

reactions occurring in the cytosol that convert glucose into pyruvate, with the concomitant production of ATP. Glycolytic intermediates have either six or three carbons. The six-carbon units are derivatives of glucose and fructose. The three-carbon units are derivatives of dihydroxyacetone, glyceraldehydes, glycerate, and pyruvate. All intermediates between glucose and pyruvate are phosphorylated. The net reaction in the transformation of glucose into pyruvate is:

$$\text{Glucose} + 2\ P_i + 2\ \text{ADP} + 2\ NAD^+ \rightarrow 2\ \text{pyruvate} + 2\ \text{ATP} + 2\ \text{NADH} + 2\ H^+ + 2\ H_2O.$$

Thus, there is a net gain of two molecules of ATP in the formation of two molecules of pyruvate from one molecule of glucose. Besides the degradation of glucose to generate ATP, the glycolytic pathway provides building blocks (carbon skeletons) for the synthesis of cellular components.

Sugars other than glucose are also feed to the glycolytic pathway (Figure 1.2). Similarly to glucose, these enter glycolysis as sugar phosphates.

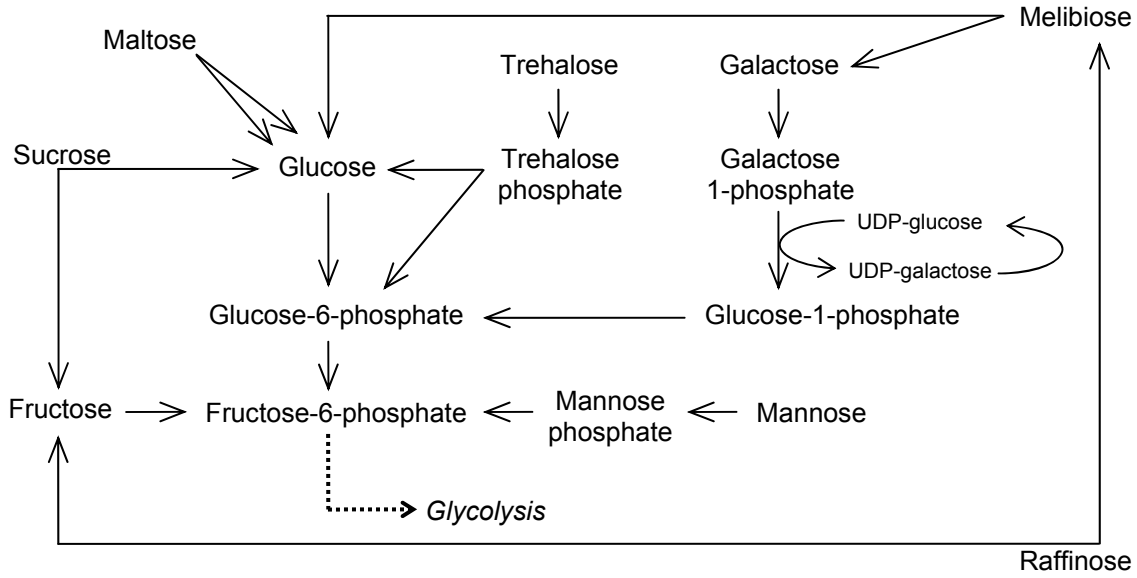

Figure 1.2 – The mode of entry of various sugars into the glycolytic pathway in *S. cerevisiae*. The catalytic steps involved in the metabolism of intracellular galactose constitute the Leloir pathway (adapted from Briggs *et al.*, 2004).

Glycolysis is active in yeast under all conditions. During growth on sugars, the direction of carbon flow is from glucose to pyruvate. During growth on oxidative carbon sources, such as ethanol, glycerol and lactate, the glycolytic pathway is reversed and used to generate

intermediates for anabolism. This process is termed gluconeogenesis. There are in yeast two gluconeogenic specific enzymes, which accomplish non-reversible glycolytic steps (Briggs *et al.*, 2004). In aerobic fermentations, when the exogenous sugar is exhausted the metabolism of yeast changes from fermentative to oxidative (ethanol-consuming). This metabolic change, called diauxic shift, results in a second period of growth at the expenses of the ethanol previously produced (which is metabolized by gluconeogenesis and respiration).

The hexose monophosphate (or pentose phosphate) pathway diverts a proportion of glucose from the main glycolytic path, returning metabolites at the level of triose-phosphate and fructose 6-phosphate. It generates reducing power in the form of $NADPH_2$, which is required for some anabolic reactions, and also provides precursors for the biosynthesis of important cellular compounds (Briggs *et al.*, 2004; Sols *et al.*, 1971).

The sequence of reactions from glucose to pyruvate is similar in all organisms and in all kinds of cells. In contrast, the fate of pyruvate is variable. In the absence of oxygen, pyruvate may be converted to ethanol (alcoholic fermentation) or to lactic acid (a kind of fermentation that occurs in several microorganims as well as in the cells of higher organisms when the amount of oxygen is limiting, like in muscle cells during intense activity). In the presence of oxygen, pyruvate may be used for respiration.

In *S. cerevisiae* growing on sugars, pyruvate can follow three main routes: (1) oxidative decarboxylation to acetyl coenzyme A (acetyl-coA); (2) carboxylation to oxaloacetate; (3) decarboxylation to acetaldehyde (Pronk *et al.*, 1996). The first route leads to respiration, which requires coupling of glycolysis to the tricarboxylic acid (TCA) cycle. Acetyl-coA, the fuel of the TCA cycle, can be synthesised from pyruvate by a direct oxidative decarboxylation, catalysed by the pyruvate dehydrogenase complex. This complex is located in the mitochondrial matrix, so the pyruvate has to be transported across the mitochondrial membranes. The withdrawal of TCA cycle intermediates for biosynthesis during growth is compensated for by the second route, i.e. the anaplerotic synthesis of oxaloacetate from pyruvate, which is catalysed by pyruvate carboxylase. The third route corresponds to alcoholic fermentation.

The first step in the conversion of pyruvate into ethanol during alcoholic fermentation is the decarboxylation of pyruvate to form acetaldehyde, with consequent release of carbon dioxide. This reaction is catalysed by the enzyme pyruvate decarboxylase. The second step is the reduction of acetaldehyde to ethanol by NADH, in a reaction catalysed by alcohol dehydrogenase. The net result of alcoholic fermentation of glucose is then:

$$C_6H_{12}O_6 + 2\ P_i + 2\ ADP + 2\ H^+ \rightarrow 2\ C_2H_5OH + 2\ CO_2 + 2\ ATP + 2\ H_2O.$$

(Glucose) (Ethanol)

NAD^+ and NADH do not appear in this equation, even though these are crucial for the overall reaction. NAD^+ is the electron acceptor in the oxidation of glyceraldehyde 3-phosphate during glycolysis and is regenerated by the reduction of acetaldehyde to ethanol. Thus, there is no net oxidation-reduction in the conversion of glucose into ethanol.

Acetaldehyde may follow an alternative route, called the pyruvate dehydrogenase bypass. In this route, acetaldehyde is converted to acetate (catalyzed by acetaldehyde dehydrogenase) that is converted to acetyl-coA (acetyl-coA synthetase). Both the enzymes involved are cytosolic and thus this bypass can be used for the synthesis of acetyl-coA, required for biosynthetic reactions, under anaerobic conditions (Briggs *et al.*, 2004; Pronk *et al.*, 1996).

In the fermentation of sugars by yeasts certain by-products also appear. The glycerol that is frequently found does not primarily result from sugar dissimilation. Rather, glycerol biosynthesis has a role in maintaining the cellular redox balance, particularly under anaerobic conditions. Although ethanol fermentation is redox neutral, excess NADH is generated by anabolic reactions, i.e. during biomass formation. Under aerobic conditions, this assimilatory NADH is reoxidized via the electron transport chain during respiration. Under conditions of extreme oxygen limitation or anaerobiosis, surplus NADH is reoxidized by means of NADH-consuming glycerol formation, which therefore acts as an essential redox valve (Rigoulet *et al.*, 2004; Taherzadeh *et al.*, 2002; Wang *et al.*, 2001). In addition to ethanol and glycerol, fermenting yeast cultures often excrete small amounts of other fermentation products, in particular organic acids (e.g. acetate and succinate), higher alcohols, esters and aldehydes. Although specific rates of formation of these compounds are often orders of magnitude lower than the rates of ethanol formation, some play an important role as (off-)flavous in alcoholic beverages (Pronk *et al.*, 1996).

As seen, fermentative and respiratory sugar metabolism diverge at the pyruvate branch-point (Pronk *et al.*, 1996). Fermentation is a far less economical process than respiration, since with the former the cell can obtain much less energy (less ATP) per mole of glucose utilized (Sols *et al.*, 1971). Only a small fraction of the energy of glucose is released in its anaerobic conversion to ethanol. Much more energy can be extracted in the respiration process, in which pyruvate is completely oxidised to carbon dioxide and water, by means of the TCA cycle and the electron transport chain (where most of the free energy in glucose is harvested) (Stryer, 1995).

In general, yeasts can utilize glucose as a carbon source. However, not all yeasts are able to ferment glucose to ethanol. The large majority of yeast strains are facultatively fermentative yeasts, i.e. can either respire sugars or ferment them to ethanol and carbon dioxide. All of these facultatively fermentative yeasts exhibit alcoholic fermentation under oxygen-limited

growth conditions. However, in many yeasts, oxygen is not the sole factor determining the contribution of respiration and fermentation to the overall rate of sugar metabolism. The diversity among facultatively fermentative yeasts with respect to the regulation of alcoholic fermentation is evident from phenomena indicated by such terms as “Pasteur effect”, “Crabtree effect”, “Kluyver effect” and “Custers effect” (named after their discoverers). These represent complex physiological regulatory mechanisms that affect the balance between fermentation and respiration (Pronk *et al.*, 1996). Overviews on each of these “effects” can be easily found in literature (see e.g. Barnett, 1997; Boulton and Quain, 2001; Dickinson and Kruckeberg, 2006; Pronk *et al.*, 1996; Snoek and Steensma, 2007). Only the Crabtree effect will be discussed here in more detail, due to its importance in industrial applications of *S. cerevisiae*.

The Crabtree effect refers to the occurrence of alcoholic fermentation (rather than respiration) of glucose under aerobic conditions. This is a characteristic of *S. cerevisiae* and some other facultatively fermenting yeasts, and occurs during aerobic batch cultivation and during aerobic glucose-limited chemostat cultivation at high dilution rates (Dickinson and Kruckeberg, 2006), as well as following transition of sugar-limited cultures to sugar-excess (Boulton and Quain, 2001; Pronk *et al.*, 1996). *S. cerevisiae* controls fermentation versus respiration primarily in response to the sugar level (Otterstedt *et al.*, 2004), switching to a mixed respire-fermentative metabolism as soon as the external glucose concentration exceeds 0.8 mM (Verduyn *et al.*, 1984). Numerous physiological differences distinguish Crabtree-positive species from Crabtree-negative species (such as *K. lactis*), but the causal factor regulating the onset of Crabtree metabolism has not yet been identified and remains controversial (Dickinson and Kruckeberg, 2006; Otterstedt *et al.*, 2004). Nevertheless, mechanisms have been proposed to explain the Crabtree effect. The proposed models are based on a limited capacity of the respiratory routes of pyruvate dissimilation, with the consequent generation of an overflow towards pyruvate decarboxylase and hence ethanol production (Otterstedt *et al.*, 2004; Pronk *et al.*, 1996; Snoek and Steensma, 2007).

The metabolism of sugars by yeast is also regulated by carbon catabolite repression (also called glucose repression) and catabolite inactivation mechanisms. Both these phenomena occur when *S. cerevisiae* is grown in the presence of glucose. Other sugars are also effective, particularly those which may be rapidly fermented such as fructose and maltose. Glucose repression is a global regulatory mechanism exerted mostly at the transcriptional level that affects the expression of various genes. Glucose also triggers inactivation and/or proteolysis (catabolite inactivation) of a number of proteins. The combination of glucose repression and catabolite inactivation mechanisms affect yeast metabolism in several ways: the expression of genes encoding enzymes required for the utilization of other sugars (galactose, maltose,

melibiose, etc) is repressed and transport proteins are inactivated; gluconeogenic enzymes are also repressed/inactivated, impairing the use of non-fermentable (glycerol, ethanol) carbon sources; the biogenesis of functional mitochondria and peroxisomes is inhibited; the respiratory pathways are inoperative, even in the presence of oxygen (Boulton and Quain, 2001; Gancedo, 1998). It may be appreciated that the visible manifestations of the glucose repression are the same as those seen in the Crabtree effect with the highly significant difference that repressed cells are not capable of respiratory growth because parts of the necessary metabolic machinery are absent (Boulton and Quain, 2001).

Growth in the absence of molecular oxygen requires adaptation of the cell for at least three reasons: (1) energy yield is usually much lower than under aerobic conditions; (2) several biosynthetic pathways require molecular oxygen (such as those for haem, sterols, unsaturated fatty acids, pyrimidines and deoxyribonucleotides); and (3) different molecules have to be transported into and out of the cell (Snoek and Steensma, 2007). Thus, cells growing anaerobically have found ways to circumvent the oxygen dependency of certain pathways, which, in certain cases, requires a redirection of metabolism. For instance, owing to the lower ATP yield of fermentation in comparison to that of respiration, a higher glycolytic flux and a higher uptake rate of sugars are necessary to maintain a high growth rate. In *S. cerevisiae*, only 23 genes were found essential for anaerobic growth and were dispensable under aerobic conditions. However, the transcription levels of ca. 500 genes differ significantly when aerobic and anaerobic cultures are compared. For many of these genes, it is unclear why they are essential or why their expression levels are up- or down-regulated under anaerobic conditions (Snoek and Steensma, 2006; Snoek and Steensma, 2007).

S. cerevisiae is usually the first choice for industrial processes involving alcoholic fermentation. The reasons for this preference include: (1) its good fermentative capacity and ethanol tolerance, allowing to produce up to 20% (v/v) ethanol (Antoni *et al.*, 2007; Cot *et al.*, 2007); (2) its GRAS status; (3) its capacity to grow rapidly under anaerobic conditions, which helps circumventing the oxygenation problems inherent to large-volume industrial fermentations (Snoek and Steensma, 2007); (4) the extensive industrial and scientific knowledge accumulated that makes it one of the best studied organisms.

The growing interest in biofuels and in particular ethanol (Antoni *et al.*, 2007; Farrell *et al.*, 2006; Hahn-Hagerdal *et al.*, 2006; Herrera, 2006; Ragauskas *et al.*, 2006; Schubert, 2006) has again attracted a lot of attention into the fermentation potential of *S. cerevisiae*. The urge to move from cane sugar and cornstarch (the raw materials currently used) towards lignocellulosic ethanol is driving the engineering of yeasts for efficient pentose (in particular xylose and arabinose) fermentation. *S. cerevisiae* is not able to ferment pentoses. Certain yeasts, such as *Pichia stipitis*, are xylose-fermenting but present poor ethanol yields and low

ethanol tolerance. Transferring the capacity to ferment pentoses to *S. cerevisiae* is therefore of prime importance, and many advances have been attained recently in this field (for reviews see e.g. Aristidou and Penttila, 2000; Hahn-Hagerdal *et al.*, 2007; Jeffries, 2006; van Maris *et al.*, 2006; van Maris *et al.*, 2007).

1.5 Brewing

1.5.1 A taste of history

Whatever its exact origins, surviving historical artefacts allow to trace brewing back to the Mesopotamians around 6000 or 7000 years ago (Anderson, 2006). Beer was already prepared by the ancient Egyptians from malted barley and a primitive type of wheat (Bamforth, 2003; Masschelein, 1997). The techniques applied by the Egyptians seem to have been quite refined. They passed on their brewery techniques to the Greeks and Romans, whose preferred drink was however wine (Bamforth, 2003). The development of brewing and the brewing industry is linked with northern Europe where cold conditions inhibited the development of viticulture (Anderson, 2006). Beer was integral to the culture of the agrarian population of northern and central Europe in the medieval and early modern period. The weaker brews were a part of everyone's diet and the stronger beers were drunk in leisure periods (Anderson, 2006). A vast variety of different beers has always been made in Europe. Each region offered its own favourite brews influenced by availability and quality of raw materials and climate. The dominant cereal in use has always been barley, the easiest to malt, although it could be supplemented or even replaced by other cereals, particularly oats and wheat. In parts of Germany and Belgium wheat beers became a specialty (Anderson, 2006). From the 10th century, the use of hops in brewing spread from Germany across Europe to replace, or at least supplement, the plethora of plants, herbs, and spices popular at that time. Hops were introduced into Britain in the 15th century and reached North America in the early 17th century (Anderson, 2006). In 1516, the *Reinheitsgebot* ("Commandment for Purity") was introduced in Bavaria, decreeing that only malt, hops and water were to be used in brewing. Yeast was later added to the list, and wheat allowed for specialty beers. The law survives in Germany to this day (Anderson, 2006; Bamforth, 2003).

For a time, the terms ale and beer were being applied to distinct beverages made by separate communities of brewers. Ale described the drink made without hops, whereas the term beer was reserved for the hopped beverage. By the 16th century, ale brewers had also come to use some hops in their brews, but at a lower level than usual for beer and an element of distinction remained (Anderson, 2006). It is a myth that lager-style products have always been

the characteristic beer of Germany. Indeed, until the 16th century ale was the main type of beer in Germany. Bottom fermentation probably started in Bavarian monasteries early in the 15th century. One of the main driving forces for the development of lager beer was an edict in 1533 that basically precluded brewing in the summer without a special dispensation. So, the emphasis shifted to the bottom fermentation practices used in winter, producing beer in sufficient quantities to store ("lager", from the German verb *lagern*, to store) until the subsequent fall (Bamforth, 2003). The defining characteristic of lager beers were the utilization of yeasts that sediment to the bottom of the vessel toward the end of fermentation and the use of low fermentation (4 to 10 ºC) and maturation (-2 to 4 ºC) temperatures. Apart from Bavaria, the rest of the world used yeasts that floated up to the surface of the fermenting wort and were accommodated to higher fermentation (15 to 25 ºC) and maturation (13 ºC) temperatures (Anderson, 2006). Later on, Bavarian brewing practices became more widely known. Some North German, Austrian and Czech brewers adopted bottom fermentation in the late 1830s. Pilsner-style lager (straw colored) has therefore emerged, proving the most popular by the end of the century. From the 1870s, the increasing availability of efficient artificial refrigeration freed lager brewers from the need for natural ice. Lager beer eventually became a world drink, spreading around the globe to Denmark, the Netherlands, the United States, Japan, China and Australia before the end of the 19th century. Only the United Kingdom and, to a lesser extent, Belgium resisted the rush to bottom fermentation (Anderson, 2006).

The industrial revolution, population growth, urbanization, social transformations and increased consumption during the 19th century prompted the rise of commercial brewing and the decline of domestic brewing. Brewing industry flourished, with beer producing facilities growing in number as well as in size in some European countries and in the United States. Breweries became highly capitalized business and major employers of labour. Its proprietors became more prominent socially and politically and welcomed the attention, stressing the importance of their industry to farming and the exchequer (quite early governments understood the opportunity that the brewing business represented for their budgets, imposing taxation to beer) (Anderson, 2006).

Many of the early advances in yeast research had strong links with brewing. For instance, Leeuwenhoek reported the observation of yeasts in fermenting beer, and Pasteur investigated brewing fermentations, in particular problems associated with industrial fermentations, publishing his famous *Études sur la Bière* in 1876. The work of Emil Christian Hansen (1842-1909) must be mentioned at this point, as it was crucial to the progress of modern brewing industry. In 1883, Hansen (who worked at the Carlsberg Laboratories in Copenhagen) developed an effective technique for obtaining pure yeast cultures. This concept was soon

applied in practical brewing, proving of major importance in standardizing yeasts for reliable fermentations. Hansen's method was also applied to the production of baker's yeast, besides being fundamental to developing microbial research (Barnett and Lichtenthaler, 2001). The 20th century brought many advances in the technology for the malting of barley and brewing, taking advantage of the scientific knowledge accumulated. The modern malting and brewing industry applies a whole spectrum of novel technical, biochemical, microbiological and genetic inventions (Linko *et al.*, 1998). These advances have made the brewing processes, along the whole production chain from barley to beer, remarkably more efficient and consistent.

1.5.2 Brewing industry

In the second half of the 20th century the brewing business went through globalisation. Multiplant brewing of major brands spread around the world, and bigger volumes got concentrated in fewer larger companies. Guiness (Ireland), Heineken (Netherlands) and Carslberg (Denmark) had been the leaders in this process, with other groups, such as Interbrew (Belgium), Scottish & Newcastle (United Kingdom) and Fosters (Australia), also following the internationalization path. Brewing globalisation continues in the 21st century, with new acquisitions every year. For many years, the biggest brewing company was Anheuser-Busch (United States), which sold 183 million hectolitre (1 hl = 100 L) worldwide in 2006 (Anheuser-Busch, 2006). In 2004, the fusion between Interbrew and AmBev (Brazil) resulted in InBev (headquartered in Leuven, Belgium) that is now the world biggest brewing company. InBev sold 247 million hl in 2006 (InBev, 2006). On the opposite hand, there has also been in many countries a trend in the establishment of smaller breweries, either microbreweries or pub breweries, producing specific beers that increase consumer's options.

Beer is nowadays drunk all over the world in all different places and circumstances. Table 1.1 shows beer production and consumption statistics in some countries.

Table 1.1 – Worldwide brewing and beer statistics, 1998[1] (after Bamforth, 2003).

Country	**Population** (million)	**Production** (million hectolitres)	**Imports** (million hectolitres)	**Exports** (million hectolitres)	**Consumption** (litre per capita)	**Average strength** (% v/v)
Australia	18.5	17.5	0.21	0.43	95.0	4.3
Belgium[2]	10.6	14.6	0.88	4.9	99.0	5.2
Brazil	165.9	88.0	0.26	0.45	52.9	–[3]
Canada	30.3	22.8	1.17	3.64	67.0	5.0
China	1 255.7	196.4	0.33	0.56	15.6	–
Czech Republic	10.3	18.3	0.154	1.9	160.8	4.5
Denmark	5.3	8.1	0.0079	2.4	107.7	4.6
Finland	5.2	4.7	0.08	0.32	79.1	4.6
France	58.7	19.8	5.3	2.4	38.6	5.0
Germany	82	111.7	2.8	8.4	127.4	–
Greece	10.4	4.0	0.19	0.3	42.0	4.9
Ireland	3.6	8.5	0.56	3.45	124.2	4.1
Italy	57.5	12.2	3.68	0.37	26.9	5.1
Japan	126.4	72.2	0.8	0.71	57.2	5.0
Netherlands	15.7	24.0	0.95	11.7	84.3	5.0
Norway	4.4	2.2	0.045	0.011	49.7	4.5
Portugal	9.9	6.8	0.29	0.55	65.3	5.2
Russia	147.4	32.5	0.73	0.047	22.5	–
Slovak Republic	5.4	4.3	0.5	0.46	84.0	4.5
South Africa	42.1	25.3	0.42	0.65	59.5	5.0
Spain	39.9	25.0	2.0	0.51	66.4	5.2
Sweden	8.9	4.6	0.534	0.041	57.3	4.0
Switzerland	7.25	3.6	0.72	0.03	59.9	4.9
United Kingdom	59.2	56.7	5.9	3.9	99.4	4.1
United States	270.3	235.5	19.1	6.5	83.7	4.6

[1]Source: Statistical Handbook, Brewers and Licensed Retailers Association, London, 2000.

[2]Includes Luxembourg, because of inaccuracies introduced by crossborder trading.

[3]A dash indicates data not available.

European breweries produce 416 million hl beer annually, which makes Europe the world's most important beer producer, as well as number one producer of malting barley, malt and hops. The total contribution of the brewing sector to the European economy in terms of value added is 57.5 billion euros (includes the impact of supply sectors to the brewing sector and the impact of the beer sale sectors). This contribution corresponds to around 0.55% of total gross domestic product (Ernst&Young, 2006).

In Portugal, there are six brewing companies. However, the beer market is dominated by the two bigger companies: UNICER (headquartered in Leça do Balio, Porto) and SCC – *Sociedade Central de Cervejas* (Vialonga, Lisbon). The history of SCC may be traced back to the 1830s with the establishment of the *Fábrica da Cerveja da Trindade* in Lisbon. In 1890,

seven breweries in Porto's area fused to create the company that would later originate UNICER. Both are now integrated in major European brewing groups, SCC in Scottish & Newcastle and UNICER in Carslberg. Total beer production in Portugal was 7.4 miillion hl in 2004. Total consumption was 6.3 million hl (corresponding to 61.7 litres per inhabitant). The total contribution to the Portuguese economy in terms of value added rising from the production and sale of beer is estimated at 1.5 billion euros (146 million euros from the breweries only). Portuguese breweries employ about 2 206 staff. However, the total impact of the brewing sector (including supply and sales sectors) in terms of employment can be estimated to be up to 122 291 employees (Ernst&Young, 2006).

In Finland, three of the fifteen breweries (including microbreweries) control around 95% of the market. The two main brewing companies are Synebrychoff and Hartwall. Beer production in 2004 was 4.6 million hl, while consumption was 4.9 million hl (about 84 litres per inhabitant). The Finnish alcoholic drinks sector is highly regulated and the taxes on alcohol (including beer) are high. Total value added due to production and sale of beer in Finland is estimated at 726 million euros (223 million euros directly from the breweries). In 2003, Finnish breweries employed 2 904 persons. The total employment due to production and sale of beer was estimated at 19 500 jobs (Ernst&Young, 2006).

1.5.3 Overview of the brewing process

The production of beer is traditionally a batch process in which several reactions take place simultaneously. The result is a variety of physical, biochemical and chemical transformations that give the product its final appearance and flavour (Masschelein, 1997). The raw materials for brewing are malted barley, water, hops and yeast. Other cereals, such as wheat, rice, rye, oats, maize and sorghum, may be used as adjuncts, or even replacing barley. Wheat malt is used in the production of special beers, for instance in Germany (*weiss* beer) and Belgium. Sorghum malt is widely used in Africa both in the brewing of native beers and as a barley malt replacement in normal beers for economic and trade reasons (Briggs *et al.*, 2004; Eaton, 2006). Sugars and syrups may also be used as adjuncts added to the wort. The brewing process is outlined in Figure 1.3. There are a number of variations of the brewing process, and alternative methods (some more traditional, some more modern) can be used to accomplish each of the individual brewing stages. A general description of each stage is briefly given below. More detailed accounts on individual stages, including raw materials and existing technologies, can be found elsewhere (see e.g. Bamforth, 2003; Briggs *et al.*, 2004; Priest and Stewart, 2006; Pryor, 1988).

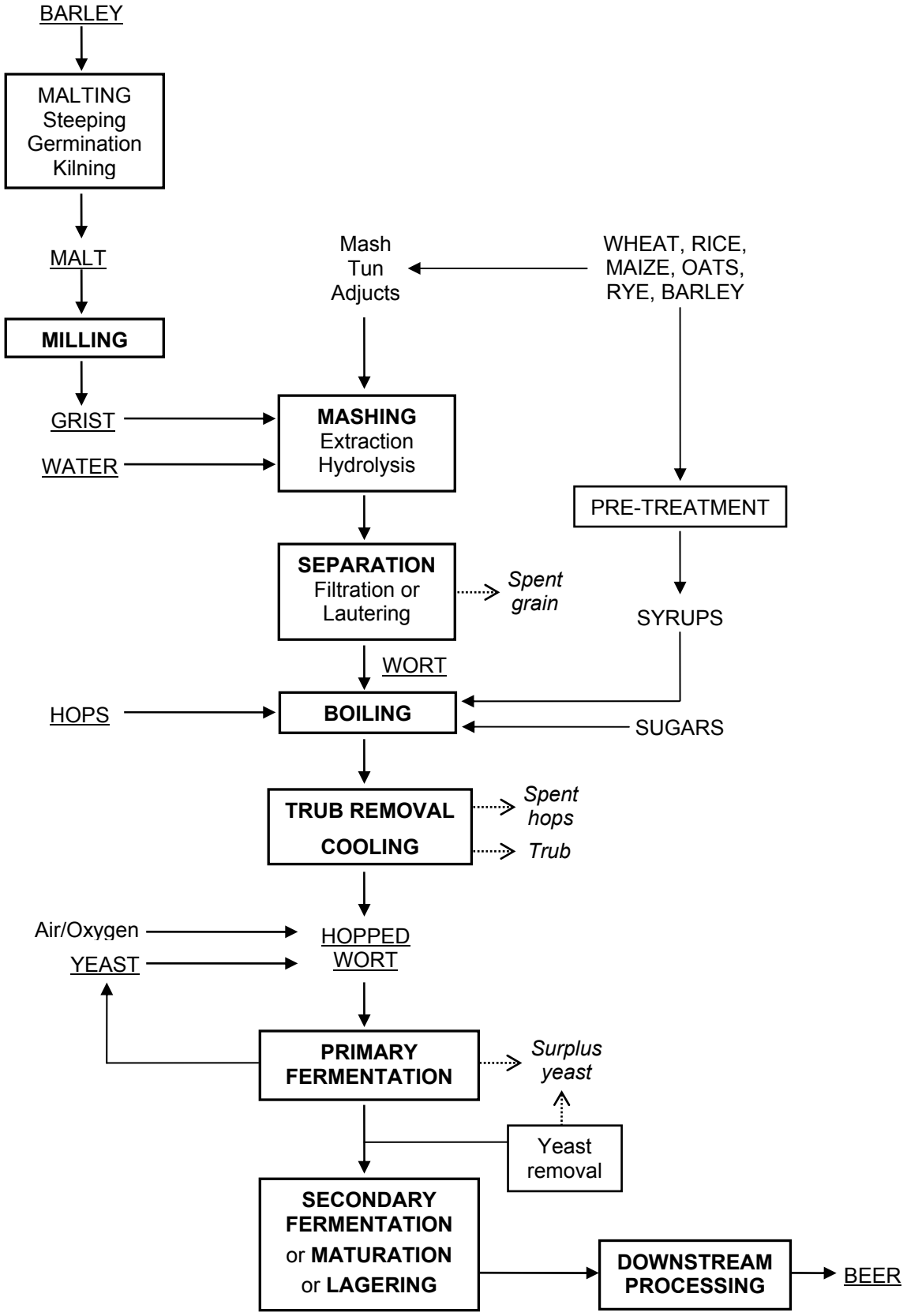

Figure 1.3 – Simplified outline of the brewing process (adapted from Linko *et al.*, 1998).

Malting is basically the initiation of germination of cereal carried to a desired point (when the grain is dried). Malts are made from selected cereal grain, usually barley. The malting process converts the raw barley into a product that is much more friable (easily crushed), with increased enzyme levels and with altered chemical and physical properties. It comprises essentially three phases: steeping, germination and kilning. First, the grain is steeped in cool water (14 – 18 ºC) until the moisture content is 42 – 46% (48 – 72 h). The water is drained occasionally or air is sucked downward through the grain bed, to ensure that the embryo in the grain receives the oxygen it needs for respiration. Germination is induced by the grain taking up the water. After steeping, the grain is drained and germination occurs to a limited extent in a cool (16 – 20 ºC) moist atmosphere. There must be turning and mixing as well as aeration of the grain to prevent the rootlets matting together and to provide sufficient oxygen for respiration. During germination, rootlets grow from the end of the grain, enzymes accumulate (in particular amylase and proteases) and so do sugars and other soluble materials. The dead storage tissue of the grain, the starchy endosperm, is partly degraded (modified), and its physical strength is reduced. In addition, high levels of starch degrading enzymes and β-glucosidases are produced. When germination and modification are sufficiently advanced, these are stopped by kilning, which consists in drying the green malt until the moisture content reaches about 3%. The heating process must be conducted carefully, in order to preserve the enzymes in the green malt, which are particularly heat-sensitive at higher moisture levels. The kilning process is therefore usually started at low temperature (around 50 ºC), which can then be raised (up to around 220 ºC) according to a ramping program dependent on the kind of malt to be produced. Besides drying the malt to preserve it, kilning is also important in the development of colour and flavour. After kilning, the malt is cooled and “dressed”, i.e. the brittle rootlets (culms, sprouts) are broken off and removed. The culms are usually used for cattle food.

The next step is the milling of the malt. The malt, sometimes mixed with adjuncts (roasted malt or barley, corn, rice) at this stage, is ground originating the grist. The grist goes into the mash tun (or mash mixer) where it is mixed with warm water. During mashing, the enzymes of malt hydrolyse the polymers: starch to mono-, di-, trisaccharides and dextrins, glucans to oligosaccharides, proteins to amino acids and peptides. There are three main mashing methods: infusion mashing, decoction mashing and double mashing. Despite the specific features of each method, different temperatures are always used in order to control the extent of proteolysis and polysaccharides hydrolysis. Mash tun adjuncts may be used. These are preparations of cereals that may be mixed with ground malt during mashing. At the end of mashing, the sweet (unhopped) wort is separated from the undissolved solids, the spent grain or draff (used for cattle food), using a lauter tun or a mash filter.

The clear (bright) wort is transferred into the kettle, or copper, in which it is boiled with hops, usually for 1 – 2 hours. Hops used for brewing are the female cones of the hop plants (*Humulus lupulus*), which contain important bittering materials and aroma components. Hops may be used whole, ground up, as pellets or as extracts. The boiling process satisfies some important objectives: sterilization of the wort; extraction of the bittering compounds from hops added early to the boil and aroma compounds from late additions; denaturation of enzymes; coagulation of excess proteins, promoted by tannin materials, to form an insoluble complex (trub or hot break); colour and flavour formation; removal of undesirable volatile compounds; and concentration of the sugars by evaporation of water, bringing the wort to the desired gravity (see definition below). When used, sugar and syrup adjuncts are added to the boiling wort. After boiling, the trub and the suspended fragments of hops have to be removed, by filtration, centrifugation or using a simple separator called whirpool. The trub is sold for cattle food (usually in a mixture with spent grains, due to its intense bitterness). The clear hopped wort is cooled to the pitching (yeast inoculation) temperature using a heat exchanger. The wort "strength" is defined by its extract, or gravity, which is a measure of the sum of fermentable sugars and non-fermentable soluble carbohydrate in wort. The most frequently used units are degrees Plato (ºP). A solution with an extract of x ºP has the same density as a solution containing x g sucrose in 100 g solution.

Before fermentation, the wort is usually aerated or oxygenated, to provide oxygen for the yeast in the initial stages of fermentation. The primary fermentation, i.e. the brewing alcoholic fermentation (discussed in the following section), starts when yeast is pitched into the wort and the final result is green (non-maturated) beer. The green beer goes through a maturation (secondary fermentation) stage, during which its flavour is refined. A key compound in maturation is diacetyl, which is always formed as a by-product in primary fermentation. The synthesis of diacetyl is closely connected with the metabolism of aminoacids. Diacetyl has an intense sweet buttery flavour that is unpleasant in lager beer. At the end of primary fermentation the concentration of diacetyl is way above the taste threshold, which is very low (0.05 $mg \cdot L^{-1}$ or less). Therefore, it is crucial to lower its concentration during beer maturation. Several solutions are available for diacetyl removal, from the traditional long lagering at low temperature to modern rapid maturation in continuous systems using immobilized yeast (Linko *et al.*, 1998).

The downstream processing of the mature beer typically involves: clarification, which is achieved by cold conditioning (chilling the beer to 0 ºC or -1 ºC makes the cold break, i.e. the precipitates formed at low temperatures, and residual yeast to progressively sediment) followed by filtration (usually using kieselguhr as filter aid) or centrifugation; stabilization (addition of antioxidants, insoluble adsorbents to remove haze precursors, foam stabilizers,

etc); dilution with de-aerated water to cut the beer to its final alcohol strength (in the case of high-gravity brewing; see section 1.5.5.); carbonation, i.e. adjustment of the carbon dioxide content of the beer; pasteurization (applied to the beer flowing into a sterile container, or to filled bottles or cans) or sterile filtration; and packaging (into bottles, cans, kegs, etc).

1.5.4 Brewer's yeast and fermentation

Wort fermentation is initiated by pitching (inoculating) the cooled hopped wort with yeast. Yeast is pitched either directly into the cooled wort in the fermentation vessel (or in a starting tank prior to transfer to the fermenter), or in-line en route from the heat exchanger to the fermenter. Aeration or oxygenation of the wort can also be performed in either of these alternative ways (Eaton, 2006). The correct amount of yeast cells must be pitched into the wort. As a rule of thumb, 10 million yeast cells must be added per millilitre of wort at 12 ºP, with proportionally more added if the wort has a higher gravity (Bamforth, 2003; Munroe, 2006).

Besides the oxygen supplied in the wort, which is consumed by the yeast in the initial phase of fermentation, the primary brewing fermentation is essentially an anaerobic alcoholic fermentation process. Brewing yeasts do not develop respiratory competence under the conditions encountered in fermentation. In the aerobic phase of fermentation, respiratory pathways are repressed because of the presence of glucose. In late fermentation, when the glucose has disappeared and its repressing effects are relieved, anaerobiosis prevents the induction of respiratory pathways (Briggs *et al.*, 2004). Nevertheless, it is accepted that promitochondria play a role in fermentation, having influence in several biochemical processes (Smart, 2007). The oxygen provided is essential for the yeast to synthesize sterols and unsaturated fatty acids, which in turn are necessary for yeast growth, as these are important components of the yeast plasma membrane (Snoek and Steensma, 2007; van der Rest *et al.*, 1995b). Being usually the limiting nutrient, the oxygen amount can be used to control yeast growth during fermentation. Wort saturated with air will contain approximately 8 ppm oxygen, depending on the temperature, wort gravity, etc (Munroe, 2006). The yeast growth is limited, with two to three divisions occurring within a typical fermentation (Smart, 2007), and therefore most of the fermentable sugars in the wort are converted to ethanol. That conversion is about 85% of the theoretical (0.538 g ethanol per g maltose); the shortfall represents the proportion of wort sugars utilized for yeast biomass formation and other metabolites (Briggs *et al.*, 2004).

The pitching yeast may derive from propagation (i.e. grown from laboratory pure culture stocks) but more often it is derived from a previous brewing fermentation. At the start of

fermentation, yeast can use the oxygen supplied in the wort to synthesise sterols and unsaturated fatty acids. During the subsequent anaerobic phase of fermentation, the pre-formed pools of these lipids, together with a small quantity supplied by wort, are progressively diluted amongst daughter yeast cells. In the yeast crop, obtained at the end of fermentation, sterol and unsaturated fatty acid levels are reduced to growth-limiting concentrations, hence, the need for oxygenation of wort in the next fermentation (Briggs *et al.*, 2004).

The practice of serial fermentation, involving cropping and re-pitching, introduces a requirement for storage of the yeast in the interval between fermentations. The duration of the storage and the conditions employed influence yeast physiological condition. In particular, the intracellular concentrations of storage carbohydrates (glycogen and trehalose) and sterols and other lipids can be influenced. Variations in the concentrations of these metabolites in pitching yeast are a cause of inconsistencies in the extent of growth during subsequent fermentation (Briggs *et al.*, 2004).

Glycogen and trehalose constitute storage carbohydrates that are accumulated in yeast when growth is restricted and in response to stresses (in the later case mainly trehalose). These carbohydrates can then be mobilized by the cell to provide maintenance energy during periods of starvation. In brewing fermentations, glycogen has been suggested to fulfil two roles. First, it provides the carbon and energy for the synthesis of sterols and unsaturated fatty acids during the aerobic phase of fermentation and, second, energy for cellular maintenance functions during the stationary phase of fermentation and during storage of yeast in the interval between cropping and re-pitching. Besides of its possible role as a storage carbohydrate, trehalose has been implicated in mechanisms of resistance to several stresses (Boulton and Quain, 2001; Briggs *et al.*, 2004).

The physiological state of pitching yeast is an important determinant of fermentation performance. Compared with other fermentations, pitching rates are comparatively high and growth extents are modest. The pitched yeast plays an active role in subsequent fermentation and a proportion may persist through the crop and be subject to further rounds of storage and re-pitching. In a modern brewery, it is usual to limit the number of serial fermentations (Briggs *et al.*, 2004). It is desirable to discard the yeast and introduce a new pure culture (propagated from reference stock cultures kept in the laboratory) into the brewery after every 10 to 15 batches (Bamforth, 2003).

The condition of the pitching yeast cells is therefore essential and, accordingly, viability (percentage of living cells) of the pitching yeast is normally measured. Sometimes the vitality ("health condition") of the yeast is also assessed, thought there are no generally accepted methods to measure vitality. In order to keep the yeast in good condition it is important to

handle it carefully during all steps in the brewery. Important steps in maintaining the vitality and viability are the proper pitching and cropping of the yeast, the propagation of the yeast from laboratory to full-scale pitching, its storage for reuse, and the techniques for acid washing the yeast to reduce contamination with other microorganisms (Eaton, 2006). Surplus yeast is a valuable coproduct and can be sold to distillers, to the yeast extract producers, the food flavourings industry, or health supplement manufacturers.

The biochemical events that occur during fermentation reflect the genotype of the yeast strain used and its phenotypic expression as influenced by the composition of the wort and the conditions established in the fermenting vessel. In order to obtain satisfactory fermentation performance and beer of a desired quality it is necessary to choose a yeast strain with a suitable genotype and manipulate the conditions to encourage appropriate metabolic behaviour (Briggs *et al.*, 2004).

Yeast uses the wort chemical components for its nutrition. The catabolism of the wort sugars provides yeast with energy and carbon skeletons for anabolic pathways during fermentation. The major sources of nitrogen in wort are amino acids, collectively referred to as free amino nitrogen (FAN), together with some ammonium ions. The metabolism of amino acids, which includes catabolism of amino acids assimilated from wort and anabolism (biosynthesis) of new amino acids, makes an important contribution to beer flavour. A number of metabolic intermediates in amino acid metabolism are also precursors in the biosynthesis of key flavour compounds of beer. The yeast requirement for phosphorus is satisfied by the assimilation of inorganic phosphate ions. Sulphur may be assimilated from both inorganic and organic (including the sulphur-containing amino acids methionine and cysteine) sources. Usually brewing wort contains adequate levels of essential mineral ions as well as of vitamins and other growth factors for yeast. The possible exception is zinc, which is commonly added to the wort (Briggs *et al.*, 2004; Munroe, 2006).

The main wort sugars are glucose (about 20% of the total), maltose (about 60%) and maltotriose (about 20%), and they are consumed in that order with some overlap. Smaller amounts of fructose and sucrose are also generally present, being consumed simultaneously with glucose (Boulton and Quain, 2001; Briggs *et al.*, 2004). Efficient brewery fermentation requires rapid and complete utilization of both maltose and maltotriose, but assimilation of these sugars is repressed and/or inactivated at high concentrations of glucose in wort. Only when the yeast has taken up approximately half of the wort glucose will maltose uptake begin. Therefore, a delay caused by glucose repression/inactivation is a major limiting factor in wort fermentation, particularly in high-gravity brewing (section 1.5.5).

Besides ethanol and carbon dioxide, a multitude of other minor products of yeast metabolism

are formed during fermentation. Many of these contribute to beer flavour and aroma. The formation of a desirable spectrum of flavour-active metabolites is influenced by the raw materials (wort composition), the process and the yeast strain used. Fermentation conditions must be controlled properly to ensure that these minor metabolic by-products are produced in desired and consistent quantities. The main flavour-active metabolites are higher alcohols, aldehydes, organic and fatty acids and esters of alcohols and fatty acids, as well as sulphur-containing compounds. These are formed as by-products during the metabolism of sugars and amino acids (Briggs *et al.*, 2004).

The progress of brewing fermentations (Figure 1.4) is most commonly monitored by measuring the decline in the wort extract. The number of yeast cells in suspension increases during the initial phase (yeast growth) and then typically drops as yeast cells start to flocculate. Ethanol production may continue after the peak in suspended yeast concentration, stopping when the final attenuation (attenuation refers to the fraction of wort carbohydrates consumed, and is calculated throughout fermentation by the difference between the original and the current extract expressed as a percentage of the original extract) is attained. As fermentation proceeds, pH drops from ca. 5.0 – 5.5 (wort) to ca. 3.8 – 4.5, slightly increasing towards the end of fermentation.

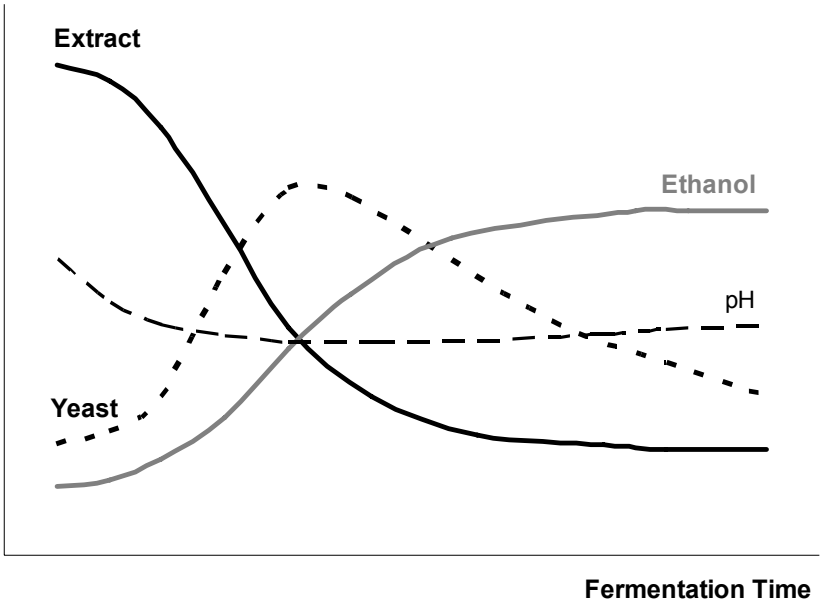

Figure 1.4 – Typical changes during a brewing fermentation.

Lager and ale beers have been traditionally distinguished both by the yeast strains used and by some features of the process. Lager strains are also called “bottom yeasts” as these

flocculate and therefore settle to the bottom of the fermenter towards the end of fermentation. Typically lagers are fermented at temperatures between 6 and 14 ºC (Bamforth, 2003). Ale strains, or "top yeasts", rise to the top of the beer and are collected at the surface of the traditional open squared fermenters. Ales are fermented at higher temperatures, usually 15 to 20 ºC (Bamforth, 2003), and thus fermentations tend to be faster than lagers. Nowadays, although traditional fermentation systems survive in smaller breweries, the differences between the production of ale and lager beers are increasingly blurred since both types are frequently fermented in cylindroconical vessels, with bottom cropping even when using ale strains. Ale and lager strains can be easily distinguished because only the latter can grow on melibiose.

Ale strains are assigned to *S. cerevisiae*. Lager yeasts were originally assigned to *Saccharomyces carlsbergensis*, but have later been included in the taxon *S. cerevisiae* and most recently in *S. pastorianus*. Lager strains appear to result from a hybridization between *S. cerevisiae* and another *Saccharomyces* yeast (more likely candidates belonging to *S. monacensis* or *S. bayanus* species). Several lines of evidence support the hybrid nature of the lager yeast genome (Smart, 2007).

Traditionally, fermenters were open squared vessels made of wood, slate or copper. After the 1960s, closed stainless steel cylindroconical vessels became the norm in modern brewing plants, and fermenters up to 6000 hl capacity replaced smaller vessels (200 to 300 hl) (Anderson, 2006). Cylindroconical vessels, as the designation indicates, have basically a vertical cylinder design with a conical bottom. This design is best suited for using bottom-fermenting yeasts, as it facilitates the cropping of flocculated yeast that sediment in the cone towards the end of fermentation. For this purpose, the included angle in the cone should be 60º (Pryor, 1988). Other advantages of the cylindroconical fermenters include: better mixing due to convection currents set up by rising gas bubbles, ease of temperature control through thermostatted jackets, more hygienic yeast cropping procedures, ease of cleaning (cleaning-in-place methods may be applied), saving space in the brewery (Bamforth, 2003). The design of these vessels should be such that there are the minimum number of openings into the vessel and a minimum number of probes, load-cells or any other impedimenta within the vessel as this aids cleaning and sterilizing (Pryor, 1988). Besides primary fermentation, cylindroconical vessels can also be used for maturation and for cold conditioning of beer.

1.5.5 High- and very high-gravity fermentations

High-gravity (HG) brewing refers to the use of concentrated wort (with higher extract) in order to obtain a concentrated beer (with higher ethanol content) that can be diluted with water to

normal strength at a later stage in the process. Normal worts have gravities of about 11 – 12 ºP, producing beers with 4 – 5% (v/v) ethanol. HG worts have gravities of 14 – 17 ºP. The use of worts above 17 ºP (18 – 25 ºP) is referred as very high-gravity (VHG) brewing. HG worts can be produced by mashing at lower water-grist ratios and/or by boosting the levels of fermentable sugar by adding syrups to the kettle boil (Bamforth, 2003). VHG worts are usually achieved by adding syrups to the wort kettle.

Reconstitution with water can occur either entirely or in part, at almost any stage of the process (Stewart *et al.*, 1997). However, in order to take fully advantage of HG brewing, most often beer is diluted to the specified ethanol content by the addition of water after fermentation and conditioning. The diluting water must be of high quality, ionically comparable to beer, deaerated to prevent oxidative damage to the beer, and preferably carbonated to the level of the beer it is diluting (Bamforth, 2003).

Popularised during the 1970s, HG brewing was progressively introduced into breweries around the world, being already the norm by the mid-1980s (Anderson, 2006). Nowadays, most of the beer produced, at least in Europe and North America, results from HG brewing. The major advantage of HG brewing is economic, resulting from increased productivity: by reducing the amount of water in the mash, increasing production demands can be met without expanding existing brewing, fermenting and storage facilities (Stewart *et al.*, 1997). Besides this more efficient use of plant facilities, advantages of HG brewing include: higher ethanol yield per unit of fermentable extract because of reduced yeast growth; use of higher adjunct rates; improved beer physical and flavour stability; beers produced from HG worts are often rated smoother in taste; greater flexibility in product type (from one “mother” liquid, a number of products can be brewed as a result of dilution and/or use of malt extracts, hop extracts and syrups); reduced energy (heating, refrigeration, etc), labour, cleaning and effluent costs (Stewart *et al.*, 1997).

HG brewing has also a number of disadvantages. Due to the more concentrated mash (increased ratio of carbohydrate to water), there is a reduction in both extract and kettle hop utilization. Modern wort separation systems overcome part of the extract recovery problems. The use of post-fermentation hop extracts help to circumvent the reduced hop utilization (Stewart *et al.*, 1997). The beer foam stability is also negatively affected in HG brewing, but some actions can be taken in order to reduce this problem (Stewart *et al.*, 1997). The main problem with HG brewing, and especially with VHG brewing (see below), is its effect on the overall yeast performance during fermentation, particularly due to the increased osmotic pressure, the elevated ethanol concentration and the modified wort nutrient balance. Nutrients that can be limiting include sterols, unsaturated fatty acids, amino acids, proteins, vitamins and certain metal ions (Stewart *et al.*, 1997). Technical developments together with the

combined use of higher yeast pitching rates and proportionately more oxygen for membrane lipids synthesis (Bamforth, 2003) has allowed circumventing the main challenges of HG brewing.

The economic advantage of HG brewing would be further improved if VHG worts could be efficiently used. However, as the wort extract is increased beyond about 18 ºP, efficient fermentation becomes increasingly difficult. Casey *et al.* (1983; 1984) reported that fermentation difficulties of VHG worts arise because of nutritional deficiencies. When supplemented with a nitrogen source, ergosterol and oleic acid, worts up to 31% dissolved solids could be fermented to produce beers up to 16.2% (v/v) ethanol. Yeast viability remained high and the yeasts could be re-pitched at least five times. Thus, the main nutritional deficiencies in VHG fermentations seem to be FAN and oxygen. Moreover, these authors proposed that brewer's yeast can tolerate 14 – 16% (v/v) ethanol, and therefore their ethanol tolerance is no different than that of wine, sake or distiller's yeast. However, wort supplementation is a practice not likely to be widely adopted by the industry. McCaig *et al.* (1992) also reported successful pilot-scale fermentation of worts up to 24 ºP. Nevertheless, there are persistent problems with VHG fermentations, including slow and incomplete fermentation, low yields of ethanol on sugars, high residual maltose and maltotriose, and low viability of cropped yeast (Huuskonen and Londesborough, 2005). Flavour matching is also critical (Walsh *et al.*, 2005). Therefore, for VHG fermentations, yeast strains are needed that ferment maltose and maltotriose rapidly and completely at high ethanol concentrations, have high viability in beers containing at least 100 $g \cdot L^{-1}$ ethanol, and produce the same aroma profile as the brewer's present strain (Huuskonen and Londesborough, 2005). Efforts have been made in order to select mutants of current production brewer's yeast strains with such characteristics (Blieck *et al.*, 2007; Huuskonen and Londesborough, 2005; Walsh *et al.*, 2005).

1.5.6 Continuous fermentations

Continuous operation provides another way to increase the productivity of beer fermentation. This solution has been considered by brewers and investigated by brewing scientists for many decades. Several systems for continuous brewing fermentation have been designed, some reaching pilot or even industrial scale. However, by the end of the 1970s most of the industrial continuous systems implemented in the past decades had been closed down (Virkajarvi, 2001). More recently, the advances in immobilised yeast technology prompted new interest in continuous fermentations. However, with the exception of a brewery in New Zealand, industrial beer fermentation persists typically as a batch process. A continuous beer

maturation system using bioreactors with immobilized yeast was developed and implemented in a brewery in Finland. The system accelerates diacetyl elimination, reducing the lager beer maturation time to 2 hours (Pajunen, 1995). Primary fermentation continuous systems with immobilized yeast have not yet reached industrial scale implementation, but much research has been done in recent years (Brányik *et al.*, 2007; Masschelein and Vandenbussche, 2001; Mensour *et al.*, 1997; Nedovic *et al.*, 2001; Pajunen *et al.*, 2000; Pilkington *et al.*, 1998; Virkajarvi and Linko, 1999).

1.6 Cheese whey and lactose fermentation

1.6.1 Cheese whey

Cheese whey is a by-product of dairy industries, particularly the watery portion that is formed during the coagulation of milk casein in cheese making or in casein manufacture. Whey is produced in large amounts and has a high polluting charge, therefore representing a significant environmental problem (Siso, 1996). On the other hand, however, whey retains about 55% of the milk nutrients, including proteins, lactose, minerals and vitamins (Kosikowski, 1979) and therefore has a vast potential as a source of added value compounds, challenging the industry to face whey surplus as a resource and not only as a waste problem.

Since about 5000 B.C., when man started making cheese, the utilization of whey has been a challenge. In the middle ages, whey was applied as a pharmaceutical drug, a component of soothing salves for burns, a skin balm, a potion to inspire vitality and to restore hair, but rarely was it used as a food for humans. As cheese production increased, the volume of whey also grew and many cheese factories were built near waterways so that most of the whey was diverted to these streams or rivers (Kosikowski, 1979). Modern times brought the awareness of the polluting problem that whey represents and the consequent regulations prohibiting its dumping into waterways and even into municipal sewage systems, whose conventional treatments are not appropriate to sufficiently reduce whey polluting charge (Kosikowski, 1979).

Whey represents about 85 – 95% of the milk volume and retains 55% of milk nutrients. Among the most abundant of these nutrients are lactose (4.5 – 5% w/v), soluble proteins (0.6 – 0.8% w/v), lipids (0.4 – 0.5% w/v) and mineral salts (8 – 10% of dried extract). Whey also contains appreciable quantities of other components, such as lactic (0.05% w/v) and citric acids, non-protein nitrogen compounds (urea and uric acid) and B group vitamins (Siso, 1996). There are two main varieties of whey, according to the procedure used for casein

precipitation: acid whey (pH < 5), resulting from the production of fresh or soft cheeses (such as cream and cottage cheese), and sweet whey (pH 6 – 7), resulting from hard (ripened) cheeses (Kosikowski, 1979; Siso, 1996; Yang and Silva, 1995). The composition of different types of whey is variable; approximate figures for the main components can be found in the literature (see e.g. Kosikowski, 1979; Pesta *et al.*, 2007; Yang and Silva, 1995).

Cheese whey represents an important environmental problem because of the high volumes produced and its high organic matter content. To make 1 kg of cheese, about 9 litres of whey are generated (Kosikowski, 1979). The world whey production is estimated to be about 82 million tons per year (Pesta *et al.*, 2007). In Portugal, the production of liquid whey is estimated to be 500 – 560 thousand tons per year (Frazão, 2001), and the largest part of it is processed by concentration and drying (Pintado and Malcata, 2007). Whey exhibits a BOD (biochemical oxygen demand) of 30 – 50 $g \cdot L^{-1}$ and a COD (chemical oxygen demand) of 60 – 80 $g \cdot L^{-1}$. Lactose is largely responsible for the high BOD and COD. Protein recovery reduces the COD of whey only by about 10 g/L (Domingues *et al.*, 1999a; Siso, 1996).

1.6.2 Whey utilisation/valorisation

Earliest ways of whey disposal included piping into oceans, funnelling into caves, spreading over fields, oxidation in lagoons or sewage systems, feeding into ruminants, etc. (Kosikowski, 1979). Disposing of whey by these means provides no valuable product, and is costly and labour demanding for the cheese manufacturer, who generally bears all the direct costs of handling and transport. Therefore, in spite of the fact that removal of whey from the premises releases pressure on the cheese manufacturing plant (Kosikowski, 1979), these solutions are not satisfactory. In order to develop integrated solutions for the cheese whey problem, it must be considered as a resource and not only as a waste effluent, in view of its large potential as a source of added value products.

Siso (1996) reported that about 50% of total world cheese whey production is treated and transformed into various food products. This percentage has most likely increased since then, due to continued research efforts in the field of whey utilization together with the pressure exerted over cheese and casein producers by aggravated legislations concerning effluent disposal. Figure 1.5 summarises some of the solutions that have been implemented or proposed for whey utilization. These solutions are briefly discussed below.

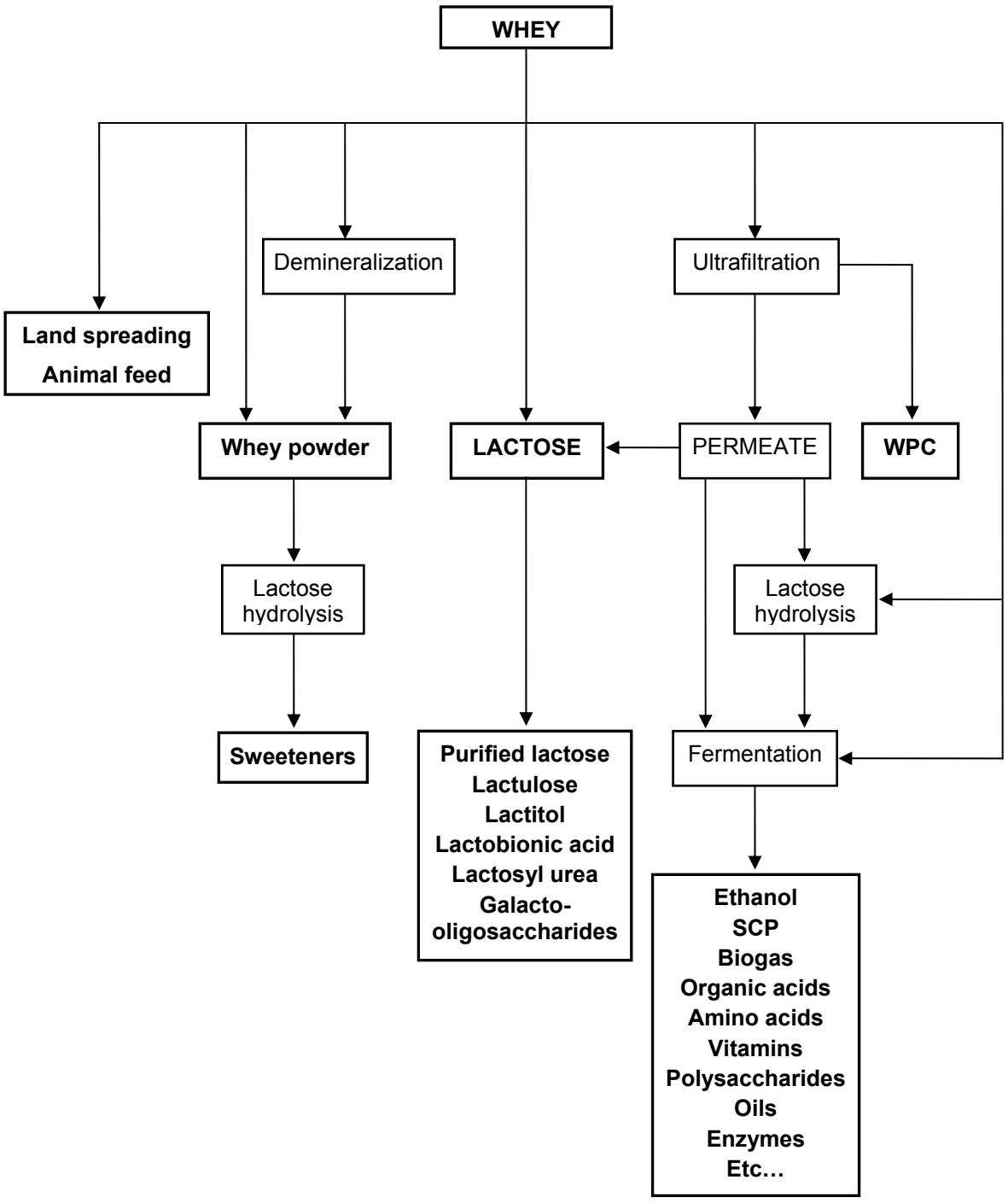

Figure 1.5 – Utilizations of cheese whey (adapted from Audic *et al.*, 2003; Siso *et al.*, 1996).

A large fraction of the whey that is processed is dried to produce cheese whey powders (Kosikowski, 1979; Yang and Silva, 1995). The quality of fresh whey is therefore maintained for a longer period of time, facilitating manipulation and transport (Siso, 1996). Whey powder is mostly used for animal feeding but smaller quantities may be also used in human foods, such as ice-creams, baked goods, cakes, sauces, milk derivatives, etc. Limitations to the use of whey powders in human food products include excessive saline taste, small protein/sugar ratio, low solubility and low sweetening power (only 40% compared to sucrose) of lactose (Siso, 1996). In Portugal, the production of whey powder is estimated to be around 6000 tons per year, which is used in animal feed (50%), baked goods and dairy industries (12%), cookies and chocolates (10%), bakery supplements (10%) and margarines (8%) (Frazão, 2001).

The first step in most procedures for cheese whey valorisation consists in the recovery of the protein fraction. Whey proteins represent about 20% of the milk proteins, having a high nutritional value (Siso, 1996) as well as reported health benefits (Beaulieu *et al.*, 2006). The most abundant proteins in whey are β-lactoglobulin (55 – 65%), α-lactalbumin (15 – 25%), immunoglobulins (10 – 15%), bovine serum albumin (5 – 10%) and lactoferrin (1 – 2%) (Beaulieu *et al.*, 2006). Separation of whey proteins is typically achieved by ultrafiltration or diafiltration to produce whey protein concentrates (WPC) with a protein content of 30 – 60% (Kosikowski, 1979; Siso, 1996). Whey proteins can be used not only as simple protein supplements, but may prove interesting for the manufacture of transformed food products due to their good solubility and functional characteristics (Kosikowski, 1979; Siso, 1996). WPC are used as supplements in dairy, bakery and beverage industries (Kosikowski, 1979). Whey proteins have also non-food uses, mainly in cosmetics and pharmaceutical products (Audic *et al.*, 2003).

During the production of WPC by ultrafiltration, high volumes of a lactose-rich stream, the permeate, are also obtained. The permeate remains a major pollutant since it retains the lactose, which is largely responsible for the whey polluting charge.

Lactose is the sugar present in the milk of most mammals. It is a disaccharide formed by galactose and glucose and is chemically defined as O-β-D-galactopyranosyl-(1-4)-β-D-glucose (Adam *et al.*, 2004; Yang and Silva, 1995). Most of the lactose produced is recovered from whey or whey permeate by a process involving crystallisation (for more details see Yang and Silva, 1995). The major uses for lactose include food ingredient, ingredient in infant formula, filler or coating agent for tablets in the pharmaceutical industry and raw material for the production of lactose derivatives (lactulose, lactitol, lactobionic acid, lactosyl urea, galacto-oligosaccharides, etc.) (Audic *et al.*, 2003; Yang and Silva, 1995). Hydrolysed lactose solutions possess greater sweetening power than lactose and have food related uses,

particularly in the confectionery and ice-cream industries replacing sucrose or starch syrup (Siso, 1996). The technology to produce hydrolysed lactose syrup is well developed and is used to produce lactose-hydrolysed milks for lactose-intolerant individuals. Chemical hydrolysis at low pH and high temperature is possible, but enzymatic hydrolysis is usually the method of choice, with enzymes from *Aspergillus* and *Kluyveromyces* species being the most commonly used (Yang and Silva, 1995).

Another major application for the lactose in whey or permeate (ca. 5% w/v) involves its use as a substrate for the production of valuable compounds by fermentation. The classical examples are ethanol (see below) and single cell protein (SCP) production in yeast-based bioprocesses, although biotechnologists have proposed a multitude of alternative bio-products (Audic *et al.*, 2003; Pesta *et al.*, 2007; Siso, 1996; Yang and Silva, 1995). Among those bio-products are: biogas (methane), organic acids (acetic, propionic, lactic, citric, gluconic, itaconic, gibberelic), amino acids (glutamic, lysine, threonine), vitamins (B12 and B2, or cobalamins and riboflavin, respectively), polysaccharides (xanthan gum, dextran, phosphomannan, pullulan, gellan), oils (lipids), enzymes (β-galactosidase, polygalactorunase) and other compounds (fructose-diphosphate, 2,3-butanediol, calcium magnesium acetate, ammonium lactate, butanol, glycerol). Lactose can be used directly by lactose-consuming microorganisms or, alternatively, pre-hydrolysed lactose solutions may be used as substrate. Large whey surplus together with the need for cheap and largely available substrates and, above all, the rapid advances in microbial biotechnology are likely to prompt further exploitation of whey lactose as fermentation feedstock to obtain value-added products.

1.6.3 Lactose-consuming microorganisms

The number of microorganisms that can use lactose as a source of carbon and energy is limited, yet including bacteria, yeasts and filamentous fungi. Bacteria have developed different strategies for the uptake and hydrolysis of lactose. The most effective implies the simultaneous phosphorylation and translocation of the sugar across the cellular membrane, existing at least two alternative mechanisms for uptake (a lactose-proton symporter and a lactose-galactose antiporter). Once inside the bacterial cell, the phosphorylated lactose is hydrolysed by a phospho-β-galactosidase (an enzyme that recognises phosphorylated lactose). When the uptake mechanism does not imply phosphorylation lactose is intracellularly cleaved by a β-galactosidase (Adam *et al.*, 2004). The regulation of lactose utilization by the *lac* operon in *Escherichia coli* has become a paradigm for prokaryotic gene regulation. The *E. coli lacZ* gene (encoding β-galactosidase) has become a very common genetic and biotechnological tool for marking gene activity and is included in different types of

cloning vectors (Adam *et al.*, 2004). Moreover, the *E. coli lacY* gene (encoding lactose permease) was the first gene encoding a membrane transport protein to be cloned into a recombinant plasmid, overexpressed (Teather *et al.*, 1978) and sequenced (Büchel *et al.*, 1980). LacY is a paradigm for the Major Facilitator Superfamily of transport proteins (Pao *et al.*, 1998) and its structure has been recently unveiled (Abramson *et al.*, 2003; Kaback, 2005). Lactic acid bacteria (includes several genera such as *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Streptococcus*) are among the most important lactose-consuming microorganisms, due to their occurrence in milk and dairy products. For example, yogurt is produced by the concerted (symbiotic) action of two types of lactic acid bacteria: *Streptococcus salivaricus* (*ssp. thermophilus*) and *Lactobacillus delbrueckii* (*ssp. bulgaricus*) (Adam *et al.*, 2004). Besides their food-related significance, the importance of lactic acid bacteria in biotechnology is extended to the production of lactic acid, e.g. from whey fermentation (Panesar *et al.*, 2007).

Filamentous fungi often utilize lactose only at very low rates (Seiboth *et al.*, 2007). In fungi there are two principal alternatives for the catabolism of lactose: (1) extracellular hydrolysis and subsequent uptake of the resulting glucose and galactose monomers and (2) uptake of the disaccharide and subsequent intracellular hydrolysis. Fungi species such as *Aspergillus nidulans*, *Neurospora crassa* or *Fusarium graminearum* follow this second strategy for lactose utilization, while others such as *Hypocrea jecorina* (*Trichoderma reesei*) hydrolyse lactose by an extracellular β-galactosidase and take up the monosaccharides (Seiboth *et al.*, 2007). The yeasts that assimilate lactose aerobically are widespread, but those that ferment lactose are rather rare (Fukuhara, 2006), including e.g. *K. lactis* (see below), *K. fragilis*, and *Candida pseudotropicalis*.

The preparations of β-galactosidase that are commercially available and rated GRAS are produced by the yeasts *K. lactis* and *K. fragilis* and by the filamentous fungi *Aspergillus niger* and *A. oryzae*. The advantage of these fungi is that they secrete the enzyme, which facilitates the recovery from the culture broth, but otherwise the yeasts produce a higher quantity of enzymatic units. Moreover, the optimum pH of fungal β-galactosidase is acid and activity at pH values higher than optimum is quite diminished, so that these enzymes are only suitable for the hydrolysis of acid wheys. The enzyme from *Kluyveromyces* (intracellular) has a near neutral pH optimum (Siso, 1996). The construction of genetically engineered yeast strains (*K. lactis* as well as *S. cerevisiae*) that secrete *K. lactis* β-galactosidase has been considered in order to avoid the costly extraction and purification processes that are necessary because of the intracellular nature of the enzyme (Becerra *et al.*, 2001; Rodriguez *et al.*, 2006). Genetically engineered *S. cerevisiae* strains with the capacity to produce and secrete *A. niger* β-galactosidase have also been reported (Domingues *et al.*, 2002; Oliveira *et al.*, 2007;

Ramakrishnan and Hartley, 1993). Bacterial β-galactosidase (primarily obtained from *E. coli*) is commercialised for analytical uses, but it is not GRAS (Siso, 1996).

1.6.4 Lactose metabolism in *K. lactis* and the *GAL*/*LAC* regulon

Similarly to other microorganisms present in milk, *K. lactis* is adapted for an efficient utilization of lactose. The ability of this yeast to metabolise lactose results from the presence of a lactose permease and a β-galactosidase (Rubio-Texeira, 2006).

The *K. lactis* lactose permease is a membrane protein of 587 amino acids encoded by the gene *LAC12* (Chang and Dickson, 1988). The lactose uptake in *K. lactis* is mediated by a transport system inducible by lactose and galactose (the inducer is intracellular galactose) (Dickson and Barr, 1983). The uptake is mediated by a carrier, being saturated at high substrate concentrations. Dickson and Barr (1983) determined a K_m of about 2.8 mM for this transport system, while Boze *et al.* (1987) reported a K_m of 1.2 – 4 mM in a different strain. The transport of lactose in *K. lactis* is an active process, requiring an energy-generating system, which permits the intracellular accumulation of lactose against a concentration gradient (Boze *et al.*, 1987; Dickson and Barr, 1983). The transport is inhibited by the proton ionophore 2,4-dinitrophenol (Boze *et al.*, 1987; Dickson and Barr, 1983), and therefore it has been suggested that the transporter operates, at least in part, by a proton symport mechanism (Dickson and Barr, 1983). In other *Kluyveromyces* species lactose uptake has also been described to proceed via a proton symport mechanism (Barnett and Sims, 1982; Carvalho-Silva and Spencer-Martins, 1990; Van den Broek and Van Steveninck, 1982). Lac12p shows sequence similarity to the *E. coli* xylose and arabinose proton symporters (Chang and Dickson, 1988) as well as significant sequence and structure homology with the *S. cerevisiae* maltose proton symporter Mal61p (Cheng and Michels, 1989), but no significant sequence similarity with the lactose permease (*lacY* gene) of *E. coli* (Chang and Dickson, 1988).

The β-galactosidase (lactase) is encoded by the *LAC4* gene (Poch *et al.*, 1992) and is regularly described to be intracellular (Dickson *et al.*, 1979; Rubio-Texeira, 2005; Sheetz and Dickson, 1980). This enzyme has a K_m for lactose of 12 – 17 mM and its pH optimum is around 7 (Dickson *et al.*, 1979).

β-galactosidase hydrolyses lactose into glucose and galactose. Intracellular glucose can enter glycolysis while galactose follows the Leloir pathway. In *K. lactis*, the metabolism of lactose and galactose are closely related. The regulatory circuit of the *GAL*/*LAC* regulon of *K. lactis* (Table 1.2) has been studied in detail (for reviews see Rubio-Texeira, 2005; Schaffrath and Breunig, 2000) particularly in comparison with the *GAL*/*MEL* regulon of *S. cerevisiae* (Table

1.2), which is one of the most intensively studied and best understood genetic regulatory circuits in yeasts and a major model for the study of eukaryotic regulation (for reviews see e.g. Johnston, 1987; Lohr *et al.*, 1995).

Table 1.2 – *GAL*/*MEL* genes of *S. cerevisiae* and *GAL*/*LAC* genes of *K. lactis*.

S. cerevisiae		***K. lactis***	
Gene	Function	Gene	Function
Structural/Catabolic genes		*Structural/Catabolic genes*	
MEL1	α-Galactosidase	*LAC12*	Lactose/galactose permease
GAL2	Galactose permease	*LAC4*	β-Galactosidase
GAL1[1]	Bifunctional galactokinase/sensor inducer	*KlGAL1*[1]	Bifunctional galactokinase/sensor inducer
GAL10	Uridine diphosphoglucose 4-epimerase	*KlGAL10*	Uridine diphosphoglucose 4-epimerase
GAL7	Galactose-1-phosphate uridylyltransferase	*KlGAL7*	Galactose-1-phosphate uridylyltransferase
GAL5[2]	Phosphoglucomutase	*KlGAL5*[2]	Phosphoglucomutase
Regulatory genes		*Regulatory genes*	
GAL4	Transcriptional activator	*KlGAL4 (LAC9)*	Transcriptional activator
GAL80	Gal4p repressor	*KlGAL80*	Gal4p repressor
GAL3	Gal80p repressor (sensor/inducer)		

[1] *GAL1* has both catabolic (galactokinase) and regulatory (sensor/inducer) functions.

[2] *GAL5* is not specific of the *GAL* regulon, having a more generalised role in carbon metabolism

S. cerevisiae cannot assimilate lactose, yet it can utilise galactose. Some *Saccharomyces* yeasts can also assimilate melibiose, which is hydrolysed to glucose and galactose by a secretable α-galactosidase encoded by the gene *MEL1*. Galactose is taken up by a permease, encoded by the gene *GAL2*. Once inside the cell, catabolism of galactose proceeds through the highly evolutionarily conserved Leloir pathway, both in *S. cerevisiae* and in *K. lactis* (Johnston, 1987; Rubio-Texeira, 2005). This pathway consists in four catalytic steps (see Figure 1.2; Table 1.2): (1) galactokinase (gene *GAL1*) phosphorylates galactose to galactose-1-phosphate; (2) uridine diphosphoglucose 4-epimerase (*GAL10*) exchanges galactose with the glucose group in UDP-glucose to create UDP-galactose, and then changes the stereochemistry of C4 in UDP-galactose, creating again UDP-glucose; (3) galactose-1-phosphate uridylyltransferase (*GAL7*) uses the glucose released in the previous step to transform galactose-1-phosphate into glucose-1-phosphate; (4) phosphoglucomutase (*GAL5*) converts glucose-1-phosphate into glucose-6-phosphate.

Despite the extensive degree of conservation in the group of genes involved in the utilization of galactose between the two yeasts, differences have arisen as a result of their evolution in different environments: *S. cerevisiae* has mainly adapted to glucose, whereas *K. lactis* has

adapted to lactose. Therefore, the two yeasts have differences in the modes of regulation that have important consequences in their overall response to carbon sources (Rubio-Texeira, 2005).

The induction of the *GAL* genes in both *S. cerevisiae* and *K. lactis* is determined by the interplay between three main GAL-specific regulatory proteins (Table 1.2): a transcriptional activator (Gal4p, also known as Lac9p in *K. lactis*), a repressor (Gal80p) and a ligand sensor (Gal3p in *S. cerevisiae*; Gal1p in *K. lactis*). This later activates *GAL* gene expression after binding galactose (the inducer) and ATP.

Gal4p (*S. cerevisiae*) and Lac9p (or KlGal4p) bind as homodimers to specific upstream activating sequences (UAS_G) in the *GAL* promoters. The DNA consensus sequence of the UAS_G is 5'-CGG(N5)A/T(N5)CCG-3' (Gödecke *et al.*, 1991; Leonardo *et al.*, 1987). The transcriptional activator seems to remain bound to the UAS_G sites in every condition, although with variable strength of binding and proportion of occupied UAS_G sites (Rubio-Texeira, 2005). *GAL4* can complement the transcriptional activation function of *LAC9* in *K. lactis* (Riley *et al.*, 1987), and conversely, *LAC9* complements the *gal4* mutation in *S. cerevisiae* (Salmeron and Johnston, 1986; Wray *et al.*, 1987). However, Gal4p did not exactly mimic LAC9p function, and vice versa. The two proteins share only three regions of significant homology (which account for about 30% of the amino acids in the proteins). These regions have been involved in nuclear localization, DNA binding, oligomerization and transcriptional activation (Salmeron and Johnston, 1986; Wray *et al.*, 1987). The absence of a higher level of homology suggests that additional regulation specifically evolved by each of the yeast species may be contributing to differences in the function of these proteins by yet uncharacterized mechanisms (Rubio-Texeira, 2005). The concentration of activator in the cell also plays an important role in the regulation of transcription, and evidences for higher levels of LAC9p in *K. lactis* than of Gal4p in *S. cerevisiae* have been reported (Zachariae and Breunig, 1993; Zachariae *et al.*, 1993; Zenke *et al.*, 1993). Thus, even though Gal4p is functionally analogous to Lac9p, specific features of each of the proteins, as well as their cellular concentration, seem to have regulatory relevance.

The main role of Gal80p is to counteract the activating function of Gal4p in the presence of carbon sources other than galactose. In the absence of galactose, Gal80p binds to Gal4p thereby physically blocking interaction of the activation domain with the transcriptional machinery (Rubio-Texeira, 2005).

In *S. cerevisiae*, Gal4p is released from Gal80p-mediated inhibition through the action of Gal3p. Induction can only take place when Gal3p binds to its two allosteric effectors: galactose and ATP. The mechanism of galactose-mediated signal transduction in *S.*

cerevisiae and the action of Gal3p have been intensively studied (reviewed by Bhat and Murthy, 2001). *K. lactis* has no homologue for the *GAL3* gene. The ligand sensor activity is performed in this case by the galactokinase, KlGal1p, which is therefore a bifunctional protein. In *S. cerevisiae*, Gal1p can also replace Gal3p function but it is a less efficient ligand sensor. Gal3p lacks galactokinase activity (Rubio-Texeira, 2005).

From a regulatory point of view, three major states, according to the carbon source available, can be observed for the *GAL*/*MEL* genes in *S. cerevisiae* and *GAL*/*LAC* in *K. lactis*: (1) repressed, in the presence of glucose; (2) non-induced/poised for induction, in respiratory carbon sources, such as glycerol; (3) induced to high levels of expression in the presence of galactose. Detailed descriptions of the complex regulatory circuits of galactose metabolism can be found in the reviews cited. In particular, Rubio-Teixeira (2005) compared the dynamics of regulation in each of these three states in *S. cerevisiae* and *K. lactis*.

Regulation of *LAC12* and *LAC4* expression in *K. lactis* is controlled by the same mechanisms that regulate *GAL* genes. *LAC12* and *LAC4* are divergently transcribed from an unusually large intergenic region, which works as promoter for the transcription of both genes. The *LAC12-LAC4* intergenic region contains four functional UAS_G elements, which are binding sites for the trans-activator Lac9p. Two functional UAS_G elements are located in front of each of the genes at almost symmetrical positions. These elements cooperate in activating transcription of both genes (Gödecke *et al.*, 1991). Gödecke *et al.* (1991) pointed that the distance of the most distal UAS_G elements to the RNA initiation sites is exceptionally large (more than 2.3 kb) in the *LAC* promoter. In *K. lactis*, as in *S. cerevisiae*, intergenic regions are usually smaller and thus regulatory elements are located closer to the transcription start. These authors proposed that *LAC4* and *LAC12* proximal sites interact to achieve maximal expression of both genes simultaneously in *K. lactis*, and that such interaction may occur via direct protein-protein contact between DNA bound Lac9p molecules or indirectly by multiple Lac9p molecules touching a common target. The four Lac9p-DNA complexes of the *LAC* promoter would therefore be assembled into a high molecular weight DNA-protein aggregate structured mainly by interacting Lac9p molecules (Gödecke *et al.*, 1991).

1.6.5 Recombinant *S. cerevisiae* for lactose fermentation to ethanol

The conversion of the lactose in cheese whey or whey permeate into fuel ethanol is hardly economically competitive with the currently established processes, using cane sugar and cornstarch as substrates, or with emerging second generation technologies using lignocellulosic biomass as raw material. However, being a waste product represents an advantage of whey over food-related fermentation feedstocks, such as corn, for ethanol

production. Moreover, the availability of diverse solutions for whey bioremediation is valuable, so that each dairy company can evaluate, according to its own specificities, the best way to deal with the environmental problem created by whey surplus. Finally, whey ethanol has food-grade quality, and therefore can find a proper market e.g. in vinegar manufacturing and in the beverage industry.

Kluyveromyces fragilis is the microorganism of choice for most commercial plants producing ethanol from whey (Siso, 1996), though other lactose-fermenting yeasts (*Kluyveromyces marxianus*, *Candida pseudotropicalis*) have also been considered (Pesta *et al.*, 2007). On the other hand, *S. cerevisiae* is usually the first choice for industrial processes involving alcoholic fermentation (see section 1.4.), but wild strains are unable to metabolise lactose. The engineering of *S. cerevisiae* for lactose utilization has therefore been addressed over the past 20 years by different strategies (reviewed by Rubio-Teixeira, 2006). However, most strains obtained displayed undesirable characteristics (such as slow growth, genetic instability or problems derived from the use of glucose-galactose mixtures) or were ineffective for ethanol production.

Many of the strategies to use *S. cerevisiae* strains for the fermentation of lactose were based on the extracellular hydrolysis of the disaccharide to produce a mixture of glucose and galactose, which could then be metabolised by *S. cerevisiae*. This could be accomplished by adding β-galactosidase into the medium (Champagne and Goulet, 1988; Terrell *et al.*, 1984), or trough the construction of recombinant *S. cerevisiae* strains that secrete β-galactosidase (e.g. from *A. niger* or *K. lactis*) to the medium (see section 1.6.3). Strategies based on the release of intracellular heterologous β-galactosidase (e.g. from *E. coli* or *K. lactis*) by other means than secretion have also been designed. The approaches used to achieve controlled release of the enzyme to the medium include yeast autolysis by overproduction of Gal4p (Compagno *et al.*, 1995; Porro *et al.*, 1992), the use of thermosensitive autolytic mutants (Becerra *et al.*, 1997; Becerra *et al.*, 2004) and cell permeabilization with toluene or ethanol (Compagno *et al.*, 1993). These later strategies may be particularly useful for the recovery of intracellular products, including β-galactosidase itself. The main disadvantage of extracellular hydrolysis approaches is the potential catabolite repression problem associated with the utilization of glucose-galactose mixtures: in the presence of glucose, the utilization of alternative carbon sources (such as galactose) by *S. cerevisiae* is impaired (see section 1.4.), which may result in diauxic growth.

Therefore, alternative strategies involving the simultaneous expression in *S. cerevisiae* of a lactose permease and an intracellular β-galactosidase were devised. The *Kluyveromyces* systems, in particular *LAC12* and *LAC4* genes of *K. lactis*, were a logical choice due to the phylogenetic proximity between the two species. Sreekrishna and Dickson (1985) were the

first to construct Lac^+ (lactose-consuming) *S. cerevisiae* strains by transfer of the *LAC12* and *LAC4* genes of *K. lactis*. A 13 kb region of the *K. lactis* genome, comprising the two genes as well as their intergenic region, was used in the construction. Thus, transcriptional expression of the genes was controlled by the endogenous *K. lactis* promoter (see section 1.6.4.). These authors only obtained Lac^+ transformants when using indirect selection (first selected for G418 resistance and then for growth on lactose). Moreover, they reported that the Lac^+ transformants had integrated 15 – 25 tandem copies of the vector containing the *LAC* genes into a host chromosome. Nevertheless, those transformants grew slowly in lactose (doubling time in lactose minimal media of 6.7 h; Sreekrishna and Dickson, 1985). Rubio-Texeira *et al.* (1998) cloned these same genes in *S. cerevisiae* but their strategy involved putting both the *LAC* genes under the control of the *CYC-GAL* promoter (a galactose-inducible hybrid promoter) and target genomic integration to the ribosomal DNA region (*RDN1* locus). The Lac^+ transformants obtained grew slowly in lactose. These transformants were crossed with wild-type strains, yielding meiotic segregants with good growth and lactose assimilation capacity, and finally two selected haploids were mated to generate a fast-growing Lac^+ diploid strain. In lactose medium, this strain exhibited a respiro-fermentative metabolism similar to that of *K. lactis*, with high biomass yield but low ethanol production (Rubio-Texeira *et al.*, 1998). The same approach was used to construct Lac^+ baker's yeast. Propagation of that yeast in cheese whey did not affect the quality of the bread or the yeast gassing power (Adam *et al.*, 1999). In our laboratory, a flocculent *S. cerevisiae* Lac^+ strain was constructed (Domingues *et al.*, 1999b) using the same plasmid (pKR1B-LAC4-1) as Sreekrishna and Dickson (1985) but a different selection procedure. The plasmid KR1B-LAC4-1 was co-transformed with a linear fragment of the plasmid YAC4 (containing the *URA3* gene) into an *ura*$^-$ strain (*S. cerevisiae* NCYC869-A3). Selection was done for *ura*- complementation in YNB-Galactose plates. Xgal (5-bromo-4-chloro-3-indolyl- β-D-galactopyranoside) was included in these plates, allowing the identification of clones with β-galactosidase activity (blue colonies). Only 4 (out of 1212) blue colonies were obtained. From these 4 transformants, only 2 kept a stable Lac^+ phenotype. One of these transformants exhibited bizarre forms and pseudo-mycelium and therefore was rejected. The other (named *S. cerevisiae* NCYC869-A3/T1, or simply T1) was selected for more detailed characterization. The original recombinant T1 grew slowly in lactose (doubling time in lactose minimal media of 5 h), with low ethanol yield (Domingues *et al.*, 1999b). However, after an adaptation period, where T1 was kept in liquid lactose medium, refreshed periodically, an increase in growth rate and in ethanol specific production rate in lactose was observed. That adapted strain was successfully used in long-term continuous lactose fermentations (Domingues *et al.*, 1999a; Domingues *et al.*, 2001) resulting in high ethanol productivities from lactose (see Table 1.3). Unexpectedly, the strain lost its improved phenotype after storage at -80 ºC: when the culture

was re-grown from -80 ºC stocks the slow growth in lactose was again observed. Thus, the adaptation period was needed even for an already adapted culture of T1 that had been kept at -80 ºC (Domingues *et al.*, 1999b). A stable evolved strain that derived from T1 by a long-term evolutionary adaptation experiment is described here (chapter 4).

Another approach to create Lac^+ *S. cerevisiae* consisted in the generation of a hybrid strain with *K. fragilis* by protoplast fusion. The fusants obtained were capable of fermenting lactose and producing about 13% (v/v) ethanol in rich medium to which lactose was fed periodically, compared to about 10% (v/v) ethanol produced by the parent *K. fragilis* strain (Farahnak *et al.*, 1986). The same technique was used to produce hybrids of *K. lactis* and *S. cerevisiae* that were able to ferment lactose in sweet and salted whey (Tahoun *et al.*, 2002).

There are a few established industrial systems to produce ethanol from whey (see Pesta *et al.*, 2007), which has been done in some countries, such as Ireland, Denmark, United States and New Zealand (Lyons and Cunningham, 1980; Pesta *et al.*, 2007; Siso, 1996). However, direct fermentation of whey or whey permeate to ethanol is generally not economically feasible because the low lactose content (ca. 5% w/v) results in low ethanol titre (2 – 3% v/v), making the distillation process too expensive. Thus, it is important to start the fermentation with high concentration of lactose, which can be achieved by concentrating the whey, e.g. by ultrafiltration, in order to obtain high ethanol titre at the end of fermentation. Besides, it is also crucial to maximise the ethanol productivity of the process. Nevertheless, in the design of a process for ethanol production from whey a compromise must be made between maximisation of ethanol titre/productivity and minimisation of residual lactose concentration in the effluent, since the purpose of the process is also waste treatment. In Table 1.3, data on ethanol production from lactose-based media reported in the literature are presented, including fermentations by natural lactose consuming yeasts and genetically modified *S. cerevisiae* strains.

Table 1.3 – Ethanol production from lactose-based media by natural lactose consuming yeasts and engineered *S. cerevisiae* strains.

Organism	Media	Bioreactor/operation type	Ethanol productivity	Ethanol titre	Reference
Kluyveromyces fragilis	Concentrated whey permeate (240 g/L lactose)	Batch (static 3 L bottles capped by air locks filled with glycerol)	0.2 g/L/h	80 g/L	Gawel and Kosikowski, 1978
K. fragilis	Peptone + deproteinized whey powder (100 g/L lactose)	6 L Bioreactor/Continuous process with cell recycling	7.1 g/L/h	47 g/L	Janssens *et al.*, 1984
Kluyveromyces marxianus	Semi-synthetic medium (57 g/L lactose) (94 g/L lactose)	1.2 L Air-lift bioreactor (external loop)/Continuous	 24.4 g/L/h 15.2 g/L/h	 29 g/L 45 g/L	Teixeira *et al.*, 1990
K. marxianus	Deproteinized whey	2 L Bioreactor/Batch 2 L Bioreactor/Fed-batch	3 g/L/h 5 g/L/h	43 g/L 58 g/L	Grba *et al.*, 2002
K. marxianus	Concentrated cheese whey powder solution	Shake-flasks	0.4 g/L/h	80 g/L	Kargi and Ozmihci, 2006
K. marxianus	Concentrated cheese whey powder solution	5 L Bioreactor/Continuous	0.7 g/L/h	32 g/L	Ozmihci and Kargi, 2007
Candida pseudotropicalis	Cheese whey + yeast extract + lactose (to 150 g/L)	5 L Bioreactor/Continuous	1.4 g/L/h	58 g/L	Ghaly and El-Taweel, 1997
S. cerevisiae (catabolite repression-resistant mutant)	Rich medium containing glucose/galactose mixtures (equivalent to lactose hydrolysate)	6 L Bioreactor/Continuous	13.6 g/L/h	70 g/L	Terrel *et al.*, 1984
S. cerevisiae-K.fragilis hybrid (protoplast fusion)	Yeast Extract/Peptone/Lactose (YPL)	0.3 L Bioreactor/Fed-batch	1.3 g/L/h	105 g/L	Farahnak *et al.*, 1986
Autolytic *S. cerevisiae* expressing *E. coli lacZ*	Yeast Nitrogen Base/Lactose	Shake-flasks	0.1 - 0.2 g/L/h	18 g/L	Porro *et al.*, 1992
Autolytic *S. cerevisiae* expressing *E. coli lacZ*	YPL + whey	2 L Bioreactor/Batch/Fed-batch	1 g/L/h	9 g/L	Compagno *et al.*, 1995
S. cerevisiae secreting *A.niger* β-galactosidase	YPL; synthetic lactose medium	Shake-flasks 2 L Bioreactor/Batch	0.14 - 0.6 g/L/h	30 g/L	Ramakrishna and Hartley, 1993
S. cerevisiae secreting *A.niger* β-galactosidase	Semi-synthetic lactose (50 g/L) medium	6 L Air-lift bioreactor/Continuous	9 g/L/h	20 g/L	Domingues *et al.*, 2005
S. cerevisiae expressing *K. lactis LAC4* and *LAC12*	Synthetic lactose (22 g/L) medium	2 L Bioreactor/Batch	0.3 g/L/h	4 g/L	Rubio-Texeira *et al.*, 1998
S. cerevisiae expressing *K. lactis LAC4* and *LAC12*	Semi-synthetic lactose (50 g/L) medium	Shake-flasks 6 L Air-lift bioreactor/Continuous	0.45 g/L/h 11 g/L/h	16 g/L 20 g/L	Domingues *et al.*, 1999
S. cerevisiae expressing *K. lactis LAC4* and *LAC12*	Cheese whey permeate (50 g/L lactose) (100 g/L lactose)	Shake-flasks 6 L Air-lift bioreactor/Continuous 2 L Bioreactor/Batch	0.68 g/L/h 10 g/L/h 1.8 g/L/h	21 g/L 20 g/L 53 g/L	Domingues *et al.*, 2001

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CHAPTER 2

DIRECT EVIDENCE THAT MALTOSE TRANSPORT ACTIVITY IS AFFECTED BY THE LIPID COMPOSITION OF BREWER'S YEAST

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2.1 INTRODUCTION

Transport systems allow the influx of nutrients and ions into the cell, as well as the excretion of metabolic products and deleterious substances to the extracellular environment. The translocation of sugars across the plasma membrane is usually mediated by carrier proteins, since sugars are highly polar molecules. *Saccharomyces cerevisiae* has been used as a model organism to study sugar transport in yeast. It consumes mono and disaccharides preferentially to any other carbon source (Lagunas, 1993).

In brewery fermentations the main wort sugars are glucose (about 20 % of the total), maltose (about 60 %) and maltotriose (about 20 %) and they are consumed in that order with some overlap (Boulton and Quain, 2001). Efficient brewery fermentation requires rapid and complete utilisation of both maltose and maltotriose, but assimilation of these sugars is repressed and/or inactivated at elevated concentrations of glucose in wort. Therefore, only when the yeast has taken up approximately 50% of the wort glucose will maltose uptake begin.

Transport across plasma membrane is the first step of hexose utilisation. The uptake of hexoses in *S. cerevisiae* is mediated by a large family of related transporter proteins (for review see e.g. Boles and Hollenberg, 1997). Two transport systems for monosaccharides have been identified in yeast, the so-called glucose and galactose (inducible) transporters that act by a facilitated diffusion mechanism. In the case of glucose transporter, which also acts upon D-fructose and D-mannose, two components with high and low affinity constants were originally suggested based on kinetic behaviour (see Lagunas, 1993). However, it is now known that there are at least seven different glucose transporters, with different kinetic properties, regulatory properties and specificities for monosaccharides (Reifenberger *et al.*, 1997). These transport systems are subjected to multiple regulatory mechanisms (Boles and Hollenberg, 1997).

The maltose uptake system of *S. cerevisiae* has been extensively studied, because of the importance of maltose in industrial processes, such as beer fermentation and bread dough leavening. Maltose is transported to the cytosol in an intact form by an active transport process and hydrolised by intracellular maltase (α-glucosidase). Maltose uptake occurs via a proton symport mechanism, with one proton being co-transported with each maltose molecule (Serrano, 1977). Maltose utilisation requires the presence of at least one of five highly homologous and unlinked *MAL* loci: *MAL1* through *MAL4* and *MAL6*. In *Saccharomyces* yeasts, maltose transporters are encoded by several genes, including *MALx1* (where x = 1-4 and 6 indicates one of the five *MAL* loci), *AGT1* (Han *et al.*, 1995; Jespersen *et al.*, 1999; Lagunas, 1993; Stambuk *et al.*, 1999), *MPH2* and *MPH3* (Day *et al.*, 2002), and *MTT1*

(Dietvorst *et al.*, 2005; Salema-Oom *et al.*, 2005). All the encoded proteins are thought to be maltose/H^+ symporters. In addition to the permease gene, maltose utilization requires the products of two other genes: maltase and an activator of transcription, encoded respectively by the *MALx2* and *MALx3* (x = 1-4 and 6) genes.

The *Saccharomyces* maltose transport systems exhibit distinct substrate specificities. The permeases encoded by *MALx1* genes have maltose and turanose as the only substrates, while the permease encoded by *AGT1* is able to mediate the uptake of a broader spectrum of α-glucosides, including isomaltose, α-methylglucoside, maltotriose, palatinose, trehalose and melezitose, in addition to maltose and turanose (Han *et al.*, 1995). Besides maltose, *MPH2*- and *MPH3*-encoded transporters are also able to mediate the uptake of maltotriose, turanose and α-methylglucoside (Day *et al.*, 2002) and the *MTT1*-encoded permease is able to transport maltotriose (Dietvorst *et al.*, 2005).

Kinetic analyses revealed that maltose transport in laboratory *S. cerevisiae* strains consists of two components: an inducible high-affinity component, with a K_m of about 4 mM for maltose, and a constitutive low-affinity component, with a K_m of 70 to 80 mM (Busturia and Lagunas, 1985; Cheng and Michels, 1991). It has been proposed that the low affinity transport is due to an experimental artifact, related with non-specific binding of the radiolabeled maltose to the cell wall and/or plasma membrane during the transport assays (Benito and Lagunas, 1992). This view is supported by the monophasic kinetics of Mal61p-mediated maltose transport in an artificial system with K_m of 4.6 mM (van der Rest *et al.*, 1995a), and by the monophasic kinetics for maltose transport (K_m of 5 mM) obtained with a strain harboring the Mal21p (Stambuk and de Araujo, 2001). These results have been obtained with laboratory strains, probably harboring a single α-glucoside transporter. The analysis of maltose transport by industrial strains has started to challenge this view. Industrial strains seem to harbor several maltose permeases with different affinities for this substrate (Stambuk and de Araujo, 2001). Ale and lager brewing strains appear to contain a high affinity and a low affinity maltose transport systems (Crumplen *et al.*, 1996; Rautio and Londesborough, 2003). Crumplen *et al*. (1996) have shown that N-ethylmaleimide inactivates the low affinity systems of both the ale and lager strains, suggesting they require proteins. These authors estimated K_m values of 2 mM and 20 mM for an ale strain, and 1 mM and 20 mM for a lager strain. Rautio and Londesborough (2003) estimated K_m values of about 3 mM and 15 – 50 mM for a lager and an ale strains.

Jespersen *et al.* (1999) screened five ale yeasts and twenty-five lager yeasts and found that all contained *MALx1* genes and all but one contained also *AGT1* genes. They found also that twenty six of the strains studied contained nucleotide sequences corresponding to *MPH2*, but none contained *MPH3*. Day *et al.* (2002) analyzed five brewing yeasts (two ale and three

lager) detecting *MALx1* genes in all the strains, *AGT1* in all but one lager strain and *MPHx* in one ale and two lager yeasts. Studies of inhibition of maltose transport by other sugars on two brewing strains indicated that the main transporters were the *AGT1*-type for the ale yeast and the *MALx1*-type for the lager yeast (Rautio and Londesborough, 2003). Vidgren *et al.* (2005) studied two ale strains and three lager strains, confirming that maltose was predominantly carried by *AGT1*-encoded transporters in the ale strains and by *MALx1*-encoded transporters in the lager strains. They found that the *AGT1* sequences in the lager strains encoded truncated polypeptides unlikely to be functional transporters.

Maltotriose is the second most abundant fermentable sugar in brewery wort, but this trisaccharide is taken up from the wort at a significantly slower rate than that observed for maltose. This slower, and sometimes incomplete, maltotriose uptake from the wort leads to some problems experienced by brewers, such as high yeast fermentable extract in the finished beer and atypical beer flavor profiles (Zheng *et al.*, 1994). The permeases encoded by the genes *AGT1*, *MPH2*, *MPH3* and *MTT1*, which are apparently widespread in brewer's yeasts, are likely to play an essential role in the fermentation of maltotriose.

The yeast plasma membrane consists mainly of lipids and proteins. The lipid bilayer is essentially built up of phospholipids and sterols. Phospholipids comprise two non-polar fatty acid chains (hydrophobic tails) oriented towards the middle of the membrane, esterified via glycerol to a polar phosphate, esterified to an organic base (hydrophilic heads). Sterols are compact rigid hydrophobic molecules with a polar hydroxyl group. Several sterols may be found in yeast but ergosterol is the most prominent. Ergosterol molecules are placed within each phospholipid monolayer and their planar rings are incorporated between phospholipid heads and tails (Höfer, 1997).

Sterols have several putative roles in yeast cells, both structural and functional. These lipids play a key role in regulating the fluidity (and also the permeability) of the plasma membrane, which, in turn, may affect the lateral movement and the activity of membrane-bound proteins. Several other functions for sterols have been reported (Boulton and Quain, 2001; van der Rest *et al.*, 1995b). Ergosterol affects the membrane fluidity by interaction of the rigid planar rings with the hydrophobic tails of the fatty acid residues. It also decreases the permeability of the membrane to small hydrophilic molecules and probably increases the flexibility and mechanical stability of the membrane (Höfer, 1997). Sterols may create an environment into which polypeptides can insert. For this bulk function, sterol auxotrophs require relatively large amounts of sterols in the medium (15 $\mu g \cdot mL^{-1}$). The role of sterols as a trigger for cell proliferation is satisfied at concentration of 1 to 10 $ng \cdot mL^{-1}$. Ergosterol can fulfill both these functions (van der Rest *et al.*, 1995b).

Sterols are synthesized using carbon devolving from glycolysis via acetyl-coA as part of the general pathway leading to the formation of branched isoprenoids. The first part of the synthesis is an anaerobic process, which involves the conversion of acetyl-coA to melavonic acid and then to squalene. Molecular oxygen is required to the formation of 2,3-epoxysqualene from squalene, followed by cyclisation to form the first sterol, lanosterol. Other sterols, including ergosterol, are formed from lanosterol in a complex pathway. Apart from the initial epoxidation of squalene, regulation by oxygen concentration occurs at several other steps on the pathways (Boulton and Quain, 2001; Ratledge and Evans, 1989). Yeast cells accumulate sterols into membranes until the bulk requirement is satisfied. Further synthesis may continue, although in this case the additional sterols are esterified to long-chain fatty acids and deposited in intracellular lipid vesicles. The most abundant sterol of the steryl ester pool is zymosterol. This sterol is not readily incorporated into membranes, being usually esterified. In aerobic batch cultures, steryl esters accumulate during the stationary phase of growth. When such cells are re-inoculated into fresh medium rapid hydrolysis of steryl esters occurs and the liberated sterols are incorporated into growing membranes (Boulton and Quain, 2001).

Fatty acids are normally found in living cells as esters, usually of glycerol or sterols (Ratledge and Evans, 1989). In *S. cerevisiae* cells the predominant fatty acyl chains are palmitoleic acid (16:1) and oleic acid (18:1), together with palmitic acid (16:0) and stearic acid (18:0). The fatty acyl package of these chains determines to a large extent the membrane fluidity. The packing increases with increasing length of the acyl chains and decreasing extent of unsaturation, which leads to a more ordered structure and a decrease in fluidity (van der Rest *et al.*, 1995b). Biosynthesis *de novo* of unsaturated fatty acids (UFA) involves formation of a saturated fatty acid followed by an oxygen-dependent desaturation reaction. As with sterols, biosynthesis proceeds from acetyl-coA (Boulton and Quain, 2001; Ratledge and Evans, 1989).

Sterol and UFA contents of brewer's yeast depend upon the availability of lipids in the wort the yeast has been grown in and, predominantly, the availability of molecular oxygen during fermentation, since molecular oxygen is required for *de novo* synthesis of sterols and UFA by yeast, as mentioned above.

In brewery fermentations both sterols and UFA must be synthesized during the initial phase, when oxygen is made available. A small proportion of the requirement for UFA is obtained from wort. Sterols may also be present in wort in small quantity, although under the conditions of brewery fermentation yeast may not be able to assimilate it. Yeast cells that are capable of sterol biosynthesis cannot take sterols from the growth medium. Only anaerobic cells and clones that are auxotrophic for sterol are able to accumulate exogenous sterol (Salerno and

Parks, 1983). Consequently, aerobic yeast (capable of sterol synthesis) does not utilize exogenous sterols and sterol uptake from the wort may only occur after the initial aerobic phase of brewery fermentation. Growth of yeast during the anaerobic phase of fermentation dilutes the pre-formed sterol pool between mother cells and their progeny. Cells divide until sterol depletion limits growth (Boulton and Quain, 2001).

Yeast cropped from industrial scale wort fermentations has low contents of UFA and sterols because it has grown with very limited access to oxygen (Aries and Kirsop, 1977; David and Kirsop, 1973; Kirsop, 1974). For this reason, wort must be aerated at the start of fermentations when, as is the usual case, cropped yeast is re-pitched to start a new fermentation. It is often stated that the low UFA and sterol contents of the plasma membrane prevent the proper function of sugar transporters, so that for the first few hours after re-pitching yeast is dependent upon its internal supplies of carbohydrate (glycogen and trehalose) because it is unable to transport wort sugars into the cells. Glycogen and trehalose are rapidly mobilised after pitching (Quain *et al.*, 1981; Reinman and Londesborough, 2000). Glycogen mobilisation is believed to provide the energy needed to drive the oxygen-dependent synthesis of sterols and UFA. Efficient transport of sugars is said to be restored and wort fermentation commence only when adequate levels of sterols and UFA are reached.

Although this scenario is frequently described, a direct demonstration that the sterol or fatty acid composition of the yeast plasma membrane affects the function of sugar transporters was not found. Quain *et al.* (1981) found no glucose uptake during the first 2 h after pitching, during which time the wort oxygen was consumed. They suggested that the lack of glucose uptake might reflect regulation of glucose transporters by the membrane lipid composition. However, although lack of glucose uptake is consistent with the proposed inability of glucose transporters to function properly in UFA- and sterol-deficient membranes, it does not prove such an inability. Because glucose transport is passive, failure to further metabolise intracellular glucose for any reason will rapidly prevent further glucose uptake. Keenan and Rose (1979) could not find a significant difference between the kinetics of glucose transport by yeast enriched with, respectively, oleyl or linoleyl residues. In contrast to glucose, the transport kinetics of several amino acids are affected by the fatty acyl composition of yeast (Calderbank *et al.*, 1984; Keenan and Rose, 1979; Mishra and Prasad, 1987). Calderbank *et al.* (1984) found that the amount of general amino acid permease, but not its affinity towards alanine, was increased in yeast enriched with linoleyl residues, and suggested that in this case the altered fatty acid composition promoted intracellular trafficking of newly synthesised permease into the plasma membrane.

In mammalian cells, the activity of the *GLUT1*-encoded glucose transporter is acutely sensitive to the phospholipid and sterol (cholesterol) composition of the membrane, as shown

both by experiments with transporter reconstituted into artificial membranes and by using cells with manipulated lipid compositions (Barnes *et al.*, 2004; Carruthers and Melchior, 1984; Yuli *et al.*, 1981). In the work described in this chapter, the sterol and fatty acid composition of yeast was manipulated by growing it, with maltose as sole carbon source, under strictly anaerobic conditions in the presence or absence of lipid supplements. The rates of maltose and maltotriose transport and subsequent fermentation are known to be strongly regulated at transcription and post-translation levels by glucose repression of α-glucoside transporters and maltases and by glucose-triggered catabolite inactivation of the transporters (Lucero *et al.*, 1997; Spencer-Martins *et al.*, 1999). The main objective was to investigate whether the rate of maltose transport is further limited by other physiological factors of practical importance during brewery fermentations.

2.2 MATERIALS AND METHODS

2.2.1 Yeast and cultivation

The yeast used was an industrial brewer's strain, A24, from VTT's collection. Possibly functional maltose transporter genes in this strain include *MAL11, MAL21, MAL31* and *MAL41*, whereas its *AGT1* gene is non-functional (Vidgren *et al.*, 2005). It also contains the recently discovered (Dietvorst *et al.*, 2005; Salema-Oom *et al.*, 2005) maltose/maltotriose transporter gene, *MTT1* (Vidgren, V., Rautio J.J. and Londesborough, J., unpublished observations).

Yeast for stocks was grown into stationary phase on YP (1% yeast extract, 2% peptone) containing 40 g maltose·liter^{-1}, collected and stored at -80 ºC as suspensions in 30% glycerol containing 200 mg fresh yeast mass·mL^{-1}. Yeast suspension (150 µL) was inoculated into 300 mL of synthetic complete (SC) medium (Sherman *et al.*, 1983) containing 40 g maltose·L^{-1} and the indicated supplements in a 500 mL bottle. SC medium consists of (mg·L^{-1}) yeast nitrogen base without amino acids, 6700; adenine, 13.5; arginine, 348; aspartate, 266; histidine, 58; myo-inositol, 36; isoleucine, 525; leucine 262; lysine, 91; methionine, 149; phenylalanine, 83; serine, 105; threonine, 119; tryptophan, 82; tyrosine, 30; uracil, 22 and valine, 117. The bottle was fitted with a magnetic stirrer, a glycerol-filled bubble trap and an inlet tube that reached below the surface of the medium. After inoculation air was purged from the medium and head space by passing CO_2 through the inlet tube and out of the bubble trap (Figure 2.1). Growths were conducted at 24 ºC. Biomass was estimated by turbidity (OD_{600}) measurements on small samples withdrawn from one of each pair of replicate bottles anaerobically by syringe through the inlet tube (Figure 2.1). Where indicated the growth media (300 mL) were supplemented with either 600 µl of a 50:50 mixture of Tween 80 (Fluka Chemika) and ethanol (giving a final concentration of 0.1% v/v Tween 80) or with 600 µL of the same Tween 80/ethanol mixture containing also 7.5 mg ergosterol·mL^{-1} (giving final concentrations of 0.1% v/v Tween 80 and 15 mg ergosterol·L^{-1}). Ergosterol was from Sigma (minimum purity, 90%).

Aerobic growths were carried out, as stated in the text, in either SC or YP medium containing 40 g maltose·L^{-1} in conical flasks fitted with porous plugs and shaken (180 rpm) at 26 ºC.

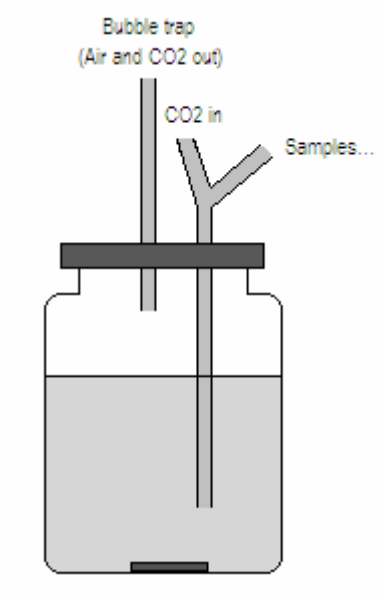

Figure 2.1 – Schematic representation of the bottles used for yeast anaerobic growths.

2.2.2 Maltose transport

Standard maltose transport assays were made essentially as earlier described (Lucero *et al.*, 1997; Serrano, 1977). Yeast were harvested by centrifugation (10 min at 5000 x g), washed with ice-cold distilled water and suspended to 200 mg fresh yeast·mL^{-1} in ice-cold 0.1 M tartrate/Tris buffer, pH 4.2. Portions of about 1 mL were equilibrated to 20 ºC for 5 min immediately before assay. Reactions were started by adding 40 µL of yeast suspension to 20 µL of 15 mM ^{14}C-maltose (900 dpm·nmol^{-1}) in 15 mL tubes with conical bottoms. [U-^{14}C]-maltose was CBF182 from Amersham Biosciences (Espoo, Finland). Reactions were stopped after 20 s by addition of 10 mL ice-cold water. The yeast was collected on a 0.45 µm HVLP filter (Millipore) and rinsed with 10 mL ice-cold water. The filter was transferred to scintillation cocktail and counted. Assays at 10 and 0 ºC were done with appropriately longer reaction times. Observed rates were at least 90% of the rates observed with 50% shorter reaction times. One U catalyses the uptake of 1 µmol of maltose per min under the stated conditions. Rates were normalised to total yeast protein, determined by extracting the yeast overnight with 1 M NaOH and assaying the extract by biuret (Gornall *et al.*, 1949), or to yeast dry mass, determined by washing the yeast with water and drying it at 105 ºC for 16 h. Protein contents were close to 40% of dry masses.

2.2.3 Lipid analyses

Yeast was harvested by centrifugation (5 min at 5000 x g) and the pellets were washed twice

with ice-cold distilled water and then suspended in ice-cold distilled water (5.0 mL water·g fresh yeast^{-1}). Four 500 µL portions were transferred to 12 mL flat-bottomed screw-cap tubes (Kimax) and centrifuged 10 min at 5000 x g. The supernatants were removed by Pasteur pipette and the pellets (83 mg fresh yeast) stored under N_2 at -20 ºC.

Duplicate samples for total fatty acids were saponified with 3 M NaOH in 50% (v/v) methanol and then esterified with 4 M HCl in methanol and the methyl esters extracted into hexane/methyl-t-butyl ether (1:1) and washed with 0.3 M NaOH. The organic phase was saturated with anhydrous Na_2SO_4 and the methyl fatty acid esters analysed by gas chromatography (GC) using an HP5890 gas chromatograph fitted with FID. The column was 30 m HP-Innowax, i.d. 0.258 mm and 0.5 µm film. Injector and detector were at 270 ºC. After 3 min at 50 ºC column temperature was raised at 25 ºC·min^{-1} to 180 ºC and then at 10 ºC·min^{-1} to 250 ºC, held for 7 min and further raised at 10 ºC·min^{-1} to 270 ºC and held for 20 min. The carrier gas was He and detector gases H_2 and synthetic air.

Duplicate samples for total sterols were hydrolysed under N_2 with 20% KOH in ethanol and sterols extracted into pentane. The pentane was evaporated at room temperature under a stream of N_2 and the sample was silylated at room temperature by adding 180 µL of a mixture of N,O-bis(trimethylsilyl)-trifluoroacetamide and trimethylchlorosilane (2:1) and analysed in an HP 6890 gas chromatograph equipped with FID and on-column injector. The column was 15 m DB-1, i.d. 0.53 mm and 0.15 µm film. After 1.5 min at 100 ºC the temperature was raised at 12 ºC·min^{-1} to 330 ºC and held for 1.0 min.

2.2.4 Northern analyses

Total RNA was isolated from yeast using TRIzol (Life Technologies, Carlsbad, CA, U.S.A.) according to the manufacturer's instructions and quantified by measuring its A_{260} assuming that 1 µg·mL^{-1} has an A_{260} of 25. RNA was denatured by treatment with glyoxal/dimethyl sulfoxide (Brown and Mackey, 1997), subjected to electrophoresis in 1% agarose, and blotted onto a nylon membrane (Hybond, Amersham). Probes were made by PCR (the 779 nucleotide *MAL61* probe started from nucleotide 361 of the *MAL61* gene and the 986 nucleotide *AGT1* probe started from nucleotide 842 of the *AGT1* gene) and labelled by the Random primed DNA Labeling kit (Roche). After hybridisation the membrane was washed and band intensities were read with a phosphorimager.

2.3 RESULTS

Typical growth curves are shown in Figure 2.2. Similar results were obtained in two other replicate experiments. In media without supplements the yeast grew slowly to a final OD_{600} of 4.1. Addition of Tween 80 alone increased the growth rate, and raised the final OD_{600} to 5.0. Addition of Tween 80 containing ergosterol further increased the growth rate and raised the final OD_{600} to 7.6. Yeast in bottles that were not sampled during the first 23 h (open symbols in Figure 2.2) did not grow more slowly than yeast in the sampled bottles, suggesting that the sampling procedure did not introduce air. Growing yeast was collected at 23.3 h by harvesting the unsampled bottles and stationary phase yeast at 48 h from the sampled bottles.

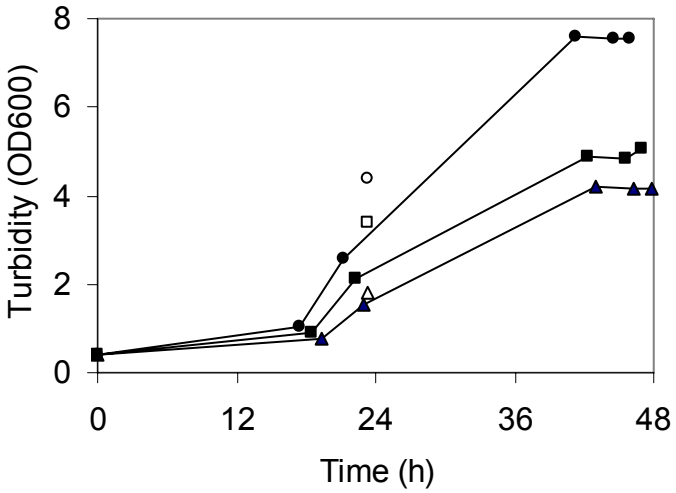

Figure 2.2 – Anaerobic growths. Media were supplemented with no addition (▲, ∆), Tween 80 alone (■, □) or Tween 80 containing ergosterol (●, ○). Solid symbols are from growths that were sampled at intervals and open symbols from growths that were not sampled until 23.3 h.

Lipid analyses and maltose transport activities of yeast samples from the Figure 2.2 growths are shown in Tables 2.1 and 2.2. As expected from earlier work (see, e.g., Keenan and Rose, 1979; Keenan *et al.*, 1982), yeast grown anaerobically without supplements contained low amounts of both palmitoleic (C16:1) and oleic (C18:1) acids, which *S. cerevisiae* can synthesise, and of linolic (C18:2) and linoleic (C18:3) acids, which *S. cerevisiae* cannot synthesise (Gonzalez and Martin, 1996; Schweizer, 1999) and had low unsaturation indices both during growth (21%) and in the stationary phase (20%) (Table 2.1). When the yeast was grown aerobically (in a shake flask) in SC containing 40 g maltose·L^{-1} to stationary phase, it contained larger amounts of total fatty acids and much higher proportions of palmitoleic and oleic acids and had an unsaturation index of 80% (the fatty acid composition and ergosterol

content of yeast grown aerobically was the same in minimal (SC) and rich (YP) media). Lauric (C12:0) and myristic (C14:0) acids accounted for much higher proportions of total fatty acids in the unsupplemented anaerobically grown yeast than in the aerobically grown control. The changes reflected absolute increases in the amounts of C12:0 and C14:0 per gram of yeast as well as decreases in the amounts of C16:1 and C18:1. Supplementation of the anaerobic growth medium with Tween 80 alone caused a large increase in the proportion of oleic acid (to 56% and 50% of total fatty acids in growing yeast and stationary phase yeast, respectively), more than doubled the absolute amount of total fatty acids and specifically decreased the proportions of lauric and palmitoleic acids. The unsaturation indices of these yeasts (62% and 56%) were still lower than that of the aerobically grown yeast. Tween 80 (polyoxyethylene-sorbitan monooleate) is a source of oleic acid. Supplementation with Tween 80 *plus* ergosterol caused similar changes as Tween 80 alone, but the absolute increase in total fatty acids was smaller (probably because the yeast grew more) and the relative increase in oleic acid was somewhat greater.

Table 2.1 – Fatty acid compositions of anaerobically grown yeasts and aerobic control.[1]

	Anaerobic growth phase			Anaerobic stationary phase			Aerobic
Lipid supplement	None	T	T+E	None	T	T+E	None
Lauric (C12:0)	19.8	1.9	1.4	14.4	2.6	2.4	1.9
Myristic (C14:0)	12.5	10.9	6.7	4.5	11.8	5.8	1.2
Palmitic (C16:0)	42.3	23.0	21.2	52.3	26.8	24.2	13.9
Palmitoleic (C16:1)	17.5	5.6	6.4	15.7	5.3	6.0	57.8
Stearic (C18:0)	4.0	2.4	2.3	8.6	3.3	3.0	3.0
Oleic (C18:1)	3.7	56.0	61.7	4.6	50.0	58.1	22.2
Linolic (C18:2)	0.1	0.2	0.3	0.1	0.3	0.3	0.1
Linoleic (C18:3)	0.1	0.0	0.1	0.1	0.0	0.1	0.1
Total fatty acids ($mg \cdot g^{-1}$)	31 ± 3	71 ± 4	56 ± 0	36 ± 2	93 ± 3	54 ± 3	69
Unsaturation index (%)	21	62	69	20	56	65	80

[1]Yeasts were grown anaerobically (Figure 2.2) with no supplements (None), Tween 80 alone (T) or Tween 80 + ergosterol (T+E) and harvested at 23.3 h (growth phase) or 48 h (stationary phase). The right hand column shows results for the same yeast strain grown in SC medium at 26 ºC without exclusion of air. Individual fatty acids are shown as percentages of total fatty acid. For anaerobic yeasts, total fatty acids as $mg \cdot [g\ dry\ yeast]^{-1}$ are means ± standard deviation of 5 samples and for the aerobic yeast the mean of duplicate assays is shown. The unsaturation index shows the sum of unsaturated C16 and C18 fatty acids as a percentage of total even chain length C12 - C18 fatty acids.

Table 2.2 shows that the ergosterol contents of yeast grown anaerobically without supplements or with Tween 80 alone were low, the contents of squalene (the last intermediate before the steps requiring oxygen in the biosynthetic pathway to sterols) were high and, consequently, the ergosterol/squalene mass ratios were low. The squalene content of the anaerobically grown yeast increased 3- to 4-fold when the yeast entered stationary phase. Supplementation with ergosterol in addition to Tween 80 raised the ergosterol content to about 1.3 mg·[g dry yeast]$^{-1}$, which is still much less than found in the same strain grown aerobically. The ergosterol content did not increase when the yeast entered stationary phase, although only a fraction of the added ergosterol was incorporated into the yeast (added ergosterol was equivalent to about 6 mg·[g dry yeast]$^{-1}$). Ergosterol uptake by yeast is an active process that normally occurs only when sterol biosynthesis is prevented (Wilcox *et al.*, 2002). Possibly the ergosterol taken up from the medium mainly enters the plasma membrane and does not form the sterol esters that accumulate as storage pools in aerobically grown yeast (Aries and Kirsop, 1977).

Table 2.2 – Squalene, ergosterol, unsaturation index and maltose transport capacity of anaerobically grown yeasts and aerobic control.[1]

	Anaerobic growth phase			Anaerobic stationary phase			Aerobic
Lipid supplement	None	T	T+E	None	T	T+E	None
Ergosterol (mg·g^{-1})	<0.3	<0.3	1.3 ± 0.1	0.2 ± 0.2	<0.3	1.4 ± 0.4	9.2
Squalene (mg·g^{-1})	2.1 ± 0.0	1.8 ± 0.6	0.8 ± 0.0	6.9 ± 0.3	7.2 ± 1.2	1.8 ± 0.0	<0.3
Erg/Squa[2]	<0.14	<0.17	1.6	<0.03	<0.04	1.3	>31
Unsaturation index (%)	21	62	69	20	56	64	80
Maltose transport at 20 °C (U·g^{-1})	11.2 ± 0.5	9.5 ± 0.1	24.5 ± 0.4	9.8 ± 0.3	2.5 ± 0.2	30.4 ± 0.2	57.6 ± 1.1
Maltose transport at 0 °C (U·g^{-1})	0.46 ± 0.01	0.29 ± 0.01	1.83 ± 0.03	0.50 ± 0.02	0.055 ± 0.015	3.10 ± 0.21	4.5 ± 0.3

[1] Yeasts were grown anaerobically (Figure 2.2) with no supplements (None), Tween 80 alone (T) or Tween 80 + ergosterol (T+E) and harvested at 23.3 h (growth phase) or 48 h (stationary phase). The aerobic sample was grown in SC containing 40 g maltose·L^{-1} in a shake flask and harvested in stationary phase. Ergosterol and squalene are reported as mg·[g dry yeast]$^{-1}$ and maltose transport as U·[g protein]$^{-1}$. Data are means ± ranges of duplicate assays.

[2] Erg/squa is the mass ratio of ergosterol to squalene.

The standard maltose transport activity measured at 20 ºC was relatively low in yeast grown anaerobically without supplements or with Tween 80 alone (Table 2.2). For yeast without supplements, maltose transport activity was slightly lower in stationary phase than during growth, but for yeast supplemented with Tween 80 alone, maltose transport activity was much smaller in stationary phase than during growth. Simultaneous supplementation with ergosterol and Tween 80 increased the maltose transport activity (measured at 20 ºC) of anaerobically grown yeast more than 2-fold during growth phase and about 3-fold in stationary phase. The biggest difference observed was between yeasts grown with supplements of Tween 80 alone or Tween 80 *plus* ergosterol: transport activity was 12-fold higher in stationary phase yeast supplemented with ergosterol and Tween 80 compared to stationary phase yeast supplemented with Tween 80 alone. Maltose transport was also measured at 0 ºC, where apparent rates were at least 10-fold smaller than at 20 ºC. However, the rates at 0 ºC were also linear (over reaction times of 2 – 4 minutes; see Materials and Methods) and so represented uptake of labelled sugar rather than mere binding to the cell surface. Greater dependencies on lipid supplementation were observed at this low temperature: supplementation with Tween 80 *plus* ergosterol increased the transport activity at 0 ºC of growing and stationary phase yeasts by 4-fold and 6-fold, respectively, and this activity was 56-fold greater in stationary phase yeasts supplemented with Tween 80 *plus* ergosterol than yeast supplemented with Tween 80 alone.

Similar results were obtained in replicate experiments (Table 2.3). The large standard deviations for ergosterol and squalene probably represent the varying success with which air was excluded from the growths. The average maltose transport activity (41.8 U·[g protein]$^{-1}$) for stationary phase yeast grown anaerobically with Tween 80 *plus* ergosterol was lower than found in yeast grown aerobically on 40 g maltose·L^{-1} in SC (Table 2.2) or YP (50 – 60 U·[g protein]$^{-1}$; data not shown) media.

Table 2.3 – Effect of lipid supplements on ergosterol and squalene levels, fatty acid unsaturation and maltose transport activity in yeast grown anaerobically into stationary phase.[1]

Lipid supplement	None	T	T+E
Ergosterol (mg.g^{-1})	0.8 ± 0.7	0.4 ± 0.2	1.8 ± 0.5
Squalene (mg.g^{-1})	3.7 ± 2.9	4.7 ± 3.6	1.2 ± 0.6
Unsaturation index (%)	27.3 ± 8.7	58.5 ± 3.5	64.7 ± 1.2
Maltose transport at 20ºC (U·g $_{protein}^{-1}$)	13.0 ± 2.9	4.9 ± 3.4	41.8 ± 11.6

[1]Yeasts were grown into stationary phase without supplements (None) or with Tween 80 alone (T) or Tween 80 *plus* ergosterol (T+E) as described in Materials and methods. Results are means ± SDs (None, T+E, n=3; T, n=2).

The 40 $g \cdot L^{-1}$ maltose carbon source in these anaerobic growths is expected to induce fully *MALx1, AGT1* and *MTT1* genes. Northern analyses were performed to check whether expression was affected by the lipid supplements. RNA from growing cells (23.3 h in Figure 2.2) gave strong *MALx1* signals, but, as expected, the messenger was barely detectable in stationary phase cells harvested after maltose was consumed (data not shown). The *MALx1* signals were quantitated by densitometry and normalised in three ways: to the A_{260} load applied to the gels (equal for all samples), to the amount of ribosomal RNA detected in the gels and to the *ACT1* signal obtained by hybridising blots to an *ACT1* probe (Table 2.4). The first two methods suggested that Tween 80 alone slightly (ca. 25%) increased expression of *MALx1* whereas Tween 80 *plus* ergosterol slightly (4 – 27%) decreased its expression. Normalisation to *ACT1* suggested changes in the opposite direction, about 40% decrease with Tween 80 alone and 55% increase with Tween 80 *plus* ergosterol. Expression of *AGT1* was not detectable, and this gene has been previously shown to be non-functional in this brewer's yeast (Vidgren *et al.*, 2005).

Table 2.4 – Relative expression of *MALx1* genes during anaerobic growth with and without lipid supplements.[1]

	No supplement	Tween 80	Tween 80 + Ergosterol
A260 normalised	100	127	96
rRNA normalised	100	123	73
ACT1 normalised	100	62	155

[1]Equal amounts (based on A260) of RNA from growing cells (23.3 h in Figure 2.2) were loaded onto gels. After electrophoresis, gels were photographed and the the ribosomal RNA (rRNA) quantitated by densitometry. After blotting, filters were probed for *MALx1* and then for *ACT1*. Hybridisation bands were quantitated by densitometry and the relative amounts of *MALx1* mRNA were calculated after normalisation to the A260 load, rRNA or *ACT1*.

These Northern results supported the *a priori* assumption that lipid supplements would not change the expression levels of *MALx1* genes, although this possibility cannot be rigorously excluded. More likely, the mechanism of the changes in maltose transport activity involves the altered lipid composition of the plasma membrane. Evidently increasing the unsaturation index of fatty acids by supplementation with Tween 80 alone was not enough to support high maltose transport activity, but supplementation with Tween 80 *plus* ergosterol raised the maltose transport activity towards that found in yeast grown under aerobic conditions, as well as increasing the unsaturation index and ergosterol content. Maltose transport in brewer's yeasts shows a high temperature coefficient below 10 ºC (Quain *et al.*, 1981; Rautio and

Londesborough, 2003), implying that physical changes in membrane lipids at low temperature impinge on the activity of maltose transporters, i.e., the transporters work poorly when the lipid surrounding them becomes more rigid. This was also suggested by the relatively larger changes in transport activity measured at 0 ºC than 20 ºC (Table 2.2). The temperature dependence of maltose transport was therefore examined in more detail in yeasts grown anaerobically with or without lipid supplements. Figure 2.3 shows combined results from independent experiments with assays at 0, 10 and 20 ºC. Matched pair T-tests showed that for the ratios of activities at 0 ºC and 20 ºC, the differences between no supplement and Tween 80 *plus* ergosterol and between no supplement and Tween alone were only just significant (p = 0.1 and 0.05, respectively) whereas the difference between Tween 80 alone and Tween 80 + ergosterol was highly significant (p = 0.01)

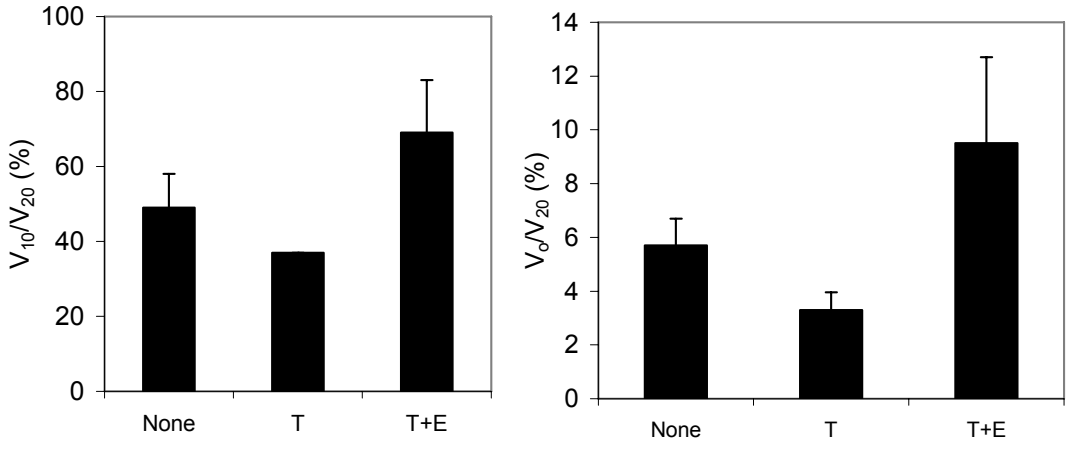

Figure 2.3 – Different temperature-dependencies of maltose transport by yeast grown anaerobically with no supplement (None), Tween 80 alone (T) or Tween 80 *plus* ergosterol (T+E). The left panel shows the transport activity at 10 ºC as a percentage of that at 20 ºC (V_{10}/V_{20}); error bars indicate the range between duplicate experiments. The right panel shows transport activity at 0 ºC as a percentage of that at 20 ºC (V_0/V_{20}); results are means ± SDs for experiments with one lot of growing cells and three or (Tween 80 alone) two lots of stationary phase cells. The differences between V_0/V_{20} values were weakly significant (p = 0.1) for no supplement *versus* Tween 80 *plus* ergosterol and highly significant (p = 0.01) for Tween 80 alone *versus* Tween 80 *plus* ergosterol (matched pair T-test).

2.4 DISCUSSION

In this brewer's yeast strain the major genes encoding functional maltose transporters belong to the group *MAL11, MAL21, MAL31* and *MAL41*. The *AGT1* gene is present but non-functional (Vidgren *et al.*, 2005) and *MTT1* is present but it is not yet known if it is functional. It is shown here that under growth conditions that were strictly anaerobic but strongly induced expression of *MALx1*, the maltose transport activity of the yeast was markedly increased by supplementation of the medium with Tween *80 plus* ergosterol (Tables 2.2 and 2.3). It can be concluded that either maltose transporter molecules cannot be properly inserted into plasma membranes that do not contain adequate amounts of sterol or that inserted transporter molecules require sterol molecules in order to fulfil their catalytic role. The results of Figure 2.3 show that the temperature-dependencies of maltose transport were greater for non-supplemented cells and cells supplemented with Tween 80 alone than for cells supplemented with Tween 80 *plus* ergosterol. This suggests that the catalytic function of transporter molecules in the plasma membrane was dependent on the lipid composition, in particular the sterol content, of the plasma membrane. Sugar transporter molecules are not simply channels across the membrane. The proteins change shape during each transport cycle (Abramson *et al.*, 2003; Huang *et al.*, 2003), which involves the surface of the protein moving against the surrounding lipid. The composition of this lipid is thus expected to alter the energetics of the transport process (Londesborough, 1980). There is also evidence (Bagnat *et al.*, 2000; Barnes *et al.*, 2004) that sterols are required to form "rafts" concerned with the transport of intrinsic membrane proteins from endoplasmic reticulum to the plasma membrane, so that it is also possible that sterol deficiency leads to a decrease in the number of transporter molecules per unit area of membrane.

Supplementation with UFA alone (Tween 80 alone) was not enough to maintain the maltose transport activity of growing cells and actually decreased the transport activity of stationary phase cells and increased the temperature-dependence of the residual activity (Tables 2.2 and 2.3, Figure 2.3). Supplementation with Tween 80 alone increased the oleic acid content of the yeast but not its sterol content, so that possibly the correct ratio of sterol to oleic acid is important for transport. It is also possible that Tween 80 alone extracted sterols from the membrane, but the data (Tables 2.2 and 2.3) at low ergosterol levels were not accurate enough to show this. The reverse experiment of growing cells under strictly anaerobic conditions with ergosterol supplement but no UFA supplement was not carried out, because it was not found a convenient way of adding ergosterol to the medium without using Tween 80 as an emulsifier. Thus, results show that efficient maltose transport requires sterols, but it is not known whether or not it also requires UFAs.

Maltose transport capacity is regulated by glucose-repression and maltose-induction of genes encoding maltose transporters and also by glucose-triggered catabolite inactivation of existing maltose transporter proteins (Lucero *et al.*, 1997; Spencer-Martins *et al.*, 1999). The present results emphasise also a third factor, namely the lipid composition, in particular the sterol content of the plasma membrane. What actually happens to the maltose transport capacity when industrially cropped yeast is pitched into aerated wort will depend upon the interaction of these three factors and upon the particular yeast strain and wort sugar composition. The maltose transport capacity of industrially cropped yeast is relatively low, partly at least because it is deficient in sterols. The sterol and UFA contents rise rapidly during the first few hours after pitching (Aries and Kirsop, 1977; David and Kirsop, 1973; Kirsop, 1974). However, in worts with high concentrations of glucose, maltose transport capacity falls during the first 20 h, presumably as a consequence of glucose-triggered catabolite inactivation, as reported by Rautio and Londesborough (2003) for a wort with a maltose:glucose ratio of 1.8. In contrast, using a wort with lower glucose content (maltose:glucose ratio of 4.8), Rautio *et al.* (2007) found that glucose-triggered catabolite inactivation of maltose transporters was ineffective and maltose transport activity peaked during the first day (Rautio *et al.*, 2007). Expression of maltose transporter genes is activated during the first day of fermentation (James *et al.*, 2003; Rautio *et al.*, 2007) and, in worts with low maltose:glucose ratio, maltose transport activity recovers and reaches a peak at about the same time as the amount of yeast in suspension reaches its maximum (Rautio and Londesborough, 2003). At this stage, maltose transporter genes are well expressed, glucose has been partially or entirely consumed (bringing an end to catabolite inactivation) and the plasma membranes contain adequate amounts of sterols and UFA. Thereafter, the specific activity of maltose transport declines steadily to the low value found in cropped yeast. During the second half of the fermentation, the remaining maltose and maltotriose must be transported into the yeast cell by transporters that are working suboptimally because the plasma membranes do not contain sufficient sterols. In particular, the daughter cells formed in the last round of cell division may be very deficient in sterols because, although their membranes may receive some sterol from the mother cell, any size increase after separation of mother and daughter occurs in an anaerobic environment where no new sterol can be made.

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CHAPTER 3

THE ADENYLATE ENERGY CHARGE AND SPECIFIC FERMENTATION RATE OF BREWER'S YEASTS FERMENTING HIGH- AND VERY HIGH-GRAVITY WORTS

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3.1 INTRODUCTION

In living cells the concentration of ATP is maintained far above equilibrium with respect to the concentrations of ADP, AMP (i.e., 5´-AMP) and inorganic phosphate, which are all potentially equilibrated by "ATPases" and adenylate kinase. The free energy available from hydrolysis of ATP is used to drive many synthetic reactions and other energy-requiring processes. The ratio of ATP, ADP and AMP concentrations is thermodynamically more important than the absolute concentration of ATP and is often expressed as the adenylate energy charge (EC), where:

$$EC = ([ATP]+0.5\times[ADP])/([ATP]+[ADP]+[AMP]).$$

The EC can vary between 0 and 1 (for reviews, see Ball & Atkinson, 1975; Chapman & Atkinson, 1977). The EC is a measure of the cell's ability to carry out the anabolic and maintenance reactions required to grow and to remain alive. For example, under conditions where absolute ATP levels fell, the rate of protein synthesis in *Escherichia coli* increased and correlated well with changes in EC over the range 0.7 to 0.9 (Swedes *et al.*, 1975). Several enzymes involved in the production or utilization of ATP (e.g. phospho-fructokinase, Reibstein *et al.*, 1986; hexokinase, Peters and Neet, 1978) are allosterically regulated by adenine nucleotides, with the result that their activities both depend on and affect the EC. The response patterns of these enzymes determined *in vitro* suggest that the EC *in vivo* should stabilise at a value between 0.8 and 0.95, i.e., in this range of EC the rates of the major ATP-producing reactions are in balance with the major ATP-consuming reactions (Chapman and Atkinson, 1977). Chapman & Atkinson (1977) concluded that the EC is near to 0.9 in most "normally metabolising" cells and suggested that the lower limit of EC permitting growth of *Saccharomyces cerevisiae* is about 0.75. Apparently, *S. cerevisiae* cells starving after aerobic growth on glucose remain viable at an EC down to 0.15 (Ball and Atkinson, 1975), whereas the viability of *E. coli* cells rapidly drops at an EC below 0.5.

During batch fermentations of brewer's wort, brewer's yeast passes through a series of different physiological states. After a lag phase, there is a phase of growth (involving consumption of ATP), during which the specific rates of ethanol production and glycolytic ATP formation are at their greatest. Under brewing conditions at about 15 ºC, about 0.25 g ethanol·[g dry yeast]$^{-1}$·h^{-1} is produced, corresponding to the production of about 1 mM ATP·sec^{-1} in the cytosol of yeast, which implies that the cytosolic ATP pool (about 5 mM) turns over every 5 seconds. Growth stops when the concentration of yeast in suspension is near its maximum. The specific rate of ethanol production usually remains high when yeast growth begins to slow, but decreases soon after growth stops. Ethanol production slows as the fermentation approaches completion and effectively stops before all fermentable sugars

have been consumed. Typically, residual maltose and maltotriose in the final beer together amount to 2 – 10 $g \cdot L^{-1}$, depending on the original gravity (sugar concentration) of the wort and the physiological condition of the yeast used to start the fermentation (pitching yeast).

In brewing fermentations, metabolism is almost entirely fermentative so that ATP must be consumed at the same rate as it is generated by glycolysis. It is, therefore, not clear why the specific rate of ethanol production remains high when the demand of growth for ATP disappears. A possible scenario is that growth slows because increasing amounts of ATP are required for maintenance reactions as the ethanol concentration and other stresses increase during the fermentation. However, it is generally accepted that in wort fermentations yeast growth ceases because of nutrient limitations, in particular because oxygen is no longer available to synthesise the sterols and unsaturated fatty acids needed to make new membranes (David and Kirsop, 1973). It remains the case that, in order for fermentation to continue after yeast growth stops, other ATP-consuming processes must operate sufficiently rapidly to remove ATP generated by glycolysis. Increasing ethanol concentrations increase the permeability of the cell membrane to protons. Considerable amounts of ATP are required to pump out these protons and maintain the cytosolic pH close to neutral (about 6.4 during wort fermentation; Rowe *et al.*, 1994).

There are few reports concerning intracellular adenine nucleotide levels during wort fermentations. Hysert *et al.* (1976) reported intracellular ATP levels of about 5 $\mu mol \cdot [g\ dry\ yeast]^{-1}$ during fermentations of brewer's worts by a lager and an ale strain. The ATP content per cell was fairly constant during fermentation but, because of the increase in cell size, $ATP \cdot [g\ dry\ yeast]^{-1}$ decreased gradually during fermentation and fell to much lower levels 2 days after the fermentation ended. Hysert and Morrison (1977) later measured the EC of ale yeast during wort fermentations and found high values (0.83 – 0.97) during a rapid brewery fermentation and low values (0.7 – 0.8) during a laboratory fermentation that was much slower. They suggested that EC measurements might be used to guide yeast management in breweries. This has not, however, become popular during the intervening 3 decades, perhaps because the experimental methods are relatively demanding and require several hours. These authors were also the first to report the appearance of extracellular ADP and ATP in beer, at levels less than a tenth of the AMP levels found by earlier workers (see, e.g., Pickett, 1974). Even the AMP levels are, however, below taste threshold. Rowe *et al.* (1994) found cytosolic ATP levels gradually rising from 7 to 12 mM through fermentation of a 10 ºP wort. Sato *et al.* (2000) reported almost constant intracellular ATP during fermentation of sake mash up to an ethanol concentration of 15%, followed by an abrupt drop when the mash was fortified by addition of ethanol to 20%. Dinsdale *et al.* (1999) reported that the EC was between about 0.8 and 0.95 during the first 20 days of a 35 day cider fermentation, although

intracellular ATP and ADP both showed large decreases.

In a series of articles (Alterthum *et al.*, 1989; Dombek and Ingram, 1987; Dombek and Ingram, 1988) from Ingram's laboratory, the specific rate of ethanol production during batch fermentation of 200 g glucose·L^{-1} in rich medium by a petite yeast strain was found to be highest during early fermentation and began to decline already when the ethanol concentration reached 10 g·L^{-1} and the culture began to enter a stationary phase. The decrease in specific rate could not be explained by decreases in cell viability, cytosolic pH, or specific activities of glycolytic enzymes (measured *in vitro*) or glucose transport. These authors reported (Dombek and Ingram, 1988) that the decrease in specific rate of ethanol production coincided with a marked increase in cytosolic AMP and decreases in ADP, ATP and EC. EC fell from about 0.7 to 0.1. These authors suggested that the decline in ATP and rise in AMP might result from increased consumption of ATP to expel protons leaking into the cell across the plasma membrane at high ethanol concentrations or from RNA degradation when cells enter stationary phase. They also demonstrated that the observed increases in AMP and the AMP/ATP ratio were enough strongly to inhibit yeast hexokinase *in vitro* and claimed that this inhibition can account for the observed decrease in ethanol production (Alterthum *et al.*, 1989).

The work presented in this chapter involved the measurement of intracellular and extracellular adenine nucleotides and the calculation of the EC through fermentations of high (15 ºP) and very high gravity (VHG, 25 ºP) worts by two lager yeast strains. The aims were to test whether, under conditions like those in a brewery fermentation, the EC correlated with yeast growth, with specific fermentation rate or with ethanol levels and whether the EC of yeast approaching the end of a wort fermentation is high enough to permit the synthesis of the new mRNA and protein molecules needed for successful adaptation to the changing environment.

3.2 MATERIALS AND METHODS

3.2.1 Yeasts and fermentations

Strain A15 is a production lager yeast (A63015) from VTT's collection. Strain Ind2 is also a production lager yeast. A15 and Ind2 were grown in the laboratory at 24 ºC in 3 L lots of 15 ºP wort in 5 L Erlenmeyer flasks on an orbital shaker. Where stated, strain Ind2 was collected from the storage tank of a local brewery within 12 h of cropping from a routine brewery fermentation of a high gravity wort. The cropped yeast slurry was conveyed on ice to the laboratory and diluted with water to 200 g centrifuged yeast·[kg slurry]$^{-1}$ before use.

The 25 ºP wort was made at VTT from malt with high maltose syrup as adjunct (40%) and contained (g·L^{-1}) maltose 120, glucose 25, maltotriose 40, fructose 5 and sucrose 2 and (mg·L^{-1}) FAN (free amino nitrogen measured as glycine equivalents) 420, Mg 170, Ca 60 and Zn 0.17. An additional 0.1 mg·L^{-1} Zn was added as $ZnSO_4$ before use. The 15 ºP wort was made from 25 ºP wort by dilution with water containing 0.2 mg·L^{-1} Zn. Yeast was grown in 15 ºP wort at 24 ºC to stationary phase and allowed to settle at 0 ºC. Most of the supernatant was decanted and the settled yeast was mixed into a smooth slurry and diluted with supernatant to 200 g fresh mass·L^{-1}. Fermentations were carried out essentially as described earlier (Rautio and Londesborough, 2003) in 100 x 6 cm stainless steel tall tubes with conical bottoms and anaerobic sampling ports 23 cm above the cones (tall tubes are designed to mimic the cylindroconical vessels used in most breweries). Well mixed yeast slurries were pitched at 10 ºC by mass into 2 L of oxygenated 15 ºP or 25 ºP wort (ca. 9 or 12 ppm O_2, respectively) to 5.0 or 8.0 g centrifuged yeast mass·L^{-1}, respectively. After pitching, the tubes were placed at 14 ºC. The 25 ºP fermentations were shifted to 18 ºC at 22 ± 2 h. For analyses of total adenine nucleotides in yeast and wort, 9 mL samples of fermenting wort were withdrawn with needle and syringe through the sampling ports and immediately (within about 20 s of starting to fill the syringe) injected into 1.0 mL of ice-cold 5.0 M perchloric acid (PCA) in tared tubes, which were re-weighed to give the sample masses. Parallel samples were similarly withdrawn, centrifuged (5 min at 13000 x g) and the supernatant injected into PCA for determinations of extracellular adenine nucleotides. Larger samples (ca. 30 mL) were also withdrawn and handled as previously described (Rautio and Londesborough, 2003) for determination of dry solids in suspension and wort density. Towards the end of the fermentations, about 200 mL of the settled yeast slurry was run out through a flap valve at the bottom of the cone, quickly mixed to a homogenous slurry and samples quenched in PCA for adenine nucleotide determinations.

Total yeast protein was determined by extracting the yeast overnight with 1.0 M NaOH and

assaying the extract according to Lowry *et al.* (1951), using ovalbumin as the standard.

For the minifermentations of Table 2, where ATP was added in mid-fermentation, 20 g lots of 25 ºP wort were pitched with industrially cropped yeast slurry as described above in 50 mL conical-bottomed Corning tubes and overlayered with 10 mL of mineral oil to limit air access. Minifermentations were followed by mass-loss. Under these conditions (with no air locks in order to facilitate ATP addition) the minifermentations proceed at the same rate as parallel 2-L scale tall tube fermentations (data not shown) until near the end, but the mass loss does not reach a definite stop unless the tubes are also fitted with air locks.

3.2.2 Fermentation calculations

The progress of wort fermentations was followed as in normal brewery practice by measuring the density of de-gassed supernatants with an Anton Paar DMA58 density meter. Apparent extracts were calculated from the densities according to Analytica EBC, Method 9.4 (EBC-European Brewery Convention, 2004).

"Extract" is a measure of the sum of fermentable sugars and non-fermentable soluble carbohydrate in wort: a solution with an extract of x ^{o}P has the same density as a solution containing x g of sucrose in 100 g of solution. "Apparent extracts" measured during fermentations are uncorrected for the effect of ethanol on the density. Apparent attenuations are the difference between the original extract (OE) and the current apparent extract (CE) divided by the original extract ([OE-CE]/OE).

Ethanol concentrations during fermentation were calculated from the original extract and current apparent extract using Holzner's tables (Goldiner *et al.*, 1966). Ethanol was also determined by quantitative distillation according to Analytica EBC, Method 9.2.1 (EBC-European Brewery Convention, 2004). Limit attenuations (which reflect the maximum possible conversion of wort carbohydrates into ethanol) were measured according to Analytica EBC, Method 8.6.1 (EBC-European Brewery Convention, 2004). Specific rates of ethanol production (g ethanol·[g dry yeast]$^{-1}$·h^{-1}) were calculated from the daily change in ethanol concentration and the average daily dry yeast concentration.

3.2.3 Adenine nucleotide assays

Samples quenched in PCA were immediately mixed with 250 µL of 0.6 M EDTA and frozen in liquid nitrogen. Each PCA extract was frozen and thawed three times and centrifuged (5 min at 13000 x g). The supernatant was collected. The pellet was washed with 5 ml of ice-cold 0.5 M PCA and the resulting supernatant added to the first supernatant. The combined

supernatants were neutralized to pH 6.5 - 7.5 with 5.0 M K_2CO_3, incubated on ice for 30 min and centrifuged (10 min at 13000 x g). The supernatant was collected in a weighed tube. The precipitate of potassium perchlorate formed during neutralization was washed with 5 mL of 0.1 M HEPES/KOH, pH 7.0, containing 15 mM EDTA, and the resulting supernatant was added to the supernatant in the weighed tube. The tube was re-weighed to give the exact mass of extract.

The ATP concentration in the extract was quantified with a commercial kit (ATP Kit SL, BioThema AB, Sweden) by the luciferin-luciferase (L-L) reaction, using an internal ATP standard, essentially as described in the kit instructions. The light emission was measured with a luminometer (Biotrace M3). When necessary, extracts were diluted with 0.1 M HEPES/KOH pH 7.0 before the L-L assays, which were made in duplicate.

ADP and AMP were quantitated after conversion to ATP, essentially as described by Lundin (2000). First, ADP was converted to ATP by the pyruvate kinase (PK) reaction, and the total ATP (corresponding to ATP+ADP) was determined by the L-L reaction. ADP was calculated as the difference between ATP+ADP and ATP alone. The PK reaction mixture was 0.1 M HEPES/KOH pH 7.0 containing 1 mM phosphoenolpyruvate, 20 mM $MgCl_2$, 40 mM KCl, and 2.8 $\mu g \cdot mL^{-1}$ PK (Sigma). The reaction was incubated at room temperature (ca. 20 ºC) for 5 – 15 min, and the ATP+ADP concentration was determined by the L-L assay. A repeat L-L assay was made after a further 5 – 10 min incubation. These assays agreed within ± 3 %.

AMP was converted to ADP by the adenylate kinase (AK) reaction. This reaction was coupled with the PK reaction, so that the ADP formed was further converted to ATP. The total adenine nucleotides in the extract (ATP+ADP+AMP) were then determined as ATP by the L-L reaction, and AMP was calculated as the difference between total adenine nucleotides and ATP+ADP. The AK+PK reaction mixture was the same as for the PK reaction with the addition of 0.25 mM cytidine triphosphate (CTP, used to accelerate the AK reaction and unable to react with luciferase; Lundin, 2000) and 31 $\mu g \cdot mL^{-1}$ AK (Sigma). The reaction was incubated at room temperature for 30 – 40 min, and the ATP+ADP+AMP concentration was determined by the L-L assay. A repeat L-L assay was made after a further 30 – 40 min incubation. These assays agreed within ± 3 %.

Intracellular adenine nucleotides levels were calculated by the difference between total adenine nucleotides (yeast + fermenting wort) and extracellular adenine nucleotides (fermenting wort). To calculate cytosolic concentrations, it was assumed 1.5 ml cytosol·[g dry yeast]$^{-1}$. For a protein content of 40% of the dry mass, this agrees with the 3.7 mL·[g protein]$^{-1}$ determined by Richard *et al.* (1996).

3.2.4 Fatty acid, sterol and squalene analyses

Samples of the pitching yeasts were harvested, washed and saponified before analyses of total fatty acids, sterols and squalene as described in chapter 2 (section 2.2.3).

3.3 RESULTS

3.3.1 Extracellular adenine nucleotides

The levels of extracellular adenine nucleotides during wort fermentations are shown in Figure 3.1. Before addition of yeast, the 15 and 25 ºP worts contained, respectively, about 0.5 and 1 µM AMP, 0.1 and 0.15 µM ADP and smaller amounts of ATP (0.002 – 0.01 µM). During fermentations, the concentration of extracellular AMP increased markedly, but increases in ADP and ATP were smaller. With laboratory-grown strain A15, the increase in AMP was greater (1.4 µM) in 25 ºP wort than in 15 ºP wort (0.4 µM). With laboratory-grown strain Ind2, the AMP increase in 25 ºP wort (1.8 µM) was similar to that observed with strain A15. However, with industrially cropped Ind2, a much larger increase (12 µM) in AMP was observed, ADP increased by 0.4 µM and ATP showed a transient increase (0.25 µM at 68 h fermentation) but then declined and was lower at the end of the fermentation than in unpitched wort.

Figure 3.1 also shows the mass action ratio ($[ADP]^2/([ATP][AMP])$) of the adenylate kinase reaction calculated for extracellular adenine nucleotides through each fermentation. Adenylate kinase is an unusually stable enzyme that equilibrates adenine nucleotides according to the reaction ATP + AMP $\Leftrightarrow$ 2·ADP, which has an equilibrium constant near 2 (2.3 at 25 ºC, 10 mM $MgCl_2$; see Bergmeyer *et al.*, 1974). With the laboratory-grown yeasts, the mass action ratios were initially close to (Figure 3.1, panels B and C) or above (8.5; not shown in Figure 3.1, panel A) the equilibrium constant and fell after 2 days to values <0.1, well below the equilibrium constant. With industrially cropped yeast, the mass action ratio (0.9, measured 3.5 h after pitching) was initially a little lower than the equilibrium constant, reached a minimum of 0.3 after 3 days, then rose to near 1.0 and remained between 0.8 and 1.0 for the rest of the fermentation.

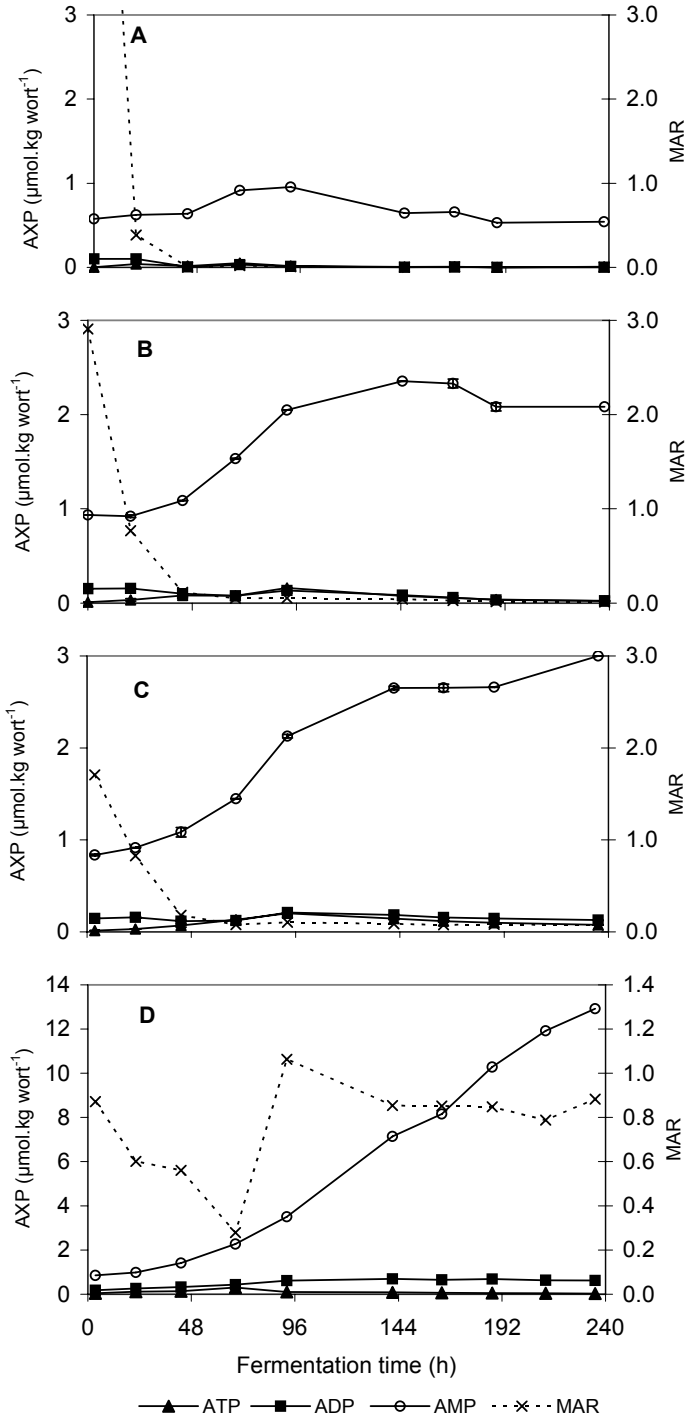

Figure 3.1 – Extracellular adenine nucleotides during wort fermentations. Adenine nucleotides (AXP; ATP, ADP and AMP) in the medium were measured during fermentation of (A) 15 ºP wort by laboratory-grown strain A15; (B) 25 ºP wort by laboratory-grown strain A15; (C) 25 ºP wort by laboratory-grown strain Ind2; (D) 25 ºP wort by industrially cropped strain Ind2. The mass action ratio (MAR= $[ADP]^2/[ATP][AMP]$) of the adenylate kinase reaction calculated from extracellular AXP concentrations is also shown. For panels B and C data are averages ± ranges (usually smaller than the symbols) from duplicate fermentations and for A and D averages of duplicate assays from single fermentations. Growth curves and apparent attenuations are shown for A and B in Figure 3.2 and for C and D in Figure 3.3.

For the laboratory-grown yeasts, the amounts of extracellular AMP at the end of the fermentations were between 1 and 5% of the maximum total adenine nucleotide (ATP+ADP+AMP) levels reached in the whole culture (intracellular + extracellular). These maximum levels were reached on Day 3, when the amounts of yeast in suspension were maximal, and are shown in Table 3.1. For the industrially cropped yeast, the final AMP level was 19% of the maximum total adenine nucleotides found in the culture. For the laboratory-grown yeasts, yeast cropped at the end of the fermentations had viabilities above 96%, but for the industrially cropped sample, yeast re-cropped from the bottom of the fermentation tubes after 189 and 236 h had viabilities of 71 and 56%. Thus, the amounts of extracellular adenine nucleotides found are consistent with an origin in dead or dying cells. Because the major intracellular adenine nucleotide was ATP (see below) whereas the major extracellular adenine nucleotide was AMP, the fate of ATP (5.2 μM) added at 72 h to minifermentations of 25 ºP wort by industrially cropped yeast was tested. After a further 18 h fermentation, the ATP was recovered quantitatively, within experimental error, 21% as ATP, 57% as ADP and 27% as AMP, and at this point the adenylate kinase mass action ratio was 1.5 for the fermentation with added ATP and 1.3 for the control fermentation (Table 3.2).

Table 3.1 – Final extracellular ATP, ADP and AMP and maximum (Day 3) total (intracellular + extracellular) adenine nucleotides during wort fermentations.[1]

Yeast[2]	Wort	ATP (μM)	ADP (μM)	AMP (μM)	$\frac{[ADP]\cdot[ADP]}{[ATP]\cdot[AMP]}$	Max. AXP[3] (μM)
A15, lab-grown	15 ^{o}P	0.008	0.003	0.54	0.002	45
A15, lab-grown	25 ^{o}P	0.021	0.024	2.1	0.013	58
Ind2, lab-grown	25 ^{o}P	0.078	0.13	3.0	0.072	58
Ind2, cropped	25 ^{o}P	0.033	0.62	13.0	0.89	67

[1]ATP, ADP and AMP concentrations are final values from the tall tube fermentations of Figure 3.1. The mass action ratios of the adenylate kinase reaction are calculated from the given values.

[2]The yeasts used were laboratory-grown A15 (A15, lab-grown), laboratory grown Ind2 (Ind2, lab-grown) and industrially cropped Ind2 (Ind2, cropped).

[3]Max. AXP shows the maximum total (intracellular + extracellular) concentration of adenine nucleotides (ATP+ADP+AMP) reached in each culture.

Table 3.2 – The fate of extracellular ATP added during fermentation of 25 °P wort.[1]

Fermentation	ATP (μM)	ADP (μM)	AMP (μM)	$\frac{[ADP]\cdot[ADP]}{[ATP]\cdot[AMP]}$
Controls	0.10 ± 0.00	0.95 ± 0.05	7.0 ± 0.3	1.3
Added ATP	1.2 ± 0.1	3.9 ± 0.4	8.4 ± 0.4	1.5
Difference	1.1 ± 0.1	3.0 ± 0.5	1.4 ± 0.6	-

[1]ATP, ADP and AMP were measured at 90 h (averages ± ranges of duplicate minifermentations) in controls and in fermentations where 5.2 μM ATP was added to the fermenting wort at 72 h. The mass action ratios of the adenylate kinase reaction are calculated from the given values. The yeast was industrially cropped Ind2.

The amount of extracellular ATP compared to total ATP was always less than 4% and usually between 0.1 and 0.3%. Extracellular ADP varied between 0.1 and 43% of total ADP and in 90% of assays it was less than 10% of total ATP (the exceptions were with industrially cropped strain Ind2 on Day 1 and Day 8). For these two nucleotides, calculation of the intracellular amount as the difference between total amount and the much smaller extracellular amount should provide reliable results, and experimental errors were apparently small. However, estimates of extracellular AMP routinely varied between 9 and 100% of the estimates of total AMP, and in 10% of cases, the estimated extracellular AMP was greater (104 – 188 %) than estimated total AMP. This probably arose because extracellular AMP as the dominant extracellular nucleotide was accurately measured whereas total AMP as a minor component of the total adenine nucleotide pool was less accurately estimated. Fortunately, errors in intracellular AMP had little effect on calculation of the intracellular EC.

3.3.2 Intracellular adenine nucleotides

Figure 3.2 shows changes in intracellular adenine nucleotides during fermentations of high gravity (15 ºP) and VHG (25 ºP) worts by lager strain A15, which is a poorly flocculent yeast that sedimented slowly at the end of fermentation. Total adenine nucleotides (ATP+ ADP+AMP) were about 6 μmol·[g dry yeast]$^{-1}$, which corresponds to a cytosolic concentration of 4 mM. The pitching yeast was grown in 15 ºP wort at 24 ºC and moved to 0 ºC 20 h before pitching (see Materials and Methods). ATP was high (5 μmol·[g dry yeast]$^{-1}$), ADP and AMP levels low, and the resulting EC was 0.92 in samples of this yeast taken immediately before transfer to 0 ºC. Similar values were found 2 h after pitching into 15 ºP wort, and ATP (4.0 – 4.3 μmol·[g dry yeast]$^{-1}$) and EC (0.85 – 0.93) remained high through the fermentation up to

an apparent attenuation of 81% (App. Att. of 0.81 in Figure 3.2, upper panel; the limit attenuation for this wort was 86%). The ATP level then dropped, ADP and AMP levels rose and EC fell from 0.85 to 0.57 during the next two days (96 to 144 h), as the apparent attenuation increased from 81% to 84%. During the final two days (144 to 188 h) there was no significant change in apparent attenuation, changes in ATP, ADP and AMP were small and the EC fell gradually to 0.5. During fermentation, total adenine nucleotides (ATP + ADP + AMP) increased from 4.7 to 6.2 μmol·[g dry yeast]$^{-1}$ at 92 h and then returned to 4.7 μmol·[g dry yeast]$^{-1}$ at 188 h. The change in total adenine nucleotides was greater when related to yeast total protein, from 10 μmol·[g protein]$^{-1}$ at the start of the fermentation to 21 μmol·[g protein]$^{-1}$ at 92 h and 11 μmol·[g protein]$^{-1}$ at 188 h (data not shown).

At 170 h, crop yeast was removed from the bottom of the cone and adenine nucleotides assayed. The cropped yeast was unavoidably exposed to air during collection and sampling, which may have altered adenine nucleotide levels. The apparent EC in the crop yeast was 0.47 (not shown in Figure 3.2), which was a little lower than the EC (0.53) of the yeast in suspension at 168 h.

Similar results were obtained during fermentation of the 25 ºP wort by the same lot of A15 yeast (Figure 3.2, lower panel). During the first 48 h there was a larger increase in ATP (from 4.0 to 6.5 μmol·[g dry yeast]$^{-1}$) and total adenine nucleotides than seen in the 15 ºP wort. The EC remained high (0.92 – 0.98) through the peak in yeast concentration and up to an apparent attenuation of at least 72% at 92 h. The EC fell when the fermentation approached completion and reached 0.55 by 168 h, when the apparent attenuation had reached its final value of 80% (the apparent attenuation limit of this wort also was 86%).

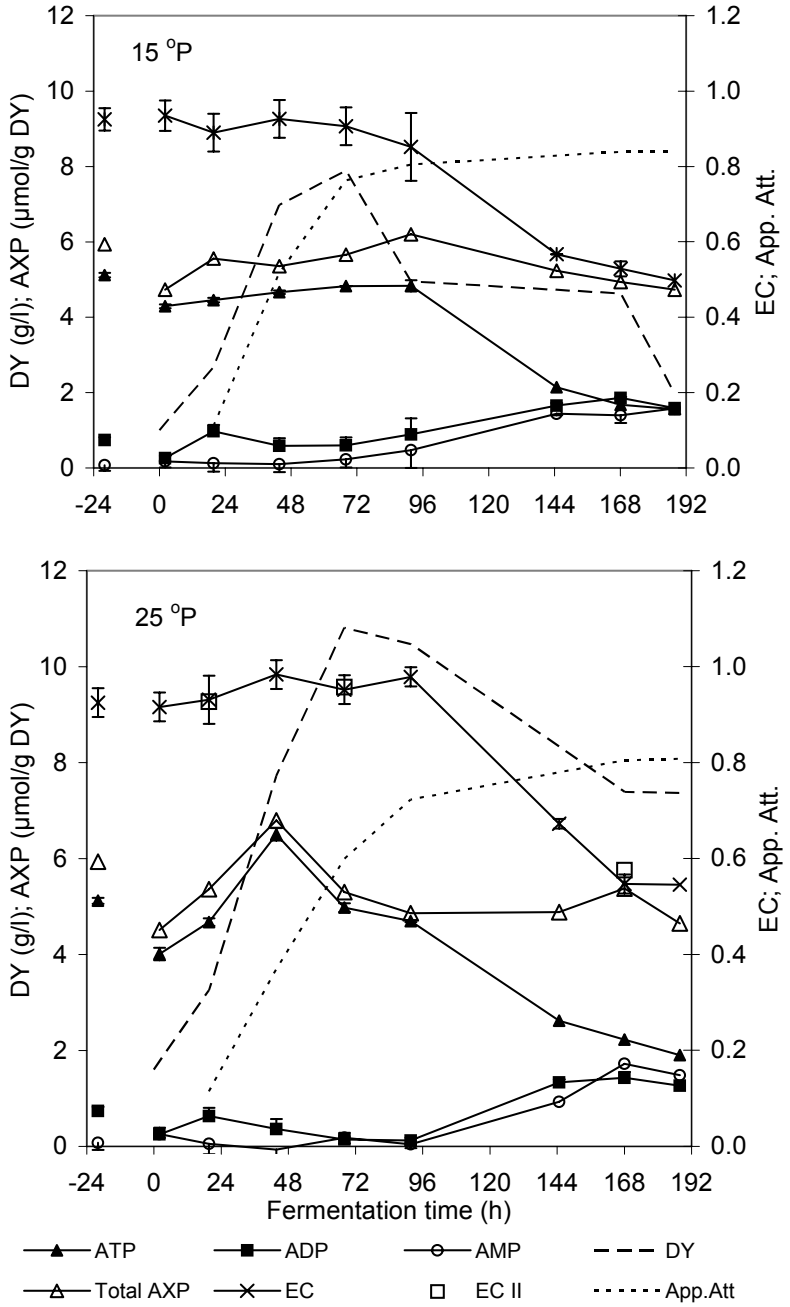

Figure 3.2 – Intracellular adenine nucleotides during fermentation of 15 and 25 ºP worts by lager strain A15. The upper and lower panels show data from the same fermentations as in panels A and B of Figure 3.1. Intracellular adenine nucleotides (AXP; ATP, ADP, AMP) and total adenine nucleotides (Total AXP = ATP + ADP + AMP) were measured and the EC calculated in samples taken during the fermentation and (at -20 h) from the propagated yeast immediately before transfer to 0 ºC (see Materials and Methods). The lower panel also shows EC data (ECII) from a replicate 25 ºP fermentation assayed at 20, 68 and 168 h. Dry yeast (DY) concentrations and the apparent attenuations (App. Att.) on a scale from 0 to 1 (i.e., 0 – 100 %) are shown. The upper panel data are from a single fermentation at 15 ºP. Error bars indicate the ranges between duplicate assays. Fermentations were pitched at about 10 ºC, and immediately shifted to 14 ºC. The temperature of the 25 ºP fermentations was further shifted to 18 ºC at 22 h.

Figure 3.3 shows fermentations of 25 ºP wort by lager strain Ind2 either grown in the laboratory (upper panel) or cropped from a brewery (lower panel). Fermentations with each lot of pitching yeast were carried out in duplicate, with essentially identical attenuation and growth profiles. The fermentations with laboratory-grown yeast were faster, more complete (i.e., reached higher apparent attenuation) and reached higher yeast concentrations, followed by more rapid sedimentation than was observed with the industrially cropped yeast. The changes in adenine nucleotides and EC values followed the same pattern observed for A15 yeast in Figure 3.2. The ECs of pitching yeasts were high (0.93 and 0.88, respectively, for laboratory-grown and industrially cropped yeasts) and remained high through the peaks in yeast concentration. For laboratory-grown yeast, the EC was still 0.78 at an apparent attenuation of 78.5%, but 24 h later dropped to 0.60 at an apparent attenuation of 79%. For industrially cropped yeast, the EC was 0.87 at an apparent attenuation of 74%, falling to 0.77 and 0.60 as the apparent attenuation rose to 76 and 77%. Yeast cropped at 189 h from the fermentation with laboratory-grown Ind2 had a viability of 96% and an EC (0.47) a little lower than that (0.56) of the yeast in suspension at the same time. Yeast re-cropped at 236 h from the fermentation with industrially cropped Ind2 had very low viability (56%) and its EC (0.39) was lower than that (0.6) of yeast in suspension at the same time (Figure 3.3).

In Figure 3.4 the EC values, calculated ethanol concentrations and specific ethanol production rates through the fermentations of Figure 3.2 are plotted against the apparent attenuations. Calculated ethanol concentrations (54.2 and 90.6 $g \cdot L^{-1}$, respectively) at the end of the 15 and 25 ºP fermentations were slightly lower than the values (55.0 and 90.9 $g \cdot L^{-1}$) determined by quantitative distillation. There was no correlation between ethanol concentration and EC: in the 25 ºP fermentation EC remained high at ethanol concentrations much higher than those reached in the 15 ºP fermentation. In the 15 ºP wort fermented at 14 ºC, the specific rate of ethanol production (g ethanol·[g dry yeast]$^{-1} \cdot h^{-1}$) was between 0.20 and 0.23 up to an apparent attenuation of 31% (when the yeast is still growing rapidly; Figure 3.2) and then fell gradually, reaching 0.02 at 78% apparent attenuation, whereas the EC was still 0.85 at 81% apparent attenuation. Above 81% apparent attenuation, the specific rate of ethanol production fell below 0.01 g ethanol·[g dry yeast]$^{-1} \cdot h^{-1}$, and the EC collapsed to 0.57 and 0.50 at 84 and 86% apparent attenuation. During the first 22 h, when both fermentations were at 14 ºC, the specific rate of ethanol production (g ethanol·[g dry yeast]$^{-1} \cdot h^{-1}$) was lower in the 25 ºP wort (0.16) than the 15 ºP wort (0.20), but then reached 0.22 at 18 ºC and 21% apparent attenuation before gradually falling to 0.05 at 65% apparent attenuation, whereas the EC was still 0.98 at 71% apparent attenuation. Above 75% apparent attenuation, the specific rate of ethanol production fell below 0.01 g ethanol·[g dry yeast]$^{-1} \cdot h^{-1}$, and the EC collapsed to 0.67 and 0.55 at 79.6 and 80.0% apparent attenuation.

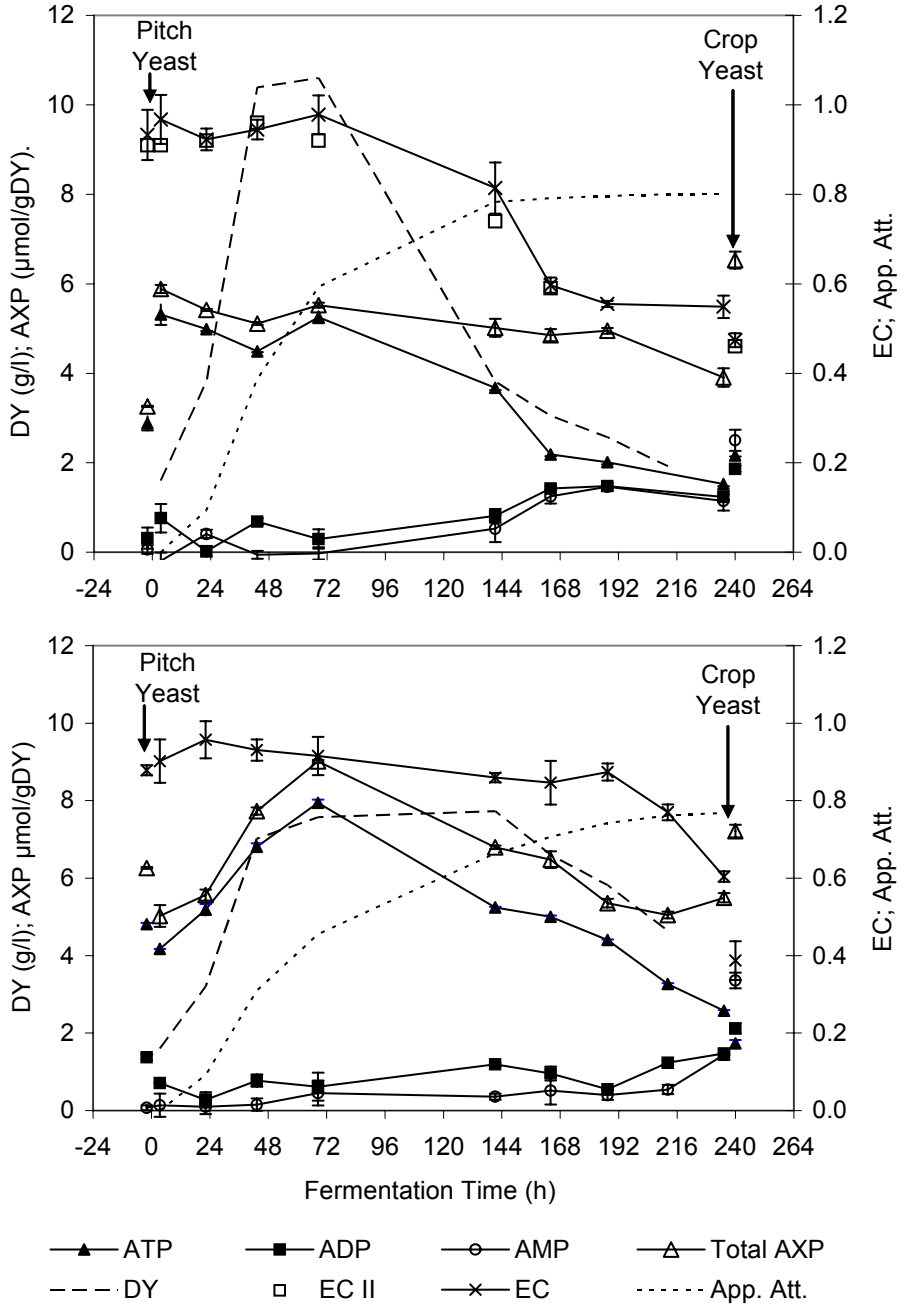

Figure 3.3 – Intracellular adenine nucleotides during fermentations of 25 ºP worts by (upper panel) laboratory-grown and (lower panel) industrially cropped lager strain Ind2. The upper and lower panels show the same fermentations as in panels C and D of Figure 3.1. Intracellular adenine nucleotides (AXP; ATP, ADP, AMP) and total adenine nucleotides (Total AXP = ATP+ADP+AMP) were measured and the EC calculated in samples from the yeast slurries immediately before pitching (plotted at -2h) and during fermentation, as well as in samples from the cropped yeasts. EC values (ECII) from a replicate fermentation with laboratory-grown yeast are also shown in the upper panel. Dry yeast (DY) concentrations and the apparent attenuations (App. Att.) on a scale from 0 to 1 (i.e., 0 – 100 %) are shown. Error bars indicate the ranges between duplicate assays. The lower panel shows results from a single fermentation. Fermentations were pitched at about 10 ºC, immediately shifted to 14 ºC and further shifted at 24 h to 18 ºC.

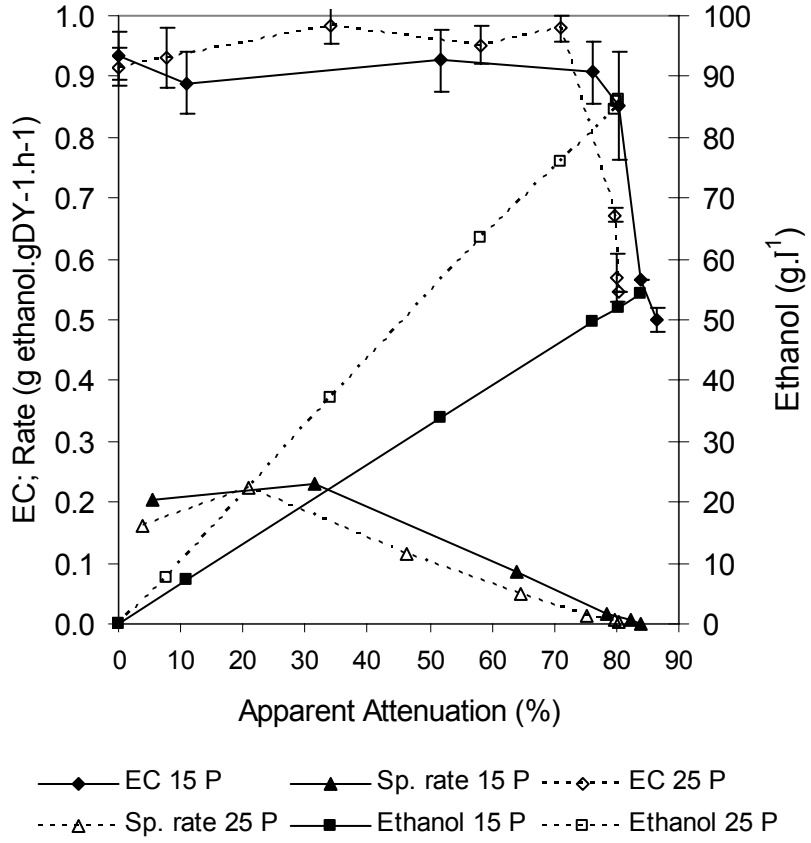

Fig 3.4 – EC, calculated ethanol concentrations and specific ethanol production rates (Sp. rate) are plotted against the apparent attenuation. Data are from the Figure 3.2 fermentations of 15 ºP (full lines and solid symbols) and 25 ºP (broken lines and open symbols) worts. EC error bars show the range between duplicate assays or (25 ºP) duplicate fermentations. The limit apparent attenuations were 86%.

Similar patterns were observed (Figure 3.5) when EC and specific fermentation rates were plotted against apparent attenuation for the Figure 3.3 fermentations of 25 ºP wort by laboratory-grown and industrially cropped lager strain Ind2. In the first day, industrially cropped yeast had a higher specific fermentation rate than the laboratory-grown yeast (0.19 and 0.16 g ethanol·[g dry yeast]$^{-1}$·h^{-1}, respectively) and both reached 0.24 g ethanol·[g dry yeast]$^{-1}$·h^{-1} at apparent attenuations of about 20%, when the yeast was growing rapidly (Figure 3.3). The specific fermentation rates then gradually fell to low values while EC remained high (for the industrially cropped yeast, EC was still >0.85 at 74% apparent attenuation when the fermentation rate was 0.02 g ethanol·[g dry yeast]$^{-1}$·h^{-1}, and for the laboratory-grown yeast, EC was still 0.78 at 78.5% apparent attenuation, when the fermentation rate was below 0.03 g ethanol·[g dry yeast]$^{-1}$·h^{-1}), followed by collapses of the EC when the fermentation rates fell below 0.01 g ethanol·[g dry yeast]$^{-1}$·h^{-1}.

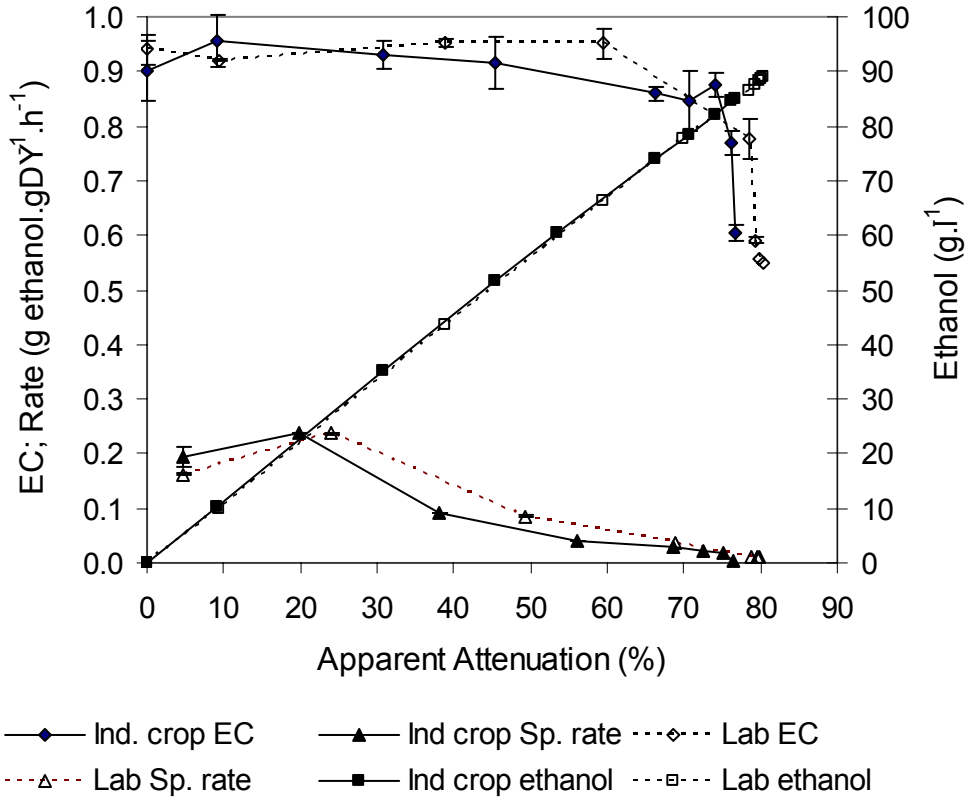

Fig 3.5 – EC, calculated ethanol concentrations and specific ethanol production rates (Sp. rate) are plotted against the apparent attenuation. Data are from the Figure 3.3 fermentations with industrially cropped (Ind crop; full lines and solid symbols) and laboratory-grown (Lab; broken lines and open symbols) lager strain Ind2. Specific rate error bars show the range between duplicate fermentations and EC error bars show the range between duplicate fermentations for the laboratory-grown yeast and between duplicate assays of a single fermentation for the industrially cropped yeast. The limit apparent attenuation was 86%.

3.4 DISCUSSION

In agreement with earlier reports (see Hysert *et al.,* 1977 and references therein) the extracellular adenine nucleotide concentrations were found to increase during the course of wort fermentations. The amounts of extracellular adenine nucleotides at the end of the fermentations varied between 1% and 19% of the maximum total (intracellular + extracellular) adenine nucleotides (reached on Day 3) and were in rough agreement with the percentages of dead cells in the yeast crop at the end of the fermentation. Thus, a quantitatively feasible origin for the extracellular adenine nucleotides is the intracellular pool in dying yeast cells. However, ATP was the dominant intracellular adenine nucleotide, whereas AMP was the dominant extracellular adenine nucleotide. ATP added to fermentations was converted within one day to a mixture of ATP, ADP and AMP (Table 3.2). Yeast cells can express broad specificity extracellular acid phosphatases (reviewed by Oshima, 1997), though the activity of these phosphate-repressible enzymes under the conditions used in the present work are not known. A plausible origin for extracellular AMP is the hydrolysis of ATP released from dead cells. The present data do not distinguish between this hypothesis and the possibility that AMP, but not ATP and ADP, can cross the membrane of healthy yeast cells. Dinsdale *et al.* (1999) reported that AMP was "excreted into the medium" during a 35 day cider fermentation, but cell viability decreased by 90%, suggesting, again, that the AMP probably came from dead cells.

With industrially cropped yeast, the concentrations of extracellular ATP, ADP and AMP were close to the equilibrium concentrations of the adenylate kinase reaction (Figure 3.1, Tables 3.1 and 3.2). Adenylate kinase is known to be released from autolysed cells and is used to estimate the extent of yeast autolysis (Driscoll *et al.*, 2002).

More extracellular AMP was found in the 25 ºP fermentations than in the 15 ºP fermentation (Figure 3.1), which is consistent with the lower viability of yeast cropped from the 25 ºP fermentations. More extracellular AMP was also found in 25 ºP fermentations with industrially cropped strain Ind2 compared to laboratory-grown Ind2, which is consistent with the lower viability, at the end of the tall tube fermentations, of the industrially cropped Ind2. This lower viability is probably related to the lower unsaturated fatty acid and sterol compositions of yeast cropped from intensely anaerobic industrial fermentations (David and Kirsop, 1973). In the present case, the fatty acid unsaturation indices (the amount of unsaturated C12 – C18 fatty acids as a proportion of total C12 – C18 fatty acids) were 77 – 80% for the laboratory-grown pitching yeasts (Ind2 and A15) and 44 – 49% for the industrially cropped Ind2 pitching yeast and the ergosterol/squalene mass ratios were > 14 for the laboratory-grown yeasts, but < 0.4 for the industrially cropped yeast. It is not possible to tell from the present data whether

the greater amount of extracellular AMP derived from industrially cropped yeast resulted only from a larger proportion of dead cells or whether AMP is more easily able to cross the membranes of viable cells with low contents of unsaturated fatty acids and sterols.

The amounts of extracellular ATP and ADP were too small seriously to disturb the estimates of intracellular ATP and ADP. However, the amount of AMP determined in the fermenting wort alone (where it could be accurately measured as the dominant adenine nucleotide) sometimes exceeded the total AMP found in wort plus cells (where, as the minor component determined by the difference between ATP+ADP+AMP and ATP+ADP, it was less accurately measurable). The concentration of intracellular AMP was so low that relatively large errors in its determination did not significantly affect the calculated EC. Because they were so low under the conditions used in this work, the present method did not define intracellular AMP levels accurately enough to describe their possible effect on enzymes subject to allosteric regulation by AMP (see, e.g., Alterthum *et al.*, 1989; Gancedo *et al.*, 1982; Reibstein *et al.*, 1986). However, the very high (up to 7 mM) intracellular AMP levels reported by Dombek & Ingram (1988) during fermentations at 30 ºC of 200 g glucose·L^{-1} were not observed at any point during the present work. Thus, the decreases in specific ethanol production rate after 20% attenuation in the fermentations reported here (Figures 3.4 and 3.5) cannot be explained by inhibition of hexokinase by high AMP, as suggested by Altherhum *et al.* (1989). High intracellular AMP (6.5 µmol·[g dry yeast]$^{-1}$, corresponding to about 4 mM) and rather low EC (0.73) were recently observed by Tai *et al.* (2007) at 30 ºC, but not at 12 ºC, during glucose-limited growth of *S. cerevisiae* in a chemostat (at 12 ºC, AMP and EC were 0.9 µmol·[g dry yeast]$^{-1}$ and 0.94). Possibly, relatively high intracellular AMP is a feature of fermentations at higher (> 20 ºC) temperatures.

The intracellular EC of the yeasts was high (>0.85) from the start of fermentation until after the yeasts stopped growing and began to sediment (Figures 3.2 and 3.3). Evidently, cessation of growth did not result from a decrease in EC caused by diversion of increasing amounts of ATP to maintenance tasks. This agrees with Rowe *et al.* (1994), who found an about 20% increase of intracellular ATP when ale yeast fermenting a 9.5 ºP wort entered stationary phase (ADP and AMP were not measured). Hysert & Morrison (1977) reported average EC values of about 0.9 and 0.75 during, respectively, the first 4 days of a brewery fermentation and the first week of a (slower) laboratory wort fermentation by an ale strain. They did not provide growth curves, but their data are consistent with EC being constant and, for the brewery fermentation, high as yeast enters stationary phase.

As well as being independent of growth rate, the EC was independent of ethanol concentration up to at least 85 g ethanol·L^{-1} (Figure 3.4). The larger concentrations of ethanol reached in 25 ºP compared to 15 ºP fermentations did not cause lower EC values. Evidently,

ethanol stress of this magnitude did not cause maintenance demands that exceeded the yeast's ability to generate ATP at a high EC. This was true (Figure 3.5) both for laboratory-grown yeast and for industrially cropped yeast that had much lower contents of unsaturated fatty acids and sterols (see above). It appears that, up to apparent attenuations of 75 – 80% (in worts with an apparent attenuation limit of 86%) and ethanol concentrations over 80 $g \cdot L^{-1}$, the yeast cells were not limited by energetic constraints, and from this point of view would still be able to respond to environmental changes by transcription of new mRNA species and translation of new proteins (protein synthesis continues at EC values as low as 0.7, at least in *E. coli;* Swedes *et al.,* 1975).

In all four conditions investigated (strain A15 in 15 and 25 ºP worts, laboratory-grown and industrially cropped strain Ind2 in 25 ºP wort), high values of EC (0.8 – 0.98) were still observed when the specific fermentation rate had fallen to values (0.02 – 0.03 g ethanol·[g dry yeast]$^{-1} \cdot h^{-1}$) that were less than 12% of the maximum specific fermentation rates (Figures 3.4 and 3.5). Only when specific fermentation rates fell to ≤ 0.01 g ethanol·[g dry yeast]$^{-1} \cdot h^{-1}$ did the EC values collapse, abruptly, to values around 0.5 to 0.6. At this point, evidently, the rate of ATP synthesis was too low to maintain a high EC. At the lower EC values, the rates of reactions using ATP are expected to decrease and a new equilibrium can be maintained, at least temporarily. However, at this stage of fermentation, cell viability begins to drop, suggesting that ATP-requiring maintenance reactions become too slow to keep the cells alive.

At the end of high gravity wort fermentations, most of the major wort sugar, maltose, has been consumed, but some remains, together with much larger amounts of maltotriose. Typically, maltotriose remains in the range 2 – 10 $g \cdot L^{-1}$ and even more after VHG fermentations. Maltotriose and maltose transporters are proton symporters, which require an energised membrane and transmembrane proton gradient maintained by the ATP-consuming proton pump. Uptake of these α-glucosides and maintenance of a high EC are mutually dependent processes. Below a certain rate of ethanol production (under the conditions used here, about 0.01 g ethanol·[g dry yeast]$^{-1} \cdot h^{-1}$), glycolytic ATP may be produced too slowly to maintain a high EC, resulting in a decreased electrochemical gradient across the plasma membrane, which further slows the uptake of α-glucosides and further decreases the EC, with a catastrophic positive feedback effect. The rapidity of this effect (Figures 3.2 and 3.3) emphasises the importance of cropping yeast for recycling to subsequent fermentations (the usual brewery practice) as soon as possible, while the beer still contains adequate concentrations of fermentable sugars. For strain A15, this collapse in EC occurred at a higher concentration of residual sugars (i.e., at a lower apparent attenuation) in the 25 ºP wort than in the 15 ºP wort (Figure 3.4) and for strain Ind2 the collapse occurred at a higher concentration of residual sugars for the industrially cropped yeast than for the laboratory-

grown yeast (Figure 3.5). In modern cylindroconical brewery fermentors, sedimented yeast, which will be cropped and used again, collects in the cone, where the yeast concentration becomes extremely high, so that decrease of maltose and maltotriose below the concentrations that support adequate fermentation rates also occurs rapidly. Continued cell viability after this point will depend upon mobilisation of the cell's own carbohydrate reserves, glycogen and trehalose. The results presented suggest that lager yeast strains might tolerate still higher ethanol concentrations (>85 $g \cdot L^{-1}$), provided that adequate amounts of readily fermentable sugars are still available (or are added) in late fermentation, because the collapse in EC and subsequent cell death appear to result not from high ethanol concentrations but from the slowness with which the yeast can ferment the residual maltotriose.

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CHAPTER 4

ADAPTIVE EVOLUTION OF A LACTOSE-CONSUMING *SACCHAROMYCES CEREVISIAE* RECOMBINANT

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4.1 INTRODUCTION

At their most basic level, the “rules” of evolution are remarkably simple: species evolve by means of random variation (via mutation, recombination, or other operators); this is followed by natural selection, in which the fittest tend to survive and reproduce, propagating their genetic material to future generations (Sauer, 2001). Hence, natural selection drives organisms towards adaptation to changing environmental conditions.

Microbial populations and single-celled microorganisms respond in a flexible manner to environmental changes. The laboratory or the fermentation tank can be regarded as an artificial environment that is in permanent change, similar to natural environments (Zelder and Hauer, 2000). Adaptation can be a genetic process: in a minority of the population changes occur that select for a few cells better adapted to the changed environment. The genetic basis of adaptation ranges from simple alterations of DNA sequences, such as point mutations, to major events in the genome structure. In a laboratory or in an industrial environment this adaptation can result in strain instability or can be used as a method to improve biotechnological processes (Zelder and Hauer, 2000).

During the past few years, efforts have been made in order to elucidate molecular mechanisms, namely genome rearrangements, involved in yeast adaptation to industrial processes (Fidalgo *et al.*, 2006; Infante *et al.*, 2003; Pérez-Ortín *et al.*, 2002; Querol *et al.*, 2003). The physiological and genetic consequences of adaptive evolution of yeasts selected after many generations of growth in glucose-limited chemostats have also been intensively studied (Adams *et al.*, 1985; Brown *et al.*, 1998; Dunham *et al.*, 2002; Ferea *et al.*, 1999). Recently, the same has been done in maltose-limited chemostat cultures (Jansen *et al.*, 2004).

Classical (non-recombinant) strain improvement has relied on random mutagenesis/screening procedures which attained many successes (Parekh *et al.*, 2000). Metabolic engineering introduced the concept of rational design into strain development strategies (reviewed by Nielsen, 2001; Ostergaard *et al.*, 2000). Inspired by natural evolution, evolutionary engineering refers to the exploitation of evolutionary principles to enhance microbial properties in a biotechnological context, provided the desired phenotype is amenable to direct or indirect selection (Sauer, 2001). Evolutionary engineering of whole cells is expected to gain relevance both as a complementary strategy in metabolic engineering for strain development and as a tool to elucidate the molecular basis of desired phenotypes (Sauer, 2001). A strain with specific properties obtained by rational metabolic engineering can be subjected to evolutionary engineering for further improvement. Likewise, evolutionary engineering can generate a strain with desirable properties that can then be further optimised by rational

metabolic design, thus integrating and complementing classical metabolic engineering strategies (Petri and Schmidt-Dannert, 2004).

Many examples of evolutionary engineering approaches have been extensively reviewed by Sauer (2001). A number of recent studies on engineering *Saccharomyces cerevisiae* for xylose metabolism (reviewed by Jeffries, 2006) highlight the importance of evolutionary engineering strategies to complement rational metabolic engineering efforts. Recombinant strains with the genetic potential to utilize the new substrate (xylose) were subjected to long-term evolution experiments (chemostats and/or serial batch cultivations), which resulted in improved strains, in particular able to ferment this pentose into ethanol (Jin *et al.*, 2005; Kuyper *et al.*, 2004; Kuyper *et al.*, 2005; Sonderegger and Sauer, 2003; Sonderegger *et al.*, 2004). Similarly, combination of metabolic and evolutionary engineering allowed the development of *S. cerevisiae* strains able to ferment arabinose, which is another widespread pentose for ethanol production. *S. cerevisiae* strains expressing bacterial genes encoding the L-arabinose pathway were not immediately able to grow on L-arabinose. However, sequential transfer of these strains in L-arabinose media enabled the selection of evolved cells that could convert this pentose into ethanol under oxygen-limited (Becker and Boles, 2003) or anaerobic (Wisselink *et al.*, 2007) conditions. Improvement of an engineered *S. cerevisiae* strain for glycerol production has also been achieved by serial transfer (Overkamp *et al.*, 2002).

Application of the evolutionary engineering principles in the selection of brewer's yeast variants suitable for very high-gravity (VHG) brewing fermentations (see chapter 1, section 1.5.5) has also been described. Huuskonen and Londesborough (2005) designed a procedure to select brewer's yeast mutants with improved ethanol tolerance and high fermentative ability (rather than growth) under the conditions encountered during VHG fermentations. Lightly mutagenised (using ethyl methanesulphonate as mutagenic chemical agent) industrial lager yeast was used for a VHG (25 ºP) fermentation. After fermentation completion, the yeast was stirred anaerobically in its own beer until nearly all cells died. During this incubation, maltose was maintained at about 50 mM and ethanol gradually increased to 140 $g \cdot L^{-1}$. Mutants selected with this strategy fermented 25 ºP worts faster and more completely than the original yeast, producing the same profile of flavour compounds. Blieck *et al.* (2007) also isolated improved variants of an industrial lager yeast strain by subjecting UV-mutagenised yeast to consecutive rounds of fermentation in very high-gravity wort (> 22 ºP). An important advantage of these improved strains is that they are not genetically modified by recombinant DNA technology (i.e., they are not genetically modified organisms, GMOs) and can therefore be introduced immediately on the market without regulatory restrictions (Blieck *et al.*, 2007).

Lactose is another sugar that cannot be metabolized by wild strains of *S. cerevisiae*.

However, the construction of *S. cerevisiae* strains with the ability to consume lactose has biotechnological interest, particularly for the valorization of cheese whey, a high pollutant by-product of dairy industries that contains about 5% (w/v) lactose (see chapter 1, section 1.6).

Kluyveromyces lactis is a natural lactose-consuming yeast. The *GAL/LAC* regulon of *K. lactis* and the *GAL/MEL* regulon of *S. cerevisiae* are closely related (see chapter 1, section 1.6.4). Despite the extensive degree of conservation in this group of genes between the two yeasts, differences have arisen as a result of their evolution in different environments: *S. cerevisiae* has adapted to glucose, whereas *K. lactis* has adapted to lactose (Rubio-Texeira, 2005). The ability of *K. lactis* to assimilate lactose depends on two genes: *LAC12*, encoding a lactose permease, and *LAC4*, encoding the enzyme β-galactosidase that catalyzes the hydrolysis of lactose into glucose and galactose. These genes are divergently transcribed from an unusually large intergenic region, which contains four functional upstream activating sites (UASs) that synergistically contribute to the activation of both genes by providing binding sites for the transcriptional activator Lac9p (also known as KlGal4p) homologous to Gal4p of *S. cerevisiae* (Gödecke *et al.*, 1991). *K. lactis GAL/LAC* and *S. cerevisiae GAL/MEL* genes are induced by galactose. Induction also occurs when lactose is the substrate, since intracellular galactose is responsible for triggering induction (for details see e.g. Rubio-Texeira, 2005).

The development of a high-productivity lactose-fermenting process using recombinant *S. cerevisiae* has been considered for ethanol production from cheese whey. In this perspective, a recombinant *S. cerevisiae* flocculent strain with the ability to express both the *LAC4* and *LAC12* genes of *K. lactis* was constructed, in previous work, using a 13 kb *K. lactis* genomic sequence that included the two genes as well as their intergenic region (Domingues *et al.*, 1999b). The original recombinant obtained metabolized lactose slowly. After a long-term adaptation experiment, in which the recombinant was cultured in liquid lactose medium, refreshed periodically, it was able to consume lactose faster with higher ethanol yield. This chapter describes comparative physiological and genetic studies of the original recombinant and the evolved strain, which aimed to identify mechanisms involved in the evolutionary adaptation of the recombinant to lactose.

4.2 MATERIALS AND METHODS

4.2.1 Strains and cultivations

The bacterial strain used for DNA preparation was *Escherichia coli* DH5α grown in Luria-Bertani (LB) medium (1% casein, 0.5% yeast extract, 0.5% NaCl). 100 mg ampicillin·L^{-1} was used for selection.

The original recombinant *S. cerevisiae* flocculent strain was named NCYC869-A3/T1 (hereafter referred as T1) and its construction has been described elsewhere in detail (Domingues *et al.*, 1999b; see also section 1.6.5) Briefly, the plasmid KR1B-LAC4-1 (Sreekrishna and Dickson, 1985) harboring the *LAC4* (β-galactosidase) and *LAC12* (lactose permease) genes of *K. lactis*, was co-transformed with a linear fragment from the plasmid YAC4 containing the *URA3* gene into the ura^- *S. cerevisiae* strain NCYC869-A3 (see below). Selection was done for ura^- complementation in YNB-plates. Xgal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was included in these plates, allowing the identification of 4 (out of 1212) transformants with β-galactosidase activity (blue colonies). Only 2 of these kept a stable Lac^+ phenotype: T1 and a second transformant which exhibited bizarre forms and pseudo-mycelium and was therefore rejected.

The other recombinant strain characterized here evolved from T1, as the result of the adaptation experiment outlined below, and is referred as T1-E.

The auxotrophic strain NCYC869-A3 (*MATα FLO1 ura3*) is a uracil-deficient mutant of the flocculent wild-type haploid strain *S. cerevisiae* NCYC869 (*MATα FLO1*) (Domingues *et al.*, 1999b). *K. lactis* CBS2359 was used for comparative studies. *Kluyveromyces marxianus* strains CBS6556 and NRRLY2415 were used as controls in some experiments.

Yeast cultivations were performed in defined mineral medium (Verduyn *et al.*, 1992) containing (g·L^{-1}) $(NH_4)_2SO_4$, 5.0; KH_2PO_4, 3.0; $MgSO_4.7H_2O$, 0.5. The concentrations of trace elements and vitamins were doubled. Final trace elements concentrations (mg·L^{-1}) were: EDTA, 30.0; $ZnSO_4{\cdot}7H_2O$, 9.0; $CoCl_2{\cdot}6H_2O$, 0.6; $MnCl_2{\cdot}2H_2O$, 1.68; $CuSO_4{\cdot}5H_2O$, 0.6; $CaCl_2{\cdot}2H_2O$, 9.0; $FeSO_4{\cdot}7H_2O$, 6.0; $Na_2MoO_4{\cdot}2H_2O$, 0.8; H_3BO_3, 2.0; KI, 0.2. The final vitamin concentrations (mg·L^{-1}) were: biotin, 0.1; calcium pantothenate, 2.0; nicotinic acid 2.0; inositol, 50.0; thiamine HCl, 2.0; pyridoxine HCl, 2.0; para-aminobenzoic acid, 0.4. The carbon source (lactose, glucose or galactose) was autoclaved separately and added after heat sterilization of the medium to a concentration of 20 g·L^{-1} (unless otherwise stated). To avoid major drops in pH during cultivation the medium was supplemented with 100 mM potassium hydrogen phthalate. Initial pH was adjusted to 4.5 with NaOH. The final pH of the cultures was in all

experiments higher than 3.7.

The cultivations were carried out in Erlenmeyer flasks filled with medium to 40% of the total volume and shaken (150 rpm) at 30 ºC. Preinocula were grown in the same conditions and used to inoculate the main cultures to an initial OD_{600} of 0.05-0.15.

During the adaptation experiment, yeast was cultivated in semi-synthetic (SS) lactose medium, which contained ($g \cdot L^{-1}$) yeast extract, 1.0; KH_2PO_4, 5.0; $(NH_4)_2SO_4$, 2.0; $MgSO_4.7H_2O$, 0.4; lactose, 20 or 50. Fermentation conditions in the 2-L bioreactor used during the last stages of the adaptation experiment were: temperature, 30 ºC; agitation speed, 150 rpm; pH control set to 4.0.

4.2.2 Adaptation experiment

The original recombinant (T1) was taken from a -80 ºC stock and spread onto SS lactose plates. Biomass washed from these plates was used to inoculate test tubes filled with 5 mL of liquid SS lactose medium (20 $g \cdot L^{-1}$ lactose). The tubes were incubated overnight at 30 ºC with gentle agitation (40 rpm) and then transferred to a 250-mL Erlenmeyer flask filled with 50 mL of SS lactose medium (20 $g \cdot L^{-1}$ lactose). This flask was incubated at 30 ºC, 40 rpm for 3 days. From this flask, 5 mL were transferred to another cultivation (same conditions), which lasted for 7 days. Samples taken from this cultivation were spread onto SS lactose plates. Biomass was again washed from these plates to inoculate test tubes filled with 5 mL of liquid SS lactose medium (50 $g \cdot L^{-1}$ lactose), which were incubated overnight (30 ºC, 40 rpm), and used to inoculate a 50 mL SS lactose (50 $g \cdot L^{-1}$ lactose) cultivation. This cultivation was incubated under the same conditions (30 ºC, 40 rpm). Taking advantage of the flocculent properties of the cells, the medium was refreshed periodically: the cultivation broth was decanted and fresh medium was added to the flocculated cells, which had sedimented to the bottom of the flask. The medium was refreshed 12 times (every 2 – 5 days), for a total of 41 days. Lactose concentration in the fresh medium added was either 20 or 50 $g \cdot L^{-1}$. After that period, 5 mL of the culture were transferred to a subsequent cultivation (grown under the same conditions), in which the medium was refreshed 2 times. The 50 mL of this cultivation were used to inoculate a 2-L bioreactor, containing 1.5 L of SS lactose medium. In this fermentation, the 20 $g \cdot L^{-1}$ lactose were consumed by the yeast in about 24 h. Samples from this fermentation were spread onto SS lactose plates. Biomass washed from these plates was again sequentially grown in 5 and 150 mL of SS lactose medium (incubation at 30 ºC, 150 rpm), and used for a second fermentation trial in the bioreactor. This time, the yeast consumed the 20 $g \cdot L^{-1}$ lactose in less than 16 h. A third fermentation trial in the bioreactor was done, following the same procedure. In this third fermentation, the yeast consumed 20 $g \cdot L^{-1}$ lactose in about 13 h. Yeast

samples were spread onto SS lactose plates. Glycerol stocks from this yeast were stored at -80 ºC. This strain was named T1-E (evolved T1).

The adaptation experiment involved growth of the recombinant for >120 generations (considering an average of 5 doublings per batch cultivation in lactose).

4.2.3 Determination of biomass and extracellular metabolites

Biomass concentration was measured by dry weight and/or absorbance methods. Biomass dry weight was determined by filtering 10 – 20 mL of yeast culture through a preweighed 0.45 μm filter, and washing with 20 mL of water. The filter was dried overnight at 104 ºC, cooled in a desiccator, and weighed. Growth was also monitored by measuring the optical density of the cell suspension at 600 nm (OD_{600}). For the flocculent strains, the biomass was deflocculated by washing twice with a 15 $g \cdot L^{-1}$ NaCl pH 3.0 solution, before reading the OD_{600}.

Lactose, glucose, galactose, ethanol and glycerol were analysed by HPLC, using a Chrompack Organic Acids column. The column was eluted at 60 ºC with 0.005 M H_2SO_4 at a flow rate of 0.6 $mL \cdot min^{-1}$. A refractive-index detector was used.

4.2.4 Flocculation assay

Yeast cells were harvested by centrifugation and the pellets were washed with 15 $g \cdot L^{-1}$ NaCl pH 3.0 solution to deflocculate. The suspension was centrifuged again and the cells were resuspended in NaCl solution to an OD_{600} of about 4. 24 mL of this yeast suspension were transferred to a 25-mL graduated cylinder, 1 mL of 100 mM $CaCl_2$ pH 3.0 solution was added and the suspension was immediately mixed by inversion 18 times. Samples of 200 μL were taken at the 20 mL level at different times. These samples were diluted with 800 μL of NaCl solution and the OD_{600} was read (Soares *et al.*, 1992). The sedimentation profiles were obtained by plotting the normalised cell concentration, defined as the ratio between actual and initial cell concentration, against the sedimentation time. The profiles presented correspond to the average of two independent assays. For each yeast suspension, a control assay was done without the addition of $CaCl_2$. In these control assays, the cell concentration in the suspension did not change significantly (±4%) over 10 min (data not shown).

4.2.5 Plasmid retention

To determine the fraction of plasmid-bearing cells, samples from the yeast culture were washed twice with a 15 $g \cdot L^{-1}$ NaCl pH 3.0 solution to deflocculate cells, appropriate dilutions

were prepared with the same solution, and aliquots were spread onto YPGal plates (1% yeast extract, 2% peptone, 2% galactose, 2% agar) supplemented with 40 $mg \cdot L^{-1}$ Xgal. Cells expressing β-galactosidase, i.e. those that still carry the plasmid, form blue colonies in Xgal plates. The fraction of plasmid-bearing cells was determined as the ratio between the number of blue colonies and the total number of colonies (blue + white).

4.2.6 β-galactosidase specific activity

Yeast cells were grown to an OD_{600} of 0.5 to 2.5. A volume correspondent to 15 to 20 OD_{600} x mL of culture was rapidly harvested by centrifugation and the cell pellet was resuspended in 3 packed cell volumes of ice-cold extraction buffer: 100 mM sodium phosphate buffer pH 7.0, 10 mM KCl, 1 mM $MgSO_4 \cdot 7H2O$, 0.28% (v/v) 2-mercaptoethanol, 1 mM EDTA, 0.3 M $(NH_4)_2SO4$, 5% glycerol, 1 mM dithiothreitol (DTT), 1 mM phenylmethanesulfonyl fluoride (PMSF), 0.5x complete EDTA-free protease inhibitor cocktail (Roche). Cells were broken by vortexing (6 cycles of 30 seconds with 1 min intervals on ice) with 4 packed cell volumes of glass beads, and then diluted with 3 volumes of ice-cold buffer Z (100 mM sodium phosphate buffer pH 7.0, 10 mM KCl, 1 mM $MgSO_4 \cdot 7H2O$, 0.28% (v/v) 2-mercaptoethanol). Cell debris was removed by centrifuging for 15 min at 13000 rpm (2 ºC) and the supernatant (extract) was used for enzyme assay and protein estimation.

The β-galactosidase activity was assayed using p-nitrophenyl-β-D-galactopyranoside (pNPG) as substrate (Miller, 1972). Briefly, 200 μL samples of appropriate dilutions of extract in buffer Z (at least 10 times dilution) were transferred to microplate wells, 50 μL of 4 $mg \cdot mL^{-1}$ pNPG were added, the plate was incubated at 30 ºC in a microplate reader (BIO-TEK Synergy HT) and the optical density at 405 nm was read over time. The supernatants from the first centrifugation were assayed for extracellular β-galactosidase activity: 50 μL of supernatant were mixed with 150 μL of buffer Z and assayed as above described for the diluted extracts. Protein concentrations in the extracts were measured according to (Bradford, 1976), using the Bio-Rad protein reagent and ovalbumin as the standard. At least 3 different dilutions of each extract were assayed for both enzyme activity and protein concentration; the coefficient of variation of each specific activity measurement was <30%. One unit of enzyme activity (U) catalyses the conversion of 1 nmol of substrate per min under the stated conditions. Specific activities were expressed as U per mg of protein.

4.2.7 General DNA methods

Standard molecular biology techniques were used basically according to the procedures

described in Sambrook and Russell (2001). Yeast was transformed by the lithium acetate method (Gietz *et al.*, 1992). *E. coli* was transformed by electroporation according to protocols from Bio-Rad. Commercial kits (Qiagen, Sigma) were used for plasmid DNA isolation from *E. coli*. Enzymes were purchased from different manufacturers and used according to the recommendations.

4.2.8 PCR amplifications and sequencing

Ribosomal DNA regions ITS1 and ITS1-5.8S rDNA-ITS2 were amplified by PCR using universal primers for fungi (White *et al.*, 1990). The primers (FLO11_Fprobe and FLO11_Rprobe) used for PCR screening for the *FLO11* gene of *S. cerevisiae* were previously described (van Dyk *et al.*, 2005).

PCR screening for *LAC4* gene was done with primers LAC4_1 (5'-AGGATTTACAGTGGGAGGAT-3') and LAC4_2 (5'-ATGTCTTGCCTTATTCCTGA-3'). For *LAC12*, primers LAC12_1 (5'-GGAAGCCTTGAACAGTGATA-3') and LAC12_2 (5'-AGACCTGCAACCTTACCTCT-3') were used.

The intergenic region between genes *LAC4* and *LAC12* (LACIR) was amplified using the primers LACIR1 and LACIR2 (Table 4.1). Primer LACIR1 is complementary to nucleotides 42 to 61 of the coding sequence of *LAC4* (Poch *et al.*, 1992). Primer LACIR2 is complementary to nucleotides 58 to 77 of the coding sequence of *LAC12* (Chang and Dickson, 1988).

The *LAC12*-LACIR-*LAC4* region of the plasmid isolated from strain T1-E was sequenced. The primers used for the sequencing reactions are listed in Table 4.1. The LACIR of the plasmid isolated from strain T1 was also partially sequenced (using primers LACIR2, LACIR3 and LACIR4). The sequence of *LAC12*-LACIR-*LAC4* in the *K. lactis* genome (GenBank CR382122, bases 1305927 to 1313578) (Dujon *et al.*, 2004) was used as reference. BLAST programs from NCBI were used for the alignments.

Table 4.1 – Primers used to sequence genes *LAC12* and *LAC4* and their intergenic region (LACIR).

Oligonucleotide	Sequence (5' → 3')
LAC12_1	AGG AGA AGA TCA AGC AGA AT
LAC12_2	TTA TTG ATA AGA TTG GTA GAA GG
LAC12_3	TTG GAA GCA CTC TTC TTC TT
LAC12_4	GTA GAT GGT TCG TGG CCT TT
LAC12_5	GAC AGA CCA AAG GGT GTT A
LAC12_6	TCC TTC AGT CAG TGT TAA TGA
LAC4_1	TAG GAA AGA GCA GAA TTT GG
LAC4_2	AAA ACC TTG ACG ACC ACT AA
LAC4_3	GGA CAA TTG TTA CGA GCT TT
LAC4_4	ACA GCT CTA CCG AAC CTT G
LAC4_5	TGA TTC AAT CTA TTA AGC ACC A
LAC4_6	CCA CCT TTC CAT AAT TTC AA
LAC4_7	CAC AAA GCC ATG TAC AAG TT
LAC4_8	TCT GGC TTT AAA GAT GGA AC
LAC4_9	CCC GTT CAT ATT AAA ATT GC
LAC4_10	ATC AGG AGG CTG ATA TTC G
LAC4_11	TGT AGT CAC GGT TAA CTC TCG
LAC4_12	CCG TTT AAA TAT TAT CAT GC
LACIR1	GCA ATC TAT TTT CGT GAA CC
LACIR2	CCC AAA GTG TCT TTA TGC TC
LACIR3	TAG TAC GGA GGG AAG AAT CC
LACIR4	AAT CAT ACC CTT CAC ACT CG

4.2.9 RNA extraction

Yeast cells for total RNA isolation were grown to an OD_{600} of 0.5 to 0.7. A volume correspondent to 15 OD_{600} x mL of culture was rapidly centrifuged (3000 rpm at 4 ºC for 3 min) and the cell pellets were immediately frozen in liquid nitrogen and stored at -80 ºC. The cells were mechanically disrupted using a ball mill (Mikro-Dismembrator S, B. Braun Biotech International). Total RNA was extracted using RNeasy Mini kit (Qiagen). The extracted RNA was quantified using the Bioanalyzer 2100 with the RNA 6000 Nano LabChip kit (Agilent), and the NanoDrop ND-1000 UV/Vis spectrophotometer (NanoDrop Technologies). The absence of contaminant DNA in RNA preparations was verified using RNA as template in real time PCR assays (see below).

4.2.10 Quantitative real time RT-PCR

One microgram of total RNA was transcribed into cDNA in a 20 µL reaction using the iScript cDNA synthesis kit (BioRad). The mRNA levels were analysed by quantitative reverse transcription-PCR (RT-PCR) using the BioRad MyIQ Real-time PCR system. Each sample was tested in duplicate in a 96-well plate (BioRad, CA, USA). The reaction mix (25 µL final) consisted of 12.5 µL of SYBR Green 2X Supermix (BioRad), 2.5 µL of each primer (250 nM final), 2.5 µL H_2O and 5 µL of 1/10 dilution of cDNA preparation. The thermocycling program consisted of one hold at 95 °C for 4 min, followed by 40 cycles of 10 s/95 °C, 45 s/56 °C. Melting curve data were then collected to verify PCR specificity and absence of primer dimers. Oligonucleotides for quantitative PCR (Table 4.2) were designed using Beacon Designer 2.0 software (PREMIER Biosoft International). The PCR efficiency of each primer pair was evaluated by the dilution series method, using sample cDNA as template.

Relative expression levels were determined using the ΔCt (threshold cycle) method, which takes into account differences in primer pair amplification efficiencies and yields more accurate data than the 2ΔΔCt method. For standardization, the results were expressed as target/reference ratio, the reference gene being genome-encoded *ACT1* or plasmid-born *bla* gene.

Table 4.2 – Oligonucleotides used for quantitative real time RT-PCR.

Oligonucleotide	Sequence	Amplicon size	Efficiency
ACT1_QPCR_F1bis	ATTATATGTTTAGAGGTTGCTGCTTTGG	285	1.98
ACT1_QPCR_R1bis	CAATTCGTTGTAGAAGGTATGATGCC		
Bla_QPCR_F13	CATTTCCGTGTCGCCCTTATTCCC	147	2.11
Bla_QPCR_R159	CTTACCGCTGTTGAGATCCAGTTCG		
LAC12_QPCR_F742	GGTCTTGTGTGTATATTTGGTTGGTTAATCCC	246	1.97
LAC12_QPCR_R987	TGCTCTGTACCTATCCGATCTCGTTCTG		
LAC4_QPCR_F1733	GTGGCTTTATCTGGGAATGGGCAAATC	278	2.03
LAC4_QPCR_R2010	CAATAAGTGGTCTGTCGTAATGAAGTCGTG		

4.2.11 Microarrays

The DNA chips were manufactured at the Biochip platform (Genopole Toulouse, France) on dendrislides (Le Berre *et al.*, 2003; Trévisiol *et al.*, 2003) using 70-mer oligonucleotides, representing 99% of the yeast genome, purchased from Operon. The list of corresponding genes can be found at http://biopuce.insa-toulouse.fr/oligosets/. Synthesis of fluorescently labeled cDNA was carried out with 5 ng of total RNA using the ChipShot™ direct labeling system from Promega then purified with the ChipShot™ Labeling and Cleanup System. This labeling method allows direct incorporation of dCTP-Cy3 or dCTP-Cy5 (Perkin-Elmer) nucleotides during the reverse transcription step. The quality of the labeled cDNA was verified by the NanoDrop® ND-1000 UV-Vis Spectrophotometer. Hybridization was carried out in an automatic hybridation chamber (Discovery™, Ventana). Microarrays were prehybridized in a solution of 1% BSA, 2x SCC, 0.2% SDS for 30 min at 42 ºC, followed by prehybridization for 30 min with the Chip Prep 1 reagent (Ventana) then 30 min with the Chip Prep 2 reagent (Ventana) at 42°C. Hybridization was done by addition of a mixture containing 180 µL of ChipHybe™ (Ventana), 10 µL of Cy3-labelled cDNA and 10 µL of Cy5-labelled cDNA. After 8 hours of hybridization at 42 ºC, the DNA chips were washed with the ChipClean (Ventana) solution. All of those steps were done automatically in the hybridization chamber. Subsequently the slides were washed manually for 5 min in a 2x SSC, 0,1% SDS solution, then for 2 min in 0.1x SSC buffer at room temperature. Finally the slides were dried with a microarray high-speed centrifuge (ArrayIt). The hybridization signal was detected by scanning the microarrays with a GenePix 4000A laser scanner (Axon Instruments) and quantified using the accompanying GenePix Pro 6.0 software. To reduce the bias due to unequal incorporation or differences in quantum efficiency of the two dyes, RNA samples from a second independent experiment were labeled by opposite dye to the first experiment (method called dye switch), and this procedure was repeated 4 times, leading to 4 independent intensity values for each gene (spots) on the microarray. This experimental design provides the opportunity to minimize the intrinsic biological noise between identical culture conditions and the technical variations inherent to the DNA microarray technology.

4.2.12 Microarray data analysis

Data analysis was performed using the BioPlot web service from the Biochip platform (Genopole Toulouse, France). This software enables to compare transcriptome data from two biological conditions and select significantly changed genes. Strain T1 was selected as the control biological condition and strain T1-E was selected as the test condition. Thus, the expression ratios were calculated as T1-E/T1.

The averages of the log-transformed ratios for the four replicate microarray slides were used for the statistical analysis. Locally weighed linear regression (lowess) analysis was performed for data normalization. Genes with significantly changed expression level were identified by combining ratio thresholds and Student's test, in order to reduce the probability of false positives and to avoid the statistical multiple test problem. Over-expression and under-expression thresholds were set to 1.5 and 0.66, respectively. Genes with p-value lower than 0.05 were considered to be significantly differentially expressed. False discovery rate with this p-value cut-off was 0.082, which gave an estimation of 14 false positives (among 173 transcripts with changed expression).

Gene descriptions and annotations were found in the *Saccharomyces* Genome Database, SGD (http://www.yeastgenome.org). Analysis tools from SGD were used.

4.3 RESULTS

4.3.1 Adaptation

The original recombinant strain (T1) was able to metabolise lactose, but rather slowly. Moreover, the flocculation performance of the recombinant was poor when compared to the host strain *S. cerevisiae* NCYC869-A3 (Domingues *et al.*, 1999b). Hence, an adaptation of this strain to growth on lactose was attempted. The adaptation experiment consisted in a serial transfer/dilution strategy in gently shaken (40 rpm) flasks (see details in Materials and Methods). This strategy was designed to keep the recombinant growing in lactose for many generations (>120), as well as to select for flocculent cells. Therefore, in some stages of the process, the medium was simply refreshed periodically in the same cultivation flask: the cultivation broth was decanted and fresh medium was added to the flocculated cells, which had sedimented to the bottom of the flask. The yeast cells recovered at the end of the process presented improved lactose fermentation performance compared to T1. These evolved cells are now considered as an independent strain, which was named T1-E.

The evolved strain culture was tested for possible contamination, particularly with *Kluyveromyces* yeasts. ITS (Internal Transcribed Spacer) regions located on the ribosomal RNA gene clusters are highly variable, allowing for species distinction even within the *Saccharomyces* genus (McCullough *et al.*, 1998). Restriction fragment length polymorphism (RFLP) analysis (with the enzymes *Eco*RI, *Hae*III, *Hind*III, *Hinf*I, *Mse*I, *Pst*I, *Rsa*I and *Taq*I) of the PCR-amplified ITS1−5.8S rDNA−ITS2 region generated identical profiles for the recombinants, T1 and T1-E, and for the host strain *S. cerevisiae* NCYC869. Results obtained with *Hae*III, *Taq*I and *Mse*I are shown in Figure 4.1. Moreover, PCR amplification of the ITS1 region resulted in a smaller product for *Kluyveromyces marxianus* than for *S. cerevisiae* (Figure 4.2). In an independent test, PCR screen for the *S. cerevisiae FLO11* gene resulted positive for strains T1, T1-E and *S. cerevisiae* NCYC869, but negative for *K. lactis* CBS2359 and *K. marxianus* CBS6556 (data not shown). These results confirmed that strains T1 and T1-E are *S. cerevisiae*, excluding the possibility of contamination during the adaptation experiment.

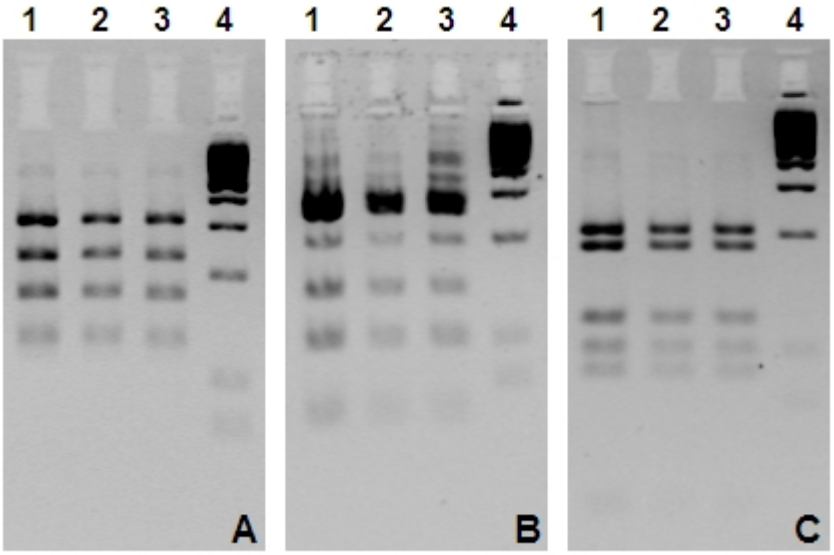

Figure 4.1 – RFLP profiles of PCR-amplified ITS1–5.8S rDNA–ITS2 region of strains *S. cerevisiae* NCYC869 (lane 1), T1 (lane 2) and T1-E (lane 3). (A) Digestions with *Hae*III. (B) Digestions with *Taq*I. (C) Digestions with *Mse*I. Lane 4 is band size marker GeneRuler 100 bp (Fermentas).

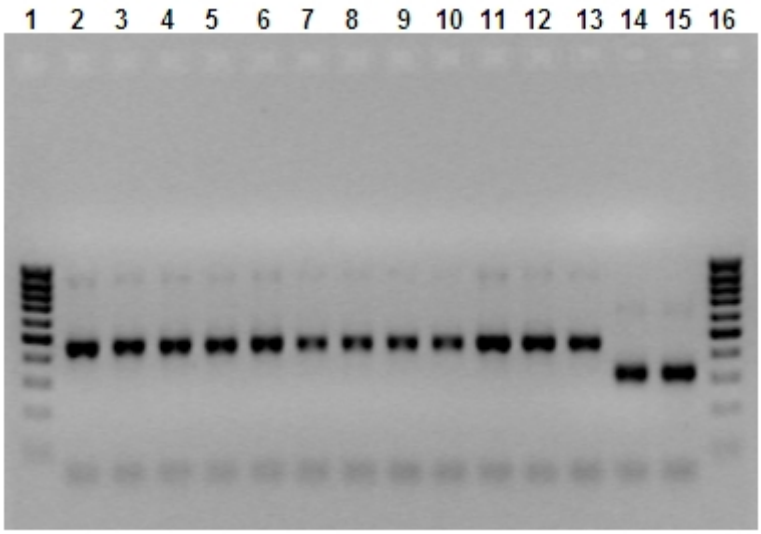

Figure 4.2 – PCR-amplification of ITS1 region of strains *S. cerevisiae* NCYC869 (lane 2), *Kluyveromyces marxianus* CBS6556 (lane 14) and *K. marxianus* NRRLY2415 (lane 15). Lanes 1 and 16 are band size marker GeneRuler 100 bp (Fermentas). PCR products obtained with strains T1 and T1-E (not in this gel) are identical to *S. cerevisiae* NCYC869. Lanes 3 – 13 correspond to PCR products obtained with brewer's yeast industrial strains.

4.3.2 Physiological characterization of the recombinants

Three single colony isolates of the evolved strain showed identical fermentation profiles (Table 4.3, Figure 4.3), indicating that T1-E is a homogenous population. The lactose fermentation performance of the evolved strain was considerably improved compared to the original recombinant. T1-E fermented lactose twice as fast as the original recombinant T1, presenting higher growth rate as well as 30% higher ethanol production, which indicated that the flux of carbon (lactose) through the fermentative pathway was higher in T1-E (Table 4.3, Figure 4.3). The differences in lactose fermentation between the evolved recombinant and *K. lactis* wild-type strain were small, though *K. lactis* grew faster and produced less ethanol (Table 4.3, Figure 4.3). Conversely, the two recombinant strains behaved similarly both in glucose and in galactose (the two products of lactose hydrolysis) cultivations (Table 4.3, Figure 4.4).

Table 4.3 – Comparison of fermentation parameters of strains T1 and T1-E in lactose, glucose and galactose cultivations, as well as *K. lactis* CBS2359 in lactose.[1]

	Lactose			Glucose		Galactose	
	T1	T1-E	*K. lactis*	T1	T1-E	T1	T1-E
μ (h^{-1})	0.14 ± 0.01	0.21 ± 0.01	0.28	0.32 ± 0.03	0.34 ± 0.03	0.15 ± 0.01	0.15 ± 0.01
X_{final} ($g \cdot L^{-1}$)	3.48 ± 0.09	2.81 ± 0.09	2.56	2.14 ± 0.03	2.13 ± 0.03	2.94 ± 0.13	2.94 ± 0.00
E_{max} ($g \cdot L^{-1}$)	7.08 ± 0.79	10.52 ± 0.04	8.86	8.19 ± 0.19	8.05 ± 0.26	7.10 ± 0.40	7.65 ± 0.64
E_{yield} (%)	53 ± 5	69 ± 1	65	81 ± 2	80 ± 2	69 ± 4	76 ± 7

[1]Cultivations were done as described in Materials and Methods. The initial lactose concentration was 25 $g \cdot L^{1}$; the initial glucose and galactose concentrations were 20 $g \cdot L^{-1}$. μ: specific growth rate; X_{final}: final biomass concentration (when sugar from medium was exhausted); E_{max}: maximum ethanol concentration; E_{yield}: ethanol conversion yield (% of the theoretical value). Data for T1 and T1-E are means ± ranges of duplicate independent cultivations, except data for T1-E in lactose, which correspond to the means ± standard deviations of triplicate cultivations with single colony isolates.

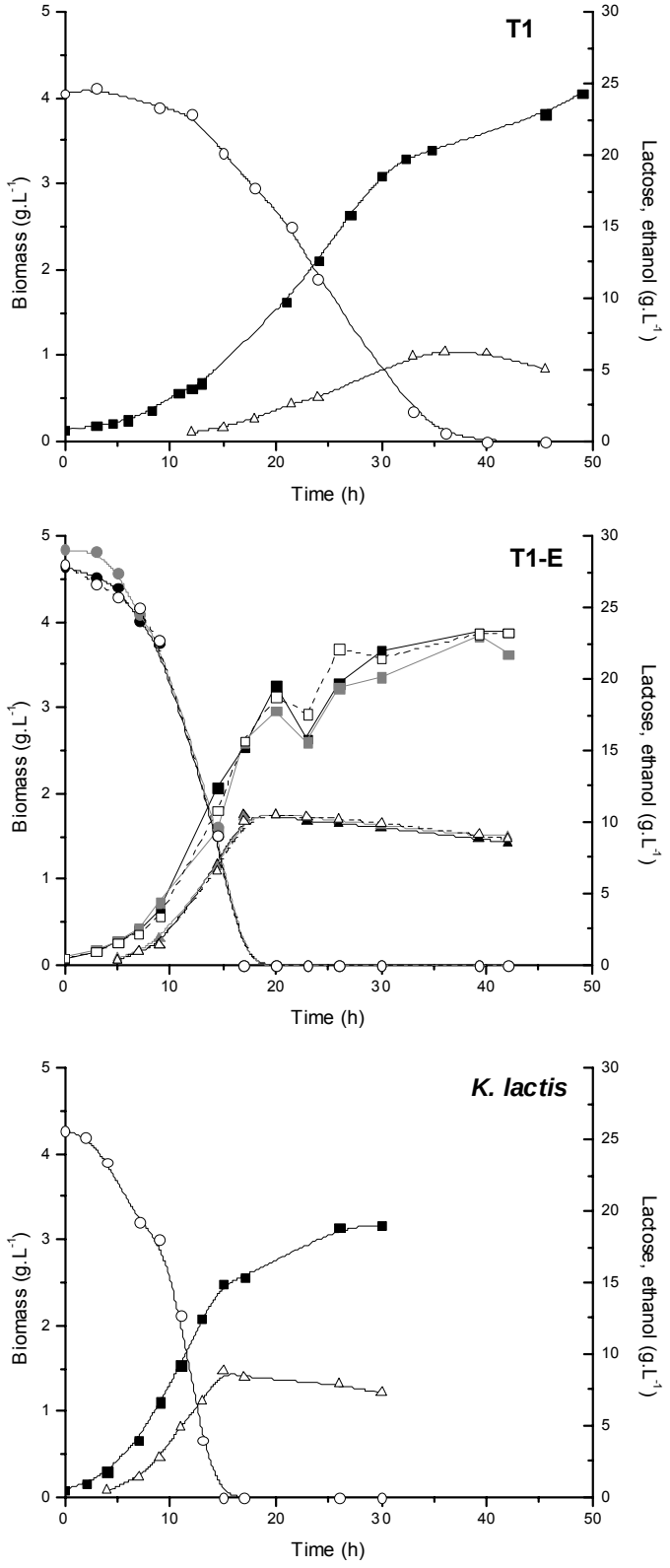

Figure 4.3 – Lactose cultivations with strains T1, T1-E and *K. lactis* CBS2359. For T1-E, triplicate cultivations with single colony isolates are shown. Lactose (●; ●; ○), ethanol (▲; ▲; Δ) and biomass (■; ■; □) concentrations were followed during shake-flask cultivations (as described in Materials and Methods).

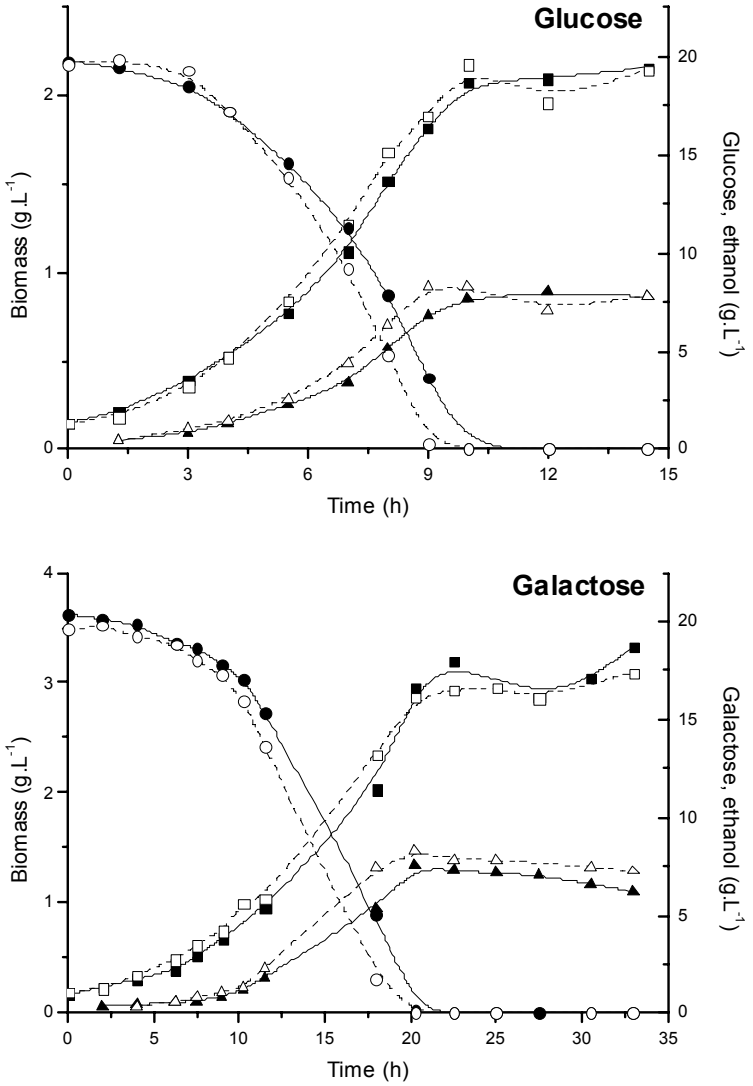

Figure 4.4 – Glucose and galactose cultivations with strains T1 and T1-E. Glucose or galactose (●, ○), ethanol (▲, ∆) and biomass (■, □) concentrations were followed during shake-flask cultivations (as described in Materials and Methods). Solid symbols/solid lines are from T1 cultures; open symbols/dotted lines are from T1-E cultures.

The adaptation experiment was also successful in the selection of cells with improved flocculation. T1-E flocculated earlier and formed much bigger flocs than T1, as could be easily observed by visual inspection of the cultivation flasks. Sedimentation profiles of lactose-grown T1 and T1-E cells are shown in Figure 4.5. When the culture reached an OD_{600} of about 2, nearly all T1-E cells showed the ability to flocculate. The flocculation performance of T1 was much weaker, although it improved in stationary phase cells compared to growing cells. Similar differences in the flocculation behaviour between the two strains were also observed in glucose and galactose cultures.

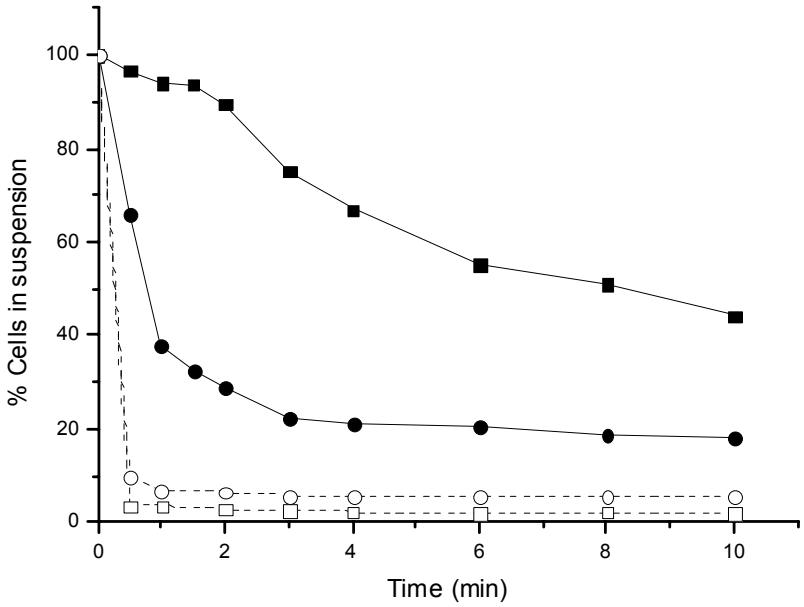

Figure 4.5 – Sedimentation profiles of T1 and T1-E cells grown in lactose. Cells were harvested during growth, at an OD_{600} of about 2 (T1-E, □; T1, ■), or at the stationary phase, after 50 h of cultivation (T1-E, ○; T1, ●).

4.3.3 Plasmid retention

Under positive selection pressure conditions, i.e., using lactose as the sole carbon source, recombinant cells need to carry the plasmid in order to retain its capacity to grow on lactose. However, in glucose and in galactose cultivations there was no selective pressure to prevent plasmid loss. Therefore, the fraction of cells expressing β-galactosidase (blue colonies in plates supplemented with Xgal) was determined, corresponding to the cells still carrying the plasmid (Table 4.4). The vast majority of the cells in T1-E cultures contained the plasmid (96 and 93% in glucose and galactose, respectively). In contrast, only 74 and 27% of T1 cells maintained the construct, in glucose and galactose cultures, respectively. For both T1 and T1-E, plasmid retention was similar in yeast samples harvested during growth phase (sampling at 7 h of growth in glucose or 6 – 11 h in galactose) and stationary phase (28 h in glucose, 30 – 50 h in galactose).

Table 4.4 – Plasmid stability (% of cells carrying the plasmid) of strains T1 and T1-E in glucose and galactose cultures. Results are means ± standard deviation of 4 independent cultivations.

Glucose		Galactose	
T1	T1-E	T1	T1-E
74 ± 6	96 ± 1	27 ± 6	93 ± 3

Thus, T1-E cells transmitted the plasmid very efficiently to their progeny, whereas plasmid transmission was less effective in T1 cultures. The instability of plasmids may be due to defects either in their replication or in their segregation into daughter cells (Maine *et al.*, 1984; Poddar *et al.*, 1999), during cell division. Additionally, in the case of T1, the carbon source apparently exerted regulation over plasmid stability, since the extent of plasmid loss was much higher in galactose than in glucose grown cells. Walsh and Bergquist (1997) also observed high plasmid stability in glucose and much lower stability in galactose, in *K. lactis* transformed with a vector carrying the bacterial *xynA* gene under the control of the *LAC4* promoter. They argued that the mitotic stability of the plasmid was influenced by the transcriptional activity of the promoter, which would be different in glucose (non-induced/repressed) and in galactose (induced).

4.3.4 Characterization of the *LAC* region in the plasmid isolated from both recombinants

To search for mutations that could explain the differences observed in the lactose fermentation phenotype of the two recombinants, the plasmid bearing the *K. lactis LAC12-LAC4* construct was isolated from the two strains and recloned in *E. coli*. Restriction analyses of these plasmids with the enzymes *Eco*RI and *Bgl*II are shown in Figure 4.6. The restriction pattern of the plasmid isolated from T1 was identical to the plasmid KR1B-LAC4-1 used for transformation. However, the plasmid isolated from T1-E showed differences. Digestions with *Xba*I, *Bam*HI and *Pst*I confirmed these observations (data not shown). Comparing the results of the restriction analyses with the restriction map of pKR1B-LAC4-1 (Sreekrishna and Dickson, 1985) it was possible to conclude that the plasmid isolated from T1-E had a deletion of 1300 – 1600 bp in the intergenic region between *LAC12* and *LAC4* genes (LACIR). PCR amplification of the LACIR confirmed the deletion in T1-E (Figure 4.7). Moreover, it was confirmed that the LACIR in T1 has the same size as in pKR1B-LAC4-1 and in the *K. lactis* genome (Figure 4.7).

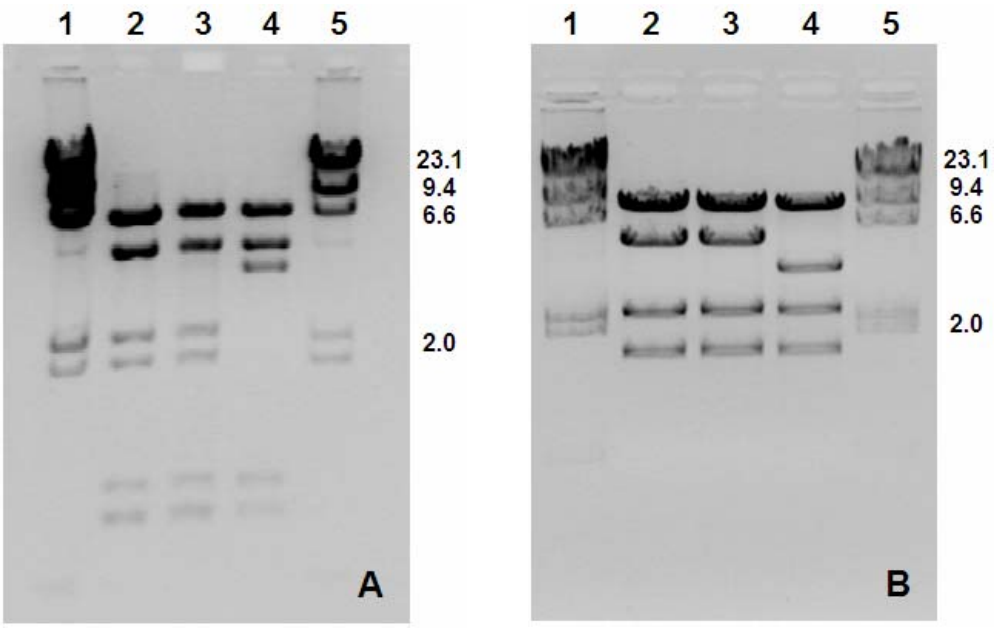

Figure 4.6 – Restriction analyses of plasmid KR1B-LAC4-1 (lane 2) and plasmids isolated from T1 (lane 3) and T1-E (lane 4). (A) Digestions with *Eco*RI. (B) Digestions with *Bgl*II. Band size marker is *Hind*III-digested λ DNA (lanes 1 and 5; size shown in kb on the right).

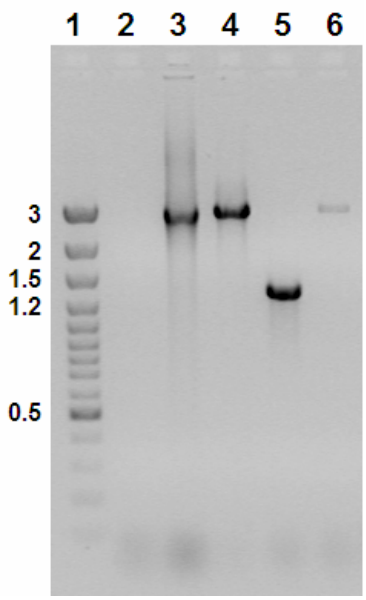

Figure 4.7 – PCR (colony PCR) amplification of the intergenic region between *LAC12* and *LAC4* genes, using primers LACIR1 and LACIR2 (Table 4.1). Lane 1: band size marker GeneRuler 100 bp Plus (Fermentas); size shown in kb on the left. Lane 2: *S. cerevisiae* NCYC869-A3 (negative control). Lane 3: *E. coli*/pKR1B-LAC4-1. Lane 4: T1. Lane 5: T1-E. Lane 6: *K. lactis* CBS2359.

The LACIR of the T1-E plasmid was therefore sequenced and compared with the same region in the published *K. lactis* genome (GenBank CR382122) (Dujon *et al.*, 2004). A deletion of 1593 bp was mapped between positions -2108 and -516 (inclusive), +1 referring to the adenosine in the *LAC4* initiation codon (Figure 4.8). Furthermore, a single nucleotide mutation was found at position -2422, which substituted the adenosine in the *K. lactis* genome sequence to a cytosine in the T1-E sequence. This substitution is interesting because it leads to a putative binding site for the transcription factor Gal4p: CGGCCCACGCAGACCCG (the A to C substitution occurred in the underlined position). The same substitution was found in the LACIR born on the plasmid isolated from T1. However, it is not known whether this putative UAS (*pU*, Figure 4.8) was functional in any of the strains.

LAC12 and *LAC4* coding sequences determined in the T1-E plasmid were 100% identical to the published sequences for these genes (Chang and Dickson, 1988; Dujon *et al.*, 2004; Poch *et al.*, 1992).

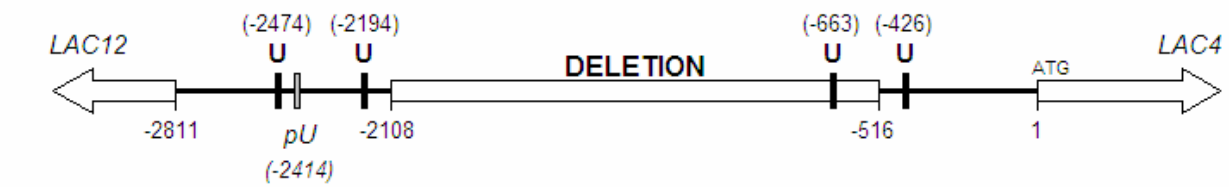

Figure 4.8 – Deletion identified in the *LAC12-LAC4* intergenic region of the plasmid isolated from the evolved strain (T1-E). The 1593 bp deletion (open box) was mapped between positions -516 and -2108, +1 referring to the adenosine in the *LAC4* initiation codon. The functional UAS elements (U) present in the LAC promoter (Gödecke *et al.*, 1991) are represented by vertical black bars and correspond to the 17 bp consensus sequence 5'-CGG(N5)A/T(N5)CCG-3' (central position of the consensus sequence is indicated between brackets). The grey vertical bar represents an additional putative UAS (*pU*) found in T1 and T1-E (see details in the text).

4.3.5 Transformation of the host strain with the plasmid isolated from T1-E did not yield Lac$^+$ transformants

Several attempts were made to transform *S. cerevisiae* strains NCYC869 (wild-type) and NCYC869-A3 (uracil-deficient mutant) with the plasmid isolated from T1-E (with the deletion). Although transformants resistant to the antibiotic G418 (used for selection) were obtained, these transformants were unable to grow on lactose. It should be stressed that the same happened in control experiments, using the original plasmid KR1B-LAC4-1. It was reasoned that genetic alterations in the T1 genome could have contributed to obtaining the Lac$^+$

phenotype, in addition to the cloning of the *K. lactis LAC* genes. Therefore, the cured strain T1 (plasmid-free colony selected after non-selective sequential growth in glucose) was used as host for transformation. With this host, transformants resistant to G418 (up to 150 $mg \cdot L^{-1}$) were again obtained. Part of the G418-resistant transformants (>10%) gave a positive PCR signal for the genes *LAC4* and *LAC12*, both in the transformations with the plasmid isolated from T1-E and with pKR1B-LAC4-1. Surprisingly, none of the transformants could grow on lactose (plates or liquid medium) or showed β-galactosidase activity (as judge by color screening in YPGal plates supplemented with Xgal).

4.3.6 *LAC4* and *LAC12* expression levels and estimation of relative plasmid copy number

It was reasoned that the deletion in the promoter region may have altered the expression of one or both genes. Thus, the levels of *LAC4* and *LAC12* transcripts in strains T1 and T1-E were compared using quantitative real-time RT-PCR. The β-galactosidase activity of the strains was also compared. Lactose transport studies with T1 and T1-E are reported in chapter 6.

The *LAC4* and *LAC12* mRNA levels were initially normalized to *ACT1* transcript levels. In lactose cultures (inducing conditions), *LAC4* mRNA level was about 2.2-fold higher in T1-E compared to T1 (Table 4.5). However, the specific β-galactosidase activity measured in T1-E was 24-fold higher than in T1, and was essentially similar to that of *K. lactis* CBS2359 (Figure 4.9). Conversely, *LAC12* transcript level was about 2.2-fold lower in T1-E compared to T1 (Table 4.5). Nevertheless, zero-*trans* lactose uptake rates were 1.1 to 1.9-fold higher in T1-E than in T1 (see chapter 6, Figure 6.2). In glucose cultures (non-induced/repressed), the levels of both *LAC4* and *LAC12* transcripts were about 10-fold lower in T1-E than in T1 (Table 4.5). This could be attributed to the decrease of plasmid copy number in the evolved strain (discussed bellow). The decrease in *LAC4* transcript level could account for the lower β-galactosidase activity observed in T1-E glucose cultures compared to T1 (Figure 4.9).

Table 4.5 – Relative expression of *bla*, *LAC4* and *LAC12* genes during growth of T1 and T1-E in lactose and in glucose (normalization with *ACT1*).[1]

	Lactose		Glucose	
	T1	T1-E	T1	T1-E
bla	100 ± 1	8 ± 0	50 ± 5	6 ± 1
LAC4	100 ± 4	225 ± 6	69 ± 2	7 ± 2
LAC12	100 ± 3	45 ± 5	66 ± 5	5 ± 2

[1]Expression levels were determined by quantitative real-time RT-PCR, as described in Materials and Methods. Expression levels of *bla*, *LAC4* and *LAC12* were normalized using *ACT1* as reference gene. T1 cells grown in lactose were used as the calibrator sample. Results shown are means ± ranges of duplicate biological cultivations. Each sample (one sample from each biological duplicate) was analysed in duplicate and the coefficient of variation (after normalization) between these technical duplicates was <30%.

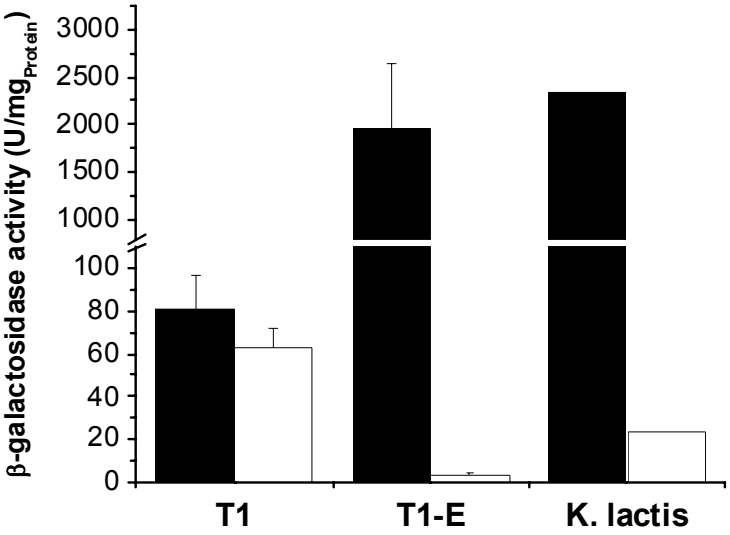

Figure 4.9 – β-galactosidase activity of strains T1, T1-E and *K. lactis* CBS2359. Yeasts were grown in mineral medium with either 2% lactose (solid bars) or 2% glucose (open bars). Cell extracts were prepared from growing cells (OD_{600} 0.5 – 2.5). Error bars (T1 and T1-E) are standard deviations of 3 (lactose) or 4 (glucose) independent cultivations. β-galactosidase activity was not detected in the supernatants from any of the cultivations.

The global differences in mRNA levels of *LAC4* and *LAC12*, obtained by normalization to the expression levels of an endogenous gene (*ACT1*, Table 4.5), could be due to differential regulation of transcription and/or variation of gene copy number, i.e. plasmid copy number, between the two strains. Therefore, to verify if the results were biased by a difference in *LAC* genes copy number, the relative plasmid copy number between the two recombinants was estimated by quantifying the transcript levels of the plasmid-born *bla* (β-lactamase) gene. *Bla*

confers resistance to ampicillin in *E. coli* and is known to be expressed in *S. cerevisiae* (Crabeel *et al.*, 1981; Roggenkamp *et al.*, 1981). Assuming that there is no differential regulation of this gene between T1 and T1-E, the relative level of *bla* expression reflects the difference in the average number of plasmid copies per cell in the culture. The expression of *bla* was higher in T1 compared to T1-E, specifically 12-fold in lactose and 8-fold in glucose cultures (Table 4.5). However, in glucose cultures, only the fraction of plasmid-bearing cells (74 and 96% for T1 and T1-E, respectively, as described above) was expressing the *bla* gene. Hence, considering only the plasmid-bearing cells, *bla* expression in glucose cultures was 11-fold higher in T1 than in T1-E, which was similar to the difference observed in lactose. These results for *bla* expression were obtained in duplicate biological independent experiments, both in lactose and in glucose, indicating that the differences observed were not due to random variation in plasmid copy number between cultivations. Furthermore, T1 showed higher resistance to the antibiotic G418 (conferred in yeast by the plasmid-encoded bacterial kanamycin resistance gene). T1 grew well on plates supplemented with 50 $mg \cdot L^{-1}$ G418, while only a few small T1-E colonies could be observed. In plates with 75 $mg \cdot L^{-1}$ G418, T1 was the only strain still able to grow. Altogether, these results indicate that plasmid copy number was lower (approximately 10-fold) in the evolved strain compared to the original recombinant.

The *LAC4* and *LAC12* mRNA levels were therefore normalized to *bla* transcript levels, which eliminates the effect of plasmid copy number variation and represent only the effect of differential regulation of the transcription of the *LAC* genes between T1 and T1-E. As illustrated by the results shown in Table 4.6, *LAC4* and *LAC12* induction factors (ratio between expression in lactose and in glucose) in T1-E were 26 and 7, respectively, whereas the induction factor in T1 was 0.7 for both genes. These results show that lactose activated the transcription of both genes in T1-E, although the induction factor was higher for *LAC4* than for *LAC12*. On the other hand, lactose did not induce the expression of the *LAC* genes in T1, which is supported by the observation that the β-galactosidase activity of T1 was similar in lactose and in glucose cultures (Figure 4.9). It is therefore proposed that the intact promoter was not able to mediate activation of the transcription of the *LAC* genes in T1 (no induction by lactose), whereas the 1593 bp deletion in the promoter region contributed to lactose activation in T1-E.

Table 4.6 – Relative expression of *LAC4* and *LAC12* genes during growth of T1 and T1-E in lactose and in glucose (normalization with *bla*).[1]

	Lactose		Glucose	
	T1	T1-E	T1	T1-E
LAC4	100 ± 3	2677 ± 211	138 ± 17	103 ± 12
LAC12	100 ± 4	531 ± 30	133 ± 21	76 ± 19

[1]Expression levels were determined by quantitative real-time RT-PCR, as described in Materials and Methods. Expression levels of *LAC4* and *LAC12* were normalized using *bla* as reference gene. T1 cells grown in lactose were used as the calibrator sample. Results shown are means ± ranges of duplicate biological cultivations. Each sample (one sample from each biological duplicate) was analyzed in duplicate and the coefficient of variation (after normalization) between these technical duplicates was <30%.

4.3.7 Transcriptome analysis

S. cerevisiae cDNA microarrays were used to compare the global gene expression profiles of T1 and T1-E cells growing under the same conditions in lactose defined mineral medium. The microarray analysis revealed 173 transcripts whose levels in T1 and T1-E differed more than 1.5 fold.

Most of the over-expressed ORFs identified (81 out of 122) were related with RNA-mediated transposition. Yeast transposons (Ty elements) encode all proteins required for their life cycle. The coding sequence of Ty elements contains two overlapping ORFs known as *TYA* and *TYB*, which are analogous to retroviral *gag* and *pol* genes, respectively. The *TYA* ORF encodes the main structural protein of virus-like particles (VLPs), while the second ORF, *TYB*, encodes the enzymatic activities that are essential for Ty retrotransposition (Roth, 2000). Retrotransposons are ubiquitous components of eukaryotic genomes. Kim *et al.* (1998) found 331 Ty elements insertions throughout the *S. cerevisiae* genome. Ty transposition is described to be induced naturally under some stress conditions, such as low temperature, UV irradiation or nitrogen starvation. Transposition may allow adaptation to an extreme environment by providing an opportunity for genetic modifications (Roth, 2000). Ty elements have been documented as agents of mutagenesis and as mediators of genome rearrangement through recombination (Adams, 2004; Kim *et al.*, 1998; Zeyl, 2004). In particular, these elements are responsible for adaptive mutations in evolving yeast populations (Adams, 2004; Zeyl, 2004). Therefore, it is not surprising to find over-expression of Ty elements genes in T1-E. This was probably a response to the stress conditions that the yeast faced during the adaptation experiment. Moreover, transposition mechanisms may even have been involved in adaptive mutations that occurred during the process.

The genes with altered expression were grouped according to GO (Gene Ontology) process

terms, using the SGD Gene Ontology Slim Mapper tool (Table 4.7). Excluding the Ty elements ORFs, 42 genes were found to be over-expressed and 51 genes were found to be under-expressed more than 1.5-fold in T1-E. From these, 8 and 19 were respectively over- and under-expressed more than 2-fold in T1-E. A fold change above 3 was found for only 2 genes: *CDC27* was over-expressed 3-fold, and *MDR1* was under-expressed 4-fold in T1-E (Table 4.8). Among the 93 transcripts with altered expression (>1.5-fold change), 23 currently have unknown biological function. The other genes are assigned to several distinct GO process terms (Table 4.7). Based on the analysis of GO trees, genes of possible interest to this study were selected within the groups in Table 4.7. These genes are listed in Table 4.8 (re-grouped according to common GO process terms).

Table 4.7 – Classification of genes with significantly changed expression level into GO process terms, according to the SGD Gene Ontology Slim Mapper Tool.[1]

GO process	Over-expressed	Under-expressed	SUM
Biological process unknown	9	14	23
Organelle organization and biogenesis	10	8	18
RNA metabolism	6	9	15
DNA metabolism	6	5	11
Transport	3	8	11
Cell cycle	3	6	9
Cytoskeleton organization and biogenesis	3	4	7
Response to stress	2	5	7
Generation of precursor metabolites and energy	4	2	6
Morphogenesis	5	1	6
Protein modification	4	2	6
Transcription	3	3	6
Protein biosynthesis	2	3	5

[1]The number of genes classified in a particular group changing 1.5-fold or more is indicated. Only groups with 5 or more genes with changed expression are shown. Terms are redundant, thus some genes are represented in more than one group.

Several genes with altered expression were found to be involved in DNA repair and recombination mechanisms, which suggests that the recombinant was exposed to DNA damaging stress during the adaptation. Genes involved in RNA metabolism were also found to be differentially expressed between the strains.

The differential expression of genes associated with (mini)chromosome stability (*ELG1*, *PLC1*, *MCM21*, and *SGO1*) is particularly relevant, because of the differences observed in plasmid copy number (see section 4.3.6) and mitotic stability (see Table 4.4) between T1 and

Table 4.8 – Genes of interest to this study with significantly changed expression (>1.5-fold) between T1 and T1-E. The ratio between expression levels in T1-E and T1 is indicated.

Gene	Ratio	p-value	Description
DNA repair / recombination / replication			
DDR48	1.61	0.0357	DNA damage responsive protein; expression is increased in reponse to heat-shock stress or treatments that produce DNA lesions
PIF1	1.57	0.0304	DNA helicase; plays a role in repair and recombination of mitochondrial DNA
ELG1	1.55	0.0218	Mutants display DNA replication defects, exhibit elevated levels of recombination and show increased levels of chromosome loss
TPP1	0.55	0.0149	DNA 3'-phosphatase that functions in repair of endogenous damage of double-stranded DNA
MND1	0.54	0.0269	Protein required for recombination and meiotic nuclear division
APN2	0.36	0.0100	Class II abasic (AP) endonuclease involved in repair of DNA damage
Response to stress			
SCH9	1.89	0.0052	Protein kinase; involved in the age-dependent response to oxidative stress during chronological cell aging
FAB1	0.65	0.0091	1-phosphatidylinositol-3-phosphate 5-kinase; vacuolar membrane kinase; generates phosphatidylinositol P2, involved in vacuolar sorting and homeostasis
PRX1	0.54	0.0202	Mitochondrial peroxiredoxin; has a role in reduction of hydroperoxides; induced during respiratory growth and under conditions of oxidative stress
MET22	0.38	9.9E-05	Bisphosphate-3'-nucleotidase, involved in salt tolerance and methionine biogenesis
Chromatin remodeling			
ITC1	1.77	0.0176	Component of the ATP-dependent Isw2p-Itc1p chromatin remodeling complex
RSC58	1.54	0.0265	58KDa Subunit of RSC (Remodel the Structure of Chromatin) Chromatin Remodeling Complex
SPT21	2.24	0.0035	Protein required for normal transcription at 2 particular histone loci (HTA2-HTB2 and HHF2-HHT2) but not at other histone loci; functionally related to Spt10p
RNA metabolism			
BRR2	2.36	0.0340	ATP-dependent RNA helicase activity; involved in nuclear mRNA splicing, via spliceosome
SMX3	1.84	0.0029	snRNP protein; involved in nuclear mRNA splicing, via spliceosome
FRS2	1.74	7.7E-04	Alpha subunit of cytoplasmic phenylalanyl-tRNA synthetase, forms a tetramer with Frs1p to form active enzyme
DED1	0.66	0.0397	RNA helicase; involved in RNA splicing and translation initiation; required for translation initiation of all yeast mRNAs
SEN34	0.65	0.0348	Subunit of the tRNA splicing endonuclease
DAL82	0.57	0.0119	Positive regulator of allophanate inducible genes; binds a dodecanucleotide sequence upstream of all genes that are induced by allophanate
CBT1	0.43	2.4E-04	Protein involved in 5' end processing of mitochondrial COB, 15S_rRNA, and RPM1 transcripts; possible role in 3' end processing of the COB pre-mRNA
Cell cycle / mitosis			
CDC27	3.04	0.0075	Anaphase-Promoting Complex/Cyclosome (APC/C) subunit, which is a ubiquitin-protein ligase required for degradation of anaphase inhibitors
GIN4	2.00	0.0370	Protein kinase involved in bud growth and assembly of the septin ring, proposed to have kinase-dependent and kinase-independent activities
CDC5	1.87	0.0302	Polo-like kinase; found at bud neck, nucleus and SPBs; has multiple functions in mitosis and cytokinesis through phosphorylation of substrates
PLC1	1.77	0.0263	Enzyme involved in kinetochore function and pseudohyphal differentiation; deletion of PLC1 causes increased minichromosome loss
BFA1	1.68	0.0089	Component of the GTPase-activating Bfa1p-Bub2p complex involved in multiple cell cycle checkpoint pathways that control exit from mitosis
CMD1	0.66	0.0163	Calmodulin; regulates Ca^{2+} independent (mitosis, bud growth, actin organization, endocytosis) and Ca^{2+} dependent processes (stress-activated pathways)
MCM21	0.54	0.0282	Protein involved in minichromosome maintenance; component of the COMA complex
CDC20	0.43	0.0017	Cell-cycle regulated activator of anaphase-promoting complex/cyclosome (APC/C), which is required for metaphase/anaphase transition
SGO1	0.41	0.0013	Component of the spindle checkpoint; required for accurate chromosomal segregation at meiosis II and for mitotic chromosome stability
Generation of precursor metabolites and energy			
PDC1	1.94	0.0043	Major of three pyruvate decarboxylase isozymes, key enzyme in alcoholic fermentation, decarboxylates pyruvate to acetaldehyde
GPM1	1.56	0.0019	Phosphoglycerate mutase; mediates conversion of 3-phosphoglycerate to 2-phosphoglycerate in glycolysis and the reverse reaction in gluconeogenesis
CDC19	1.52	0.0098	Pyruvate kinase; catalyzes the conversion of phosphoenolpyruvate to pyruvate, the final step in glycolysis; also involved in the cell division cycle
PDC6	1.54	0.0027	Minor isoform of pyruvate decarboxylase, key enzyme in alcoholic fermentation, decarboxylates pyruvate to acetaldehyde
Transport			
NUP82	0.58	0.0291	Essential nuclear pore complex (NPC) subunit; transport of macromolecules between the nucleus and the cytoplasm occurs through the NPC
MDR1	0.25	8.6E-06	GTPase activating protein (GAP) for Ypt6; involved in recycling of internalized proteins and regulation of Golgi secretory function

T1-E. *MCM21* is involved in minichromosome maintenance. Mutations in this gene are reported to have caused a decrease in the stability of a minichromosome, together with an increase in the copy number of the minichromosome in cells carrying it. Therefore, it was concluded that the mutants were defective in the segregation, rather than replication, of the minichromosome (Poddar *et al.*, 1999). Mutations in gene *PLC1* have also been described to result in increased levels of minichromosome loss and chromosome missegregation (Lin *et al.*, 2000). The altered expression of other genes involved in cell cycle control and mitosis regulation may also be directly or indirectly related to plasmid stability changes.

Two genes that code for components of chromatin remodelling complexes (*ITC1* and *RSC58*) were over-expressed in T1-E. Moreover, *SPT21* was also over-expressed in T1-E. This gene affects the transcription of yeast histone genes. Mutations in *SPT21* greatly decrease the transcript levels of two of the four histone loci in *S. cerevisiae* (Dollard *et al.*, 1994). Altered histone levels can cause effects on transcription and chromatin structure. Alterations in chromatin structure, particularly of the plasmid, may have been relevant to the adaptive process of the recombinant to lactose. These alterations may be related with the higher stability of the plasmid in T1-E. Moreover, chromatin structure may influence the activity of the *LAC* genes promoter region (LACIR), in which a deletion occurred during the adaptation (Figure 4.8).

CMD1, the single gene encoding calmodulin in *S. cerevisiae*, was under-expressed 1.5-fold in T1-E. Calmodulin, a small Ca^{2+} binding protein that is found in all eukaryotic organisms, has many functions in yeast. This protein plays essential roles in mitosis and bud growth, and is also required for endocytosis in yeast. It can perform these functions in a Ca^{2+} free form. Calmodulin also participates in Ca^{2+} dependent stress activated signalling pathways (Cyert, 2001).

Two genes of the glycolytic pathway (*GPM1* and *CDC19*) were over-expressed in T1-E. These genes encode enzymes involved in the last steps of glycolysis: *GPM1* encodes phosphoglycerate mutase, which mediates the conversion of 3-phosphoglycerate to 2-phosphoglycerate during glycolysis (and the reverse reaction during gluconeogenesis); *CDC19* encodes pyruvate kinase, which catalyses the conversion of phosphoenolpyruvate to pyruvate (the final step in glycolysis). Over-expression of these glycolytic enzymes is consistent with the higher growth and lactose consumption rates presented by the evolved strain. Moreover, *PDC1* and *PDC6* genes (encode isoforms of pyruvate decarboxylase) were over-expressed in T1-E. Pyruvate decarboxylase is a key enzyme in alcoholic fermentation, which converts pyruvate into acetaldehyde (that is further converted into ethanol). Over-expression of pyruvate decarboxylase is in agreement with the observation that the flux of carbon (lactose) through fermentation was higher in the evolved strain, which presented

higher ethanol productivity and lower biomass yield.

The microarray analysis did not find significant differences in the expression of genes specific for galactose metabolism (*GAL* genes). Furthermore, no significant differences were found in the expression of *FLO* genes (related with flocculation).

4.4 DISCUSSION

A recombinant *S. cerevisiae* flocculent strain with the genetic potential to utilize lactose was previously constructed (Domingues *et al.*, 1999b). Using a simple evolutionary engineering process, consisting of serial transfer/dilution in lactose medium for >120 generations, an evolved recombinant was isolated that fermented lactose 2-fold faster than the original recombinant T1, presenting higher ethanol yield and improved flocculation.

A similar process of adaptation had already been attempted before with the recombinant T1, which resulted in a comparable improvement of the lactose fermentation phenotype. That adapted strain was successfully used in long-term continuous lactose fermentations (Domingues *et al.*, 1999a; Domingues *et al.*, 2001). However, the strain lost its apparently adapted phenotype after storage at -80 ºC. When cultures stored at -80 ºC were re-grown, the limitation on lactose consumption (T1 phenotype) was again observed (for details see Domingues *et al.*, 1999b). Presumably the outcome of that former adaptation experiment was a heterogeneous population containing adapted and non-adapted cells, and during the storage/re-growth phase non-adapted cells were consistently selected. The evolved strain described here (T1-E) resulted from an independent adaptation experiment and its fermentative characteristics were maintained after -80 ºC storage.

The evolved strain T1-E showed improved lactose fermentation phenotype, but otherwise displayed identical performance as the original recombinant T1 on the fermentation of glucose and galactose. This suggested the occurrence of mutations in lactose-specific genes, rather than in pathways affecting sugar metabolism in general. Accordingly, two major molecular events that specifically targeted the lactose metabolism system in T1-E were identified. The first event was a 1593 bp deletion in the intergenic region between *LAC4* and *LAC12* (LACIR) in the plasmid isolated from the evolved recombinant. The second event was a reduction of the plasmid copy number by about 10-fold in T1-E as compared to the non-evolved strain T1. Moreover, the transcriptional analysis revealed that lactose strongly induced the expression of *LAC4* and *LAC12* in the evolved strain, whereas, in the original recombinant, these genes were expressed at the same level in lactose and in glucose cultures. This indicates that the *K. lactis* LAC promoter was unable to mediate induction by lactose of the *LAC* genes in *S. cerevisiae*, and that the 1593 bp deletion in the intergenic region between *LAC4* and *LAC12* apparently restored induction. A comparative analysis of the transcriptomes of T1 and T1-E growing in lactose, using *S. cerevisiae* cDNA microarrays, found few differences in the *S. cerevisiae* genome transcriptional expression between the two strains.

The *K. lactis LAC12-LAC4* intergenic region contains four functional upstream activating sites (UASs), which are binding sites for the trans-activator Lac9p, the *K. lactis* homologue of *S.*

cerevisiae Gal4p (also known as KlGal4p). Two functional UAS elements are located in front of each of the genes at almost symmetrical positions (see Figure 4.8). Gödecke *et al.* (1991) pointed that the distance of the most distal UAS elements to the RNA initiation sites is exceptionally large (more than 2.3 kb) in the LAC promoter. In *K. lactis*, as in *S. cerevisiae*, intergenic regions are usually smaller and thus regulatory elements are located closer to the transcription start. These authors proposed that *LAC4* and *LAC12* proximal sites interact to achieve maximal expression of both genes simultaneously in *K. lactis*, and that such interaction may occur via direct protein-protein contact between DNA bound Lac9p molecules or indirectly by multiple Lac9p molecules touching a common target. The four Lac9p-DNA complexes of the LAC promoter would therefore be assembled into a high molecular weight DNA-protein aggregate structured mainly by interacting Lac9p molecules.

In the recombinant strains described here, the trans-activator is *S. cerevisiae* Gal4p, which substitutes for the *K. lactis* Lac9p. Lac9p and Gal4p bind to the same DNA consensus sequence: 5'-CGG(N5)A/T(N5)CCG-3' (Gödecke *et al.*, 1991; Leonardo *et al.*, 1987). *GAL4* can complement the transcriptional activation function of *LAC9* in *K. lactis* (Riley *et al.*, 1987), and conversely, *LAC9* complements the *gal4* mutation in *S. cerevisiae* (Salmeron and Johnston, 1986; Wray *et al.*, 1987). However, Gal4p did not exactly mimic Lac9p function, and *vice versa*. Even though Gal4p is functionally analogous to Lac9p, specific features of each of the proteins (which share only three regions of significant homology, accounting for about 30% of the amino acids), as well as their cellular concentrations, seem to have regulatory relevance (Rubio-Texeira, 2005; Zachariae and Breunig, 1993; Zachariae *et al.*, 1993; Zenke *et al.*, 1993; see also chapter 1, section 1.6.4). Hence, a reasonable interpretation of the results shown here is that Gal4p cannot effectively replace Lac9p in the LAC promoter. It is therefore proposed that the deletion in the intergenic region between *LAC4* and *LAC12* altered the architecture of the promoter giving rise to a favorable structure for transcriptional activation by the *S. cerevisiae* Gal4p. The removal of sequences involved in transcription repression mechanisms existing in the deleted region cannot be excluded.

Gödecke *et al.* (1991) measured *LAC4* and *LAC12* transcriptional levels in a *K. lactis* mutant (JA6-LR0) with a deletion – positions -670 to -1540 from the *LAC4* initiation codon – similar to the one found in T1-E. That deletion decreased *LAC4* and *LAC12* expression levels in the mutant to about 80% and 60%, respectively, relative to the wild-type strain. This argues in favour of the suggestion that the positive effect over transcription of the deletion in the recombinant T1-E is specific for *S. cerevisiae*.

It is noteworthy that one of the UAS elements of the promoter was located within the deleted region in T1-E. However, although one of the UAS elements was deleted, the new arrangement of the other three may be favorable to activation by Gal4p. It is interesting that

the deleted UAS is the one that presented lower affinity to Lac9p, which correlated with its lower activation potential for *LAC4* (Gödecke *et al.*, 1991).

The spatial distribution of UAS elements within a promoter influences the pattern of transcription of the genes regulated (Bram *et al.*, 1986; Gödecke *et al.*, 1991; Leonardo *et al.*, 1987). Promoter architectural mechanisms have been implicated in the modulation of regulatory relevant protein-DNA and protein-protein interactions (Giniger and Ptashne, 1988; Melcher and Xu, 2001). Melcher and Xu (2001) reported that correctly spaced Gal4p binding sites stabilize otherwise transient higher order Gal80p multimers, which are probably necessary for complete repression of transcription. Chromatin organization is also implicated in transcription regulation. Chromatin structure is perturbed in the neighborhood of expressed genes, particularly in the neighborhood of promoters (Felsenfeld *et al.*, 1996). The nucleosomal changes in *S. cerevisiae GAL* promoter regions have a role in transcriptional control, namely regulating UAS accessibility (Lohr *et al.*, 1995; Lohr, 1997).

Unexpectedly, the copy number of the plasmid bearing the *LAC* construct was lower in T1-E than in T1. Possibly, the transformant originally selected (T1) owed its ability to grow on lactose to its high plasmid content. Considering that under the control of the intact *K. lactis* LAC promoter the *LAC* genes were expressed at low (non-induced) levels, it is conceivable that only transformants with high dosage of these genes would be able to sustain viability/growth on lactose. Furthermore, during T1 lactose cultivations, cells with higher plasmid copy number would be selected, since these would have a clear advantage. In fact, Sreekrishna and Dickson (1985) found that only the transformants that had integrated the *LAC* genes at high copy numbers (15 – 25 tandem copies) were able to grow on lactose. Despite the high dosage of *LAC* genes (under the control of the *K. lactis* LAC promoter), those strains grew slowly in lactose (doubling time in lactose minimal media was 6.7 h; Sreekrishna and Dickson, 1985).

An interesting consequence of high plasmid copy number is the high number of UAS elements, due to many copies of the LAC promoter. Hence, UAS occupancy by Gal4p in T1 must be limiting. The number of UAS elements in T1-E cells is lower, due to decreased plasmid copy number and to the deletion of one UAS in the LAC promoter.

The results suggest that tuning of the expression levels of the *LAC* genes in the evolved recombinant was accomplished by a fine molecular interplay between decreased copy number of both genes and different levels of transcriptional induction by lactose for *LAC4* and *LAC12*, resulting from a large deletion in the intergenic region between the two *LAC* genes. Changes in gene copy number (Adams, 2004; Brown *et al.*, 1998) as well as mutations in *cis*-regulatory sequences have been identified as genetic events involved in yeast adaptive

responses to environmental stresses. Brown *et al.* (1998) showed that a *S. cerevisiae* strain evolved in glucose-limited chemostat for 450 generations had multiple tandem duplications involving the high-affinity hexose transport genes *HTX6* and *HTX7* (the duplicated genes had the upstream promoter of *HTX7* and the coding sequence of *HTX6*). Recently, Fidalgo *et al.* (2006) found that a 111 bp deletion within a repression region of the *FLO11* gene promoter (possibly the largest *S. cerevisiae* promoter with about 3 kb), which significantly increases its expression, was determinant to confer the ability to float to wine "flor" yeasts (an adaptive mechanism of these yeasts to gain direct access to oxygen).

Besides these two identified mechanisms it is not possible to exclude the existence of additional mutations related to the improvement of the lactose fermentation phenotype. Surprisingly, transforming the original host or cured (plasmid-free) T1 strain with the plasmid isolated from T1-E did not yield Lac^+ transformants. These results emphasise that lactose utilisation in *S. cerevisiae* is a complex trait, which is not easily achieved by transfer of the *LAC* genes. Only Domingues *et al.* (1999b) and Sreekrishna and Dickson (1985) have addressed the construction of Lac^+ *S. cerevisiae* recombinants by cloning the *K. lactis LAC* genes under the control of the endogenous promoter (LACIR). Sreekrishna and Dickson (1985) obtained Lac^+ transformants only when using indirect selection (first selected for G418 resistance and then for growth on lactose), and when the *LAC* construct integrated in 15 – 25 tandem copies. The strategy devised by Domingues *et al.* (1999b) for construction of the original recombinant T1 also involved indirect selection (for ura- complementation and for β-galactosidase activity in galactose plates containing Xgal) using co-transformation with two different plasmids, and yielded only 2 (out of 1212) transformants with a stable Lac^+ phenotype.

The studies discussed in this chapter illustrate the usefulness of simple evolutionary engineering approaches in the improvement of genetically engineered strains that display poor efficiency. The next chapter describes the results obtained in batch fermentations with high concentrations of lactose (up to 200 $g \cdot L^{-1}$), demonstrating that the evolved recombinant T1-E presents decisive advantages over other engineered *S. cerevisiae* strains for the fermentation of lactose based-media.

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CHAPTER 5

FERMENTATION OF HIGH CONCENTRATIONS OF LACTOSE TO ETHANOL BY THE EVOLVED *SACCHAROMYCES CEREVISIAE* RECOMBINANT

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5.1 INTRODUCTION

Cheese whey is a by-product of the dairy industries, particularly the watery portion that is formed during the coagulation of milk casein in cheese making or in casein manufacture. Whey is produced in large amounts and has a high polluting charge, due to its high organic matter content, representing a major environmental concern. On the other hand, whey retains many of the milk nutrients and therefore has a vast potential as a source of added value compounds, challenging the industry to face whey surplus as a resource and not only as an environmental problem (see chapter 1, section 1.6.1).

The first step in most procedures for cheese whey valorisation consists in the separation of the protein fraction (ca. 1%), typically by ultrafiltration, to produce whey protein concentrates (Kosikowski, 1979; Siso, 1996). A lactose-rich stream, the permeate, is also obtained during this process. The permeate remains a major pollutant since it retains the lactose, which is largely responsible for the whey polluting charge. Lactose in permeate (ca. 5%) may be used as a substrate for the production of valuable compounds by fermentation. The classical examples are ethanol and single cell protein (SCP) production in yeast-based bioprocesses, although biotechnologists have proposed a multitude of alternative bio-products (see chapter 1, section 1.6.2). The existence of large amounts of whey surplus together with the need for cheap and largely available sugar-containing substrates and, above all, the rapid advances in microbial biotechnology will probably stimulate further exploitation of whey lactose as fermentation feedstock to obtain value-added products.

Recently, the production of bio-ethanol for fuel has gained renewed interest (Farrell *et al.*, 2006; Hahn-Hägerdal *et al.*, 2006; Ragauskas *et al.*, 2006; Schubert, 2006). Being a waste product, whey has advantage over food-related fermentation feedstocks, such as corn, for bio-ethanol production. In addition, lactose fermentation strongly reduces the whey polluting charge, contributing to solve the environmental problem created by whey surplus. Moreover, whey ethanol has food-grade quality, and therefore can find a proper market e.g. in vinegar manufacturing and in the beverage industry. Direct fermentation of whey or whey permeate to ethanol is hardly economically competitive with the currently established processes (using cane sugar and cornstarch as substrates) or with emerging second generation technologies (using lignocellulosic biomass as raw material), mainly because the low lactose content (ca. 5% w/v) results in low ethanol titre (2 – 3% v/v), making the distillation process too expensive. Concentration of whey lactose, e.g. by ultrafiltration, is an option to obtain higher ethanol titres. In a number of recent articles (Kargi and Ozmihci, 2006; Ozmihci and Kargi, 2007a; Ozmihci and Kargi, 2007b; Ozmihci and Kargi, 2007c; Ozmihci and Kargi, 2007d), Kargi and Ozmihci have proposed the use of cheese whey powder as an alternative source of

concentrated lactose to the production of ethanol. High ethanol concentrations (10 – 12% v/v) may be obtained by fermentation of concentrated lactose solutions (up to about 200 $g \cdot L^{-1}$ lactose) thus reducing distillation costs. Microbial strains are needed that can efficiently convert such high concentrations of lactose into ethanol, allowing the development of fermentation processes that reach high ethanol titres as well as high ethanol productivities. Such processes must be designed to minimise residual lactose at the end of fermentation, since one of the major motivations for whey utilization is to reduce/eliminate its polluting charge (Yang and Silva, 1995).

Kluyveromyces fragilis has been the microorganism of choice for most industrial plants producing ethanol from whey (Siso, 1996), though other lactose-fermenting yeasts (*Kluyveromyces marxianus* or *Candida pseudotropicalis*) have also been considered (Pesta *et al.*, 2007). *Saccharomyces cerevisiae* is usually the first choice for industrial processes involving alcoholic fermentation, mainly because of its good fermentative capacity, ethanol tolerance, capacity to grow under anaerobic conditions and extensive industrial use (see chapter 1, section 1.4). However, this yeast is unable to metabolise lactose. The engineering of *S. cerevisiae* for lactose utilization has been addressed over the past 20 years by different strategies (see chapter 1, section 1.6.5). However, most strains obtained displayed undesirable characteristics (such as slow growth, genetic instability or problems derived from the use of glucose-galactose mixtures) or were ineffective for ethanol production, as is the case of *S. cerevisiae* strains expressing both the *LAC4* (β-galactosidase) and *LAC12* (lactose permease) genes of *Kluyveromyces lactis* (Rubio-Texeira *et al.*, 1998; Rubio-Texeira *et al.*, 2000; Sreekrishna and Dickson, 1985), with the exception of the strain T1-E used in this work.

This chapter describes the results obtained in batch fermentations with mineral medium containing high concentrations of lactose (up to 200 $g \cdot L^{-1}$), using the evolved *S. cerevisiae* recombinant strain, T1-E, that was described in the previous chapter. The aims were to determine the highest ethanol titre and highest batch productivity that can be reached with this strain, as well as the initial lactose concentration that allows to maximise both ethanol titre and productivity while attaining low lactose residual at the end of fermentation. The results of a batch fermentation of 3-fold concentrated cheese whey by T1-E are also presented, confirming that the strain is an interesting alternative for whey fermentations.

5.2 MATERIALS AND METHODS

5.2.1 Yeast

The yeast used was the evolved *S. cerevisiae* recombinant strain, T1-E, described in the previous chapter.

5.2.2 Media and fermentations

Fermentations were performed in the defined mineral medium described by Verduyn *et al.* (1992), but with doubled concentrations of trace elements and vitamins (see chapter 4, section 4.2.1). The lactose was autoclaved separately and added after heat sterilization of the medium to a concentration of 20 – 200 $g \cdot L^{-1}$, as indicated.

Bioreactor fermentations were done in a 600 mL bubble column with H/D (height/column diameter) ratio of 2.1, filled with 400 mL of defined mineral medium. The temperature and pH were maintained at 30 ºC and 4.0, respectively. An air flow rate of 1.0 vvm (adjusted and controlled by a mass flow controller) was applied using a sintered porous plate (with a diameter of 3 cm) located at the bottom of the bioreactor. A magnetic stirrer was used to aid in keeping the entire reactor volume well-mixed. The yeast for inoculation was grown in 100 mL Erlenmeyer flasks filled with 40 mL of defined mineral medium containing 50 $g \cdot L^{-1}$ of lactose. After incubation at 30 ºC and 150 rpm for 20 – 30 h, the cell suspension was aseptically pumped into the bioreactor to start the fermentation. The initial working volume after inoculation was therefore 440 mL.

Shake-flask fermentations were done in 500 mL Erlenmeyer flasks filled with 200 mL of defined mineral medium and incubated at 30 ºC and 150 rpm. To avoid major drops in pH during cultivation the medium was supplemented with 100 mM potassium hydrogen phthalate. The initial pH was adjusted to 5.5 with NaOH. The final pH was higher than 4.3 in all fermentations. Pre-inocula were grown in 250 mL Erlenmeyer flasks filled with 100 mL of defined mineral medium containing 20 $g \cdot L^{-1}$ lactose for about 30 h (30 ºC, 150 rpm), and appropriate volumes were used to inoculate the fermentations to an initial OD_{600} of 0.1 – 0.15.

For batch fermentation of 3-fold concentrated cheese whey, 600 g of whey powder obtained from a Portuguese dairy were dissolved with 2.2 L of warm water and the solution was further warmed to boiling temperature. The precipitate was removed by filtering with a cloth followed by centrifugation. 1.5 L of the cheese whey powder solution was then transferred to the bioreactor and sterilized by autoclaving. Fermentation was performed in a 2-L bench-top

bioreactor (Bioengineering). The temperature was maintained at 30 ºC and the pH at 4.0 by automatic addition of ammonia. The agitation speed was set at 150 rpm. An air flow rate of 0.1 vvm (adjusted and controlled by a mass flow controller) was applied during the cultivation. The yeast for inoculation was grown in a shake-flask (filled with defined mineral medium containing 20 $g \cdot L^{-1}$ lactose), as described above.

5.2.3 Analytical procedures

Biomass and extracellular metabolites were determined essentially as described in chapter 4 (section 4.2.3).

Yeast (biomass) growth was monitored by measuring the optical density of the cell suspension at 600 nm (OD_{600}). The biomass was deflocculated by washing 2 – 5 times with 15 $g \cdot L^{-1}$ NaCl solution pH 3.0 prior to OD_{600} measurements. A standard curve of OD_{600} against cellular dry weight (DW) was previously constructed, and used to estimate the biomass concentration. Biomass DW was determined by filtering 10 mL of yeast culture through a pre-weighed 0.45 µm filter and washing with 20 mL of water. The filter was dried overnight at 104 ºC, cooled in a desiccator, and weighed.

Lactose, ethanol and glycerol were analyzed by HPLC, using a Chrompack Organic Acids column. The column was eluted at 60 ºC with 0.005 M H_2SO_4 at a flow rate of 0.6 $mL \cdot min^{-1}$. A refractive-index detector was used.

5.2.4 Determination of fermentation parameters

Maximum specific growth rates (µ) were calculated from numerically smoothed experimental biomass concentration data (Boltzmann fit function), using the definition $\mu=(1/X)\cdot(dX/dt)$, in which X is the biomass concentration and t is the fermentation time. The Microcal™Origin® software (Northampton, U.S.A) was used for numerical and differential calculation.

Biomass conversion yield was calculated by the ratio between the final biomass concentration (at the time of lactose exhaustion) and the initial lactose concentration. Ethanol conversion yield was calculated by the ratio between the maximum ethanol concentration and the initial lactose concentration. Ethanol yield was also expressed as a % of the theoretical yield, i.e. the yield considering a production of 4 mol of ethanol per mol of lactose consumed (equivalent to the production of 0.538 g of ethanol per g of lactose consumed). Ethanol productivity was defined as the ratio between ethanol concentration and fermentation time.

5.3 RESULTS AND DISCUSSION

Parameters measured in aerated batch fermentations in the bioreactor (bubble column) with mineral medium containing initial lactose concentrations ranging from 5 to 200 $g \cdot L^{-1}$ are shown in Table 5.1. Lactose was consumed completely in all the fermentations. The maximum specific growth rate increased from 0.20 h^{-1} with 5 $g \cdot L^{-1}$ initial lactose to about 0.35 h^{-1} with 10 and 20 $g \cdot L^{-1}$ initial lactose. With higher lactose concentrations the growth rate was lower (0.14 – 0.19 h^{-1}), indicating that there was substrate inhibition. The final biomass concentration, measured at the moment when lactose in the medium was exhausted, increased with increasing initial lactose concentrations (up to 20 g biomass$\cdot L^{-1}$ in the fermentation with 200 $g \cdot L^{-1}$ initial lactose). The biomass conversion yield on lactose was highest (0.31 g biomass$\cdot$[g lactose]$^{-1}$) with initial lactose concentration of 5 $g \cdot L^{-1}$, strongly declining for 10 and 20 $g \cdot L^{-1}$ initial lactose (to 0.16 and 0.19, respectively). A further drop (to 0.07 – 0.10) was observed for higher initial lactose concentrations. The maximum ethanol concentration produced increased linearly with increasing initial lactose concentration (Figure 5.1), reaching 57 g ethanol$\cdot L^{-1}$ with 200 $g \cdot L^{-1}$ initial lactose. However, the ethanol yields were only 44 – 59% of the theoretical value (i.e. assuming a production of 0.538 g of ethanol per g of lactose consumed). These low yields most likely resulted from the high aeration rates used in the bioreactor fermentations. The high air flow rate used was motivated by operational reasons, i.e. to obtain bulk mixing, particularly of the flocculent biomass, in the bioreactor. The maximum ethanol productivity increased with increasing initial lactose concentration up to 150 $g \cdot L^{-1}$ (reaching 1.6 g ethanol$\cdot L^{-1} \cdot h^{-1}$ at 150 $g \cdot L^{-1}$initial lactose). Further increase in initial lactose to 200 $g \cdot L^{-1}$ did not improve productivity.

Table 5.1 – Variation of fermentation parameters with the initial lactose concentration in the fermentations carried out in the bubble column bioreactor with defined mineral medium.

Initial lactose concentration *($g \cdot L^{-1}$)*	**5**	**10**	**20**	**50**	**100**	**150**	**200**
Max. specific growth rate *(h^{-1})*	0.20	0.34	0.36	0.14	0.19	0.18	0.16
Final biomass concentration *($g \cdot L^{-1}$)*	1.7	1.7	4.0	4.3	7.5	14	20
Biomass conversion yield *($g \cdot g_{lactose}^{-1}$)*	0.31	0.16	0.19	0.08	0.07	0.10	0.10
Max. ethanol concentration *($g \cdot L^{-1}$)*	1.3	2.3	6.5	16	32	43	57
Ethanol conversion yield *($g \cdot g_{lactose}^{-1}$)*	0.26	0.24	0.32	0.31	0.32	0.29	0.29
Ethanol conversion yield *(% of theoretical)*	48	44	59	57	59	53	54
Max. ethanol productivity *($g \cdot L^{-1} \cdot h^{-1}$)*	0.10	0.19	0.35	0.59	1.2	1.6	1.5

Figure 5.1 – Variation of the maximum ethanol concentration obtained in the bubble column bioreactor fermentations with the initial lactose concentration.

The profiles of biomass growth, lactose consumption and ethanol production during the fermentations with higher initial lactose concentrations (20 – 200 $g \cdot L^{-1}$) are shown in Figure 5.2. Interestingly, the fermentations with 50 – 150 $g \cdot L^{-1}$ initial lactose reached completion in about the same time (27 h) and the highest ethanol concentration was reached at the point of lactose exhaustion. In the fermentation with 200 $g \cdot L^{-1}$ initial lactose, there was a longer (ca. 25 h) lag phase for lactose consumption, possibly caused by high osmotic pressure, followed by fast consumption (between around 25 h and 40 h of fermentation) during which most lactose (ca. 140 $g \cdot L^{-1}$) was consumed. The highest ethanol concentration (57 $g \cdot L^{-1}$), ethanol conversion yield (68% of the theoretical) and ethanol productivity (1.5 $g \cdot L^{-1} \cdot h^{-1}$) were reached at approximately 40 h, when the lactose residual was still ca. 40 $g \cdot L^{-1}$. From that point on, lactose consumption was much slower, which may have been caused by product (ethanol) inhibition. Lactose exhaustion occurred only at about 60 h, when the ethanol productivity was already much lower (0.8 $g \cdot L^{-1} \cdot h^{-1}$). The overall ethanol yield, considering the production of 57 $g \cdot L^{-1}$ ethanol from 200 $g \cdot L^{-1}$ lactose, was 53% of the theoretical (Table 5.1).

The data from the fermentations with initial lactose concentrations of 5 – 50 $g \cdot L^{-1}$ (Table 5.1; Figure 5.2) have been used to construct a multi-route, non-structural kinetic model to describe the fermentation of lactose to ethanol by this yeast strain (Juraščík *et al.*, 2006).

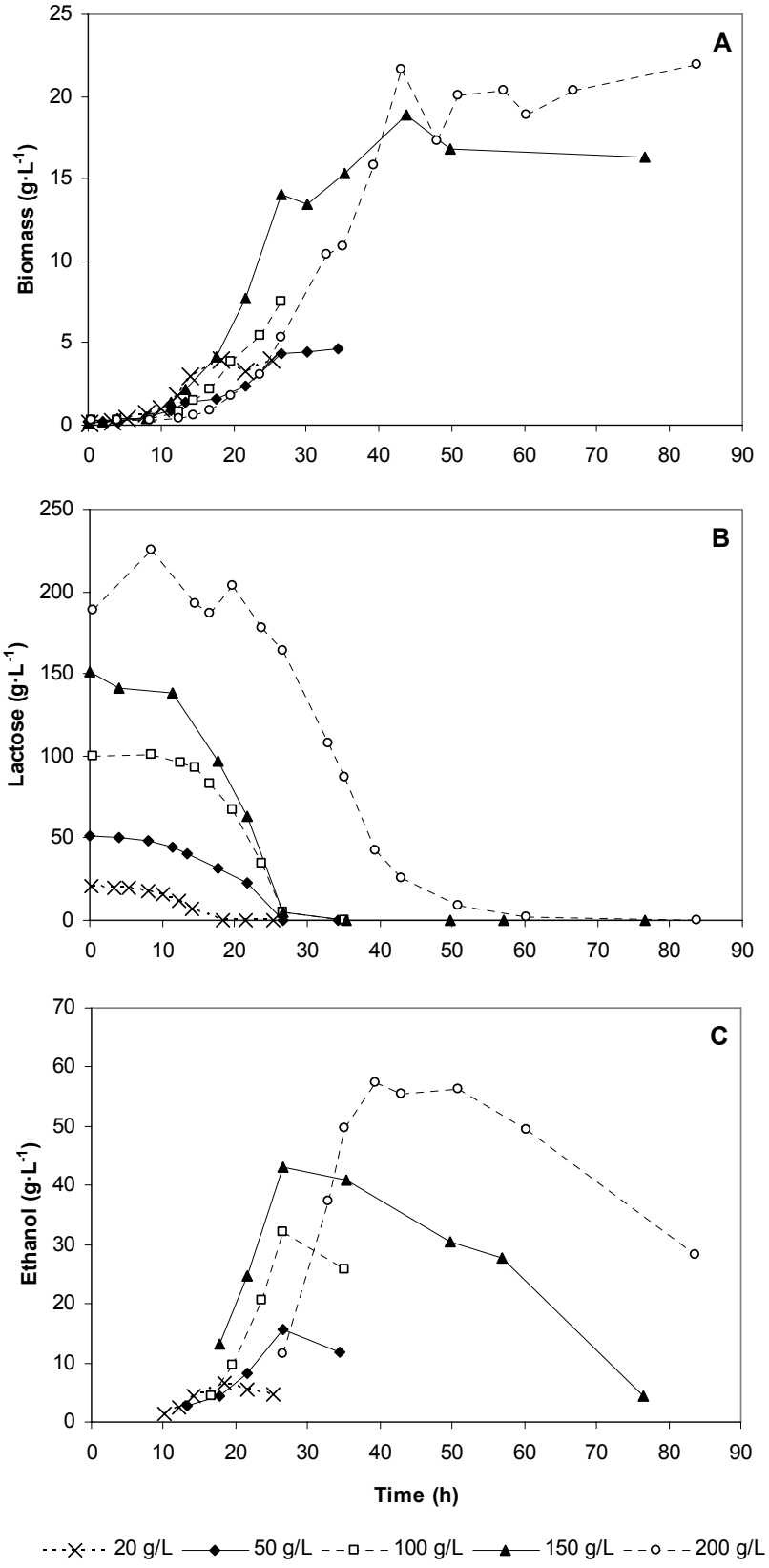

Figure 5.2 – Profiles of biomass growth (A), lactose consumption (B) and ethanol production (C) during fermentations in the bubble column bioreactor with initial lactose concentrations of approximately 20, 50, 100, 150 and 200 g·L^{-1}.

The recombinant strain T1-E was also tested in shake-flask fermentations with initial lactose concentrations of 100 – 200 $g \cdot L^{-1}$. The yeast completely consumed 114 $g \cdot L^{-1}$ initial lactose in about 24 h, producing 48 $g \cdot L^{-1}$ ethanol. This corresponds to an ethanol productivity of 2 $g \cdot L^{-1} \cdot h^{-1}$ and an ethanol conversion yield 78% of the theoretical. The data obtained in duplicate fermentations with about 150 $g \cdot L^{-1}$ initial lactose concentration are shown in Figure 5.3. The yeast produced 55 – 59 $g \cdot L^{-1}$ ethanol in about 28 h. At this point, ethanol productivity was highest (2 $g \cdot L^{-1} \cdot h^{-1}$). However, there was still a lactose residual of 17 – 34 $g \cdot L^{-1}$, which was further consumed attaining a final residual of < 1.5 $g \cdot L^{-1}$ lactose after about 45 h. At 40 – 45 h, ethanol concentration was highest (ca. 63 $g \cdot L^{-1}$) but ethanol productivity had decreased to 1.5 $g \cdot L^{-1} \cdot h^{-1}$. The ethanol conversion yield was 78 – 84% of the theoretical yield. Figure 5.4 shows the fermentation profiles obtained with an initial lactose concentration of 200 $g \cdot L^{-1}$. Ethanol productivity was maximal (1.7 $g \cdot L^{-1} \cdot h^{-1}$) at 27 h, corresponding to an ethanol concentration of 46 $g \cdot L^{-1}$ and a lactose residual of 95 $g \cdot L^{-1}$. Ethanol concentration was highest (66 $g \cdot L^{-1}$) after 48 h of fermentation, corresponding to a productivity of 1.4 $g \cdot L^{-1} \cdot h^{-1}$. A lactose residual of about 50 $g \cdot L^{-1}$ remained unconsumed. Yeast growth was only slightly slower with 200 $g \cdot L^{-1}$ initial lactose than that with 150 $g \cdot L^{-1}$ initial lactose (Figures 5.3 and 5.4). Expectedly, alcoholic fermentation was accompanied by glycerol biosynthesis (Figures 5.3 and 5.4), which has an essential role in maintaining yeast redox balance particularly under anaerobic growth conditions (Rigoulet *et al.*, 2004; Taherzadeh *et al.*, 2002; see also chapter 1, section 1.4).

The ethanol yields were much higher in the shake-flask fermentations (about 80% of the theoretical) than in the bioreactor fermentations (< 60%). This probably resulted from lower oxygen availability for yeast in the shake-flasks (conditions of micro-aeration), leading to higher sugar flux towards fermentative metabolism. In the shake-flasks, ethanol decreased only slightly after peak concentration (Figures 5.3 and 5.4), which may have resulted from evaporation. Under these conditions, oxygen limitation probably impaired ethanol respiration after lactose depletion. Conversely, in the well-aerated bioreactor fermentations the ethanol decreased markedly after lactose exhaustion (Figure 5.2, panel C), most likely because it was consumed by the yeast cells by respiration.

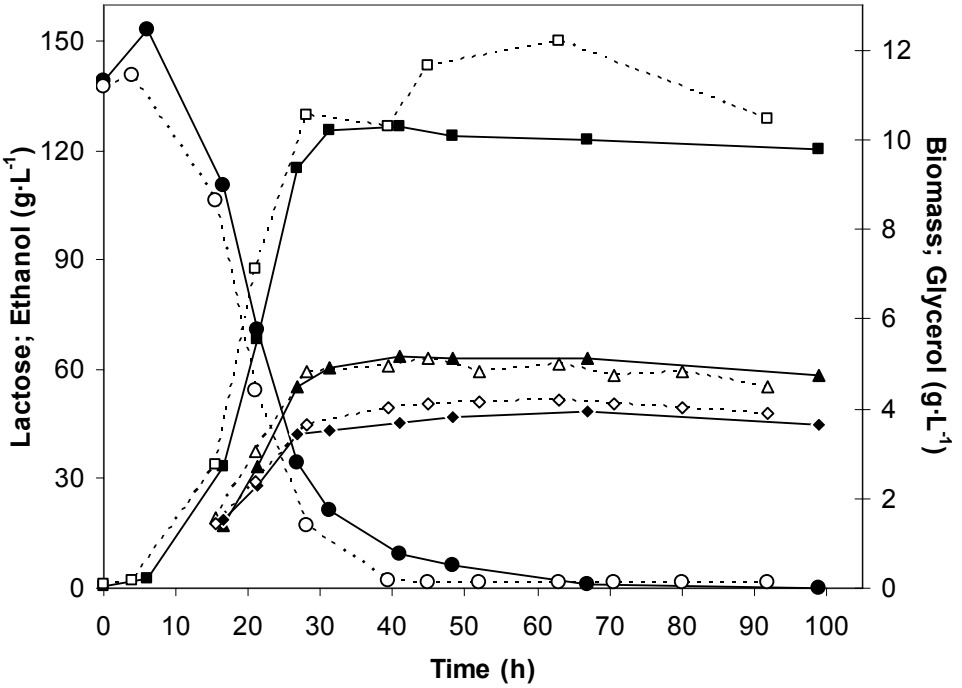

Figure 5.3 – Profiles of biomass (■; □) growth, lactose (●; ○) consumption, and ethanol (▲; Δ) and glycerol (♦; ◊) production during shake-flask fermentations with initial lactose concentration of approximately 150 g·L^{-1}. Data from duplicate fermentations are shown.

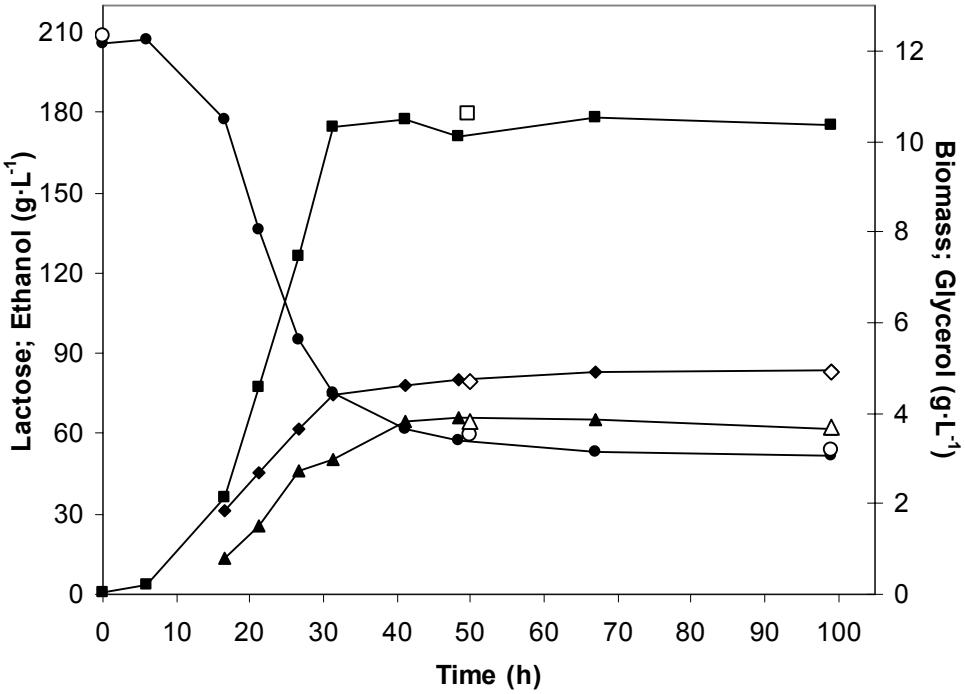

Figure 5.4 – Profiles of biomass (■) growth, lactose (●) consumption, and ethanol (▲) and glycerol (♦) production during shake-flask fermentation with initial lactose concentration of approximately 200 g·L^{-1}. Data from a duplicate fermentation (open symbols) are shown at 0, 50 and 99 h.

In the design of processes for ethanol production from cheese whey or whey permeate a compromise must be made between maximisation of ethanol titre and productivity and minimisation of the residual lactose concentration in the effluent, since the purpose of the process is also waste treatment. The recombinant *S. cerevisiae* strain T1-E consumed rapidly and completely 150 $g \cdot L^{-1}$ of lactose in either well-aerated (bioreactor) or micro-aerated (shake-flasks) batch fermentations, producing 5.5% (v/v) and 8.0% (v/v) of ethanol, respectively (Figures 5.2 and 5.3). Increasing the initial lactose concentration to 200 $g \cdot L^{-1}$ resulted in slower, yet still complete, consumption of lactose in the well-aerated bioreactor fermentations (Figure 5.2). Conversely, the yeast was unable to totally consume 200 $g \cdot L^{-1}$ of lactose in the micro-aerated shake-flask fermentations (Figure 5.4). This may be related with the yeast's inability to synthesise proper amounts of ergosterol and unsaturated fatty acids under the oxygen limiting conditions found in the shake-flask fermentations. It is well established that yeast needs oxygen in order to synthesise unsaturated fatty acids and ergosterol (see chapter 2), and lower amounts of these lipids in the plasma membrane have been correlated with lower ethanol tolerance (Aguilera *et al.*, 2006; Alexandre *et al.*, 1994a; Alexandre *et al.*, 1994b). The ethanol yields were considerably higher in the micro-aerated fermentations. The results suggest that an initial lactose concentration of 150 $g \cdot L^{-1}$ represents the best compromise to obtain high ethanol titre (8% v/v) and productivity (1.5 $g \cdot L^{-1} \cdot h^{-1}$) at the end of fermentation (i.e. with a lactose residual < 1.5 $g \cdot L^{-1}$). With an initial lactose concentration of 114 $g \cdot L^{-1}$ the ethanol productivity was considerably higher (2 $g \cdot L^{-1} \cdot h^{-1}$) but the ethanol titre attained was much lower (6.1% v/v).

The ethanol productivity obtained in this work with strain T1-E was higher than that reported for batch or fed-batch fermentations with other lactose-consuming recombinant *S. cerevisiae* strains: 0.3 $g \cdot L^{-1} \cdot h^{-1}$ (Rubio-Texeira *et al.*, 1998); 0.14 to 0.6 $g \cdot L^{-1} \cdot h^{-1}$ (Ramakrishnan and Hartley, 1993); 1 $g \cdot L^{-1} \cdot h^{-1}$ (Compagno *et al.*, 1995); 1.3 $g \cdot L^{-1} \cdot h^{-1}$ (Farahnak *et al.*, 1986). The utilization of high initial lactose concentrations enabled also to attain higher ethanol titres than previously obtained with recombinant *S. cerevisiae*, with the exception of the work of Farahnak *et al.* (1986) that reported an ethanol titre of 13% (v/v).

The evolved strain T1-E was also tested for the fermentation of cheese whey. Cheese whey powder solution (CWPS) was used in order to get 3-fold concentrated whey (corresponding to about 150 $g \cdot L^{-1}$ lactose). In this batch fermentation, a low aeration rate (0.1 vvm) was used. The yeast was able to grow and flocculate in CWPS and consumed nearly all the lactose (residual lactose < 3 $g \cdot L^{-1}$) in about 120 h, producing 55 $g \cdot L^{-1}$ of ethanol and 5 $g \cdot L^{-1}$ of glycerol (Figure 5.5). The overall ethanol yield was 72% of the theoretical conversion yield, and the productivity was 0.46 $g \cdot L^{-1} \cdot h^{-1}$.

The results presented here show that the evolved recombinant strain T1-E is the most

efficient lactose-fermenting *S. cerevisiae* strain reported in the literature, providing an attractive alternative for the fermentation of lactose-based media. Being highly flocculent, this strain is particularly suitable for application in high cell density fermentation systems. One of the main advantages of flocculation, which traditionally has been used by brewers during lager beer fermentations, is that flocculated cells are more easily separated from the broth at the end of the fermentation. Flocculation has several other advantages for the development of fermentation processes (reviewed by Domingues *et al.*, 2000). In particular, it may be considered a simple and cheap cell immobilization method, allowing the accumulation of high cell densities inside a bioreactor, thus increasing the process productivity. For processes operated in continuous, flocculation facilitates the retention of the biomass inside the bioreactor.

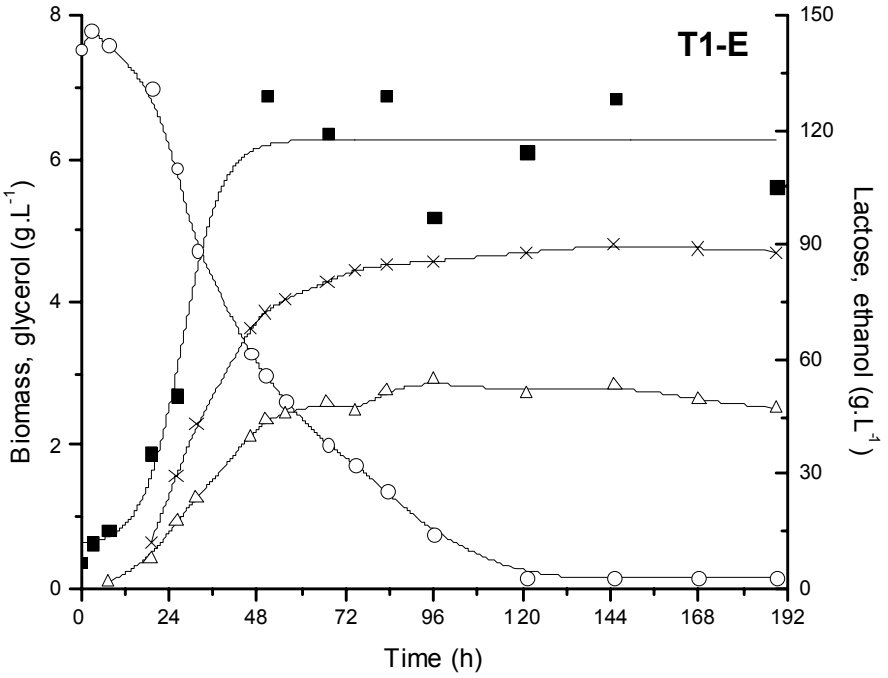

Figure 5.5 – Fermentation of 3-fold concentrated cheese whey. Lactose (○), ethanol (Δ), biomass (■) and glycerol (x) concentrations were followed during batch fermentation in a 2-L bioreactor with low aeration (0.1 vvm), as described in Materials and Methods.

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CHAPTER 6

STIMULATION OF ZERO-*TRANS* RATES OF LACTOSE AND MALTOSE UPTAKE INTO YEASTS BY PRE-INCUBATION WITH HEXOSE TO INCREASE THE ADENYLATE ENERGY CHARGE

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6.1 INTRODUCTION

Transport systems mediate the exchange of substances between the cell and the extracellular environment. Sugars are highly polar molecules that are transported across the plasma membrane by carrier proteins. The uptake of sugars in yeast can occur by a number of different mechanisms (Lagunas, 1993; Van der Rest *et al.*, 1995; Weusthuis *et al.*, 1994). Facilitated diffusion systems rely on the concentration gradient of the solute over the membrane and therefore do not require metabolic energy. These systems are widespread among yeasts, but do not allow the uptake of sugars against a concentration gradient. Other systems, however, drive the uphill transport of solutes using the energy supplied by electrochemical gradients of other solutes, in particular ions, between the external solution and cell interior. Protons are the most common driving solute for transport systems in microorganisms and plants (Lagunas, 1993). Solute/H^+ symports couple the transport of solute into the cell with the thermodynamically favourable transport of protons into the cell. The proton motive force that drives protons into the cell results from the transmembrane electrochemical gradient of protons (Δp). Δp has two components, the difference in pH, ΔpH, between the (usually acidic) medium and the near neutral cytosol, and the membrane potential, ΔΨ, with the cytosol some 50 – 200 mV negative compared to the cell exterior (Hauer and Höfer, 1978; Serrano, 1991; Slayman *et al.*, 1989). In *Saccharomyces* yeasts, Δp is largely generated by the plasma membrane ATPase (Pma1p), which is the major membrane protein and pumps protons out of the cell with a stoichiometry of 1 proton/ATP hydrolysed to ADP (reviewed by Ambesi *et al.*, 2000; Van der Rest *et al.*, 1995).

The plasma membrane ATPase is therefore a primary transport system that converts the chemical energy stored in ATP into the Δp (Van der Rest *et al.*, 1995). The Δp supplies the driving force for several membrane-associated processes, in particular for proton-dependent secondary transport systems. The membrane potential will only contribute to drive transport processes if the transport of the proton-sugar-carrier complex is electrogenic, i.e. if a net charge is transported per cycle of carrier movement during sugar uptake, as is the case of the co-transport of sugars with protons (Hauer and Höfer, 1978; Komor and Tanner, 1976). Electroneutral uptake mechanisms like proton-anion symport or proton-cation antiport are independent of the membrane potential (Komor and Tanner, 1976). The plasma membrane ATPase accounts for a large proportion of ATP consumption during yeast growth, at least 10 to 15% and over 25% during fermentative growth on actively transported disaccharides such as maltose (Weusthuis *et al.*, 1993) or lactose, where one proton must be pumped out for every sugar molecule entering the cell.

The ability of the yeast *Kluyveromyces lactis* to metabolise lactose results from the presence

of a lactose permease and a lactase (β-galactosidase). The β-galactosidase is thought to be intracellular (Dickson *et al.*, 1979; Rubio-Texeira, 2005; Sheetz and Dickson, 1980) and hydrolyses lactose into glucose and galactose. Lactose uptake by *K. lactis* is mediated by a transport system inducible by lactose and galactose (Dickson and Barr, 1983) and is an active process that permits the intracellular accumulation of lactose against a concentration gradient (Boze *et al.*, 1987; Dickson and Barr, 1983). A lactose/H^+ symport is believed to be responsible in this and related *Kluyveromyces* species (Barnett and Sims, 1982; Carvalho-Silva and Spencer-Martins, 1990; Dickson and Barr, 1983; Van den Broek and van Steveninck, 1982).

The maltose uptake system of *Saccharomyces cerevisiae* has been extensively studied, because of the importance of maltose in industrial processes, such as beer production. Maltose uptake occurs via a proton symport mechanism, with one proton being co-transported with each maltose molecule (Serrano, 1977). In *Saccharomyces* yeasts, maltose transporters are encoded by several genes, including at least *MALx1* (where x = 1-4 and 6 and indicates one of five 5 *MAL* loci, each on a different chromosome), *AGT1* (Han *et al.*, 1995; Jespersen *et al.*, 1999; Lagunas, 1993; Stambuk *et al.*, 1999) and the relatively recently discovered *MTT1* (Dietvorst *et al.*, 2005; Salema-Oom *et al.*, 2005). All the encoded proteins are thought to be maltose/H^+ symporters. Some can carry other α-glucosides as well as maltose.

Zero-*trans* rates of sugar uptake into yeasts are most often measured using cells harvested, washed to remove growth media and stored at 0 − 5 ºC in nutrient-free buffer for minutes or hours before assay. Rapid handling and storage at low temperatures are attempts to preserve the, often non-constitutive, transporters in the state existing at the moment of harvest. These starved cell suspensions are then assayed, after equilibration to assay temperature, either by short (typically 5 – 30 s) incubation with radiolabelled sugar followed by determination of the radioactivity incorporated into the cells or by rather longer incubation in weakly buffered solutions of the sugar, during which the pH changes caused by the operation of sugar/H^+ symports are recorded (see, e.g., Alves-Araújo *et al.*, 2007; Bisson and Fraenkel, 1983; Lucero *et al.*, 1997; Nobre *et al.*, 1999; Serrano, 1977; Walsh *et al.*, 1994a). This chapter reports studies of lactose transport by *K. lactis* and two recombinant *S. cerevisiae* strains expressing *K. lactis LAC* genes (strains T1 and T1-E; see chapter 4). The zero-*trans* lactose uptake rates measured using yeast harvested while growing on lactose and suspended in nutrient-free buffer were several fold smaller than the lactose consumption rates at the time of harvest. Short pre-incubation of the starved cell suspensions with glucose or fructose immediately before the uptake assays increased the lactose uptake rates. This stimulation of lactose transport correlated with increases in the yeast ATP level and adenylate energy

charge (EC = ([ATP] + 0.5×[ADP])/([ATP] + [ADP] + [AMP])) during the pre-incubation with glucose. Similar observations were made for maltose transport in brewer's yeast.

6.2 MATERIALS AND METHODS

6.2.1 Yeasts

The lactose-consuming yeasts used were *K. lactis* strain CBS2359 and the two *S. cerevisiae* recombinant strains, T1 and T1-E, described in chapter 4. Strain A15 (A63015) is an industrial brewer's (lager) yeast from VTT's collection (also used in the work reported in chapter 3). The maltose-negative, ura3-Δ laboratory strain, S150-2B was transformed with a multicopy plasmid carrying *URA3* and a *MALx1* gene isolated from strain A15, yielding the strain S150-2B/MALx1. The *MALx1* gene is expressed from a *PGK1* promoter. Construction of this strain will be described elsewhere (Vidgren *et al., in preparation*). It was grown on minimal medium lacking uracil and containing 2% glucose.

Yeasts for stocks were grown into early stationary phase (A15, S150-2B/MALx1) or late growth phase (lactose-consuming strains), harvested and stored at -80 ºC as suspensions in 30% glycerol containing 200 mg fresh yeast mass · mL^{-1}.

6.2.2 Radioactive sugars

[D-glucose-1-^{14}C]-lactose and [U-^{14}C]-maltose were CFA278 and CBF182, respectively, purchased from Amersham Biosciences (Espoo, Finland). According to HPLC analysis ^{14}C-glucose accounted for about 2% of the total label in the [D-glucose-1-^{14}C]-lactose used.

6.2.3 Lactose fermentations and lactose transport assays

The lactose-consuming yeasts were grown in the defined mineral medium described by Verduyn *et al.* (1992), but with doubled concentrations of trace elements and vitamins (see chapter 4, section 4.2.1). The lactose was autoclaved separately and added after heat sterilization of the medium to a concentration of 20 to 25 $g \cdot L^{-1}$, as indicated. To avoid major drops in pH during cultivation the medium was supplemented with 100 mM potassium hydrogen phthalate. The initial pH was adjusted to 4.5 with NaOH. The cultivations were carried out in Erlenmeyer flasks filled with medium to 40% of the total volume (0.5, 1.0 or 2.0 L). Media were inoculated with glycerol stocks to an initial OD_{600} of 0.03 to 0.10 and shaken (150 rpm) at, usually, 30 ºC (18 ºC when so stated).

Some fermentations were sampled at 1 – 3 h intervals for biomass and residual lactose determinations. Lactose consumption rates were calculated from the change in the residual lactose concentration and the average dry yeast concentration, over the interval when dry

yeast concentration increased from about 0.6 to 1.5 $g \cdot L^{-1}$, which corresponded to an increase in lactose consumption from about 15% to 60% (10 – 20% for *K. lactis* at 18 ºC) of the initial lactose. Over shorter time intervals within this frame, specific consumption rates fluctuated markedly (up to ± 50 %), but with no pattern reproducible between experiments. These fluctuations probably resulted from random errors in the small differences between large lactose concentrations, so only more reliable data from longer time intervals was used.

Yeast samples for zero-*trans* lactose uptake assays were harvested when the dry yeast concentration was 0.6 – 1.5 $g \cdot L^{-1}$ (the same interval used to calculate the lactose consumption rates, see above) by centrifugation (5 min at 9000 x g), washed twice with ice-cold water and suspended to 200 mg fresh yeast mass · mL^{-1} in ice-cold 0.1 M tartrate/Tris buffer, pH 4.2. The strongly flocculating strains T1 and T1-E were washed with ice-cold 15 $g \cdot L^{-1}$ NaCl solution pH 3.0, instead of water. Zero-*trans* rates of lactose uptake were determined at 2 mM or 20 mM lactose and 18, 20 or 30 ºC essentially as earlier described for maltose uptake (Lucero *et al.*, 1997; Serrano, 1977; see also chapter 2, section 2.2.2). Portions of yeast suspension (0.3 – 0.6 mL) were equilibrated to 18 or 20 ºC for 5 min or to 30 ºC for 10 min, immediately before assay. Reactions were started by adding 40 µL of equilibrated yeast suspension to 20 µL of labeled lactose solution (6 mM ^{14}C-lactose at about 1000 $cpm \cdot nmol^{-1}$ for assays at 2 mM lactose, or 60 mM ^{14}C-lactose at about 100 $cpm \cdot nmol^{-1}$ for assays at 20 mM lactose). Reactions were stopped after 10 s, by adding 10 mL of ice-cold water. Assays were done in duplicate and linearity with respect to time was confirmed by 15 and/or 20 s assays. Rates determined with these longer times were >90% of those determined with 10 s assays, with the exception of *K. lactis* assayed at 30 ºC with 20 mM lactose. In this case, rates calculated from 15 s and 20 s assays were, respectively, about 85% and about 65% of those calculated from 10 s assays, suggesting that the results from 10 s assays may also have been significantly smaller than the true initial rates. Zero time assays (which give the amount of radioactivity on the membranes after zero seconds incubation of yeast in the reaction mixture) were performed by adding first the 10 mL ice-cold water to the 20 µL of ^{14}C-lactose solution and then adding the 40 µL of yeast suspension. Rates were normalised to yeast dry mass, determined by washing the yeast with water and drying it overnight at 105 °C.

For pre-incubations with glucose (or fructose), 500 µL of yeast suspension was equilibrated to 18 or 20 ºC for 5 min or to 30 ºC for 10 min and then mixed with 20 µL of 280 or 700 mM hexose to give 11 or 27 mM hexose in the yeast suspension. After further incubation for the times indicated, zero-*trans* sugar uptake was assayed as described above, so that the final reaction mixtures contained ≤ 7 mM or 18 mM hexose. In control experiments, water was added to the yeast suspension instead of hexose solution. Possible instantaneous effects (e.g. competitive inhibition) of hexose on lactose transport were checked by assays in which

hexose was mixed with the labeled lactose before addition of the yeast suspension to give final hexose concentrations up to 110 mM.

To determine the effect of glucose-stimulation on the kinetic parameters of lactose uptake, *K. lactis* grown at 18 ºC was assayed at 18 ºC with lactose concentrations between 0.5 and 20 mM. The suspension of harvested yeast was used directly or pre-incubated with 27 mM glucose for 5 and 10 min before assay with ^{14}C-lactose. Differences in rate between the 5 min- and 10 min-pre-incubated samples were <5%. Replicate experiments were done with independently grown lots of yeast suspensions. V_{max} and K_m were calculated by the direct linear plot of Eisenthal and Cornish-Bowden (1974).

6.2.4 Maltose transport assays

Strain A15 was grown in Erlenmeyer flasks filled to 40% of their total volume with YP (1% yeast extract, 2% peptone) medium containing 40 g·L^{-1} maltose and shaken (150 rpm) at 24 ºC. For strain S150-2B/MALx1 minimal medium lacking uracil and containing 20 g·L^{-1} glucose was used. Yeasts were harvested when the dry yeast concentration was 1.0 – 2.3 g·L^{-1} (OD_{600} of 3 – 6.5), washed and suspended in 0.1 M tartrate/Tris, pH 4.2, as described above. Zero-*trans* rates of maltose uptake were determined, with or without glucose-stimulation, at 5 mM maltose (about 1000 cpm·nmol^{-1}) unless stated otherwise and 20 ºC as described above for lactose uptake.

6.2.5 Trehalose and glucose analyses

Trehalose was extracted from washed cells by boiling in water for 10 min and converted to glucose by treatment with trehalase (Sigma-Aldrich, Helsinki, Finland). Glucose was assayed enzymatically with hexokinase and glucose-6-phosphate dehydrogenase (both from Sigma-Aldrich).

6.2.6 Preparation of spheroplasts

Spheroplasts were prepared by modifications of standard methods (see, e.g., Schwencke *et al.*, 1983). The *S. cerevisiae* recombinants, T1 and T1-E, were grown to an OD_{600} of 2.0 – 3.0 and harvested by centrifugation (5 min at 5000 x g). The yeast pellet was suspended to 100 mg fresh yeast · mL^{-1} in 0.1 M EDTA/2% mercaptoethanol, pH 7.0, and incubated at 30 °C for 30 min. Yeast cells were collected and washed with 10 – 15 mL of 1 M sorbitol in buffer A (25 mM potassium phosphate buffer, pH 6.5, containing 2 mM $MgCl_2$, 1 mM EDTA and 0.1 mM

DTT) and resuspended to 40 mg fresh yeast · mL^{-1} in buffer A. Pepstatin A and PMSF were added to final concentrations of 10 $\mu g \cdot mL^{-1}$ and 170 $\mu g \cdot mL^{-1}$, respectively. Zymolyase 100T (Seikagaku, Japan) was added to a final concentration of 30 $\mu g \cdot mL^{-1}$ and the cell suspension was incubated at 30 °C until spheroplast formation (estimated by the decreasing OD_{600} of periodic dilutions of 100 µL samples into 3 mL of water) was nearly complete. The spheroplast suspension was centrifuged (10 min at 1000 x g) and the supernatant was collected. The pellet was washed with 1 M sorbitol/buffer A containing pepstatin A and PMSF and the resulting supernatant was combined with the previous supernatant to give the cell wall/periplasmic fraction. The pellet was then suspended in 10 – 15 mL of buffer A containing pepstatin A and PMSF and the spheroplasts allowed to lyse to give the cytoplasmic fraction. For *K. lactis* CBS2359, the sorbitol concentration was increased to 1.2 M and the zymolyase concentration decreased to 6 $\mu g \cdot ml^{-1}$ to avoid premature lysis of the spheroplasts, and the incubation time was extended to 60 min.

6.2.7 Enzyme assays

The β-galactosidase activity was assayed using p-nitrophenyl-β-D-galactopyranoside (pNPG) as substrate (Miller, 1972). Briefly, 800 µL samples of appropriate dilutions (at least 1:10) of cell extract in buffer Z (100 mM sodium phosphate buffer, pH 7.0, 10 mM KCl, 1 mM $MgSO_4$, 0.28% (v/v) 2-mercaptoethanol) were transferred to a spectrophotometer cuvette and the reaction was started by adding 200 µL of 4 $mg \cdot mL^{-1}$ pNPG. The reaction at room temperature (ca. 23 ºC) was followed by reading the absorbance at 405 nm over time. The assay was calibrated by reading the A_{405} of standard solutions (0.01 – 0.2 mM) of p-nitrophenol in the same buffer.

Phosphoglucoisomerase (PGI) was assayed by coupling its catalysed conversion of fructose-6-phosphate to glucose-6-phosphate with the activity of glucose-6-phosphate dehydrogenase (G6PDH). The conversion of NADP to NADPH was followed by the change in A_{340} at room temperature (ca. 23 ºC). The reaction mixture consisted of 50 mM HEPES/KOH, buffer pH 7.5, containing 10 mM $MgCl_2$, 0.1 mM EDTA, 0.4 mM NADP, 10 mM fructose-6-phosphate and 1.5 $U \cdot mL^{-1}$ G6PDH (Sigma).

Invertase activity was assayed by following the glucose released from sucrose. The invertase reaction mixture consisted of 50 mM sodium acetate buffer, pH 4.5, containing 20 mM sucrose. Reaction was started by adding the extract and stopped after 10 or 20 min incubation at room temperature (ca. 23ºC), by transfer to a boiling water bath for 5 min. The glucose formed during this reaction was then measured using hexokinase and G6PDH.

One unit of enzyme activity (U) catalyses the conversion of 1 µmol of substrate per min under

the stated conditions. Specific enzyme activities are expressed as U per g of fresh yeast mass.

Total protein was determined by the Lowry method (Lowry *et al.*, 1951), using ovalbumin as the standard.

6.2.8 Adenine nucleotide analyses

Intracellular adenine nucleotides were determined as described in chapter 3. For analyses of total adenine nucleotides (intra- *plus* extracellular) in fermentations, 9 mL samples were collected and immediately injected into 1.0 mL of ice-cold 5.0 M perchloric acid (PCA). Parallel samples (20 – 40 mL) were withdrawn, centrifuged (5 min at 10000 x g) and 9 mL of the clear supernatant was injected into 5.0 M PCA for determinations of extracellular adenine nucleotides. The yeast pellet was washed with ice-cold water and dried overnight at 105 °C, for yeast dry mass determination. Intracellular adenine nucleotides levels were calculated by the difference between total adenine nucleotides (intra- *plus* extracellular) and extracellular adenine nucleotides.

Changes in adenine nucleotides during glucose stimulation were assayed using yeast harvested and suspended to 200 mg fresh yeast mass · mL^{-1} in ice-cold 0.1 M tartrate/Tris buffer, pH 4.2, as described above for the transport assays. For analyses of total adenine nucleotides, 1.5 mL portions of the yeast suspension were first equilibrated to 20 ºC for 5 min or to 30 ºC for 10 min, and 60 µL of 700 mM glucose were then added (27 mM final glucose concentration). After further incubation for up to 20 min at 20 ºC or 30 ºC, the yeast suspension was quenched with 8.5 mL of ice-cold 0.59 M PCA. For estimation of extracellular adenine nucleotides, 100 µL of 700 mM glucose were added to 2.5 mL of yeast suspension previously equilibrated to 20 ºC for 5 min or to 30 ºC for 10 min. The suspension was incubated at the respective temperature for a further 10 min, filtered through a 0.45 µm membrane, and 1.5 mL of the filtrate were added to 8.5 mL of ice-cold 0.59 M PCA. Extracellular ATP levels were very low (< 0.6%) compared to intracellular levels. Extracellular ADP and AMP levels were also low, but sometimes accounted for up to 20% of the total (intra- *plus* extracellular) levels. Nevertheless, these extracellular levels were sufficiently low not to disturb calculations of the EC by more than 0.01. Control experiments were done in which water was added to the yeast suspension instead of glucose solution.

PCA extracts were handled and adenine nucleotides assayed using firefly luciferase essentially as described in chapter 3.

6.3 RESULTS

6.3.1 Lactose consumption rates during fermentations

Figure 6.1 shows specific rates of lactose consumption during shake flask fermentations of lactose by *K. lactis* or the recombinant *S. cerevisiae* strains, T1 and T1-E. Specific consumption rates were calculated from the changes in lactose concentration and the average dry mass concentration over the interval when dry yeast concentration increased from about 0.6 to 1.5 $g \cdot L^{-1}$ (see Materials and Methods). *K. lactis* and the evolved *S. cerevisiae* recombinant, T1-E, exhibited about 2.5-fold higher lactose consumption rates than the non-evolved recombinant, T1. For *K. lactis*, specific rates of consumption were about 2.2-fold higher at 30 ºC than 18 ºC.

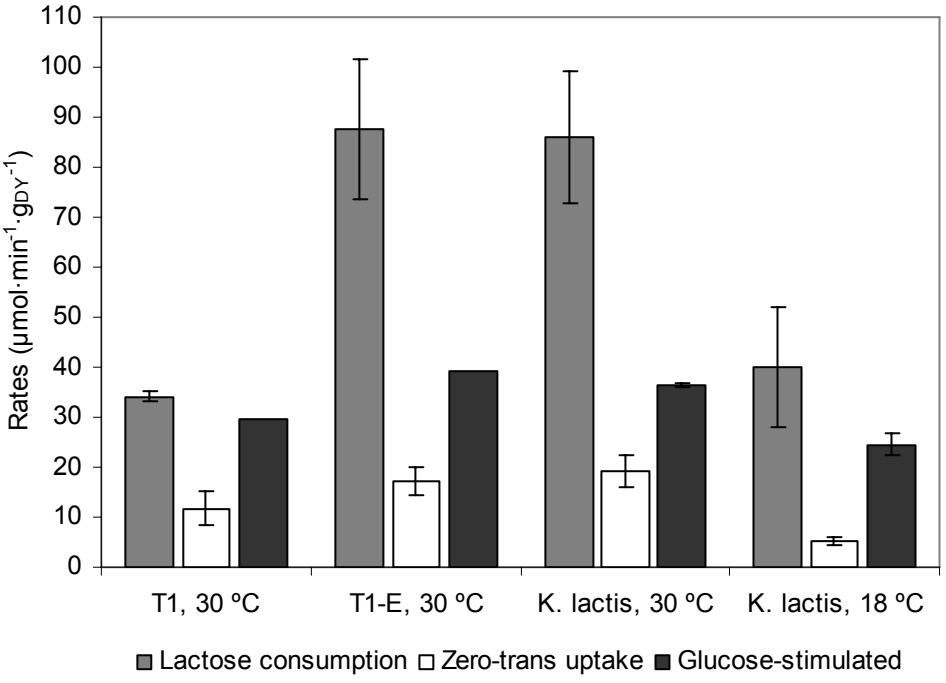

Figure 6.1 – Lactose consumption rates (grey columns) during fermentation for recombinant *S. cerevisiae* strains T1 and T1-E at 30 ºC and for *K. lactis* strain CBS2359 at 30 and 18 ºC. The zero-*trans* uptake rates at 20 mM lactose (white columns) and the glucose-stimulated rates (dark columns) are also shown for direct comparison. The assay temperature (same as in fermentation) is indicated. Lactose consumption rates are the averages with standard deviations (SDs) for 2 – 5 independent fermentations. Zero-*trans* rates are the averages with SDs of 3 – 8 determinations with independently grown yeast suspensions. Glucose-stimulated rates are the zero-*trans* lactose (20 mM) uptake rates obtained after optimal pre-incubation of the yeast suspension with 27 mM glucose (single experiments for T1 and T1-E, and averages with SDs of 2 – 3 independent experiments for *K. lactis*).

6.3.2 Zero-*trans* lactose uptake rates

For uptake assays, the yeasts were harvested from lactose fermentations when the dry yeast concentration was between 0.6 and 1.5 $g \cdot L^{-1}$ (i.e. in the same interval used to calculate the specific rates of lactose consumption shown in Figure 6.1). After washing and suspending in ice-cold tartrate/Tris, pH 4.2, the yeasts were equilibrated to assay temperature and zero-*trans* rates of lactose uptake were determined (Figure 6.2). At 2 mM lactose, zero-*trans* uptake rates were 2- to 3-fold higher at 30 than at 20 ºC. At 30 and 18 ºC, rates were 1.5- to 2-fold higher at 20 mM than 2 mM lactose, which is consistent with K_m values of 1 to 3 mM (Boze *et al.*, 1987; Dickson and Barr, 1983). The zero-*trans* rates at saturating lactose concentration (20 mM) were clearly lower than those of lactose consumption in fermentations at the same temperature (Figure 6.1). The highest difference (7.5-fold) was for *K. lactis* at 18 ºC, while the lowest (2.9-fold) was for T1 at 30 ºC.

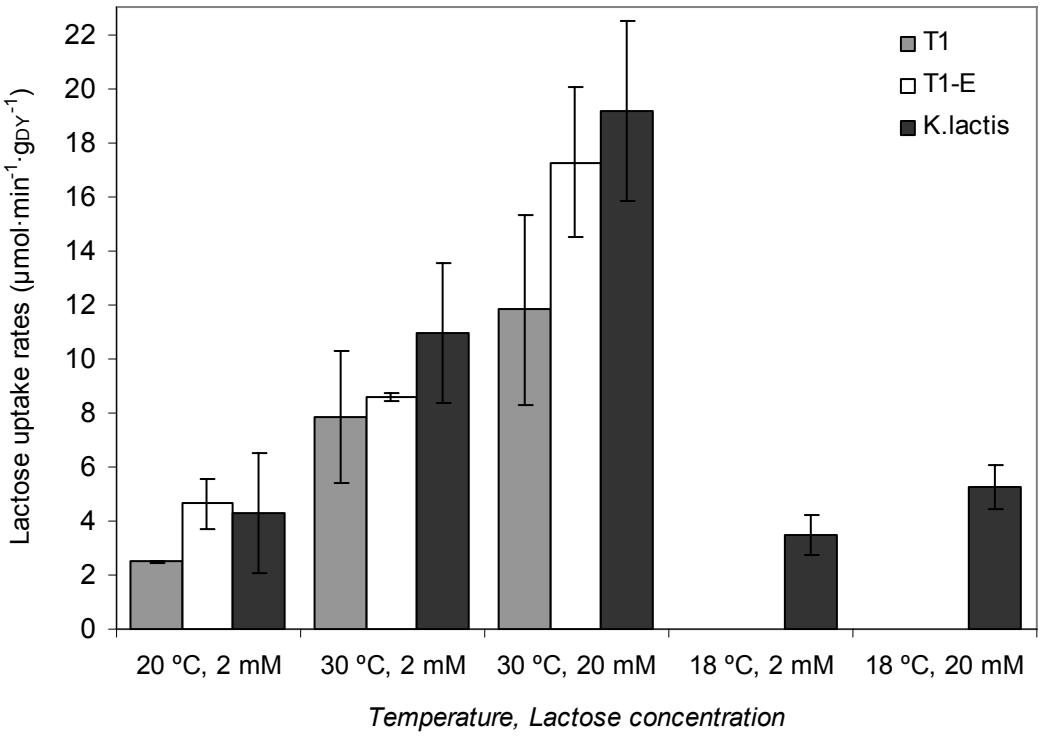

Figure 6.2 – Zero-*trans* lactose uptake rates of recombinant *S. cerevisiae* strains T1 (grey columns) and T1-E (white columns) and *K. lactis* strain CBS2359 (dark columns). The three yeasts were grown at 30 ºC and the rates were measured, by transport assays using ^{14}C-labelled lactose, at 20 ºC with 2 mM lactose and at 30 ºC with 2 and 20 mM lactose, as indicated. *K. lactis* was also grown at 18 ºC and assayed at 18 ºC with 2 and 20 mM lactose. Error bars are the SDs of 2 – 8 determinations with independently grown yeast suspensions.

Eliminating the known differences in experimental conditions between the fermentations and the zero-*trans* assays did not decrease the large differences in their specific rates. For example, when harvested yeast (T1-E) was suspended in and zero-*trans* assays performed in defined mineral medium (pH 4.5) without lactose or in potassium hydrogen phthalate, pH 4.2, the zero-*trans* uptake rates were the same or 20 – 40% smaller than those measured in tartrate/Tris buffer, pH 4.2. Evidently, zero-*trans* uptake rates determined under conditions (temperature, pH, buffer composition and saturating lactose concentration) very close to those in fermentation could not account for the observed lactose consumption rates.

6.3.3 β–galactosidase activity is intracellular

Carvalho-Silva and Spencer-Martins (1990) reported a cell-bound extracellular β-galactosidase activity in some, but not all, *Kluyveromyces marxianus* strains. It was therefore re-investigated whether *K. lactis* strain CBS2359 and the lactose-utilising recombinant *S. cerevisiae* strains showed extracellular β-galactosidase activity, which might account for the discrepancy between zero-*trans* lactose uptake rates and lactose consumption rates. No activity (< 0.05 U·[g fresh yeast]$^{-1}$) was detected in supernatants from the cultivations, showing that β-galactosidase was not excreted to the medium in a stable form. To investigate a possible cell-wall or periplasmic β-galactosidase, spheroplasts were prepared and the activities of β-galactosidase, PGI and invertase were compared in the cell wall/periplasmic and the cytoplasmic fractions (Table 6.1). Cytoplasmic β-galactosidase activity was 21-fold higher in T1-E than T1 (in good agreement with the results shown in chapter 4, section 4.3.6, figure 4.9) and 1.5-fold higher in T1-E than *K. lactis*. The activity of the cytosolic marker enzyme, phosphoglucoisomerase (PGI), in the periplasmic fraction was 7 – 12% of its total (cytoplasm + periplasm) activity, indicating that some spheroplasts broke during preparation. Invertase activity was 2 to 5-fold higher in the cell wall/periplasmic fraction than in the cytoplasm, as expected for this mainly cell wall-bound enzyme (Perlman *et al.*, 1984). For T1 and T1-E, the proportions of total activity found in the periplasmic fractions were lower (1.5 – 2%) for β-galactosidase than for PGI (7 – 10%), indicating that β-galactosidase was intracellular in these recombinant strains. For *K. lactis*, the proportion of β-galactosidase in the periplasmic fraction was slightly higher (15%) than that (12%) of PGI, but the difference was close to experimental error. These results show that the proportions of total β-galactosidase activity outside the cell membrane were very small compared to the intracellular activities for all three yeasts. The β-galactosidase activities shown in Table 6.1 were determined at pH 7.0, and β-galactosidase from *K. lactis* has at least 10-fold smaller activity at pH 4.5 than pH 7.0 (Dickson *et al.*, 1979; Kim *et al.*, 1997; Numanoglu and Sungur, 2004). The absolute amounts of possibly extracellular enzyme activity at the pH during fermentation

(pH < 4.5) were therefore also small compared to the lactose transport activities, and cannot account for the discrepancy between lactose consumption rates and zero-*trans* uptake rates.

Table 6.1 – Enzyme activities and total protein in broken spheroplasts (cytoplasmic fraction) and in the cell wall/periplasmic fraction from the recombinant *S. cerevisiae* strains T1 and T1-E and *K. lactis* strain CBS2359. Except for invertase, results are averages ± SDs of 2 – 4 assays. (The percentages of totals found in the periplasmic fraction are shown in brackets.)

	T1		T1-E		*K. lactis*	
	Cytoplasm	Periplasm	Cytoplasm	Periplasm	Cytoplasm	Periplasm
β-galactosidase ($U \cdot g_{Fresh\ Yeast}^{-1}$)	13.3 ± 0.7	0.2 ± 0.1 (1.5%)	272 ± 19	5.5 ± 1.3 (2.0%)	179 ± 29	32.6 ± 6.0 (15%)
PGI ($U \cdot g_{Fresh\ Yeast}^{-1}$)	261 ± 15	28.1 ± 2.5 (9.7%)	155 ± 7	11.7 ± 1.1 (7.0%)	128 ± 3	17.8 ± 0.4 (12%)
Invertase ($U \cdot g_{Fresh\ Yeast}^{-1}$)	3.7	7.2 (66%)	2.5	11.5 (82%)	0.5	1.9 (79%)
Protein ($mg \cdot g_{Fresh\ Yeast}^{-1}$)	65.4 ± 2.0	63.8 ± 3.0 (49%)	61.6 ± 6.9	61.6 ± 5.4 (50%)	52.3 ± 3.2	70.4 ± 4.6 (57%)

6.3.4 Stimulation of zero-*trans* lactose uptake by incubation of the yeast suspension with glucose or fructose

Because lactose uptake by *Kluyveromyces* yeasts occurs by a proton symport (Barnett and Sims, 1982; Carvalho-Silva and Spencer-Martins, 1990; Dickson and Barr, 1983), it depends on the transmembrane proton motive force that is generated by the plasma membrane H^+/ATPase. The low ATP levels and adenylate energy charge (EC) expected (Ball and Atkinson, 1975) in yeasts harvested before diauxie and then starved, even briefly, might restrict zero-*trans* lactose uptake. Yeast suspensions were therefore pre-incubated with glucose for various times immediately before assaying lactose uptake. At 30 ºC, incubation of *K. lactis* suspensions with 11 or 27 mM glucose caused a rapid (30 s) increase of about 60% in uptake of 20 mM lactose (Figure 6.3). Peak stimulation (about 90%) was seen between 2 and 4 min, followed by a decline over the next 26 min. Without glucose addition, the uptake rate was stable for the first 5 min after the 10 min pre-incubation, and then declined by about 50% over the next 25 min. For the initial concentration of 27 mM glucose, 90% of the glucose in the yeast suspension was consumed within 10 min (Figure 6.4). Fructose (27 mM) had essentially the same effect as glucose, causing a 1.8- to 2-fold stimulation of lactose uptake (30 °C, 20 mM lactose) between 1 and 10 min after its addition to the suspensions (Figure

6.5). Similar results were obtained with the recombinant *S. cerevisiae* strains, T1 and T1-E (stimulations of 2- to 2.3-fold by 2 to 5 min pre-incubation with 11 or 27 mM glucose). The largest stimulation observed was 4.9-fold, for *K. lactis* harvested from lactose fermentation at 18 ºC and assayed at 20 mM lactose and 18 ºC, after pre-incubation with 27 mM glucose.

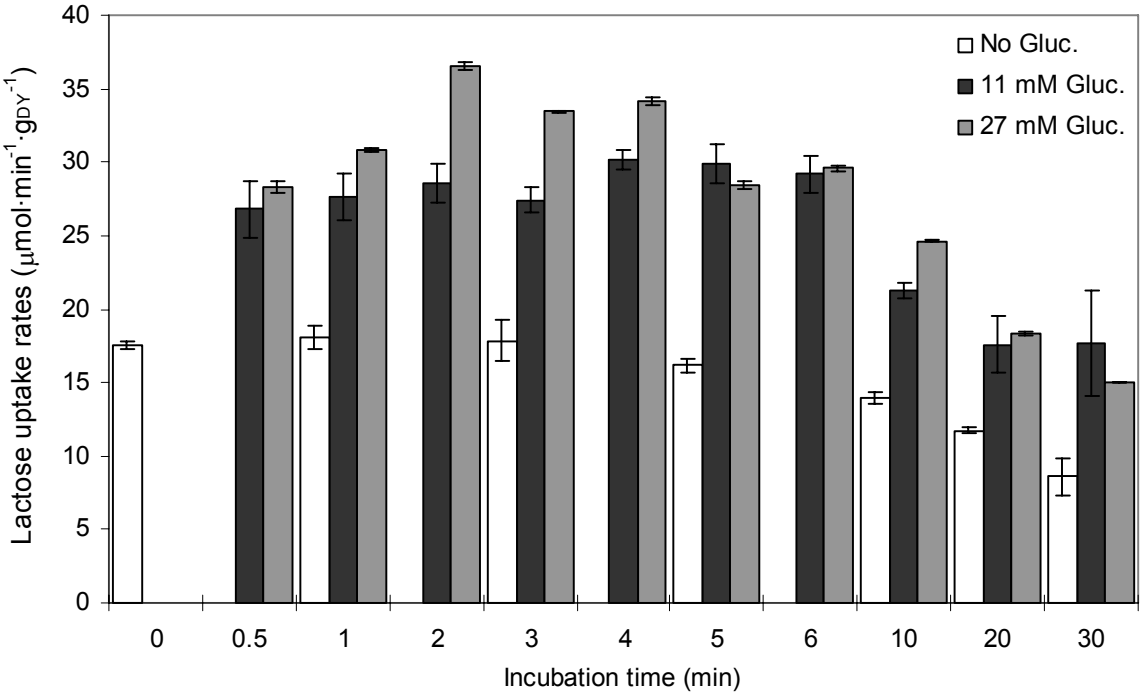

Figure 6.3 – Stimulation of *K. lactis* CBS2359 zero-*trans* lactose uptake rates by pre-incubation of the yeast suspension with glucose. The yeast suspension was first equilibrated for 10 min at 30 °C, after which glucose was added to a final concentration of 11 mM (dark columns) or 27 mM (grey columns). Lactose uptake assays (30 °C, 20 mM lactose) were then performed after further incubation of the suspension at 30 °C for the times indicated. Results of control experiments, without glucose addition, are also shown (white columns). Error bars show the range of duplicate assays.

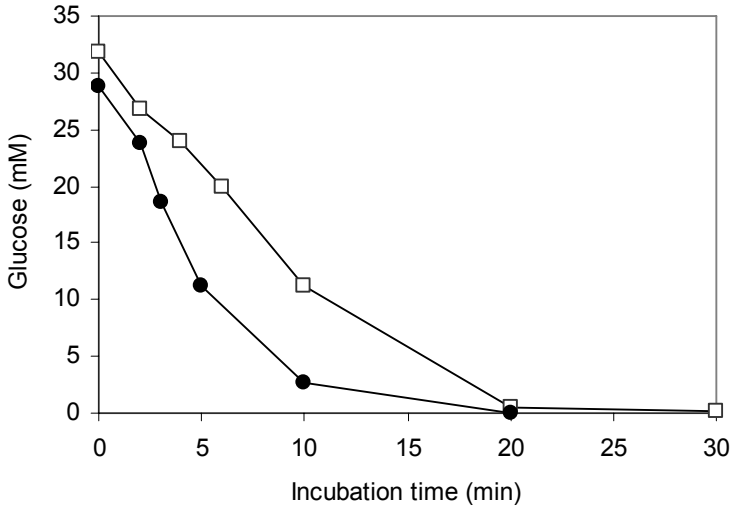

Figure 6.4 – Glucose concentration in the yeast suspension during incubation of *K. lactis* CBS2359 at 30 °C (●) or brewer's yeast A15 at 20 °C (□). Yeast suspension was first equilibrated to the proper temperature (10 min at 30 °C or 5 min at 20 °C) and glucose was then added. Samples from the yeast suspension were taken and immediately filtered through a 0.45 µm membrane. Glucose was quantified in the filtrate.

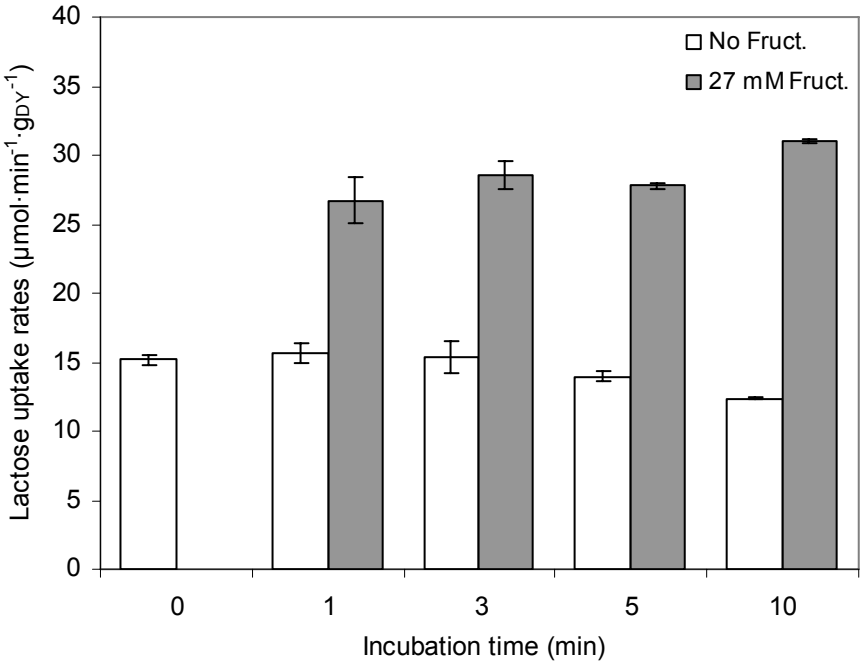

Figure 6.5 – Stimulation of *K. lactis* CBS2359 zero-*trans* lactose uptake rates by pre-incubation of the yeast suspension with fructose. The yeast suspension was first equilibrated for 10 min at 30 °C, after which fructose was added to a final concentration of 27 mM (grey columns). Lactose uptake assays (30 °C, 20 mM lactose) were then performed after further incubation of the suspension at 30 °C for the times indicated. Results of control experiments, without fructose addition, are also shown (white columns). Error bars show the range of duplicate assays.

These 1.6- to 4.9-fold stimulations were not observed when glucose was added to the ^{14}C-lactose solution before addition of the yeast suspension. In this case, instead of pre-incubation with glucose before the uptake assay, the yeasts were exposed to the same final concentrations of glucose (7 or 18 mM) or to higher concentrations (up to 110 mM) only during the 10 s uptake assay. Within experimental error (about ± 15%) no activations were observed with *K. lactis*, T1 or T1-E under these conditions. At 20 mM lactose, inhibition by glucose under these conditions was also quite small (< 20% at 110 mM glucose for *K. lactis*).

For *K. lactis* grown and assayed at 18 ºC, glucose stimulation (5 – 10 min at 27 mM glucose) increased the V_{max} by 4.9-fold (from 5.5 ± 0.6 to 27 ± 2 μmol·min^{-1}·g dry yeast^{-1}; mean ± range of duplicate determinations), and caused a smaller increase in K_m (from 1.0 ± 0.1 to 1.8 ± 0.0 mM; mean ± range of duplicate determinations) (Figure 6.6).

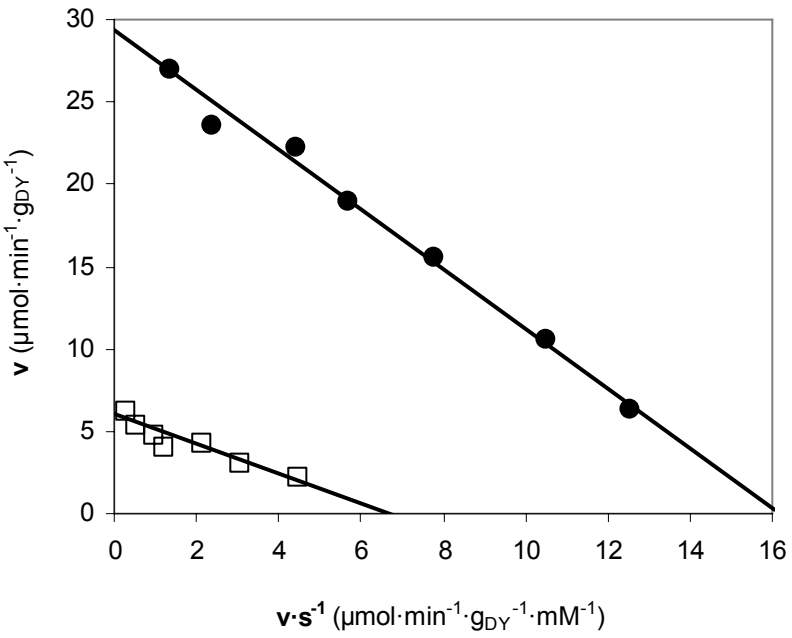

Figure 6.6 – Eadie-Hofstee plots of unstimulated and glucose-stimulated rates of zero-*trans* lactose uptake at 18 ºC by *K. lactis* CBS2359 grown at 18 ºC. Zero-*trans* lactose uptake rates (v) were measured without glucose stimulation (□) and after stimulation with 27 mM of glucose (●) for 5 – 10 min. Lactose concentrations (s) were varied from 0.5 to 20 mM. The trend lines are drawn for the median V_{max} and K_m estimates calculated according to Eisenthal and Cornish-Bowden (1974). Data are from one of two replicate experiments.

The zero-*trans* lactose uptake rates obtained after stimulation with glucose for the optimal time (e.g., about 2 min for *K. lactis* at 30 ºC) are shown in Figure 6.1 (dark columns). For the

S. cerevisiae recombinant T1 at 30 ºC, the glucose-stimulated zero-*trans* rates reached nearly 90% of the lactose consumption rates during fermentation and for *K. lactis* at 18 ºC, they reached 60% of the (average) lactose consumption rates. However, for the recombinant T1-E and for *K. lactis* at 30 ºC, glucose-stimulated zero-*trans* rates were only 45 and 42%, respectively, of the lactose consumption rates.

6.3.5 Stimulation of zero-*trans* maltose uptake by incubation of starved yeasts with glucose

Maltose uptake by *S. cerevisiae* also occurs by a proton symport (Serrano, 1977). Pre-incubation of brewer's yeast suspensions with glucose increased their maltose transport capacity (Figure 6.7). The stimulation (at 20 ºC) was slower than observed for lactose transport by the lactose-utilizing yeasts at 30 ºC, and reached a maximum of 1.8-fold after 10 min. After 10 min, the yeast had consumed 65% of the glucose, and nearly all after 20 min (Figure 6.4). During incubation without glucose, the maltose uptake rate was stable for the first 6 min and showed a small decrease over the next 24 min. Glucose (final concentration in the assay of 18 mM) added to the ^{14}C-maltose solution before addition of the yeast suspension, caused only slight inhibition (about 7%) of the maltose uptake. For a laboratory yeast containing a single maltose transporter encoded by a *MALx1* gene (S150-2B/MALx1; see Materials and Methods), stimulation by glucose increased the V_{max} by about 50%, but had no significant effect on the K_m for maltose (unstimulated and glucose-stimulated K_m values of 4.7 ± 0.3 mM and 4.2 ± 0.8 mM, respectively; averages ± ranges, n=2). For strain S150-2B/MALx1, glucose (at final concentrations of 11 or 28 mM) added to the ^{14}C-maltose solution before the yeast suspension had no effect on the maltose uptake rates.

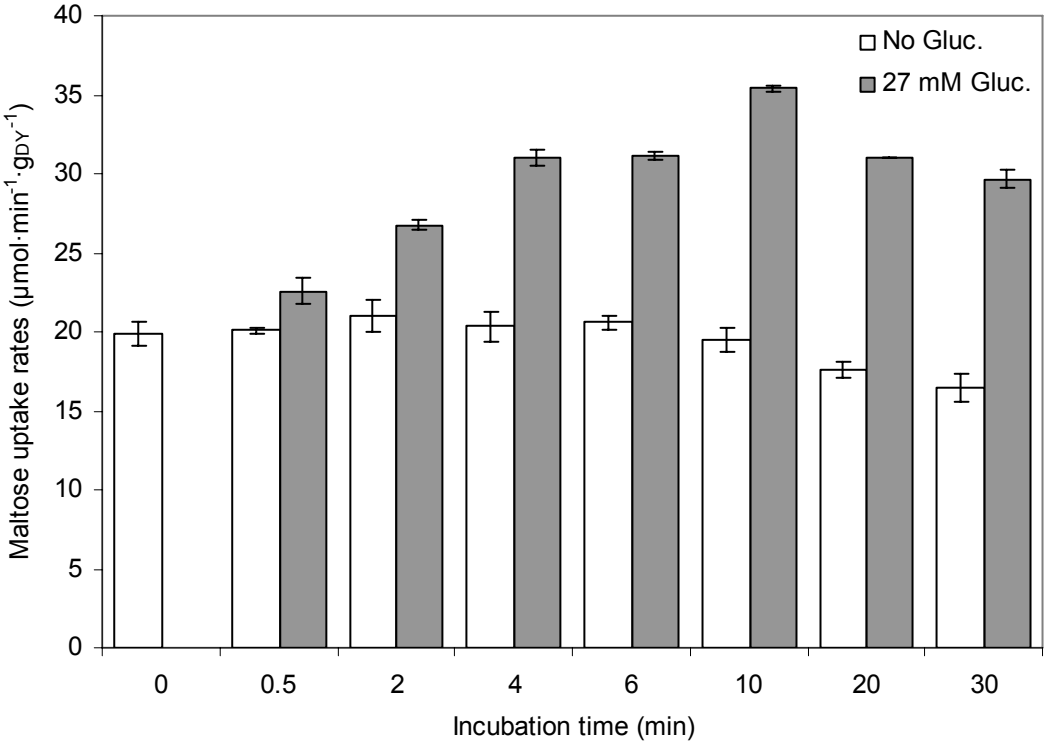

Figure 6.7 – Stimulation of brewer's yeast (strain A15) zero-*trans* maltose uptake rates by pre-incubation of the yeast suspension with glucose. The yeast suspension was first equilibrated for 5 min at 20 °C, after which glucose was added to a final concentration of 27 mM (grey columns). Maltose uptake assays (20 °C, 5 mM maltose) were then performed after further incubation of the suspension at 20 °C for the times indicated. Results of control experiments, without glucose addition, are also shown (white columns). Error bars show the range of duplicate assays.

The maltose transport activity of yeasts harvested during growth on sugars was unstable during storage at 0 ºC, whereas yeasts harvested after diauxie seemed to retain their activity for longer during storage (data not shown). S150-2B/MALx1 cells harvested at OD_{600} 3.6 contained < 1 mg trehalose · [g dry yeast]$^{-1}$, whereas those harvested, after diauxie, at OD_{600} 9.5, contained 8 – 9 mg trehalose · [g dry yeast]$^{-1}$. For S150-2B/MALx1 harvested at OD_{600} 3.5 to 5.0 during growth on glucose (20 g·L^{-1}), maltose transport activity decreased about 3-fold during two days' storage on ice. However, when maltose transport assays were made after pre-incubation with glucose (27 mM for 5 – 8 min at 20 ºC as described above), stored cells were stimulated more (2- to 3.5-fold) than freshly harvested cells (ca. 50%) (Figure 6.8). This suggests that most of the activity loss during storage at 0 ºC is not caused by instability of the transporter molecules, but by changes in the metabolic status of the yeast.

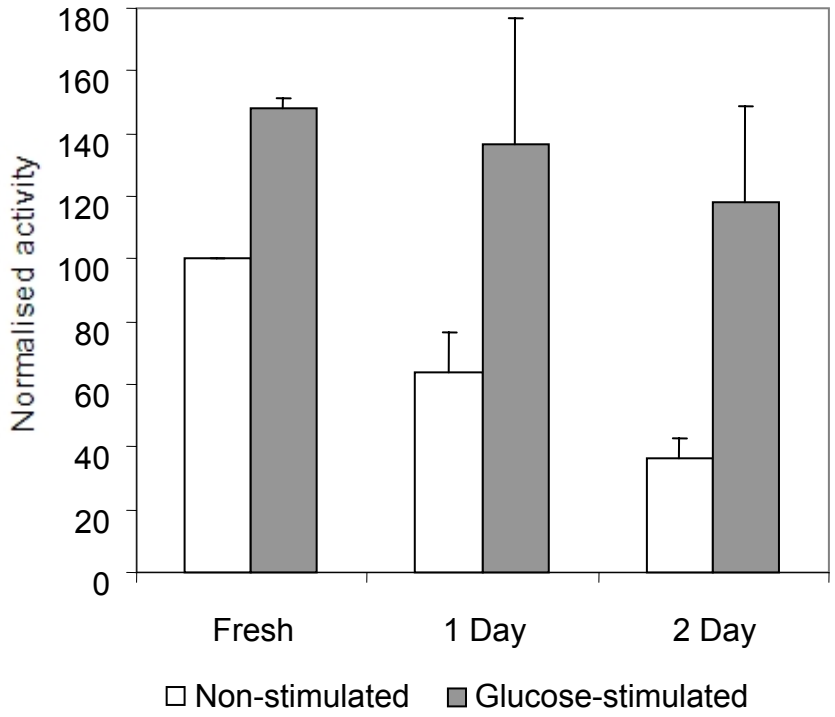

Figure 6.8 – Decay of non-stimulated and glucose-stimulated maltose transport during storage at 0 ^{o}C of S150-2B/MALx1 yeast harvested from YP/2 % glucose during growth on glucose (at OD_{600} of 3.5 – 5). Yeasts were harvested and assayed after about 2.5 h (Fresh) and after storage for 24 h (1 Day) or 48 – 52 h (2 Day). Transport activities were normalised by setting the non-stimulated activity of each fresh yeast to 100. Results are averages ± SDs for 3 independent suspensions of fresh yeast and 2 day old yeast and 2 independent suspensions of 1 day old yeast.

6.3.6 Intracellular adenine nucleotides levels during incubation of starved yeasts with glucose

Intracellular ATP levels (3.8 µmol·[g dry yeast]$^{-1}$) and EC (0.61 to 0.64) were low in the starved *K. lactis* suspensions prepared for zero-*trans* assays. During incubation with 27 mM glucose at 30 ºC, the ATP concentration increased from 3.8 to 7.8 µmol·[g dry yeast]$^{-1}$ in the first 30 s, reached 8.6 µmol·[g dry yeast]$^{-1}$ after 10 min and then fell to 7.8 µmol·[g dry yeast]$^{-1}$ after 20 min, by which time all the glucose was consumed (Figure 6.9). Similarly, EC increased to 0.96 within 30 s and remained above 0.9 for 20 min (but fell to 0.82 at 20 min in a replicate experiment). The ADP level (initially 2.6 µmol·[g dry yeast]$^{-1}$) became very low already after 30 s and did not start recovering until 20 min. The negative ADP values obtained at 0.5 and 10 min result from experimental error in measuring the small difference between

ATP *plus* ADP and ATP alone. Apparently negative ADP values were set to zero for calculation of EC, but have no significant effect on that calculation. AMP also decreased during the first 30 s (from 1.5 to about 0.5 µmol·[g dry yeast]$^{-1}$) and remained low thereafter. The total adenine nucleotide pool (ATP+ADP+AMP) increased a little (from 7.9 to 9.0 µmol·[g dry yeast]$^{-1}$) during the pre-incubation with glucose.

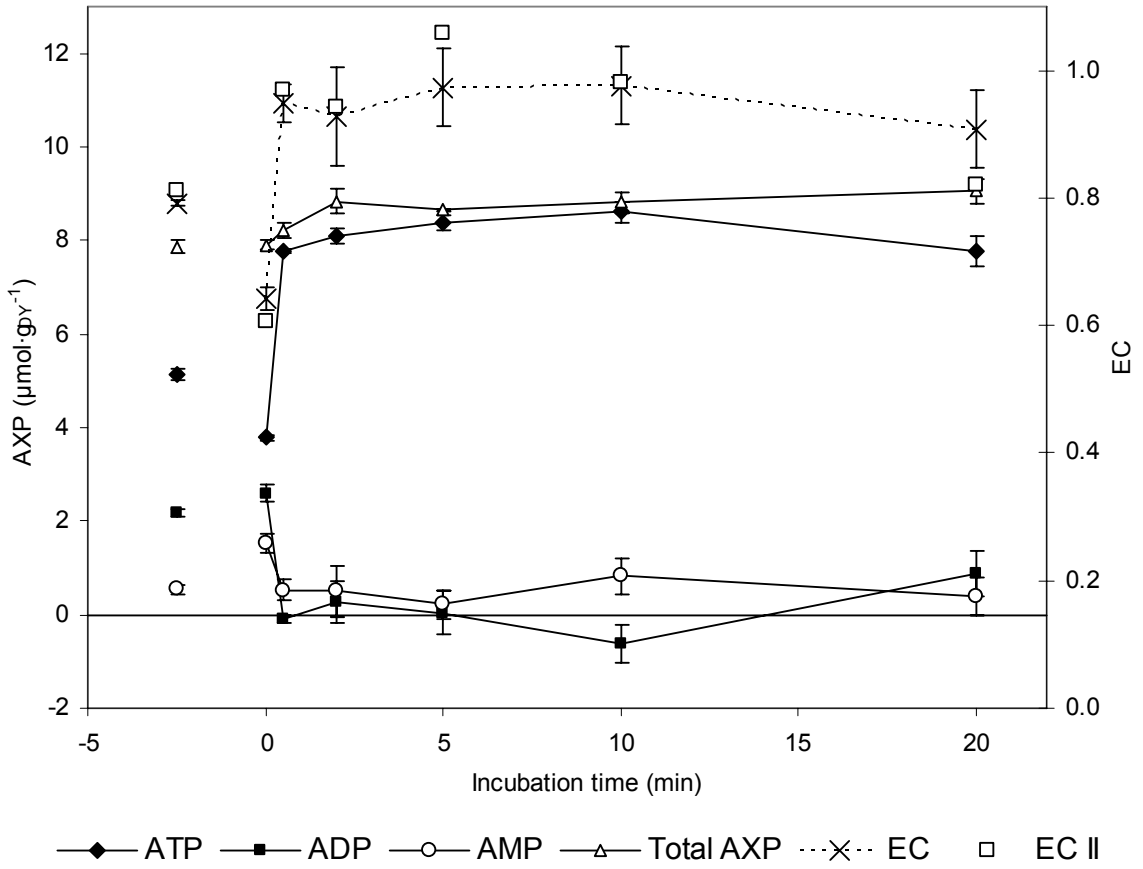

Figure 6.9 – Intracellular adenine nucleotide levels during incubation of a buffered and starved suspension of *K. lactis* CBS2359 with glucose at 30 °C. Portions of the suspension were pre-equilibrated for 10 min at 30 °C, after which glucose was added (at 0 min) to 27 mM. Intracellular adenine nucleotides (AXP; ATP, ADP, AMP) and total adenine nucleotides (Total AXP = ATP + ADP + AMP) were measured and the EC calculated after incubation with glucose for the times indicated and (plotted at -2.5 min) in samples taken from the yeast culture immediately before harvesting. For the incubation with glucose, error bars indicate the ranges between duplicate assays. For the culture, error bars correspond to the ranges between two samples. EC data (EC II) from a replicate experiment using independently grown yeast are also shown.

The adenine nucleotides levels in the culture were also determined, just before harvesting the

yeast (plotted at -2.5 min in Figure 6.9). The EC in the culture was 0.79 – 0.81, while in the buffered yeast suspension equilibrated to 30 °C for 10 min (0 min in Figure 6.9) it was 0.61 – 0.64. In control experiments without glucose addition, incubation of the yeast suspension at 30 °C for a further 5 or 10 min (after the initial 10 min equilibration) lead to a further decrease in EC to 0.39 – 0.47, with ATP decreasing to 1.9 – 2.4 μmol·[g dry yeast]$^{-1}$ (data not shown).

For brewer's yeast, incubation with 27 mM glucose at 20 °C caused similar changes in adenine nucleotide levels (Figure 6.10) as observed for *K. lactis*, except that the changes were slower at the lower incubation temperature (as also was the stimulation of maltose transport; compare Figures 6.3 and 6.7). The main increase in ATP (from 2.6 to 6.0 μmol·[g dry yeast]$^{-1}$) and EC (from 0.52 to 0.9) took place over 2 min, rather than the 30 s seen with *K. lactis* at 30 ºC. For brewer's yeast (at 20 ºC), the ADP level never fell below that of AMP whereas for *K. lactis* (at 30 ºC) the ADP level was below that of AMP (and even undetectable) during the first 10 min of incubation with glucose. With the present data it is not possible to know if this difference is species-dependent or temperature-dependent. During incubation of brewer's yeast suspension at 20 °C without glucose for a further 10 min (after the initial 5 min equilibration to 20 ºC), ATP decreased by 25% and EC fell to 0.4 (data not shown).

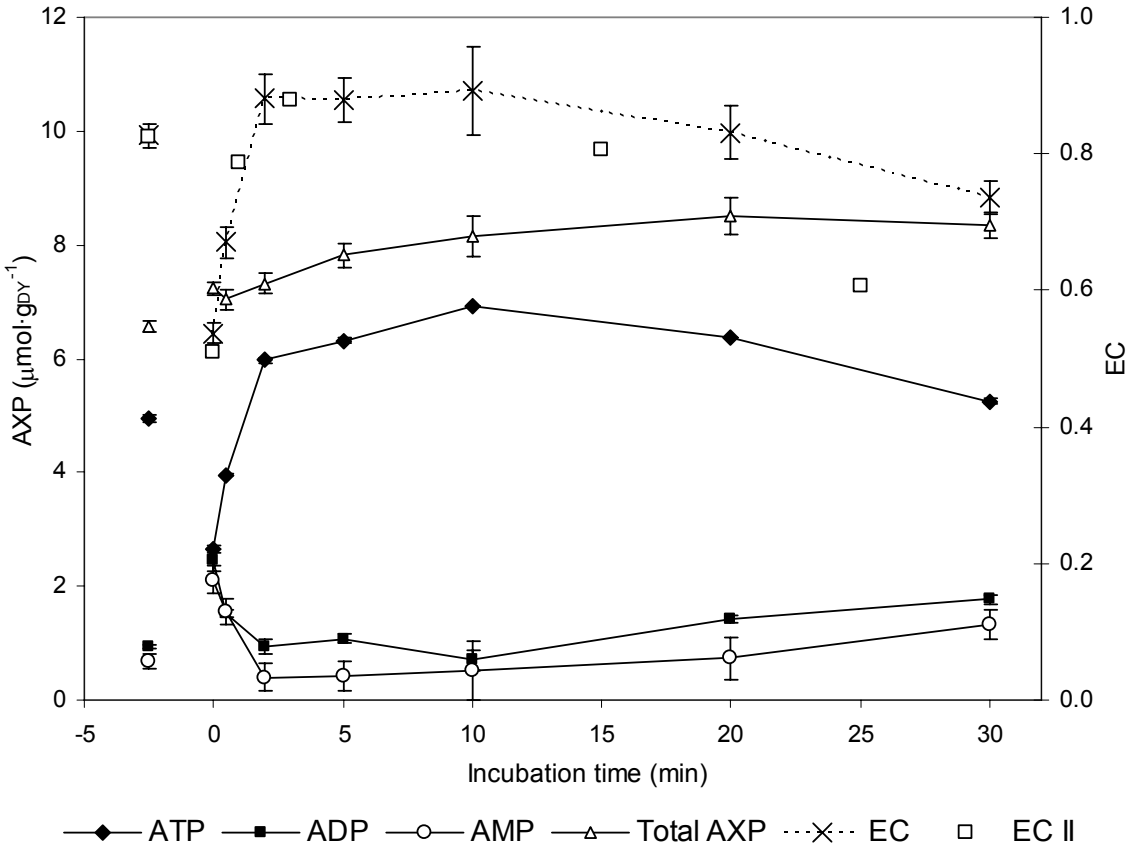

Figure 6.10 – Intracellular adenine nucleotides levels during incubation of a buffered and starved suspension of brewer's yeast (strain A15) with glucose at 20 °C. Portions of the suspension were pre-equilibrated for 5 min at 20 °C, after which glucose was added (at 0 min) to 27 mM. Intracellular adenine nucleotides (AXP; ATP, ADP, AMP) and total adenine nucleotides (Total AXP = ATP + ADP + AMP) were measured and the EC calculated after incubation with glucose for the times indicated and (plotted at -2.5 min) in samples taken from the yeast culture immediately before harvesting. For the incubation with glucose, error bars indicate the ranges between duplicate assays. For the culture, error bars correspond to the ranges between two samples. EC data (EC II) from a replicate experiment using independently grown yeast are also shown.

6.4 DISCUSSION

When yeasts were harvested from sugar fermentations before diauxie and suspended in nutrient-free buffer, short incubations of the starved yeasts with glucose increased their intracellular ATP and adenylate energy charge (EC) and simultaneously increased the V_{max} for active transport of lactose or maltose. For lactose, a relatively smaller increase in K_m also occurred, but for maltose the K_m was unchanged. The lactose transport results (with *K. lactis* and two recombinant strains of *S. cerevisiae*) are consistent with earlier work by Van den Broek *et al.* (1987), who showed that the rate of lactose transport by *K. marxianus* closely correlated with intracellular ATP levels when these were manipulated in various ways, including pre-incubation with glucose (to increase ATP) or antimycin A (to decrease ATP). Also for the glycerol/H^+ symport of *S. cerevisiae* short (20 s to 5 min) pre-incubations of starved cells with glucose increased the V_{max} by about 30% with no change in K_m (Lages and Lucas, 1997; possible changes in adenylate nucleotides were not examined by these authors). However, the results presented here with maltose transport (by a brewer's lager strain) appear to contradict work by Serrano (1977), who found that the rate of maltose transport by *S. cerevisiae* S-13 did not change when intracellular ATP was almost completely (≥ 98 %) depleted by pre-incubation with antimycin A and 2-deoxyglucose. Serrano's (1977) growth and assay conditions were very similar to the ones used in the present work (cells harvested from YP/2 % maltose during growth on maltose, and stored at low temperature in tartrate/Tris, pH 4.2, before assays at 20 ºC in the same buffer). A possible explanation for the apparent discrepancy between Serrano's and this work's results is that Serrano (1977) examined the effect of further decreasing ATP and the EC below the levels in starved yeast suspensions whereas here it was examined the effect of increasing ATP and the EC in such cells to the levels found in actively growing cells. In the experiments described here, increasing the EC from 0.4 to 0.67 had little effect on maltose transport, but increased transport rates occurred when the EC was raised to between 0.74 and 0.9 (Figure 6.11). For lactose transport, the rate did not increase significantly until the EC was above 0.8. Serrano (1977) showed that maltose transport was dependent upon an electrochemical gradient of protons across the cell membrane even when the extracellular concentration of maltose exceeded its intracellular concentration. Therefore, it is suggested that starved yeast cells can maintain an electrochemical gradient of protons sufficient to support a basal level of maltose transport activity even when ATP is depleted, but that increasing the EC to the levels (ca. 0.9) found in actively fermenting cells enhances both the Δp and the transport rate.

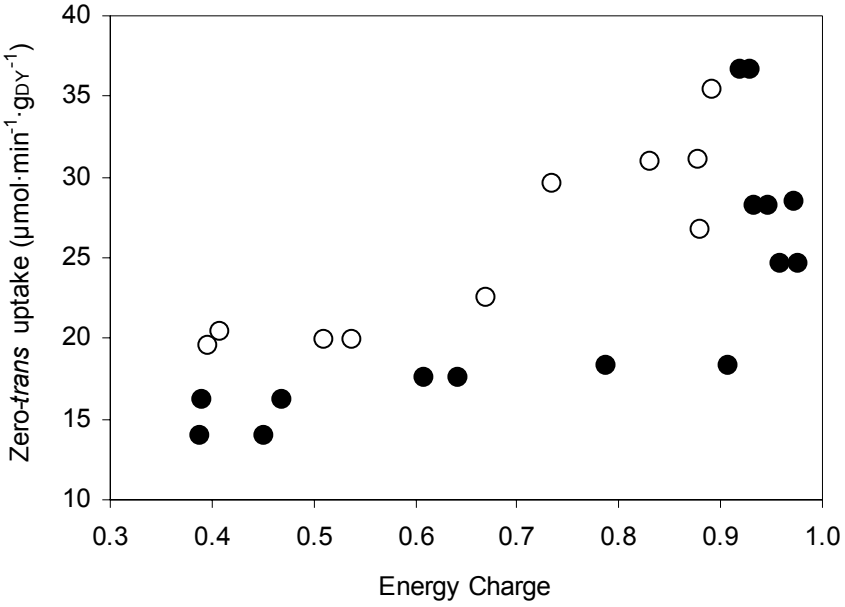

Figure 6.11 – Correlation between the lactose uptake rates in *K. lactis* at 30 ºC (●) or maltose uptake rates in brewer's yeast (strain A15) at 20 ºC (○) and the adenylate energy charge.

In the present work, yeast cells that were growing on lactose or maltose were harvested and suspended in buffer lacking nutrients. Ball and Atkinson (1975) showed that yeast cells growing on glucose were unable to maintain high ATP and EC when transferred to medium without glucose (the EC fell to below 0.6). In contrast, cells harvested after diauxie, when they are growing aerobically on ethanol produced earlier, can maintain EC values between 0.8 and 0.9. The different behaviours may be explained by the accumulation of reserve carbohydrates, glycogen and trehalose, beginning, respectively, just before or at diauxie (Lillie and Pringle, 1980) and the appearance of oxidative phosphorylation. Probably a drop in ATP and EC cannot be avoided when attempting to measure the activity of sugar/H^+ symports in yeast growing on the same sugar, because residual sugar must be removed from the cell suspensions before carrying out zero-*trans* uptake assays. Short (2 min) aeration of yeast cells before zero-*trans* assays has been used (Diderich *et al.*, 1999), but is not likely to restore cytosolic ATP levels in repressed cells harvested during growth on sugars and lacking reserve carbohydrates and a functional electron transport chain. Treatments to restore ATP and EC levels must be designed to avoid both biosynthesis of new transporter molecules and inactivation of existing transporters. It is known that maltose transporters are subject to glucose-triggered catabolite inactivation (see, e.g., Lucero *et al.*, 1993; Lucero *et al.*, 2002; Medintz *et al.*, 1996). The present method (5 – 10 min incubation with ≤ 27 mM glucose) will not induce synthesis of new disaccharide transporters and is fast enough to avoid extensive

catabolite inactivation. Typically, maltose transporters disappear with a half life of 1.3 h when exposed to 100 mM glucose at 30 ºC (Lucero *et al.*, 1993), corresponding to < 10% loss of activity in 10 min.

The impetus for the present work was the finding that zero-*trans* uptake rates of lactose in *K. lactis* and in lactose-consuming *S. cerevisiae* recombinants were too small (by factors of 3 to 8) to account for the lactose consumption rates observed during shake-flask fermentations. Others have reported similar discrepancies. For example, Alves-Araújo *et al.* (2007) found a V_{max} for zero-*trans* maltose uptake of 0.66 nmol·s^{-1}·[mg dry yeast]$^{-1}$ compared to an estimated maltose consumption rate of 1.8 ± 0.3 nmol·s^{-1}·[mg dry yeast]$^{-1}$. Even for the facilitated transport of glucose, 5 s zero-*trans* assays were inhibited when the level of cytosolic ATP was decreased by respiratory inhibitors (Walsh *et al.*, 1994b). In this case, the mechanism cannot be a decrease in Δp and is thought to be inhibition by intracellular glucose, which accumulates at low ATP levels. No inhibition was seen when reaction times were short enough (200 ms) to prevent significant accumulation of glucose.

No evidence was found for extra-cellular β-galactosidases that might resolve the discrepancy between the rates of lactose consumption and zero-*trans* uptake. Making the zero-*trans* reaction mixtures closer to the composition of the fermentation medium did not increase the zero-*trans* rates. Stimulation of zero-*trans* uptake by pre-incubation with glucose nearly resolved the discrepancy for the slower lactose-fermenting recombinant *S. cerevisiae*, T1, where the glucose-stimulated zero-*trans* rate was 29.8 ± 0.2 µmol·min^{-1}·[g dry yeast]$^{-1}$ and the lactose consumption rate was 34 ± 1.0 µmol·min^{-1}·[g dry yeast]$^{-1}$ (Figure 6.1). Similarly, for *K. lactis* at 18 ºC, the glucose-stimulated zero-*trans* rate (24.6 ± 2.3 µmol·min^{-1}·[g dry yeast]$^{-1}$) was close to the error limits of the lactose consumption rate during fermentation (40 ± 12 µmol·min^{-1}·[g dry yeast]$^{-1}$). However, for the faster fermenting recombinant, T1-E, and for *K. lactis* at 30 ºC, the glucose-stimulated zero-*trans* rates were still markedly lower (2.2- to 2.4-fold) than the observed rates of lactose consumption (Figure 6.1). At the high transport rates involved (80 to 100 µmol·min^{-1}·[g dry yeast]$^{-1}$) the 10 s zero-*trans* assays were possibly too long to measure true initial rates (at least for *K. lactis*; see Materials and Methods). Assays by rapid reaction methods (e.g., 200 ms; Walsh *et al.*, 1994b) are needed to determine whether the true initial rates can account for the observed rates of lactose consumption under these conditions, or whether there is still some unknown factor involved in lactose uptake.

The increases in K_m values for lactose caused by glucose-stimulation were relatively small and have little practical consequence for industrial fermentations, where the concentrations of lactose (or maltose) are much greater than their K_m values. The lactose K_m of about 2 mM corresponds to 0.7 g·L^{-1} and the maltose K_m of about 5 mM to 1.8 g·L^{-1}, which are low concentrations from an industrial view point, met only at the end of fermentations. On the

other hand, the increases in V_{max} values translate directly into increased rates at all lactose or maltose concentrations.

Rautio and Londesborough (2003) reported close agreement between the specific rates of maltose consumption and zero-*trans* uptake assays (measured with each day's yeast in each day's wort) during the early and middle stages of fermentations of brewer's wort by brewer's yeast, and concluded that maltose uptake was the dominant factor controlling the rate of maltose consumption under these conditions. In the final stages of these fermentations, the specific rates of maltose consumption were up to 50% lower than those determined by zero-*trans* assays, indicating that other factors also exerted significant control over the fermentation rate. The present results (see Figure 6.7) suggest that the maximum maltose uptake rates were probably about 60% higher than estimated by the zero-*trans* assays of Rautio and Londesborough (2003), so that also in early and mid fermentation the rate of maltose consumption was limited by other factors as well as transport. The importance of maltose transport to the speed of wort fermentations is shown by the acceleration obtained when maltose transport capacity is increased (Kodama *et al.*, 1995; Vidgren *et al.*, unpublished results).

In conclusion, the results reported in this chapter show that zero-*trans* uptake assays with yeast samples harvested from sugar fermentations and then washed and starved before assay, can seriously underestimate the capacity of sugar/H^+ symports. Short pre-incubation with moderate concentrations of glucose provides a quick way, allowing little possibility for new synthesis or degradation of transporters, to approach more closely the sugar/H^+ symport capacity of the actively metabolising cells.

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CHAPTER 7

CONCLUSIONS AND FUTURE PERSPECTIVES

This thesis focused on the yeasts used in two alcoholic fermentation processes: primary brewing fermentation and fermentation of lactose (particularly lactose derived from cheese whey) to ethanol using engineered *Saccharomyces cerevisiae* strains.

The work with brewer's yeast was developed in the perspective of increasing the knowledge of the physiology of the yeast under the specific environment of brewery fermentations, where the yeast experiences constantly changing conditions. The studies done addressed two specific issues that had not yet been effectively studied: first, the effect of yeast lipid composition on the activity of the maltose transporters and second, the yeast energetic condition throughout the different phases of brewing fermentations.

It is often stated that in some phases of brewery fermentations the low unsaturated fatty acid (UFA) and sterol contents of the plasma membrane prevent the proper function of sugar transporters, though a direct demonstration that the sterol or fatty acid composition of the yeast plasma membrane affects the function of sugar transporters could not be found in the literature. The results reported in chapter 2 directly demonstrate for the first time that the lipid composition of brewer's yeast affects the activity of maltose transporters. These results show that under growth conditions that were strictly anaerobic but strongly induced expression of *Malx1* genes, the maltose transport activity of the yeast was markedly increased (at least 2- to 3-fold) by supplementation of the medium with UFA plus ergosterol. Moreover, the temperature-dependencies of maltose transport were greater for non-supplemented cells and cells supplemented with UFA alone than for cells supplemented with UFA plus ergosterol. Supplementation with UFA alone was not enough to maintain the maltose transport activity of growing cells and actually decreased the transport activity of stationary phase cells. Therefore, it can be concluded that either maltose transporter molecules cannot be properly inserted into plasma membranes that do not contain adequate amounts of sterol or that inserted transporter molecules require sterol molecules in order to fulfil their catalytic role. The results of the temperature-dependencies of maltose transport suggest that the catalytic function of transporter molecules in the plasma membrane was dependent on the lipid composition, in particular the sterol content, of the plasma membrane.

Maltose transport capacity is known to be regulated by glucose-repression and maltose-induction of the genes encoding maltose transporters and also by glucose-triggered catabolite inactivation of existing maltose transporter proteins. The results in chapter 2 emphasise a third factor, namely the lipid composition, in particular the sterol content of the plasma membrane, showing that proper function of maltose transporters requires adequate amounts of ergosterol in the yeast. This effect has consequences on brewery fermentations and may partly explain the low maltose (and maltotriose) uptake rates at the beginning of fermentation

and during the second half of the fermentation. In particular, the daughter cells formed in the last round of cell division may be very deficient in sterols because, although their membranes may receive some sterol from the mother cell, any size increase after separation of mother and daughter occurs in an anaerobic environment where no new sterol can be made.

The lager yeast studied may express several functional maltose transporters (*MAL11*, *MAL21*, *MAL31*, *MAL41*, *MTT1*; see chapter 2). It may be interesting to investigate the effect of the sterol content of the plasma membrane on the activity of individual maltose transporters (e.g. using genetically modified strains expressing only one of the maltose transporters, such as the strain S150-2B/*MALx1* used in chapter 6). In particular, it may be of interest to compare the effect of sterol composition in lager and ale strains, where maltose is believed to be predominantly transported by *MALx1*- and *AGT1*-encoded permeases, respectively. The effect of lipid composition in maltotriose transport, a process very relevant in the later stages of brewery fermentations, should also be studied.

There are few reports concerning the energetic state of brewer's yeast during wort fermentations. A methodology was developed and used to measure intracellular and extracellular adenine nucleotides throughout the different phases of wort fermentations under conditions similar to those in a brewery. Thereby, the adenylate energy charge (EC) of the yeast was calculated through fermentations of high- and very high-gravity (VHG) worts by two lager yeast strains. The results reported in chapter 3 show that ATP was the dominant intracellular adenine nucleotide, while AMP was the dominant extracellular adenine nucleotide. Larger extracellular AMP amounts correlated with lower viability of the yeast cropped at the end of fermentations. A possible origin for the extracellular AMP is the hydrolysis of ATP released from dead cells, though the present data can not distinguish between this hypothesis and the possibility that AMP, but not ATP and ADP, can cross the membrane of viable yeast cells.

In brewing fermentations, metabolism is almost entirely fermentative so that ATP must be consumed at the same rate as it is generated by glycolysis. The intracellular EC of the yeasts was high (>0.85) from the start of fermentation until after the yeasts stopped growing and began to sediment. Thus, cessation of growth did not result from a decrease in EC caused by diversion of increasing amounts of ATP to maintenance tasks. The EC was also independent of ethanol concentration up to at least 85 g ethanol·L^{-1}; ethanol stress of this magnitude did not cause maintenance demands that exceeded the yeast's ability to generate ATP at a high EC. This was observed both for laboratory-grown yeast and for industrially cropped yeast that had much lower contents of unsaturated fatty acids and sterols. Up to apparent attenuations of 75 – 80% (in worts with an apparent attenuation limit of 86%) and ethanol concentrations

over 80 $g \cdot L^{-1}$, the yeast cells did not seem to be limited by energetic constraints, and from this point of view would still be able to respond to environmental changes by transcription of new mRNA species and translation of new proteins. Only when specific fermentation rates fell to ≤0.01 g ethanol·[g dry yeast]$^{-1} \cdot h^{-1}$ did the EC values collapse to around 0.5 to 0.6. At this point, evidently, the rate of ATP synthesis was too low to maintain a high EC. At this stage of fermentation, cell viability begins to drop, suggesting that ATP-requiring maintenance reactions become too slow to keep the cells alive.

These results have some consequences for brewery practices. At the end of high-gravity wort fermentations most of the maltose has been consumed, but some remains, together with much larger amounts of maltotriose (typically, maltotriose remains in the range 2 – 10 $g \cdot L^{-1}$ and even more after VHG fermentations). Maltotriose and maltose transporters are proton symporters, which require an energised membrane and transmembrane proton gradient maintained by the ATP-consuming proton pump. Uptake of these α-glucosides and maintenance of a high EC are mutually dependent processes. Below a certain rate of ethanol production (under the conditions reported in chapter 3, about 0.01 g ethanol·[g dry yeast]$^{-1} \cdot h^{-1}$), glycolytic ATP may be produced too slowly to maintain a high EC, resulting in a decreased electrochemical gradient across the plasma membrane, which further slows the uptake of α-glucosides and further decreases the EC, with a catastrophic positive feedback effect. The results in chapter 3 illustrate the rapidity of this effect, emphasising the importance of cropping yeast for recycling to subsequent fermentations (the usual brewery practice) as soon as possible, while the beer still contains adequate concentrations of fermentable sugars. The results further suggest that lager yeast strains might tolerate still higher ethanol concentrations (>85 $g \cdot L^{-1}$), provided that adequate amounts of readily fermentable sugars are still available (or are added) in late fermentation.

The results described in chapter 3 were obtained with two lager strains that, overall, presented similar patterns of EC changes throughout fermentation. It would be interesting to compare these results with results obtained with ale brewing strains. Another intriguing question relates to what is the yeast doing with the ATP available in the later stages of fermentation, when the demand of growth for ATP disappears but the EC is still high. It would be interesting for example to study if there is still considerable synthesis of new proteins during late fermentation and, if so, what kind of proteins are being preferentially synthesised by the yeast.

The work on lactose fermentation dealt with the improvement and characterization of *S. cerevisiae* recombinant strains for efficient lactose to ethanol fermentation processes. A new strain with great potential was obtained using a simple evolutionary engineering approach and

its performance was tested in batch fermentations using high concentrations of lactose.

A flocculent lactose-consuming *S. cerevisiae* recombinant had been constructed in previous work, which involved the transfer of a 13 kb *K. lactis* genomic sequence that included the genes *LAC4* (β-galactosidase) and *LAC12* (lactose permease) as well as their intergenic region, an unusually large intergenic region that works as a promoter for the divergent transcription of both genes. This original recombinant (strain T1) was able to grow on lactose, but its lactose fermentation and flocculation performances were rather poor. It was therefore subjected to an evolutionary engineering process (serial transfer/dilution in lactose media), designed to keep the recombinant growing in lactose for many generations (>120), as well as to select for flocculent cells, as described in chapter 4. This experiment yielded an evolved recombinant (strain T1-E) that consumed lactose 2-fold faster producing 30% more ethanol, and that flocculated earlier and formed bigger flocs than T1.

A series of physiological and genetic studies, reported in chapter 4, were done to compare the original recombinant and the evolved strain. Two molecular events that targeted the *LAC* construct in the evolved strain were identified: (1) a 1593 bp deletion in the intergenic region (promoter) between *LAC4* and *LAC12*, and (2) a decrease of the plasmid copy number by about 10-fold compared to the original recombinant. The results suggest that the intact promoter (endogenous *K. lactis* promoter) was unable to mediate induction by lactose of the transcription of *LAC4* and *LAC12* in the original recombinant T1, whereas the deletion identified established transcriptional induction of both genes in the evolved recombinant T1-E. Thus, it was proposed that tuning of the expression of the heterologous *LAC* genes in the evolved recombinant was accomplished by interplay between decreased copy number of both genes and different levels of transcriptional induction for *LAC4* and *LAC12*, resulting from the changed promoter structure. The experiments described in chapter 4 illustrate the usefulness of simple evolutionary engineering approaches in the improvement of genetically engineered strains that display poor efficiency.

The evolved recombinant strain, T1-E, was tested in batch fermentations with mineral media containing concentrations of lactose up to 200 $g \cdot L^{-1}$ (reported in chapter 5). At initial concentrations up to 150 $g \cdot L^{-1}$, lactose was consumed rapidly and completely in both well aerated and micro-aerated batch fermentations. The maximum ethanol titre reached was 8% (v/v) and the highest ethanol productivity was 1.5 – 2 $g \cdot L^{-1} \cdot h^{-1}$, which is higher than reported with other lactose-consuming recombinant *S. cerevisiae* strains. The evolved strain was also able to ferment 3-fold concentrated cheese whey (about 150 $g \cdot L^{-1}$ lactose), consuming nearly all lactose (residual lactose < 3 $g \cdot L^{-1}$) and producing about 7% (v/v) ethanol with a productivity of 0.46 g of ethanol$\cdot L^{-1} \cdot h^{-1}$. This yeast is the most efficient lactose-fermenting *S. cerevisiae*

recombinant strain reported in the literature, providing an interesting alternative for the fermentation of lactose-based media, such as cheese whey or whey permeate. The strain is highly flocculent, a property that makes it particularly suitable for application in high cell density fermentation systems.

Future work with T1-E may involve the exploitation of its highly flocculent phenotype for the development of a high cell density continuous fermentation process for the production of ethanol from demineralised concentrated whey or whey permeate. Such a process could result in much higher ethanol productivity than obtained in batch systems. High-lactose whey may be supplemented with cheap nutrient sources (e.g. corn steep liquor), which may benefit yeast performance and consequently process efficiency. Another line of work may involve efforts further to improve the ethanol production capacity of T1-E. The strain has not been able to produce an ethanol titre above 8% (v/v), which suggests that it has low tolerance to ethanol. The use of evolutionary engineering strategies may allow the selection of mutants of T1-E with enhanced ethanol tolerance.

Finally, chapter 6 described studies of lactose transport by *K. lactis* (strain CBS2359) and the two *S. cerevisiae* lactose-consuming recombinants (T1 and T1-E) as well as maltose transport by brewer's yeast. The impetus for these studies was the finding that, for all three lactose-consuming yeasts investigated, zero-*trans* uptake rates of lactose, measured by standard methodology, were too small (by factors of 3 to 8) to account for the lactose consumption rates observed during shake-flask fermentations. This standard methodology uses cell suspensions harvested and stored at low temperature (0 to 5 ºC) in nutrient-free buffer and, therefore, starved before they are assayed using radiolabeled sugar. The results reported in chapter 6 suggest that the electrochemical proton potential that drives transport through sugar/H^+ symports is significantly lower in these starved yeast suspensions than in actively metabolising cells, and, therefore, that the results of the zero-*trans* assays with such starved yeast suspensions can seriously underestimate the capacity of sugar/H^+ symports. This emphasises the need to interpret carefully the results of zero-*trans* uptake assays, which, in contrast to this work's observations, are frequently considered to provide a good estimate of the transport capacity of cells under cultivation conditions. A short incubation (1 to 7 min) with glucose (10 to 30 mM) increased the low intracellular ATP and adenylate energy charge characteristic of starved cells to the levels found in actively fermenting yeast cells, and simultaneously increased the activity (the V_{max} value) of the lactose or maltose transporters by factors of 1.5 to 5. This glucose stimulation method provides a quick way, allowing little possibility for new synthesis or degradation of transporters, to approach more closely the sugar/H^+ symport capacity of the actively fermenting cells.

Despite being the most studied of the traditional yeast fermentation processes, the accumulated knowledge of yeast activity in brewing fermentations is still limited, and thus, there is plenty of room for studies like those described in this thesis, which, to my point of view, must unite the efforts and interests of academia and industry. In fact, research efforts on the activity of yeasts under the specific environments found in industrial alcoholic fermentation processes, are likely to produce developments in both fundamental aspects of yeast physiology and applied aspects with implications for industrial fermentation practices. The topic of lactose fermentation has occupied biotechnologists at least for the last 40 years, and industrial plants for ethanol production from whey have been established in some countries. Nevertheless, it is still an attractive area from the industrial point of view, as illustrated by the recent announcement that a German dairy company (Müllermilch) is building a plant near Dresden to produce 10 million litres of bio-ethanol per year from dairy by-products. This substantiates the importance from an applied standpoint of developing better lactose-fermenting microorganisms, such as the recombinant *S. cerevisiae* strain reported in this thesis.