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Virve Vidgren

Maltose and maltotriose transport into ale and lager brewer's yeast strains

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Abstract

Maltose and maltotriose are the two most abundant sugars in brewer's wort, and thus brewer's yeast's ability to utilize them efficiently is of major importance in the brewing process. The increasing tendency to utilize high and very-high-gravity worts containing increased concentrations of maltose and maltotriose renders the need for efficient transport of these sugars even more pronounced. Residual maltose and maltotriose are quite often present especially after high and very-high-gravity fermentations. Sugar uptake capacity has been shown to be the rate-limiting factor for maltose and maltotriose utilization. The aim of the present study was to find novel ways to improve maltose and maltotriose utilization during the main fermentation.

Maltose and maltotriose uptake characteristics of several ale and lager strains were studied. Genotype determination of the genes needed for maltose and maltotriose utilization was performed. Gene expression and maltose uptake inhibition studies were carried out to reveal the dominant transporter types actually functioning in each of the strains. Temperature-dependence of maltose transport was studied for ale and for lager strains as well as for each of the single sugar transporter proteins Agt1p, Malx1p and Mtt1p. The *AGT1* promoter regions of one ale and two lager strains were sequenced by chromosome walking and the promoter elements were searched for using computational methods.

The results showed that ale and lager strains predominantly use different maltose and maltotriose transporter types for maltose and maltotriose uptake. Agt1 transporter was found to be the dominant maltose/maltotriose transporter in the ale strains whereas Malx1 and Mtt1-type transporters dominated in the lager strains. All lager strains studied were found to possess an *AGT1* gene encoding a truncated polypeptide unable to function as maltose transporter. The ale strains

were observed to be more sensitive to temperature decrease in their maltose uptake compared to the lager strains. Single transporters were observed to differ in their sensitivity to temperature decrease and their temperature-dependence was shown to decrease in the order Agt1≥Malx1>Mtt1. The different temperature-dependence between the ale and lager strains was observed to be due to the different dominant maltose/maltotriose transporters ale and lager strains possessed. The *AGT1* promoter regions of ale and lager strains were found to differ markedly from the corresponding regions of laboratory strains and instead were similar to corresponding regions of *S. paradoxus*, *S. mikatae* and natural isolates of *S. cerevisiae*. The ale strain was found to possess an extra MAL-activator binding site compared to the lager strains. This could, at least partly, explain the observed differential expression levels of *AGT1* in the ale and lager strains studied. Moreover, the *AGT1*-containing *MAL* loci in three *Saccharomyces sensu stricto* species, *i.e. S. mikatae*, *S. paradoxus* and the natural isolate of *S. cerevisiae* RM11-1a were observed to be far more complex and extensive than the classical *MAL* locus usually described in laboratory strains.

Improved maltose and maltotriose uptake capacity was obtained with a modified lager strain where the *AGT1* gene was repaired and placed under the control of a strong promoter. Integrant strains constructed fermented wort faster and more completely, producing beers containing more ethanol and less residual maltose and maltotriose. Significant savings in the main fermentation time were obtained when modified strains were used. In high-gravity wort fermentations 8-20% and in very-high-gravity wort fermentations even 11–37% time savings were obtained. These are economically significant changes and would cause a marked increase in annual output from the same-size of brewhouse and fermentor facilities.

Preface

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Espoo, December 2010

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List of publications

This thesis is based on the following original publications, referred to in the text by their Roman numerals I–IV.

I Vidgren, V., Ruohonen, L., and Londesborough, J. 2005. Characterization and functional analysis of the *MAL* and *MPH* loci for maltose utilization in some ale and lager yeast strains. *Appl. Environ. Microbiol.* 71: 7846–7857.

II Vidgren, V., Huuskonen, A., Virtanen, H., Ruohonen, L., and Londesborough, J. 2009. Improved fermentation performance of a lager yeast after repair of its *AGT1* maltose and maltotriose transporter genes. *Appl. Environ. Microbiol.* 75: 2333–2345.

III Vidgren, V., Multanen, J.-P., Ruohonen, L., and Londesborough, J. 2010. The temperature dependence of maltose transport in ale and lager strains of brewer's yeast. *FEMS Yeast Res.* 10: 402–411.

IV Vidgren, V., Kankainen, M., Londesborough, J. and Ruohonen, L. Identification of regulatory elements in the *AGT1* promoter of ale and lager strains of brewer's yeast. Submitted to *Yeast* 2010.

List of abbreviations

AA	apparent attenuation
ADP	adenosine diphosphate
AGT1	transporter gene (alpha-glucoside transporter)
ATP	adenosine triphosphate
BGL2	beta-glucanase gene
BLASTN	basic local alignment search tool nucleotide
bp	base pair(s)
Can1	arginine transporter, confers canavanine resistance
CE	current apparent extract
Chr	chromosome
CoA	coenzyme A
COMPASS	complex proteins associated with Set1
DNA	deoxyribonucleic acid
FSY1	fructose transporter gene, fructose symport
Fur4	uracil permease, 5-fluorouracil sensitivity
GAL	galactose (utilization)
GMO	genetically modified organism
HG	high-gravity
Hxt	hexose transporter
IPR	intellectual property rights
K_m	Michaelis-Menten constant

MAL	maltose (utilization)
MEL	melibiose (utilization)
Mig1	multicopy inhibitor of *GAL1* promoter
MPH	transporter gene (maltose permease homologue)
mRNA	messenger ribonucleic acid
MTT1	transporter gene (mty1-like transporter)
MTY1	transporter gene (maltotriose transport in yeast)
NCBI	National Center for Biotechnology Information
OE	original extract
ORF	open reading frame
°P	degree Plato (measure of the sum of dissolved solids in wort)
PCR	polymerase chain reaction
PEST	peptide sequence rich in proline (P), glutamic acid (E), serine (S) and threonine (T)
PGK	phosphoglycerate kinase
PMA1	gene for plasma membrane ATPase
SbAGT1	*Saccharomyces bayanus*-derived *AGT1*
SER2	phosphoserine phosphatase gene
Set1	histone methyltransferase
SGD	*Saccharomyces* Genome Database
SUC	sucrose (utilization)
TAT2	gene for tryptophan amino acid transporter
TCA	tricarboxylic acid
V_{max}	maximum velocity
v/v	volume/volume
VHG	very-high-gravity
w/v	weight/volume

1. Introduction

Beer is one of the oldest biotechnological products. It has been manufactured for thousands of years and nowadays beer brewing is an important field of industry. In Finland alone, 400–425 million litres of beer is sold yearly (statistics of years 2003–2009). This is approximately 80–90 litres of beer consumed per person annually (www.panimoliitto.fi/panimoliitto/tilastot). After understanding the role of yeast in the fermentation process in the early nineteenth century (reviewed by Boulton and Quain, 2001), there has been continuously increasing interest in improving and accelerating the brewing process, for example by means of developing better performing yeast strains.

In the fermentation process, sugars of the wort are converted to ethanol and carbon dioxide by the metabolism of the yeast cell. A major factor determining the rate and extent of the fermentation is the utilization rate of sugars. A lot of effort has been made to accelerate the fermentation of maltose and maltotriose sugars, which usually are not consumed immediately at the beginning of the fermentation but instead have a rather long lag phase before their utilization is initiated. Sometimes maltose and especially maltotriose are left unfermented at the end of the main fermentation. This lowers the efficiency of the process and also has an impact on the final quality of the beer by impairing the flavour. Delay in the utilization of maltose and maltotriose is mostly due to the fact that glucose is the preferred sugar for yeast as a carbon and energy source. When there is glucose present the utilization of alternative fermentable sugars is hindered. Mechanisms by which glucose causes this delay occur by catabolite repression and catabolite inactivation of enzymes and transporters that are needed for the utilization of alternative sugars.

Several studies have shown that the rate-limiting step in the utilization of maltose and maltotriose is the transport capacity of sugars into the yeast cell (Kodama *et al.*, 1995; Rautio and Londesborough, 2003; Meneses *et al.*, 2002; Alves

et al., 2007). Improving the ability of yeast cells to transport maltose and maltotriose has been the subject of many studies. Over the last years new maltose/maltotriose transporters have been identified and characterized (Day *et al*., 2002a; Salema-Oom *et al*., 2005; Dietvorst *et al*., 2005) or just identified and not yet characterized (Nakao *et al*., 2009). Ways to improve the transport efficiency have been obtained, for example by over-expressing the corresponding maltose or maltotriose transporter genes (Kodama *et al*., 1995; Stambuk *et al*., 2006). These strains have been shown to have improved sugar uptake capacity and are able to intensify the fermentation process. However, since these strains are genetically modified their commercial use in the breweries is not, at least yet, accepted because of the current negative attitude towards GMO of consumers. Nonetheless, these strains have given important knowledge about the bottlenecks of the fermentation process and information has been gained in how the process could be improved and what magnitude of intensification could be obtained.

Efficient utilization of sugars is even more important nowadays when there is a tendency to move to a greater extent to ferment high-gravity (HG) or even very-high-gravity (VHG) worts, which have increased concentrations of sugars compared to traditional worts. Incomplete utilization of sugars, especially maltotriose, is sometimes a problem even in standard fermentations and even more when HG and VHG worts are used (Piddocke *et al*., 2009).

1.1 Outline of malting and brewing processes

A schematic diagram of malting and brewing processes is presented in Figure 1. Malt is the main starting material in the brewing process together with water and hops. Malt is produced from barley grains by a three-phase malting process including steeping, germination and kilning. In steeping, barley grains are soaked in water to obtain the right moisture content. After that, germination is carried out in carefully controlled temperature, moisture and aeration conditions. Kilning is performed to stop the biochemical reactions in the kernel and to produce a dry product. The main purpose of malting is so that the natural enzymes in the barley grain are activated. These enzymes then assist the conversion of the storage carbohydrate material, starch, composed mainly of amylose and amylopectin, to fermentable sugars. Degradation of starch starts during malting and continues at wort production phase.

In wort production, malt is first milled to release the contents of the grains. In mashing, milled malt is suspended in water and heated to prepare an aqueous

extract. During mashing the malt components are solubilized and hydrolysed by the enzymes produced during germination. Heating disrupts the crystalline structure of starch granules and makes them susceptible to attack by amylases. Most of the degradation of starch to fermentable sugars takes place during mashing where α- and ß-amylases degrade it. The ß-amylases are more heat labile than the α-amylases and thus their activity is lost in high temperature mashes. In the production of lager beer the mash mixture is heated gradually to certain temperatures (50–72°C), which are suitable for enzymatic reaction, whereas in traditional ale brewing a single mashing temperature (65°C) is used. Conditions used in mashing, especially the temperature range, have a significant effect on the sugar spectrum formed. The combined action of α- and ß-amylases produces mostly maltose and to a lesser extent maltotriose and glucose. Also, a significant share of undegraded dextrins remain. The debranching enzyme, limit dextrinase, which is present in barley and is activated during germination, is able to convert branched dextrins into linear glucose polymers, which can after that be degraded by other amylolytic enzymes. However, limit dextrinase is heat labile and is rapidly denatured during mashing. After mashing, solids are removed, and clarified wort is obtained.

In the next step, wort is boiled together with hops. Liquid sugar syrup adjuncts, if used, are added at this point before boiling. Boiling serves many purposes. It sterilizes the wort and inactivates malt enzymes. It also assists with clarification and removes substances that would interfere with downstream processes. After boiling, solids in the form of trub and any hop material are separated from the hot wort. After that, wort is cooled and delivered to the fermentation vessel.

Before the main fermentation, wort is oxygenated. Oxygenation is important for yeast cells to be able to synthesize sterols and unsaturated fatty acids, which are necessary for the correct composition of yeast membranes. These lipids cannot be synthesized under anaerobic conditions and thus yeast must rely on lipids synthesized at the early phases of the fermentation during the rest of the fermentation. At the moment yeast is added (pitching), the main fermentation starts. During the main fermentation, fermentable sugars are converted by the yeast metabolism to ethanol, CO_2 and to a minor extent to higher alcohols, organic acids and esters. Quite soon yeast cells have used up all the oxygen and conditions change to anaerobic. It is characteristic of brewer’s yeast that even under aerobic conditions metabolism is both respiratory (oxidative phosphorylation) and fermentative (substrate level phosphorylation). Ethanol is therefore formed

also during the aerobic phase. The main fermentation has reached its end when the major part of the fermentable sugars has been used. In some cases fermentation stops earlier when there still is a significant amount of fermentable sugars present but for some reason yeast cells are not able to utilize them further.

Ale and lager fermentations differ in several respects. Lager fermentations are performed with lager strains, which perform better at low temperatures. Main fermentations performed with lager strains last approximately 7–10 days and are carried out at 6–14°C. Whereas main fermentations with the ale strains are carried out at higher temperatures, 15–25°C, and need less time to be completed. Ale and lager strains differ also in their flocculation and sedimentation performance. Ale strains tend to float and ferment on top of the beer. Whereas lager strains tend to form flocs, which sediment to the bottom of the fermentation vessel at the late stages of the fermentation. Yeast cells can be collected from the fermentation tank at the end of the main fermentation. The yeast cells collected can be stored and used to repitch a new main fermentation.

The product of the main fermentation is called green beer. It is not potable since it contains unwanted flavour components like diacetyl. For maturation of the beer flavour, secondary fermentation is needed. Removal of diacetyl is the rate-limiting step in the maturation of beer. Maturation requires the presence of viable yeast cells since diacetyl must be taken into and metabolised by the remaining yeast cells. For lager strains the secondary fermentation, which is performed traditionally near 0°C, is a slow process taking approximately 1 to 3 weeks. However, with use of an immobilized yeast technique it is possible to significantly reduce the secondary fermentation time (Pajunen *et al*., 1991). For ale beers instead only three to four days maturation at 4°C is needed.

After the secondary fermentation, downstream processing, *i.e*. filtration and pasteurization (or strerile filtration) and finally bottling takes place.

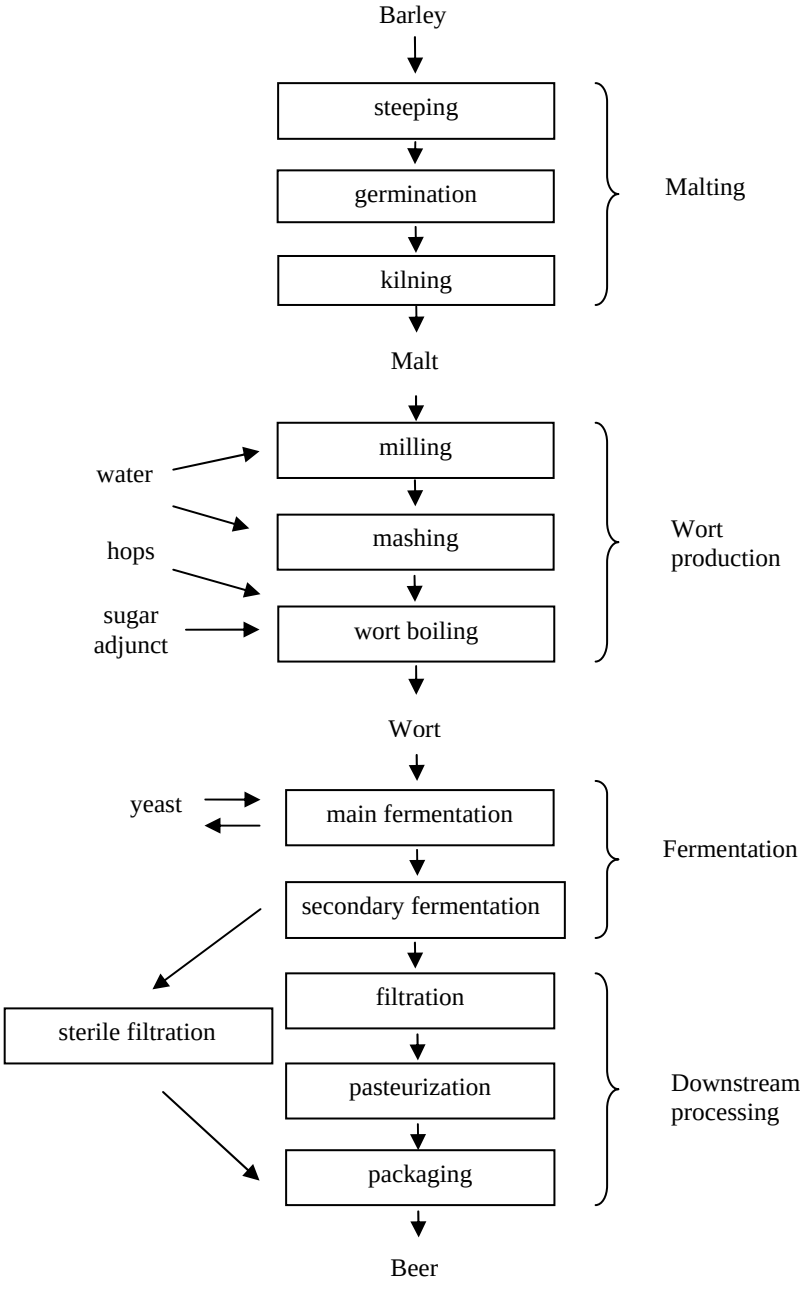

Figure 1. Shematic diagram of malting and brewing processes.

Some brewing terms are introduced below.

Extract, Degrees Plato is a measure of the sum of dissolved solids in wort, *i.e.* mostly fermentable sugars plus nonfermentable soluble carbohydrates of wort: a solution with an extract of x°P has the same density as a water solution containing x g of sucrose in 100 g of solution.

Apparent extracts measured during fermentation and not corrected for ethanol density. Apparent extracts can be corrected to real extracts if the ethanol concentration is separately determined.

Attenuation measures the proportion of carbohydrates that have been consumed from wort.

Apparent attenuation is the difference between the original extract (OE) of the wort and the current apparent extract (CE) divided by the original extract ([OE-CE]/OE).

Apparent attenuation limit is apparent attenuation measured after exhaustive fermentation with excess yeast, measure of total amount of fermentable sugars in wort.

1.2 Brewer's yeast strains

Brewer's yeast strains are divided into ale (*Saccharomyces cerevisiae*) and lager (*Saccharomyces pastorianus*, earlier referred to as *S. carlsbergensis*) strains. "Top-fermenting" ale strains are ancient strains, which have been used in beer brewing for thousands of years. "Bottom-fermenting" lager strains emerged presumably only a few hundred years ago when the low temperature fermentation technique was introduced in Bavaria (Hornsey, 2003). Since ale strains have been in use for a longer time than lager strains their diversification is much greater. Chromosomal fingerprinting showed that lager strains throughout the world essentially have only one or two basic fingerprints with small differences between the strains. Instead ale strains didn't have any common form of fingerprint (Casey, 1996).

Ale strains constitute a broad variety of strains, most of which seem to be closely related to *S. cerevisiae* (Kobi *et al*., 2004; Tornai-Lehoczki and Dlauchy, 2000). However, it has been shown recently that there are strains included, *e.g.* isolated from Trappist beers, which are actually hybrids between *S. cerevisiae* and *S. kudriavzevii* (González *et al*., 2008). Also, some other strains previously classified as *S. cerevisiae* may be hybrids (Querol and Bond, 2009). All lager strains are regarded as hybrids of two species. Parental species of the lager

hybrid were most probably diploids, which fused to generate an allotetraploid strain (Aigle *et al*., 1983). One component of the hybrid has uniformly been described as *S. cerevisiae* but there have been different suggestions for the other component during the last decades. However, in recent years it has been confirmed that lager strains are actually hybrids of *S. cerevisiae* and *S. bayanus* (Naumova *et al*., 2005; Caesar *et al*., 2007; Dunn and Sherlock, 2008). Moreover, *S. bayanus* strains consist of two subgroups, *i.e*. *S. bayanus* var. *uvarum* and *S. bayanus* var. *bayanus* and it has been shown that the *S. bayanus* component in lager strains is more related to *S. bayanus* var. *bayanus* (Nakao *et al*., 2009). Genomes of lager yeasts are reported to be dynamic and able to undergo rearrangements (Smart, 2007). Changes such as chromosome loss and/or duplications have resulted in unequal numbers of chromosomes in the present-day strains, a state referred to as aneuploidy (Querol and Bond, 2009). Also, copies of each sister chromosome are not necessarily identical, for example sister chromosomes derived from *S. cerevisiae* have diverged from each other with time. The hybrid lager strain formed between *S. bayanus* and *S. cerevisiae* species probably had selective advantage in cold brewing temperatures. Cryophilic performance of lager yeasts is suggested to derive from characteristics of *S. bayanus* (Sato *et al*., 2002). However, it has been observed that the parental species *S. cerevisiae* and *S. bayanus* are less capable of metabolizing the available sugars to ethanol at cold brewing temperatures than the hybrid (Querol and Bond, 2009). Thus, it seems that the combination of parental types is needed for efficient fermentation performance at low temperatures. The hybrid genome of lager yeast is suggested to confer a high degree of resistance to various stresses such as temperature, low pH, high alcohol concentrations, high osmotic pressure and anaerobiosis stress met during the fermentation (Querol and Bond, 2009).

Recent genome-wide sequencing of a lager strain WS34/70 further confirmed that lager brewing yeast is a hybrid between *S. cerevisiae* and *S. bayanus*. Part of the WS34/70 genome was observed to be related to the *S. cerevisiae* genome, whereas another part of WS34/70 was observed to be highly similar to *S. bayanus*. In the genome of WS34/70 there were both *S. cerevisae* and *S. bayanus*-type chromosomes found as well as 8 hybrid chromosomes consisting partly of *S. cerevisiae* and partly of *S. bayanus*. Presence of hybrid chromosomes shows that the hybrid genome has reorganized markedly after the hybridization event (Nakao *et al*., 2009). Dunn and Sherlock (2008) also report that significant reorganization of the hybrid genome took place after the hybridization event. They divide lager strains into two subgroups, which they show originate from two

separate hybridization events between *S. cerevisiae* and *S. bayanus*. They propose that in both events the *S. cerevisiae* partner was a different, but closely related, ale strain and hybridization was followed in group 1 by a loss of large portion of *S. cerevisiae* genome whereas in group 2 the loss of the *S. cerevisiae* portion of the genome was minor (Dunn and Sherlock, 2008). The loss of portions of the *S. cerevisiae* genome indicates that these parts, at least, of the *S. cerevisiae* genome were redundant in the hybrid strains under the conditions of fermentation at low temperature in which the hybrids have further evolved. It also appears that lager strains vary in the copy number of the parental chromosomes and the number and type of hybrid chromosomes they possess (Querol and Bond, 2009).

Physiological differences between ale and lager strains are most probably an outcome of their considerable genetic difference. Ale strains are called top-fermenting because they form a head yeast at the top of the wort during fermentation, whereas bottom-fermenting lager strains flocculate and sediment to the bottom of the fermentation tank in the late phase of fermentation. This difference has been explained by the different surface hydrophobicity between ale and lager strains. Ale strains are suggested to be more hydrophobic and because of this more able to adhere to CO_2 bubbles and to form yeast heads at the top of the fermentor (Dengis *et al*., 1995). However, recent process development has somewhat changed these features. Use of large cylindroconical fermenting vessels and selection have resulted in some ale yeast becoming bottom-fermenting (Boulton and Quain, 2001).

Optimum growth temperature for the ale strains is higher than for the lager strains (Giudici *et al*., 1998). The ale strains also ferment better at higher temperature (approximately 20°C) than the lager strains, which prefer 6–14°C for their optimum performance (Bamforth, 1998). This difference can, at least partly, be explained by their different capability for sugar utilization at low temperatures. Both maltose and maltotriose utilization were observed to be affected more in an ale strain compared to a lager when temperature was decreased from 14°C to 8°C (Takahashi *et al*., 1997).

Ale and lager strains differ in their sugar utilization abilities and this has been one method for their classification. The most pronounced difference is the ability of lager strains to utilize melibiose (disaccharide of galactose and glucose subunits). Lager strains possess *MEL* genes, which encode the melibiase enzyme, which is secreted into the periplasmic space of the yeast cell and is able to hydrolyse melibiose (Boulton and Quain, 2001; Turakainen *et al*., 1993). Lager

yeast strains also possess the *FSY1* gene encoding a fructose transporter not present in the ale strains (Gonçalves *et al*., 2000). It has been also shown that the lager strains use maltotriose more efficiently than the ale strains and less residual maltotriose is usually left after lager fermentation (Zheng *et al*., 1994a).

1.3 Carbohydrates of wort

A typical sugar spectrum for 11–12°Plato wort is shown in Table 1. Worts supplemented with sugar adjuncts have markedly changed sugar concentrations as described in section 1.10. Wort contains both fermentable (accounting for 70–80%) and non fermentable (20–30%) carbohydrates. Of fermentable sugars, the most abundant is maltose, which is a disaccharide of two glucose subunits joined together via α-1,4-linkage. Maltose accounts for 60–65% of the total fermentable sugars. Two other main sugars of wort are glucose and maltotriose, each accounting for approximately 20% of the total fermentable sugars. Maltotriose is a trisaccharide consisting of three glucose subunits joined together via α-1,4-linkages. Both maltose and maltotriose are hydrolysed by the yeast to glucose subunits by an intracellular α-glucosidase enzyme (maltase) capable of hydrolysing terminal 1,4-linked α-D-glucoside residues with a release of α-D-glucose. The α-glucosidase has the same affinity for both of these sugars (Zastrow *et al*., 2000) (K_m 17 mM for both, Needleman *et al*., 1978).

In addition to the three main sugars, there is a minor amount of sucrose (disaccharide of glucose and fructose subunits) and fructose present in the wort. The unfermentable fraction of wort consists mostly of dextrins which are carbohydrates with four or more glucose subunits linked by α-1,4 or α-1,6 glycosidic bonds. In addition to dextrins unfermentable fraction contains a fraction of β-glucans (polysaccharides consisting of glucose molecules linked together by β-1,3 and β-1,4 bonds) and a small fraction of pentose sugars such as arabinose and xylose.

Table 1. Typical sugar spectrum of 11–12°Plato wort. Share of each sugar is shown as a percentage (%) (modified from Stewart, 2009).

Wort concentration	11–12°Plato
Maltose	50–60
Maltotriose	15–20
Glucose	10–15
Sucrose	1–2
Fructose	1–2
Total fermentable sugars	70–80
Total dextrins	20–30

1.4 Sugar uptake and assimiliation during fermentation

The barrier between the outside and inside of the yeast cell consists of cell wall, plasma membrane and periplasmic space, which is located in between these two. The cell wall of the yeast cell is porous and sugars are able to pass through it. Thus, it is the plasma membrane that forms a barrier between the inside and outside of the yeast cell. Sugars do not freely permeate biological membranes and cellular uptake of sugars requires the action of transporter proteins. Sugar transporters specifically bind their substrate sugar and subsequently carry it into the yeast cell. Some of the sugar transporters are highly specific whereas some have a wide substrate range (Bisson *et al*., 1993; Lagunas, 1992). Sugar transporters mediate two types of transport processes in the yeast cells: energy-independent facilitated diffusion, in which solutes are transported down a concentration gradient, and energy-dependent transport via proton symport mechanism where solutes can be accumulated also against the concentration gradient (Bisson *et al*., 1993; Lagunas, 1992).

Brewer's yeasts can utilize a wide variety of sugars but when several sugars are present simultaneously yeast tend to use them in sequential manner. Most easily assimilated sugars, *i.e*. monosaccharides glucose and fructose, are used first (Fig. 2). Both glucose and fructose are carried into the yeast cell by members of the hexose transporter (HXT) family that consists of 18 transporters (Wieczorke *et al*., 1999). Hxt transporters mediate energy-independent facilitated diffusion of glucose and fructose. Uptake of both glucose and fructose is initiated at an early phase of the fermentation. Hxt transporters are more efficient

carriers of glucose compared to fructose and, for this, glucose is taken up faster than fructose (D'Amore *et al*., 1989a). Thus, glucose is usually used up before fructose (Meneses *et al*., 2002), even if the initial concentration of glucose was higher. Differently to other sugars, sucrose is usually not carried into the yeast cell but is hydrolysed in the periplasmic space by the secreted invertase enzyme encoded by the *SUC* genes (Hohmann and Zimmermann, 1986). Hydrolysis of sucrose to glucose and fructose by invertase and slower uptake of fructose compared to glucose may even cause a transient increase in the concentration of fructose at the beginning of the fermentation (Meneses *et al*., 2002).

Glucose is the substrate preferred over all the other carbohydrates by the yeast and in the presence of glucose uptake of other less preferred sugars, like the maltose and maltotriose, is delayed. The most important mechanisms by which glucose causes this delay are catabolite repression and catabolite inhibition (discussed in more detail in chapter 1.9). Usually, uptake of maltose starts only when approximately 60% of the glucose has been utilized (D'Amore *et al*., 1989a).

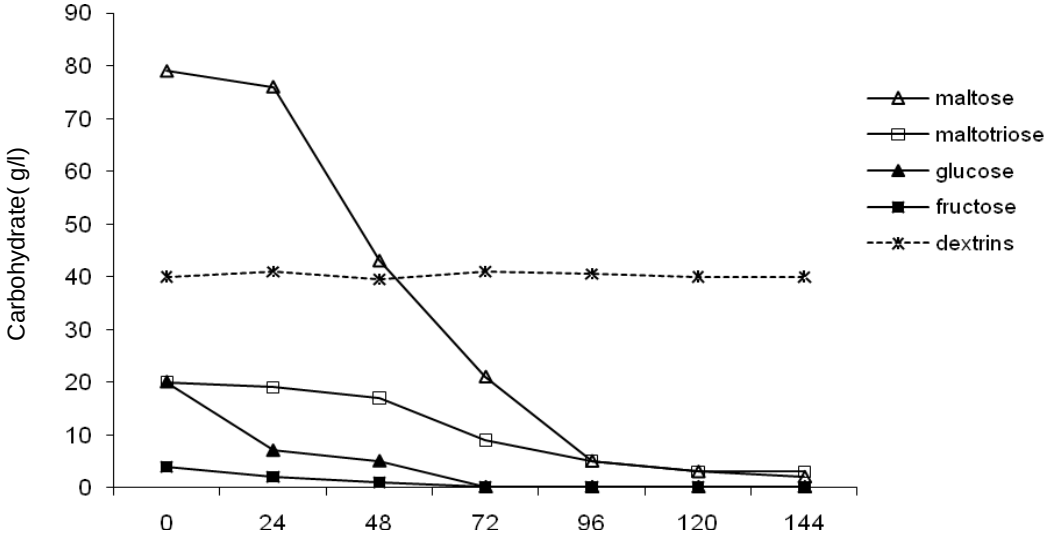

Figure 2. Order of uptake of sugars by yeast from wort (modified from Stewart, 2009).

A schematic representation of sugar uptake by brewer's yeast cell is shown in Figure 3. Maltose and maltotriose are carried into the yeast cell by energy-dependent transport through a symport mechanism, in which one proton is co-transported with each maltose or maltotriose molecule (Serrano, 1977; van Leeuwen *et al*., 1992). The driving force for this transport is an electrochemical transmembrane proton gradient generated largely by plasma membrane ATPase, which pumps protons out of the cell with a stoichiometry of 1 proton/ATP hydrolysed to ADP.

Maltotriose does not have its own specific transporters, but is transported with some, but not all, of the maltose transporters (Han *et al*., 1995; Day *et al*., 2002a; Salema-Oom *et al*., 2005). Most of the transporters capable of carrying both of these sugars carry maltose more efficiently than maltotriose (Han *et al*., 1995; Day *et al*., 2002a) and thus its uptake is faster. Competition for the same transporters and maltose being the preferred substrate leads to maltotriose being utilized only after most of the maltose has been assimilated.

Several studies have shown that the overall fermentation rate of maltose and maltotriose is correlated with their maltose and maltotriose transport activity and correlates poorly with maltase activity (Meneses *et al*., 2002; Rautio and Londesborough, 2003; Kodama *et al*., 1995; Alves *et al*., 2007). Transport rather than hydrolysis is therefore the rate limiting step in the utilization of these two sugars.

The higher polysaccharides dextrins are not utilized by brewer's yeasts and contribute to the beer flavour by imparting fullness. Attempts have been made to utilize dextrins, for example, by introducing appropriate enzymes into the brewing yeast by genetic engineering or by addition of dextrinase enzyme to the wort (Hammond, 1995). Both of these approaches have been successful in the production of diet beer.

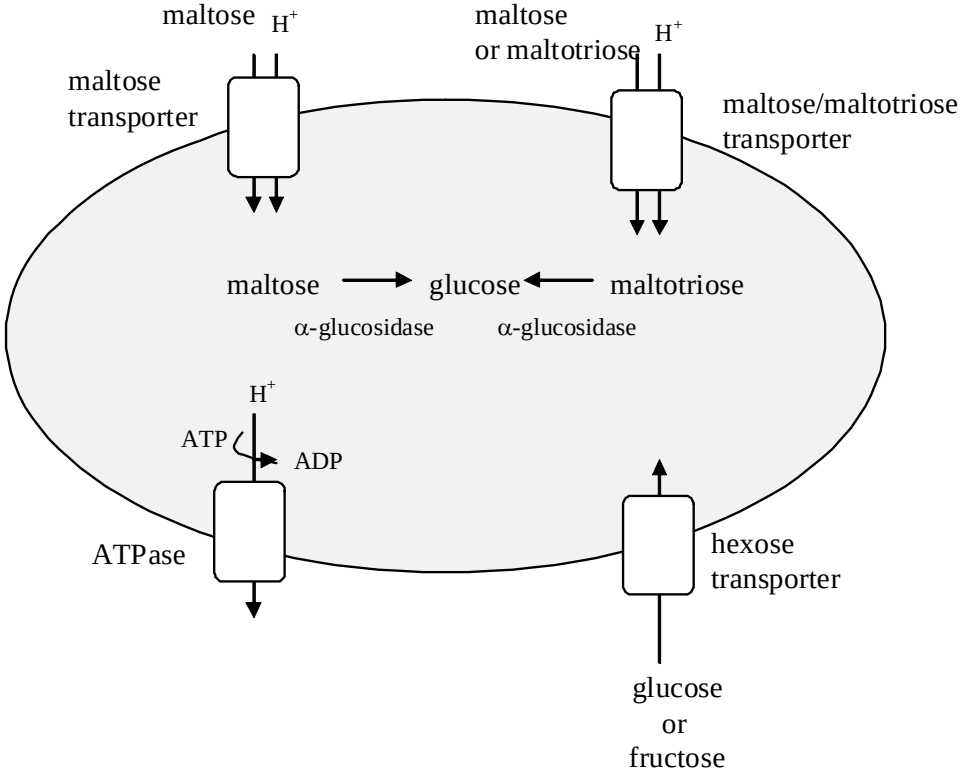

Figure 3. Uptake of wort sugars by brewer´s yeast.

The sugar uptake profile of brewer's yeast differs markedly from that of laboratory strains. Laboratory strains are not usually able to use maltose or maltotriose at all. In laboratory strains, sucrose hydrolysis by invertase is delayed by glucose (Meijer *et al.*, 1998), mostly because glucose represses the expression of the *SUC2* gene encoding invertase (Neigeborn and Carlson, 1984). In contrast, many brewer's yeast strains are characterized by rapid depletion of sucrose in the presence of glucose (D'amore *et al.*, 1989b; Meneses *et al.*, 2002) implying that invertase activity is constitutive. In brewer's yeast strains direct uptake of sucrose also occurs. Agt1 transporters are able to carry sucrose with high affinity (Salema-Oom *et al.*, 2005) and, once inside the cell, α-glucosidase is able to hydrolyse it to subunits (Needleman *et al.*, 1978). However, since *AGT1* genes are known to be glucose repressed, there is practically no importance in direct sucrose uptake in the brewery fermentations because, by the time glucose re-

pression is lifted, sucrose has already been hydrolysed by invertase. In addition the lager strains have been shown to possess specific fructose transporters. These are fructose/proton symporters encoded by the *FSY1* gene not present in the ale strains (Gonçalves *et al.*, 2000). Glucose is known to repress also the *FSY1* genes (Rodrigues de Sousa *et al.*, 2004) so that direct fructose transport does not have significance in brewery fermentations for the same reason as described for the sucrose direct transport.

Glucose, transported into the yeast cell by Hxt transporters or produced by intracellular hydrolysis of maltose and maltotriose, has the same fate, *i.e.* it is channelled to glycolysis. Also, fructose can enter directly to the glycolysis pathway after its phosphorylation to fructose 6-phosphate. In glycolysis, glucose is degraded to pyruvate and energy in the form of ATP is produced. Pyruvate intermediate is a branchpoint where respiration or fermentation is selected. Pyruvate can either be converted into acetyl-CoA, the fuel of the TCA-cycle (respiration), or be decarboxylated and reduced to ethanol (fermentation). In principle, oxygen availability will determine whether yeast respires or ferments pyruvate. However, despite fully aerobic conditions some yeast including brewer's yeast can exhibit alcoholic fermentation.

A further level of complexity in maltotriose utilization by *S. cerevisiae* yeast cells was revealed by Zastrow *at al*. (2000) who observed that several industrial strains could utilize maltotriose only aerobically, *i.e*. grow on this carbon source in the absence of ethanol production. However, Londesborough (2001) showed that two brewer's yeast strains could grow anaerobically on pure maltotriose as sole carbon source, but the lag phase was very long. Salema-Oom *et al*. (2005) concluded that the relative fraction of maltotriose fermented versus respired is strain-dependent and varies with the efficiency of maltotriose transport into the cell. Salem-Oom *et al*. (2005) suggested that this is because the rate of glycolysis is diminished when maltotriose transport occurs slowly and reduced glycolytic flux leads to an increase in respirative metabolism.

1.5 Factors affecting maltose and maltotriose uptake efficiency

Ability to utilize maltose and maltotriose varies widely between different brewer's yeast strains. Widest variation is seen in the ability to utilize maltotriose (Dietvorst *et al.*, 2005; Meneses *et al.*, 2002), *i.e*. there are strains with

severe difficulties, whereas some of the strains utilize it fast and efficiently (Meneses *et al*., 2002).

Both maltose and maltotriose transport velocities have been observed to decrease significantly at late phase of cultivation in both ale and lager strains (Zheng *et al*., 1994b). In the late fermentation, sugar uptake is generally inhibited by deteriorated circumstances, *i.e*. increased ethanol concentration, nutrient deprivation, inhibited yeast metabolism, *etc*. Guimarães *et al*., (2006) have shown that maltose transport activity is affected by the lipid composition of the yeast. The proper function of maltose transporters was shown to require adequate amounts of ergosterol in the yeast. This effect may partly explain the low maltose (and maltotriose) uptake rates in the secondary half of brewery fermentations when the sterol content of the yeast has fallen. Inactivation of plasma membrane transporters has been connected also to nitrogen starvation in resting cells (laboratory strains) (Riballo *et al*., 1995; Peñalver *et al*., 1998). Nitrogen starvation was observed to lead to endocytosis and degradation of Mal61 transporters expressed in laboratory strains (Lucero *et al*., 2002). However, it is not known if this phenomenon takes place also in brewer's yeast cells. Since several different causes seem to deteriorate the maltose and maltotriose uptake at late phases of fermentation, an early onset and high rate of maltose and maltotriose utilization is important.

Fermentation temperature is also an important factor affecting the uptake capacity of maltose and maltotriose. Raising the fermentation temperature from 15 to 21°C increased markedly the rate of maltotriose utilization in both ale and lager strains (Zheng *et al*., 1994a). Takahashi *et al*. (1997) observed that when temperature was raised from 8 to 14°C there was no significant effect on the glucose utilization but the maltose and maltotriose utilization rates were both increased.

Maltose and maltotriose uptake velocities have been shown to be dependent on pH of the medium. An external pH rise from 5.5 to 7.0 decreased maltose uptake from 8.7 to 0.4 nmol/min/mg dry wt (Van Leeuwen *et al*., 1992). Similar results were obtained by Visuri and Kirsop (1970) for both maltose and maltotriose uptake. Visuri and Kirsop (1970) suggest that the pH optimum for the uptake of both maltose and maltotriose is pH 5.

Wort extract changes (in range between 7°P to 15°P) did not have a notable effect on glucose, maltose or maltotriose utilization ability in either ale or lager strains (Takahashi *et al*., 1997). However, when wort osmotic pressure was increased with sorbitol (15–30% w/v) significant decrease in the maltotriose up-

take was observed in the lager strains indicating that in very-high-gravity wort lager strains may have lowered maltotriose uptake ability (Zheng *et al*., 1994a).

1.6 Kinetics of maltose and maltotriose transport

The transporters work practically like enzymes. They show specific binding for the substrates after which they catalyse uptake of the substrate and, while doing so, undergo some conformational change. Affinities and maximal velocities can be determined for transporters similarly as for enzymes. Because of the finite number of binding sites, both enzymes and transporters are saturable. There can be several substrates for each transporter and in this case they inhibit each others' binding to the transporter proteins. Analogously to the velocity of enzymatic reactions sugar transport velocity also follows Michaelis-Menten kinetics. Reaction velocity approaches a maximum when substrate concentration is increased. If the initial rate of the reaction is measured over a range of substrate concentrations (denoted as [S]), the reaction rate (v) increases as [S] increases. However, as [S] gets higher, the enzyme becomes saturated with substrate and the rate reaches V_{max}, the enzyme's maximum rate. K_m is defined as the substrate concentration where reaction velocity is ½ V_{max}.

Some sugar transport systems, like glucose transport in *S. cerevisiae*, exhibit biphasic kinetics, where there appear to be two distinct K_m values (Busturia and Lagunas, 1986). Biphasic kinetics has also been observed for maltose transport in both ale and lager strains. A high affinity system with a K_m of 1.3–4 mM and V_{max} of 28 nmol/min/μg dry wt and a low affinity system with K_m 15–70 mM and V_{max} of 17–20 nmol/min/μg dry wt have been described for both ale and lager strains (Crumplen *et al*., 1996; Rautio and Londesborough, 2003). Some authors have suggested that the low-affinity component for maltose transport is due to the function of low affinity maltose transporters such as Agt1 and Mtt1 (Salema-Oom *et al*., 2005; Alves *et al*., 2008). Alves *et al*. (2008) studied natural isolates of *S. cerevisiae* strains and observed that they exhibited biphasic maltose transport kinetics with both high (K_m 5 mM) and low affinity (K_m 30 mM) systems. For maltotriose transport only the low affinity (K_m 36 mM) system was observed. When the *AGT1* gene was deleted from these strains, maltotriose transport ability was completely lost as well as the maltose low affinity transport. Thus, Agt1 transporters seem to be responsible for the low affinity maltose transport system as well as for maltotriose transport in these strains.

For maltotriose transport, only the low affinity component has most often been found in both *S. cerevisiae* and in brewer's yeast strains (Zastrow *et al*., 2001; Salema-Oom *et al*., 2005; Alves *et al*., 2008). An exception is a study by Zheng *et al*. (1994b) where it is reported that in both ale and lager strains there exists also a high affinity system for maltotriose transport, which was observed to be almost completely inhibited by maltose.

Two approaches have been used to measure the maltose or maltotriose uptake into yeast cells. In the first approach uptake studies are performed with [^{14}C] labelled maltose or maltotriose and velocity of the transport is calculated from the radioactivity remaining inside the yeast cells after Zero-*trans* transport assay (Lucero *et al*., 1997). Another approach is to calculate the rate of H^+ symport activity determined from the increased alkalinity of the medium due to concomitant uptake of protons with sugars (Serrano, 1977).

1.7 Maltose and maltotriose transporters

At present there are four different types of maltose and/or maltotriose transporters characterized from *S. cerevisiae* and/or *S. pastorianus*. These are Malx1, Agt1, Mphx and Mtt1 transporters. Substrate ranges determined in different studies for each of the transporters are shown in Table 2. Michaelis-Menten constants K_m and V_{max} for each transporter are shown in Table 3. K_m and V_{max} values have been obtained by cloning a single transporter gene and expressing the gene from a plasmid in a laboratory strain lacking endogenous α-glucoside transporter activity. Thus, affinities and V_{max} values can be compared between maltose and maltotriose when a study is performed with a single construct. Whereas results obtained in different studies with different constructs are only indicative, since there can be differences in the expression levels of genes, stability of the transporters in the plasma membrane, *etc*. so that the number of transporters per g of yeast in each case is not known.

Table 2. Substrate range of α-glucoside transporters.

	Maltose	**Turanose**	**Maltotriose**	**Trehalose**	**α-methyl glucoside**	**Sucrose**	**Reference**
Agt1	√	√	√	√	√	√	Salema-Oom *et al*., 2005; Han *et al*., 1995
Mtt1	√	√	√	√	-	-	Salema-Oom *et al*., 2005
Mphx	√	√	√	-	√	n.d.	Day *et al*., 2002a
Mal31	√	√	√	-	-	-	Day *et al*., 2002a
Mal31	√	√	-	-	-	-	Salema Oom *et al*., 2002
Mal61	√	√	-	-	-	-	Han *et al*., 1995

√ = substrate,
- = not a substrate
n.d. = not determined

Table 3. Affinities and maximal velocities of transporters.

	Maltose		**Maltotriose**		**Reference**
	K_m (mM)	V_{max}	K_m	V_{max}	
Mal21	4.7 ±0.2	115 ±3	n.d.	n.d.	Stambuk and de Araujo, 2001
Mal31	4.2 ± 1.1	42	5.7 ± 1.0	41	Day *et al*., 2002b
	4.3 ± 0.6	n.d.	335 ± 165	n.d.	Multanen, 2008
Mal61	2–4	n.d.	n.d.	n.d.	Han *et al*., 1995
	2.7 ± 0.6	36	7.2 ± 0.9	40	Day *et al*, 2002b
Agt1	5–10	n.d.	n.d.	n.d.	Han *et al*., 1995
	17.8 ±1.3	13.2 ± 0.5	18.1 ± 1.8	7.8 ± 0.3	Stambuk and de Araujo, 2001
	5.1 ± 0.6	41	4 ± 0.7	39	Day *et al*., 2002b
	30	n.d.	36 ±2	n.d.	Alves *et al*., 2008
Mphx	4.4 ± 0.5	39 ± 4.2	7.2 ± 1.0	49 ± 5.4	Day *et al*., 2002a
Mtt1	61–88	n.d.	16–27	n.d.	Salema-Oom *et al*., 2005
	41 ± 0	n.d.	23 ± 4	n.d.	Multanen, 2008

V_{max} = nmol/min/µg dry wt; n.d.= not determined

1.7.1 Malx1 transporters

Both ale and lager strains usually possess several copies of *MALx1* (maltose utilization) genes in their genomes (Jespersen *et al*., 1999). Several *MALx1* genes (*MAL11, MAL21, MAL31* and *MAL61*) have been cloned and sequenced. Most of them are derived from laboratory strains but *MAL61* gene was originally isolated from a lager strain (Needleman *et al*., 1984). All these genes have very similar sequences and they are observed to encode transporters bearing 98% identity at amino acid level, thus suggesting a conserved nature for Malx1 transporters. Lager strains also possess *S. bayanus*-derived Malx1 transporters approximately 80% identical to corresponding *S. cerevisiae* transporters (Nakao *et al*., 2009).

Malx1 transporters are reported to be high affinity maltose transporters (K_m 2–5 mM) (see Table 3; Day *et al*., 2002b; Han *et al*., 1995; Stambuk and de Araujo, 2001). It has been shown in many studies that the substrate range of Malx1 transporters is restricted to maltose and turanose (Han *et al*., 1995; Salema-Oom *et al*., 2005; Alves *et al*., 2008; Multanen, 2008; Duval *et al*., 2010) and that maltotriose is not carried by Malx1 transporters. This view has been challenged by Day *et al*. (2002b) who claimed that Mal61 and Mal31 transporters are actually able to carry maltotriose as efficiently as maltose. The reason for the significantly different results obtained by Day *et al*. could be due to the analysis method used. In results shown in Table 3 only Day *et al*. and Multanen measured the uptake of radioactive maltotriose whereas other authors have used the H^+ influx rate measurement method to assay maltotriose uptake. Dietvorst *et al*. (2005) have shown that commercial [^{14}C] maltotriose from the same supplier that Day *et al*. were using in their study actually is not pure but is heavily contaminated with [^{14}C] maltose and [^{14}C] glucose residues. It has been shown that use of commercially available [^{14}C] maltotriose without further purification can overestimate the rate of maltotriose transport by more than four-fold (Dietvost *et al*., 2005). It has been suggested that, due to the contaminations, maltotriose transport was strongly overestimated by Day *et al*. (Alves *et al*., 2008). In the study of Multanen, [^{14}C] maltotriose used has been further purified and results by Multanen actually show that Mal31 can't carry maltotriose. However, Day *et al*. (2002b) claim that they have verified and determined by chromatography that no degradation has occurred in [^{14}C] maltotriose used. Another possibility to explain conflicting results is that Mal31 and Mal61 transporter proteins used in the study of Day *et al*. have some changed amino acids that significantly affect

their sugar carrying ability. It is known that even one amino acid change in transporter protein can have a significant effect on sugar uptake characteristics (Smit *et al*., 2008).

1.7.2 Agt1 transporters

Charron and Michels (1988) isolated a *MAL1* locus with a maltose transporter gene clearly distinct from *MAL11*, as observed by restriction mapping and Southern analysis. This allele was referred to as *MAL1g* and the maltose transporter gene located in it was characterized later by Han *et al*. (1995). This new transporter gene was referred to as *AGT1* (alpha-glucoside transporter) since it was found to carry several different α-glucosides (Han *et al*., 1995). Its preferred substrates were observed to be trehalose and sucrose with K_m 8 mM for both. Significantly lower affinity (K_m 20 to 35 mM) was detected for maltose, maltotriose and even lower affinities for α-methylglucoside, turanose, isomaltose, palatinose and melezitose (Han *et al*., 1995.). The Agt1 transporter was observed to be an α-glucoside/proton symporter (Han *et al*., 1995; Stambuk *et al*., 1999) similar to Malx1 transporters.

Recently, results of the whole genome sequencing of lager strain WS34/70 have revealed that the WS34/70 strain possesses another putative maltose/maltotriose transporter not earlier described (Nakao *et al*., 2009). There was an ORF found, referred to as LBYG13187 and believed to be the *S. bayanus* homologue of *S. cerevisiae AGT1*. This is because its closest homology showed 79% identity to the *AGT1* sequence in the *Saccharomyces* Genome Database (Nakao *et al*., 2009). Here we call this gene *Sb-AGT1*, although nothing is yet known about its functionality and sugar carrying properties.

1.7.3 Mphx transporters

MPHx (maltose permease homologue) genes were originally identified by *S. cerevisiae* (laboratory strain) genomic sequence data clustering and grouping. Two ORFs, YDL247w and YJR160c, were grouped with a cluster of maltose transporter genes and referred to as *MPH2* (YDL247w) and *MPH3* (YJR160c) (Nelissen *et al*., 1995). These ORFs have identical sequences but are located on different chromosomes, *MPH2* is located on chromosome IV and *MPH3* on chromosome X. Sequence identity of *MPHx* to *MALx1* and *AGT1* genes is 75% and 55%, respectively (Day *et al*., 2002a). Day *et al*. (2002a) cloned *MPHx* gene

from a lager strain and characterized its ability to transport sugars. Day *et al.* showed that Mphx transporters are able to carry maltose, maltotriose, α-methylglucoside and turanose. Rather high affinities for both maltose (K_m 4.4 mM) and maltotriose (K_m 7.2) were observed. There are no reports on whether Mphx transporters function as α-glucoside/proton symporters as with other maltose/maltotriose transporters.

Several studies have questioned the role of Mphx transporters in maltose and maltotriose transport. Jespersen *et al.* (1999) have suggested that Mphx transporters most probably play a secondary role in the maltose uptake since they have not been found in functional analysis screenings performed but were identified via genomic sequencing of a laboratory strain. Moreover, Alves *et al.* (2008) have shown that *MPH2* and *MPH3* genes derived from a laboratory strain do not allow efficient transport of maltotriose. Duval *et al.* (2010) suggest that *MPHx* genes probably have little influence on maltotriose (and maltose) utilization since in 21 brewer's yeast strains included in their study, the utilization of maltotriose (maltose) didn't correlate with the presence of *MPHx* genes. Whereas there was significant correlation observed in the presence of other maltose/maltotriose transporter genes.

1.7.4 Mtt1 transporters

A new type of maltose and maltotriose transporter gene was identified in 2005 by two independent research groups (Salema-Oom *et al.*, 2005; Dietvorst *et al.*, 2005). Both groups found the transporter gene by screening genomic libraries of lager strains for the ability of cells to grow on maltotriose either aerobically (Salema-Oom *et al.*, 2005) or when the respiration of the cell was blocked by antimycin A (Dietvorst *et al.*, 2005). Salema-Oom *et al.* (2005) referred to the new α-glucoside gene they found as Mty1 (maltose transport in yeast) and Dietvorst *et al.* (2005) as Mtt1 (mty1-like transporter) because they noted the similarity to the *MTT1* sequence deposited by Salema-Oom in EMBL gene bank before publication. *MTY1* and *MTT1* genes are identical in their sequence and are hereafter referred to as *MTT1*. *MTT1* share 90% and 54% identity to *MALx1* and *AGT1* genes, respectively. Mtt1 transporters can carry maltose, maltotriose, trehalose and turanose but trehalose is the preferred substrate (Salema-Oom *et al.*, 2005).

Interestingly, Mtt1 displays higher affinity (K_m 16–27 mM) for maltotriose than for maltose (K_m 61–88 mM). This is a unique characteristic among all α-glucoside transporters and this feature makes Mtt1 particularly important in re-

gard to brewery fermentations. Mtt1 transporters were shown to be α-glucoside/proton symporters (Salema-Oom *et al*., 2005), similar to Malx1 and Agt1 transporters.

Dietvorst *et al*. (2005) also obtained an altered version of *MTT1* in the screening. This version lacks 66 base pairs from the 3'–end of *MTT1* gene but instead contains 54bp of the cloning vector. This altered version referred to as *MTT1alt* was found to encode maltose/maltotriose transporter with more efficient uptake of maltotriose than the original *MTT1* encoded version. The ratio of maltotriose uptake versus maltose uptake was also observed to be raised with this altered version in favour of maltotriose. Increase in transport ability could be due to the deletion of catabolite inactivation signal as discussed in the chapter 1.9.

1.8 *MAL* loci

Genes required for maltose and maltotriose utilization are located in *MAL* loci (except *MPHx* and *Sb-AGT1*). In the classical *MAL* locus there are three genes, all of which are needed for the efficient utilization of maltose. There is a gene encoding the maltose transporter, a gene encoding α-glucosidase (maltase) and a gene encoding the activator protein needed for the efficient expression of the two other genes of the locus. Structure of the classical *MAL* locus is shown in Figure 4. There are five known *MAL* loci in *S. cerevisiae*; *MAL1* (located on chromosome VII), *MAL2* (Chr III), *MAL3* (Chr II), *MAL4* (Chr XI) and *MAL6* (Chr VIII). Genes of the locus are referred to as *MALx1* for maltose transporter (where x refers to the *MAL* locus, *i.e* 1 to 4 and 6), *MALx2* for maltase and *MALx3* for MAL-activator encoding gene. The regulatory protein from one locus can act in *trans* to activate *MALx1* and *MALx2* genes from another locus. Laboratory strains are not usually able to use maltose or maltotriose at all because the *MAL* loci they possess are non-functional due to presence of non-functional MALx3 activators (Bell *et al*., 2001). Glucose is known to repress *MAL* genes in a Mig1-mediated manner. There are Mig1 binding elements present in promoters of all the three genes of the locus (Hu *et al*., 1995; Wang and Needleman, 1996). Maltose is an inducing agent of *MAL* genes. It has been suggested that MAL-activators are bound by maltose and this yields a conformation with functional activity (Wang *et al*., 1997). Active conformation would then be capable of entering the nucleus and/or activate the transcription (Danzi *et al*., 2000).

There are usually several *MAL* loci present in each yeast strain. *MAL* loci studied by restriction fragment analysis and Southern hybridization studies are

shown to be highly similar in their structure (Charron *et al*., 1989). Also, sequence data obtained has revealed highly conserved sequences for at least *MALx1* and *MALx2* genes between different loci and also between different strains.

All *MAL* loci are located near telomeres. Regions near telomeres are known to be more prone to chromosomal rearrangements since recombination events between different chromosomes are common near telomeres (Bhattacharyya and Lustig, 2006). In addition, genes that are located close to the telomeres can become transcriptionally repressed by an epigenetic process known as telomeric silencing, *i.e.* variation in chromatin structure near the telomeres leading to the silencing of genes located in this region (Pryde and Louis, 1999; Loney *et al*., 2009). The role of chromatin remodelling in the regulation of expression of *MAL* genes has been reported (Houghton-Larsen and Brandt, 2006; Dietvorst and Brandt, 2008). It has been observed that telomeric silencing does not occur uniformly but there is significant variation between different strains (Pryde and Louis, 1999). A specific complex consisting of several subunits, the COMPASS complex is known to be involved in the telomeric silencing in the yeast cells (Miller *et al*., 2001). In a study where the COMPASS complex was rendered non-functional, strain-dependent differences in the telomeric silencing of *MAL* genes were observed. The *MAL* genes were found to be strongly silenced in some strains, whereas in other strains the non-functional COMPASS complex did not cause any changes in the expression of *MAL* genes (Houghton-Larsen and Brandt, 2006). It has also been observed that in a single strain some chromosome ends are more prone to the telomeric silencing than others (Loney *et al*., 2009). Thus, it is possible that the different *MAL* loci, located at different chromosome ends, are not uniformly regulated by telomeric silencing.

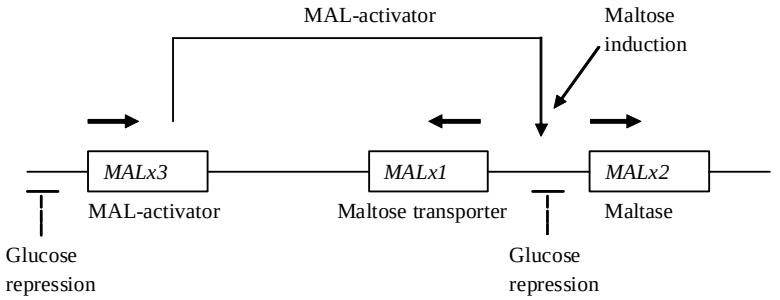

Figure 4. Structure of classical *MAL* locus.

In some *MAL1* loci there is a gene encoding a different type of transporter than *MAL11*. This gene, 57% identical to *MALx1* transporter, is referred to as *AGT1* and it has changed characteristics for sugar transport (Han *et al*., 1995). *MALx2* and *MALx3* genes were found upstream and downstream of the *AGT1* gene, respectively, referring otherwise conventional *MAL* locus. Also, the *MTT1* gene was observed to be located in a conventional *MAL* locus, at the place of *MALx1* gene, since *MALx2* and the *MALx3* genes were found upstream and downstream of the *MTT1* encoding sequence, respectively (Salema-Oom *et al*., 2005). The *MTT1* gene has been mapped to chromosome VII at right sub-telomeric region (Nakao *et al*., 2009) similarly to *MAL1* locus. Possibly *MAL13-MTT1-MAL12* is another version of *MAL1* locus but this remains to be verified in the future work. As an exception, *MPHx* and *SbAGT1* genes are not located in conventional *MAL* loci but exist as single genes without *MALx2* or *MALx3* genes in proximity (Day *et al*., 2002a; Nakao *et al*., 2009).

The *MALx1* and *MALx2* genes share a divergent promoter region (Bell *et al*., 1997). Similarly, there is a divergent promoter region also for *AGT1-MAL12* (SGD) and *MTT1-MAL12* (Salema-Oom *et al*., 2005; Dietvorst *et al*., 2005) gene complexes. Gene clustering and divergent promoters are found in the yeast genome like, for example, *GAL1-GAL10*. In many cases the two genes either function in the same metabolic pathway or the functions of their products are related. The regulation of neighboring genes by a common promoter element and regulatory proteins allows efficient and coordinate gene expression (Beck and Warren, 1988). *MALx1* and *MALx2* genes are coordinately induced several hundred-fold by maltose and repressed dramatically immediately following glucose addition (Vanoni *et al*., 1989). To mediate glucose repression there are two Mig1 binding sites found in the *MALx1-MALx2* divergent promoter regions (Hu *et al*., 1995) and one Mig1 binding site in *MALx3* promoters (Wang and Needleman, 1996). For maltose-based induction there are three binding sites in the *MALx1-MALx2* divergent promoter region for the MAL-activator (Levine *et al*., 1992, Sirenko *et al*., 1995). The *AGT1-MALx2* divergent promoter region has also three binding sites for the MAL-activator but only one binding site for the Mig1 element (SGD). The *MTT1-MALx2* intergenic region hasn't been studied in detail but it has been observed that sequence identity to the *MALx1-MALx2* divergent promoter region is very high ~99% (in ~540 bp upstream region from the start of the available *MTT1* gene sequence) (Dietvorst *et al*., 2005). The same promoter elements are most probably found in *MTT1-MALx2* intergenic region.

Although *MPHx* genes are not located in *MAL* loci and have significantly different promoter sequences compared to *MALx1* and *AGT1* promoters (43-45% identity, respectively), *MPHx* genes have been shown to be glucose repressed and maltose and maltotriose induced. There is a single MAL-activator binding site in the promoter region of *MPHx* as well as one putative Mig1 binding site (Day *et al*., 2002a). It has also been shown that *MPHx* genes need a MAL-activator for induction (Day *et al*. 2002a). *MPHx* genes actually showed very similar expression profile to *MALx1* and *AGT1* genes when expression was studied in repressing or inducing conditions (Day *et al*., 2002a).

Some *MAL* loci are known to possess extra copies of one or more *MAL* genes (Charron *et al*., 1989; Michels *et al*., 1992). Moreover, now that more sequence data has started to emerge from various whole genome sequencing projects of *S. cerevisiae* and other *Saccharomyces sensu stricto* strains, it has actually been observed that *MAL* loci are not so conserved as the classical model of the locus would suggest. There are, for example, *MAL* loci found where there are several copies of each *MAL* locus gene present, but not in equal numbers, for example there is a *MAL* locus found where there are three *MALx2*, two *MALx3*, one *AGT1* and one *MALx1* gene present in the same *MAL* locus (present in same continuous contig sequence spanning approximately 22 kbp region thus referred here as *MAL* locus) of *S. cerevisiae* RM11-1a strain. Interestingly, both *AGT1* and *MALx1* genes are located in the same *MAL* locus in this case (*S. cerevisiae* RM11-1a sequencing project, Broad Institute of Harvard and MIT (http://www.broad.mit.edu)).

1.9 Catabolite repression and inactivation

When glucose is present, the enzymes, transporters, *etc*. required for the utilization of alternative carbon sources are synthesized at low rates or not at all. This phenomenon is known as carbon catabolite repression or simply catabolite repression or glucose repression. Catabolite repression allows yeast to use the preferred (most rapidly metabolizable) carbon and energy source first (Gancedo, 1998).

Catabolite repression of maltose and maltotriose utilization is mostly mediated by the repression of gene expression although glucose has been shown to interfere also with the stability of at least α-glucosidase mRNA (Federoff *et al*., 1983). Repression of gene expression is mediated in a Mig1p mediated manner. Mig1p is a DNA-binding transcriptional repressor regulating the expression of several genes in response to glucose. As explained earlier, promoters of *MAL*

locus genes possess binding sites for Mig1 (Han *et al*., 1995; Dietvorst *et al*., 2005; Day *et al*., 2002a). These promoters also have MAL-activator binding sites and competition in the promoters between Mig1 and MAL-activator binding has been suggested to mediate the balance between repression versus induction (Wang *et al*., 1997). Probably MAL-activators are not able to bind when Mig1 repressors already cover the promoter. For example Kodama *et al*. (1995) have shown with a lager strain that when the MAL-activator was over-expressed from a multicopy plasmid, no increase in the *MALx1* and *MALx2* expression was seen under glucose repressive conditions (Kodama *et al*., 1995). Part of the glucose repression is due to a secondary effect, *i.e.* glucose represses *MALx3* genes, which in turn causes a lower level of induction of *MALx1* and *MALx2* genes (Hu *et al*., 1995).

In addition to the Mig1-dependent repression mechanism, a Mig1-independent mechanism has also been described. It was detected that in mig1 deletion strains, with constitutive MAL-activator expression, glucose repression was not completely alleviated (Hu *et al*., 2000). This could be because the MAL-activator needs intracellular maltose to obtain its active conformation (Wang *et al*., 1997). Another option is that when glucose is present, active conformation of the MAL-activator can't be obtained (Hu *et al*., 1995).

Maltose transport is affected also by catabolite inactivation. In particular, catabolite inactivation means glucose-triggered inactivation and/or proteolysis of proteins. By analogy to catabolite repression, this phenomenon has been called catabolite inactivation. Catabolite inactivation is a common mechanism for a number of plasma membrane proteins, which are observed to be removed from the plasma membrane and inactivated by glucose under different physiological conditions (Medintz *et al*., 1996). Catabolite inactivation has been mostly studied in laboratory strains but it has also been shown to occur at least in an ale strain (Rautio and Londesborough, 2003). Addition of glucose to maltose fermenting cells causes a rapid and irreversible loss of the ability to transport maltose (Görts *et al*., 1969). Maltose transporters but not maltase enzyme is subject to catabolite inactivation (Federoff *et al*., 1983; Rautio and Londesborough 2003), as expected since catabolite inactivation is particularly connected to the plasma membrane proteins.

There is an endocytosis and degradation targeting signal found in the N-terminal cytoplasmic domain of the Mal61 protein (Medintz *et al*., 2000). This signal sequence is referred to as the PEST sequence since it is rich in proline, glutamate, aspartate, serine and threonine. Glucose-triggered phosporylation of

serine and threonine residues in the PEST sequences is suggested to be associated with regulated degradation (Medintz *et al*., 2000). This occurs by rapid inactivation of the transporter from the membrane by endocytosis and subsequent vacuolar proteolysis (Riballo *et al*., 1995). Mal61 transporter mutants lacking the PEST sequence exhibit significantly reduced rates of glucose-induced proteolysis (Medintz *et al*., 2000). The PEST sequence has been reported to be present also in the Mal31 transporter (Day *et al*., 2002a) and is most probably found in other Malx1-type transporters due to the high conservation of sequences. Mal21p has been shown to be more resistant to glucose-induced degradation than Mal31p and Mal61p. This has been suggested to be due to D46G and L50H conversion in its amino acid sequence (Hatanaka *et al*., 2009).

Agt1 transporters lack the PEST signal sequence (Day *et al*., 2002a; Dietvorst *et al*., 2005). Nonetheless, Hollantz and Stambuk (2001) have shown that glucose induces a rapid catabolite inactivation of the Agt1 transporter. Possibly there is some other internalization signal present in the Agt1 protein. Dietvorst *et al*. (2005) observed that in Mtt1 transporter there is a RSTPS protein motif present, which they suggest to be an internalization signal. This signal sequence was missing in the altered version (Mtt1alt) of the transporter, which possessed increased maltose and maltotriose transport rates. This possibly suggests that Mtt1 type transporters are also under catabolite inactivation through this RSTPS signal motif.

It has been shown that yeast adaptation to maltose utilization diminishes the glucose regulation of maltose uptake. Cells pre-grown on maltose and harvested while the sugar is still present in the medium are more adapted to utilize maltose and maltotriose during the early stages of fermentation (Ernandes *et al*., 1993). Hazell and Attfield (1999) showed that pulsing yeast with maltose to induce expression of *MAL* genes prior to inoculation to mixed sugar medium enhanced sugar fermentation. The reason for this is probably that due to pre-growth on maltose there is already a significant amount of maltose transporters present in the yeast plasma membrane and thus yeast cells are able to start utilization of maltose earlier. This also suggests that glucose inactivation doesn't occur remarkably in these strains.

Glucose repression and glucose inactivation of the maltose transporters, result in rather strict exclusion of maltose from the cell (since there are no maltose transporters in the plasma membrane). This result is due to an inducer exclusion effect (Hu *et al*., 1995), *i.e.* while glucose is present, the inducing agent maltose is not transported into the cell in sufficient amounts to be able to create the in-

ducing effect. However, maltose transport at very low levels has been shown to suffice for the inducing effect (Hu *et al.*, 2000) so that practically complete exclusion of the inducer must be obtained for this effect.

Glucose regulation may be difficult to overcome completely since it is mediated at different levels and by different mechanisms. However, it has been shown by Meneses *et al*. (2002) that the actual rate of maltose or maltotriose transport into the cell once it begins is of major importance. Strains with efficient maltose and maltotriose transport can perform better than strains with early onset but not as efficient transport (Meneses *et al*., 2002).

1.10 High-gravity brewing

In traditional brewing, worts with original gravity of 11 to 12°Plato are fermented to beers with 4 to 5% (v/v) ethanol. In high-gravity brewing, concentrated wort with higher original gravity is fermented. Higher sugar concentration in the wort results in higher final alcohol concentrations and formation of stronger beer. Beer produced is diluted at some point to produce finished beer of desired alcohol content. Breweries often use carbohydrate adjuncts (*e.g*. sugar syrups) when making high-gravity worts. High-gravity wort contains more fermentable sugars for each degree plato of extract.

High-gravity brewing was originally introduced in the United States in the 1950s and is now a very common practice worldwide. Today, most major beer brands are made by fermenting high-gravity (14–18°P) worts and there is effort to increase wort strength into the very-high-gravity (VHG) range, *i.e.* a specific gravity of 18–25°P. There are several advantages in high-gravity brewing. Production capacity increases, *i.e*. more beer is produced with the same size of brewhouse and fermentation facilities at a given time. The closer to the final stage (packaging) that the concentrated beer is diluted, the more economical benefit is obtained. Use of high-gravity brewing decreases energy consumption of brewhouse and gives savings in labour and cleaning costs per unit volume of beer produced. With high-gravity brewing the capacity of existing plants can be increased 20–30% (Boulton and Quain, 2001) or even up to 50% if very-high-gravity worts are used (Blieck *et al*., 2007).

Attemps to use VHG worts have faced some problems. Beer fermentation using initial wort gravities above 18°P has a negative impact on yeast performance and often results in sluggish fermentations, *i.e*., lower specific growth rate and a longer lag phase before initiation of ethanol production (Pátková *et al*., 2000;

Piddocke *et al*., 2009). Also, unacceptably high concentrations of sugars, especially maltotriose, often remain in the final beer, with consequent decreases in yield and lower and variable product quality. There are several reasons linked to the deteriorated performance of yeast in very-high-gravity brewing. One of the reasons is increased osmotic pressure at the beginning of the fermentation due to high sugar concentration. Elevated osmotic pressure has been shown to have an effect on the uptake of maltose and maltotriose (whereas glucose uptake is only modestly affected) (Stewart *et al*., 1995). Decreased oxygen availability due to decreasing solubility of oxygen with increasing wort gravity could be one reason for decreased sugar uptake (D'Amore *et al*., 1991). An adequate amount of oxygen is needed for the synthesis of membrane lipids, which are necessary for efficient maltose/maltotriose transporter function.

Towards the end of the fermentation ethanol concentrations in HG and VHG wort fermentations are high. There is also a shortage of nutrients. It has been claimed that nutritional deficiency is a more severe problem than high alcohol concentrations as when nutritional deficiencies are remedied it has been demonstrated that brewer's yeast tolerates high ethanol concentrations well (Jakobsen and Piper, 1989). It has also been shown that when a nitrogen source, ergosterol, and oleic acid are added to worts it is possible to use even 31°P extract wort and beer up to 16.2% (v/v) ethanol can be produced (Casey *et al*., 1984).

Traditionally, brewers recycled yeast indefinitely from fermentation to fermentation so that present brewer's yeast strains result from hundreds of years of selection in traditional brewer's worts. However, this selection stopped some 40 years ago when brewer's started to store their strains as pure cultures. Nowadays, even if serial repitching is used, propagation of the yeast is nonetheless started from stored pure cultures at regular intervals. Thus natural evolution of yeast strains by selection for better performance in high-gravity conditions doesn't take place.

Sugar adjuncts are any added source of fermentable sugars used in the brewing process. Most often sugar syrups of different forms are used. Syrups are characterized by their concentration, purity, colour and sugar spectrum. Thus they can range from almost pure glucose syrup, which is entirely fermentable, to crude hydrolysates of cereal starch, which have been subjected to various amylases and thus contain a mixture of fermentable sugars and dextrins. Less pure cereal extract may also include significant concentrations of nitrogen. In Table 4, typical sugar spectra of VHG worts obtained by addition of two different types of sugar syrups are shown. Addition of barley syrup results in high-glucose wort

whereas addition of high-maltose syrup raises mostly the share of maltose. Addition of maltose-rich syrup as opposed to glucose-rich syrup has been shown to result in more balanced fermentation performance (Piddocke *et al*., 2009).

When sugar adjuncts are added it must be taken into consideration that this modifies the nutrient balance. For example nitrogen concentration is diluted. This leads to modified carbon to nitrogen ratio, which in turn if changed dramatically, can change the flavour profile (Piddocke *et al*., 2009).

Table 4. Typical composition of very-high-gravity worts (Huuskonen *et al*., 2010). In high-glucose wort barley syrup and in low-glucose wort high-maltose syrup was used as an adjunct.

Composition or characteristic of wort	**High-glucose 25°P wort**	**Low-glucose 25°P wort**	**All-malt 18°P wort**
Glucose (g /l)	62	25	13
Fructose (g/l)	5	4	3
Maltose (g /l)	105	120	80
Maltotriose (g /l)	24	40	24
Total conc of fermentable sugars (g/l)	198	193	121
Maltose/glucose ratio (g/g)	1.7	4.8	6.2
pH	4.9	5.04	4.98

1.11 Effect of temperature change on the plasma membrane and transporters embedded in it

Composition of the plasma membrane is essential for transporter function. This is because the transporter proteins are associated with specific lipids of the plasma membrane and are dependent on these lipids for their structural integrity and function (Bogdanov *et al*., 2002; Opekarová and Tanner 2003). Actually transporters can be regarded as protein-lipid complexes.

The existence of functional lipid rafts in the plasma membrane of yeast has been shown (Bagnat *et al*., 2000). Lipid rafts are less fluid membrane microdomains whose main components in yeast are sphingolipids and ergosterol. Many transporter proteins have been shown to be located in the lipid rafts, like, for example, uracil permease Fur4p (Hearn *et al*., 2003), tryptophan permease Tat2p (Umebayashi and Nakano, 2003), arginine/H^+ symporter Can1p and ATPase

Pma1 (Malínská *et al*., 2003). It has been shown that proteins located in the lipid rafts need certain amounts of ergosterol and/or sphingolipids for both efficient delivery to the plasma membrane and for their subsequent stabilization and function in the plasma membrane (Bagnat *et al*., 2000; Hearn *et al*., 2003; Mitsui *et al*., 2009). It has been suggested that proteins localized in lipid rafts associate with lipid rafts already in the endoplasmic reticulum and lipid rafts are essential for the delivery of these newly synthesized proteins to the plasma membrane (Bagnat *et al*., 2000; Hearn *et al*., 2003). Also, after the delivery to the plasma membrane these protein remains in the lipid rafts.

At the optimum growth temperature for a strain, membranes are colloidal solutions of lipids and proteins in a fluid (liquid crystalline) phase. Proteins embedded in the membrane, like transporters, function efficiently only when the membrane is in the fluid phase and loose their activity when the phase changes to rigid (McElhaney, 1982). It is possible to measure membrane fluidity with probes such as 1,6-diphenyl-1,3,5-hexatriene, which are fluorescent only in the liquid phase. A decrease in the temperature or an increase in pressure lead to a decrease in the mobility of these fluorescent molecular probes, and is interpreted as decrease in the membrane fluidity (MacDonald *et al*., 1988). Studies performed with such probes with membrane preparation have shown that there is no evidence of sharp phase transition with temperature but rather a continuous change of membrane fluidity when temperature decreases (Beney and Gervais, 2001).

The occurrence of phase-transition depends on the membrane lipid composition (*e.g*., the presence and type of sterols and fatty acids) and on external factors, such as the osmotic pressure (Guyot *et al.*, 2006). For example, ergosterols determine to a large extent the rigidity of the plasma membrane (van der Rest *et al*., 1995a). The lower the temperature the more unsaturated the membrane fatty-acid composition is (Watson, 1978). Increased portion of unsaturated lipids in the membranes is suggested to protect against the temperature decrease and reduce the temperature at which the membrane undergoes the phase change. Many organisms regulate their membrane lipid composition in response to environmental temperature so that membrane fluidity can remain optimal. The degree of lipid unsaturation has been observed to be affected by growth temperature in *S. cerevisiae* strains, *i.e*. the degree of unsaturation increased significantly (doupled) at 13°C compared to that at 25°C (Torija *et al*., 2003).

The rates of most enzyme-catalysed reactions approximately double for each 10°C rise in temperature because of more collisions between reactants per time

(until the denaturation temperature is reached). In chemical reactions a relationship between the temperature and velocity is usually expressed with the Arrhenius equation. In an Arrhenius plot the logarithm of transport velocity is plotted against the inverse temperature (1/T). For a single rate-limited thermally activated process, an Arrhenius plot gives a straight line. However, reactions catalysed by integral membrane proteins usually exhibit non-linear Arrhenius plots with increased temperature-dependence at lower temperatures. This is usually interpreted as reflecting change of state ("freezing") of the lipids around the catalytic protein.

The substrate-translocation mechanism most commonly ascribed to secondary membrane transporters is the alternating access mechanism. In this mechanism, the transporter is suggested to have two major alternating conformations: inward-facing and outward-facing. In any moment, a single binding site in a polar cavity is accessible to any one side of the membrane (Huang *et al.*, 2003) and thus change of shape of the transporter requires that it moves against the surrounding lipid (Abramson *et al.*, 2003). When temperature decreases and plasma membrane becomes more rigid, conformational change occurring between inward and outward conformations is probably more tedious.

2. Materials and methods

Materials and methods for studies performed in publications I–IV are described in each publication. Transcriptional analysis results represented in Figure 5 were performed as described in Rautio *et al*. 2007. Industrial strains used in I–IV are listed in Table 5.

Table 5. Industrial strains used in the studies of I–IV.

Strain	**Origin**
Lager strains	
A-60012	Weihenstephan 1
A-62013	Weihenstephan 294
A-63015 (A15)	Nordic brewery
A-66024 (A24)	Nordic brewery
A-82064	Nordic brewery
A-85072	Nordic brewery
A-95143	Nordic brewery
WS34/70	Weihenstephan
CMB33	Belgium
Ale strains	
A-10179 (A179)	UK brewery
A-10180 (A180)	UK brewery
A-101181 (A181)	UK brewery
A-60055	NCYC 1200
A-60056	NCYC 240
A-75060 (A60)	Nordic brewery
A-93116	NCYC 1087
Baker's yeasts	
B-62001	Nordic baker's yeast
B-62003	Nordic baker's yeast
Distiller's yeasts	
C-72051	Nordic distillery
C-77076	Nordic distillery
C-91180A	Nordic distillery

3. Results and discussion

3.1 *MAL* locus distribution and integrity in brewer's yeast strains (Paper I, IV)

Distribution of *MAL* loci in the genomes of brewer's yeast strains was studied by hybridizing chromosome blots with *MAL* locus probes. In studies described in paper I, *AGT1*, *MALx1*, *MALx2* and *MALx3* probes were used to study the distribution of the *MAL* loci genes in several brewer's and in some laboratory yeast strains. Two specific *MALx3* probes for the detection of divergent *MALx3* sequences, ca. 75% identical to standard *MALx3* gene, found in the *MAL1* and the *MAL3* loci, respectively, were also used. These probes hybridize to *MAL33* and to the *MAL13* activator, here called *MAL13*(*AGT1*), found in the *MAL1* loci that contain *AGT1*. Earlier studies on *MAL* locus distribution in the brewer's yeast strains have been performed with α-glucoside transporter, *MALx1* and *AGT1* probes solely and thus do not give information on whether the two other genes of the locus are also present (Jespersen *et al*., 1999; Meneses and Jiranek, 2002). In the present study, the distribution of the *MAL* loci in the genomes of yeast strains, as well as the integrity of each *MAL* locus was revealed. It was observed that the *MAL* loci are highly conserved in possession of all three genes of the locus. Apart from two exceptions, only *MAL* loci with all three genes were found. These exceptions were weak hybridization of *MAL62* probe to chromosome IX in all brewer's yeast strains, while no hybridization of other probes (*MALx1*, *AGT1* or *MALx3*) to this chromosome was observed. Based on the S288C laboratory strain sequence (SGD), there is an ORF, 73% identical to the *MAL62* gene, present independently without other *MAL* loci genes, on chromosome IX. It is possible that the hybridization observed occurred with this ORF, which possibly has higher similarity to the *MAL62* probe in the brewer's yeast

strains. Another exception was very weak binding of the *MAL61* probe but not *MAL62* and *MAL63* probes to chromosome VIII, where the *MAL6* locus maps, in the brewer's yeast strains. However, in lager strain A72 all three probes bound strongly to the *MAL6* locus indicating that the locus is entire in this strain.

Some studies have shown that there can be extra copies of one or more of the three genes of the *MAL* locus present. For example, there are two MAL-activator encoding genes present in the *MAL6* loci of some yeast strains (Charron *et al*., 1989). In the S288C strain there is an extra *MALx2* gene, *i.e.* verified ORF YGR287c, 75% identical to *MAL12*, encoding isomaltase, present in the *MAL1* locus (*Saccharomyces* Genome Database data). Also partial tandem duplication of *MAL3* locus genes has been described where the copy number of *MAL31* and *MAL33* genes are increased compared to the *MAL32* genes (Michels *et al*., 1992). In the present study, hybridization intensities were observed to vary between the *MAL* loci with a single probe. Unequal hybridization might be due to extra copies of the *MAL* genes in certain loci as explained above. It also might be due to unequal distribution of the *MAL* loci in sister chromosomes, especially in lager strains, which are polyploid. Thirdly, unequal labelling could be due to the sequence divergence between the loci but since, at least *MALx1* and *MALx2* genes deriving from different *MAL* loci have been shown to be almost identical this option does not seem probable. Unequal labelling observed between the *MAL* loci was measured by taking the *MAL3* locus as a reference locus and the intensity of the hybridization signals obtained with *MAL61* and *MAL62* probes at each *MAL* locus were calculated relative to the intensities obtained at the *MAL3* locus (I, Table 4). In the lager strain A15, the signal intensities normalized this way were similar for both probes, *MAL61* and *MAL62*, suggesting that at each *MAL* locus of strain A15 the ratio of *MALx1* and *MALx2* genes is the same as in its *MAL3* locus. Thus, in the A15 strain the different signal intensities between the loci probably reflect different copy numbers of the *MAL* loci present (*MAL1>MAL3>MAL4>MAL2>MAL6*), *i.e*. there are more chromosomes VII where *MAL1* locus is present than chromosomes II where *MAL3* locus is present *etc*. For the other brewer's yeasts, the normalized signals at *MAL1* and *MAL2* loci were markedly stronger with the *MAL62* probe than with the *MAL61* probe. This would imply that there are more copies of *MALx2* compared to the *MALx1* in *MAL1* and *MAL2* loci than in the reference *MAL3* locus. A24 differed from other strains by having particularly strong signal strengths at *MAL2* (I, Table 4; I, Fig. 4), suggesting that *MAL2* is the most abundant *MAL* locus in this strain

and that in A24 either several copies of chromosome III carry the *MAL* locus or there is tandem duplication of *MAL* genes in some of the chromosomes III.

Both the *AGT1* and *MAL11* genes were present in all the strains studied (I, Table 3) on chromosome VII (I, Fig. 3), as has been described earlier for other brewer's yeast strains (Jespersen *et al*., 1999; Meneses and Jiranek, 2002). There possibly are two types of chromosomes VII in brewer's yeast strains, *i.e*. one possessing *AGT1-MALx2-MALx3* type *MAL1* locus and another possessing *MAL11-MALx2-MALx3* type *MAL1* locus. This is based on the observation that in the laboratory yeast strains either the *AGT1* or *MALx1* gene is found at each time in the *MAL1* locus together with *MALx2* and *MALx3* genes (Naumov *et al*., 1994; Charron and Michels, 1988). However, recently after obtaining sequence data from whole genome sequencing projects of several *S. cerevisiae* yeast strains, contig sequences have been presented where, for example, both *AGT1* and *MALx1* genes are located 10 kbp apart from each other on the same continuous stretch of DNA (IV, Fig. 3) (*S. cerevisiae* strain RM11-1a sequencing data http://www.broadinstitute.org/annotation/genome/saccharomyces_cerevisiae.3/Home.html). Thus, another option would be that *AGT1* and *MALx1* genes are actually both located on chromosome VII and are not allelic. Lager strain WS34/70 whole genome sequencing results do not answer the question of localization of *MAL11* and *AGT1* genes in respect to each other since there was no *MALx1* sequence mapped on chromosome VII of WS34/70. This is most probably because there are gaps in the sequence (sequence coverage was estimated to be 95.8%) and the *MAL11* gene might be in the non-sequenced 4.2%.

If *AGT1* and *MAL11* are alleles of each other, unequal labelling would be expected for the *MAL1* locus in the hybridization studies. There should be *MAL1* loci with *AGT1* sequences, which do not bind the *MAL61* probe but are accompanied by *MAL12* genes that do bind the *MAL62* probe. In all other brewer's yeast strains, except in A15, unequal labelling between *MALx1* and *MALx2* probes was observed in the *MAL1* locus (I, Table 4) that could imply the allelic nature of *MAL1* and *AGT1* genes. In the A15 strain, more or less equal binding of *MAL61* and *MAL62* probes to the *MAL1* locus was observed. This probably suggests a different organization of the *MAL1* locus in this strain like, for an example, an *AGT1* gene not accompanied by *MAL12* sequences.

A common characteristic of all brewer's yeast strains in the present study was that they all possessed *MAL1, MAL3* and *MAL4* loci. This was in accordance with the results obtained by Jespersen *et al*. (1999) and Meneses and Jiranek (2002), who found that *AGT1*, *MAL11, MAL31* and *MAL41* genes were present

in practically all ale and lager strains in their studies. Distribution of the *MAL2* locus was observed to be different between ale and lager strains. *MAL2* locus was found in all lager strains but only in one of the ale strains (A60 strain) (I, Table 3). In studies of Jespersen *et al*. (1999) and Meneses and Jiranek (2002) it was observed that the *MAL21* gene was found in 60% of the lager strains whereas all ale strains lacked this gene. Some *MAL21* gene-encoded transporters have been observed to possess amino acid changes, which make them resistant to glucose inactivation, *i.e*. endocytosis of maltose transporters from the plasma membrane caused by glucose (Hatanaka *et al*., 2009). Possession of such a *MAL2* locus would be beneficial and probably increase maltose uptake capacity. The *MAL6* locus was observed to be the least common. Only lager strain A72 possessed it in the present study and none of the ale or lager strains in the studies of Jespersen *et al*. (1999) and Meneses and Jiranek (2002).

Hybridization of *MAL* locus probes to other than known *MAL* loci was observed (I, Table 3). All three probes *MALx1*, *MALx2* and *MALx3* hybridized to ~1.3 Mbp chromosome in the A60 ale strain. In *S. cerevisiae* there is no chromosome of this size and therefore it is not known which chromosome this band represents. Binding with all three probes was slightly weaker than observed with other *MAL* loci suggesting that sequence homology is probably lower. Jespersen *et al*. (1999) found also hybridization of *MAL61* probe to this same size of chromosome (~1.3 Mbp) in one lager strain. They studied if the hybridization could be explained by chromosome size polymorphism of chromosome VII, which is in the same size range and contains *MAL* locus. However, the 1.3 Mbp chromosome was not detected with probes for the *BGL2* or *SER2* genes mapping to the chromosome VII (Jespersen *et al*., 1999). Taken together, these results suggest that there seems to be a new, not earlier identified *MAL* locus localized in this 1.3 Mbp chromosome found in both ale and lager strains. This putative *MAL* locus seems to be even more often present in the brewer's yeast genomes than the rarely met *MAL6* locus.

3.2 *AGT1* gene of lager strains encodes a non-functional transporter (Paper I, III)

The *AGT1* gene of all lager strains studied was found to possess an extra nucleotide at position 1183 leading to the frame shift mutation and formation of truncated polypeptide of 394 amino acids instead of the full length 616 amino acid protein (I). It was observed that this truncated polypeptide was unable to transport

maltose (III). It was also shown that *AGT1*, possessing the frame shift mutation, was characteristic for lager strains as it was found in all nine studied lager strains but in none of the ale, baker's or distiller's strains studied (III, Table 1).

The *AGT1* genes deriving from ale strains, A60 and A179, were observed to encode a full-length maltose transporter that was functional, *i.e.* Agt1 transporters of ale strains were observed to be high affinity maltose transporters strongly inhibited by maltotriose (85% and 79% inhibition in A60 and A179, respectively) and trehalose (94% and 85% inhibition in A60 and A179, respectively) (Paper III) as expected for a broad specificity Agt1 transporter.

3.3 Presence of *MPHx*, *MTT1* and *SbAGT1*genes (Paper I, III)

The presence of *MPHx* genes was studied similarly by hybridizing chromosome blots with the *MPHx* probe. It was found that the *MPH3* gene was missing in all the brewer's yeast strains but was present in the laboratory strains (I, Table 3; I, Fig. 5). In accordance, the *MPH3* gene was also found to be missing in the brewer's yeast strains studied by Jespersen *et al*. (1999) and Meneses and Jiranek (2002). The *MPH2* gene was observed to be differently distributed among the ale and lager strains. It was present in most of the lager strains but usually missing from the ale strains. In the A15 strain the *MPHx* probe hybridized to Chromosome VII/XV duplet instead of the known localizations for the *MPH2* and *MPH3* genes on chromosomes IV and X, respectively. Also Jespersen *et al*. found hybridization of the *MPHx* probe to the VII/XV duplet region in some of the lager strains. This suggests that, in addition to previously identified *MPH2* and *MPH3* loci, there is another *MPHx* locus present in the lager strain genome either on chromosome VII or XV.

At the time our chromosome blot hybridization studies were performed (I) *MTT1* gene had not yet been identified (first time reported 2005). The presence of the *MTT1* gene in relevant strains was performed as a separate analysis by PCR later on (III). It was found that *MTT1* was present in all lager strains studied but was missing in the ale strains (III, Table 1). *MTT1* gene was also present in some but not all baker's and distiller's yeasts. Nakao *et al*. (2009) have suggested that the *MTT1* gene is derived from the *S. bayanus* component of the lager yeast strain but since *MTT1* was found also in baker's and distiller's yeasts, which are related to *S. cerevisiae*, that option does not seem plausible.

Presence of the *Sb-AGT1* gene in ale and lager strains was screened for by PCR analysis (III). *Sb-AGT1* was found to be present in all lager strains studied

but missing from ale strains (III, Table 1). Sequencing of the *Sb-AGT1* gene of A15 and A24 lager strains revealed both of them to be 100% identical to *Sb-AGT1* of WS34/70 (Nakao *et al*. 2009) (III). Based on sequence analysis, *Sb-AGT1* seems to encode a full-length Sb-Agt1 protein with 85% identity at amino acid level to Agt1 protein. Substrate specificity or any sugar transport characteristics of this Sb-Agt1 protein haven't, however, yet been characterized. Possibly Sb-Agt1 protein has a role in maltose/maltotriose transport in lager strains but this remains to be elucidated in future work.

3.4 *MAL* and *MPHx* genotypes of laboratory strains (Paper I)

Laboratory strains differed from brewer's yeast strains in that they seem to possess only *MAL1* (*AGT1-MALx2-MALx3*) and *MAL3* loci, except the CEN.PK2-1D strain where the constitutive *MAL2-8c* locus has been integrated to its genome to obtain a mal-positive phenotype (Rodicio and Zimmermann, 1985). In the S288C strain both of the genes encoding MAL-activator, *MAL13* and *MAL33*, are known to be non-functional (Bell *et al*., 2001). This renders the S288C strain unable to utilize maltose, producing a Mal-negative phenotype. Also, other laboratory strains (except CEN.PK2-1D with constitutive *MAL* locus) of this study were observed to be Mal-negative. It is possible that possession of defective activators in *MAL1* and *MAL3* loci is a common characteristic for laboratory strains. The *MPH3* gene was found to be present in the genomes of all laboratory strains. There was also very weak hybridization to chromosome IV where the *MPH2* locus maps with the *MPHx* probe.

3.5 More prevalent α-glucoside transporter genotypes for ale and lager strains (Paper I, III)

Taking the genotype determination results together, the more prevalent genotype in regard to α-glucoside transporter genes can be represented as *agt1*, *Sb-AGT1*, *MAL11*, *MAL21*, *MAL31*, *MAL41*, *MPH2*, *MTT1* for a lager strain and *AGT1*, *MAL11*, *MAL31* and *MAL41* for an ale strain, respectively. The *AGT1* gene was shown to be non-functional in all studied lager strains and for this it is marked as defective in the genotype description above. It is also possible that some other of these α-glucoside transporter genes encode non-functional proteins. For example, functionality of Mphx transporters has been questioned (Duval *et al*., 2010).

In addition, nothing is known about the functionality of Sb-Agt1 transporter yet. There can also be strain-dependent differences in the functionality of each transporter. In general, lager strains seem to have wider distribution of α-glucoside transporter encoding genes in their genome. This was expected because of the hybrid nature of lager strains, *i.e.* they possess transporters originally derived from both species. Actually, there can even be more α-glucoside transporter genes present in the genomes of lager strains than now detected since, for example, the *S. bayanus* derived *MALx1* genes are not necessarily recognized with probes used, *i.e.* probes designed based on *S. cerevisiae* sequences.

3.6 Expression of α-glucoside transporter genes *AGT1*, *MALx1* and *MPHx* in brewer's yeast strains (Paper I, II)

The ale and lager strains were observed to differ markedly in their expression of *MALx1* and *AGT1* genes in all conditions tested [induced (maltose), repressed (glucose) or in the presence of both inducing and repressing sugars]. *MALx1* genes were strongly expressed in all three lager strains studied, whereas their expression in the ale strains was very weak (I, Fig. 6). In contrast to *MALx1*, the expression of *AGT1* gene was observed to be strong in the ale strains and very weak in the lager strains (I, Fig. 7). In these expression studies it is possible that *Sb-AGT1* expression would not have been detected because of low sequence identity (79%) to the probe. These results are consistent with the study of Meneses and Jiranek (2002), where it was shown that *AGT1* expression in a lager strain was hardly detectable whereas in the five ale strains of the study, strong *AGT1* gene expression was observed. The *MTT1* gene was not included in these studies since it was not yet identified at that time. However, it has been shown that in the brewery fermentations with a lager strain, *MTT1* expression has a very similar pattern to *MALx1* gene expression (Rautio *et al*., 2007). As a conclusion, *MALx1* and possibly also the *MTT1* genes seem to be the most dominantly expressed transporter genes in the lager strains, whereas the *AGT1* gene is dominantly expressed in the ale strains. It is seen that the α-glucoside transporter genes present most often in the genomes of ale and lager strains are not necessarily the most strongly expressed. For example, ale strains generally possess three different *MALx1* genes (*MAL11*, *MAL31* and *MAL41*) and the *AGT1* gene but nonetheless *AGT1* gene expression was observed to be much stronger compared to *MALx1*.

MPHx expression was not detected in any conditions (repressed, induced, or in the presence of both repressing and inducing sugar) in any of the ale or lager strains of the present study (I). This is in contrast to the studies of the Day *et al*., (2002b) who found strong expression of the *MPHx* gene in similar conditions. In the expression studies of Day *et al*., *MPHx* gene was cloned from a lager strain and expressed under its own promoter from a multicopy plasmid in a laboratory strain. Strong expression of *MPH2*, induced by both maltose and maltotriose and repressed by glucose, was observed (Day *et al*., 2002b). Also during wort fermentations with different lager strains strong expression of *MPHx* genes has been observed (James *et al*. 2003; Gibson *et al*., 2008). It is possible that the expression of *MPHx* genes is strain-dependent and in the three lager strains included in the present study it does not occur. Another explanation would be that the *MPHx* probe used in the present study was somehow defective although its sequence as well as radioactive labelling was verified.

Meneses and Jiranek (2002) have shown that there are strain-dependent differences in the degree of glucose repression of the *AGT1* and *MALx1* genes. They observed that in ale strains able to constitutively utilize maltose, the *AGT1* (or *MALx1*) genes were expressed constitutively and seemed not to be under glucose repression. In the present study, strong glucose repression of the *MALx1* and *AGT1* genes as well as strong induction by maltose was observed in all strains (I, Fig. 6; I, Fig. 7). In shake flask cultivations glucose repression was still present when glucose concentration was 5 g/l and only at a glucose concentration below 2 g/l was strong expression of *MALx1* and *AGT1* genes detected, even if the inducing sugar maltose was present at a concentration of ~10 g/l in the cultivation where both inducing and repressing sugars were present simultaneously (I, Fig. 6, sugar concentrations not shown). In shake flask cultivations the starting concentration for glucose was 20g/l and glucose was the sole fermentable sugar present, except in the mixed sugar cultivation where the inducing and repressing sugars, maltose and glucose, were both present at starting concentrations of 10 g/l. In brewery fermentations sugar concentrations are higher, for example, in 24°P wort used in studies described in paper II, glucose concentration was 24 g/l and concentration of total fermentable sugars as high as 180 g/l. Expression studies performed with samples collected during the main fermentation of VHG wort (Rautio and Londesborough, 2003) differed significantly from those obtained from shake flask cultivations. In a lager strain, strong coordinate increase in the expression of *MALx1*, *MALx2* and *MTT1* was observed when there was still a high concentration of glucose present (>20 g/l). Similar strong

expression of *MALx1* was observed also in the A15 strain when glucose concentration was still correspondingly high (Rautio and Londesborough, 2003). One explanation for this significant difference could be that maltose concentration is much higher in the VHG wort (24°P), 80–120 g/l, compared to the maltose concentration in shake flask cultivations (10 g/l). Thus, the inducing effect caused by maltose could be stronger in wort fermentations and possibly somehow overtake the glucose repression. In addition, there is also a significant amount of maltotriose present in VHG wort (20–40 g/l). Maltotriose is suggested to be a better inducer of maltose/maltotriose transporters than maltose (Salema-Oom *et al*., 2005; Dietvorst *et al*., 2005).

3.7 Effect of amino acid changes in the Agt1 sequence on maltose and maltotriose uptake (Paper I)

It has been shown that even a single change in the amino acid sequence of Agt1p can have significant effects on maltose and maltotriose transport ability. Smit *et al*. (2008) have reported two specific amino acid changes I505T and T557S that render the Agt1 transporter in *S. cerevisiae* unable to carry maltotriose. Whereas we observed in our studies that L117P change in the Agt1 sequence rendered the Agt1 transporter unable to carry maltose (unpublished results). *AGT1* genes were sequenced from A15, A24, A60 and A179 strains (I). There were several amino acid changes detected when translated sequences were compared to Agt1p of SGD data bank (I, Table 6). Altogether there were 33 amino acid changes between the SGD Agt1 sequence and the brewer's yeast sequences, 13 common to all of the brewer's yeast strains, 5 specific for the lager strains, and 15 specific to one or both ale strains. Agt1 tranporters of lager strains are known to be non-functional due frame-shift mutation but in ale strains these sequence changes most probably lead to at least slight differences in the characteristics of maltose and/or maltotriose uptake between these transporters. Interestingly, some of the amino acid changes described by Smit *et al*. (2008) were found in the ale strains of the present study. One of them was V548A change (referred to as V549A in Smit *et al*. (2008) because they use different alignment where Mal31p sequence is also included), which was found in the ale strain A60 of the present study (I, Table 6). According to Smit *et al*. (2008) V549A amino acid change decreased maltotriose transport capacity 3-fold. The second common change was T557S change (referred in our study as T556S, see above), which is reported to lead to the complete inability to carry maltotriose (Smit *et al*., 2008). The A179 ale

strain was observed to possess this change. The third change described by Smit *et al*. (2008), *i.e*. I505T change that as well renders Agt1 transporter unable to carry maltotriose was not found in the strains of the present study.

Although the changes described by Smit *et al*. (2008) change significantly maltotriose transport, ability of Agt1 transporter to uptake maltose, was observed to be not affected. It was actually shown that maltose transport was even more efficient by Agt1 transporter with T557S change than with the ordinary Agt1p (Smit *et al*., 2008). One of the amino acid changes that decrease or even completely abolish maltotriose transport ability of Agt1p were present in both ale strains of the present study. These strains are not known to be closer relatives to each other than ale strains are in general. If possession of such changes is characteristic of ale strains, it could, at least partly, explain the slower utilization rate of maltotriose observed in ale strain fermentations compared to those performed with lager strains (Zheng *et al*., 1994a). Ale strains are not known to possess other transporters able to carry maltotriose since they do not possess Mtt1 transporters and only rarely possess Mphx transporters.

3.8 Maltose and maltotriose uptake kinetics (Paper I)

Competitive inhibition studies of maltose uptake can be used to deduce the transporter types that are actually present in the plasma membranes of yeast cells. Trehalose is known to be a substrate for Agt1 and Mtt1 transporters but not for Mphx and Malx1 transporters (Han *et al*., 1995; Day *et al*., 2002a; Salema-Oom *et al*., 2005) and trehalose is therefore expected to inhibit competitively maltose transport by the Agt1 and Mtt1 transporters but not affect transport by the Malx1 and Mphx transporters. After growth on maltose, strong inhibition of maltose transport by trehalose for both ale strains, 82% for A60 and 88% for A179, was observed (I, Fig. 1). Lager strains showed more moderate inhibition in their maltose transport capability by trehalose, 30% for A24 strain and 55% for A64 strain, whereas maltose transport in A15 strain was not affected (I, Fig. 1).

Corresponding results were obtained when maltose uptake inhibition was performed with maltotriose and α-methylglucoside. Strong inhibition of maltose uptake was observed in the ale strains (average inhibition 59–66% with maltotriose and 41–74% with α-methylglucoside) whereas only moderate inhibition in the lager strains (12–24% with maltotriose and 10–23% with α-methylglucoside) was observed (I, Table 1).

Taken together, these kinetic results suggest that when these brewer's yeast strains are grown on maltose, high specificity maltose transporters, such as Malx1p, account for 0–15% of the maltose transport capacity of ale strains but for 40–80% of the maltose transport capacity of lager strains. The inhibition studies are in accordance with expression results of *AGT1* and *MALx1* genes in these strains. Both expression and inhibition studies suggest that maltose transport by the two studied ale strains was mainly mediated by broad specificity α-glucoside transporters that can carry also trehalose, maltotriose and α-methylglucosides, whereas transport by the three lager strains involved mainly Malx1 type transporters.

3.9 Improved fermentation performance of lager yeast strain after repair and 'constitutive' expression of its *AGT1* gene (Paper II, IV)

It has been earlier shown that fermentation performance of brewer's yeast can be enhanced by constitutively over-expressing *MAL61* maltose transporter gene in the brewer's yeast cells (Kodama *et al*., 1995). Significantly increased maltose uptake rates were observed with the *MAL61* over-expression strain and as significant as 30% reduction in the total fermentation time could be obtained in VHG wort (24°P) fermentation performed with the strain constructed. It was also observed that *MAL61* over-expression did not have effect on the glucose or maltotriose utilization pattern, which remained the same between the host strain and the modified strain. This result once more supports the prevailing view that maltotriose is not carried by *MALx1* transporters. Also, a baker's yeast strain over-expressing *AGT1* gene has been constructed (Stambuk *et al*., 2006). This over-expression strain carried maltotriose three-fold faster compared to the parent strain. Also, uptake of other α-glucosides, trehalose and sucrose, was increased by approximately three-fold.

In both of these over-expression strains described, the *MAL61* and *AGT1* genes are expressed from a multicopy plasmid where a gene conferring G418 antibiotic resistance for selection is present. Thus, constant presence of antibiotic in growth media is necessary to prevent the loss of the plasmids. For industrial-scale fermentations, maintenance of such selection is not desirable and not even economically profitable. Moreover, as the finished product, packaged beer, can possibly contain some residual yeast, use of antibiotic marker genes is not suitable. In addition, beer would contain antibiotics, which is not acceptable. For

this, another strategy, better suited for modification of food microbes, was used, *i.e*. stable integration of a cassette containing the desired gene and promoter to the genome of the host strain. Antibiotic resistance conferring plasmids was temporarily used to facilitate the screening phase of the transformants. During the co-transformation event, yeast tend to take inside both the integrating fragment and the selection plasmid. Selection plasmid is later removed from the cells when cultivated in the absence of selection (Blomqvist *et al*., 1991). As a result, stable integrant strains are obtained, which are not dependent on any additional agent for their stability. It has been earlier shown that brewer's yeast can be engineered this way and stable brewer's yeast strains have been obtained by using this method (Blomqvist *et al*., 1991; Liu *et al*., 2004).

3.9.1 Construction of integrant strain with repaired *AGT1* gene under the control of *PGK1* promoter

The approach of the present study to intensify the lager strain fermentation capacity was to repair the defective *AGT1* gene of the lager strain and at the same time put the *AGT1* gene under the control of the constitutive *PGK1* promoter. The latter was to avoid the glucose repression directed towards α-glucoside transporters at the early phases of the fermentation. Since brewer's yeast is a food microbe, it is important and desired that it does not contain any heterologous DNA. For this, an integration cassette was constructed in such a way that it contained only *S. cerevisiae* (ale) or *S. pastorianus* (lager) DNA. Structure of the integration cassette is shown in paper II, Figure 1. Lager strain A15 was co-transformed with integration cassette and antibiotic G418 resistance conferring plasmid pKX34. Homologous recombination, which occurs in *S. cerevisiae* spontaneously (Szostak *et al*., 1983) was expected to take place at the *AGT1* promoter and in the *AGT1* gene region, *i.e*. at both ends of the integration cassette leading to the integration of the cassette to the *AGT1* locus.

Selection of transformants pre-grown on glucose was performed on G418 plates by screening the ability of clones to grow on maltotriose when respiration was blocked with antimycin A (Dietvorst *et al*., 2005). It has been observed that there is a long lag phase before yeast pre-grown on glucose adapts to fermentative growth on maltotriose (Londesborough, 2001). Transformants containing a constitutively expressed maltotriose transporter gene were expected to start fermentative growth on maltotriose earlier. Colonies appearing first on the selection plates (II, Table 2) were collected and analysed by Southern blot analysis. This

analysis revealed that all the first appearing 36 colonies were transformants. Thus, the selection strategy was very efficient. Secondly, it was observed that there were two types of transformants. Most of the transformants (80%) contained both an endogenous *AGT1* gene and a transformed *AGT1* gene with *PGK1* promoter. The rest of the transformants contained only the transformed *AGT1* with *PGK1* promoter but not the endogenous *AGT1* (II, Fig. 2). This suggested that there must be at least two loci where *AGT1* resides in the genome of the lager strain.

PCR analyses confirmed that the integration had occurred as planned and as a result a chimeric *AGT1* gene was produced having sequence from nucleotide 1 up to 1478 from the A60 lager strain and the rest is the original A15 sequence previously present in the locus. Several transformants were sequenced at the corresponding region to verify that the second cross-over had occurred 3' to the frameshift in the A15 sequence. The actual cross-over place was observed to vary between different transformants but in most cases the crossover occurred 3' to the frame shift. In Integrant 1 there were two versions of the cross-over site. One cross-over site was found 5' to the frame shift and another 3' to the frame shift. This implies that there must actually be more than two *AGT1* loci present in the lager strain genome, *i.e*. at least three *AGT1* loci since there were two versions of the integrated *AGT1* gene and in addition also the native *AGT1* gene found in Integrant 1. Tandem integration was observed not to have occurred in any of the integrants studied as verified by Southern hybridization (II, Fig. 2). The resultant chimeric *AGT1* gene encodes Agt1 protein with up to 492 amino acids deriving from A60 lager strains and the rest from A15 strain. In this way the changed amino acid (V548A) of the ale strain A60, which has been reported to lead to lowered maltotriose transport velocity (Smit *et al*., 2008), is excluded from the resultant protein.

After the screening, the plasmid pKX34 was removed from the transformed strains. Removal of the plasmid was verified by Southern blot analysis with plasmid sequences. As a result, integrant strains, which contain only *S. pastorianus* and *S. cerevisiae* own DNA were obtained. Transformed strains were observed to be genetically stable, *i.e.* when they were grown in non-selective conditions over the several successive (more than 100) generations they still contained changed phenotypes (II, Table 4).

3.9.2 Characterization of the integrant strains

Integrant strains showed increased expression levels of the *AGT1* gene and the pattern of expression was rather similar when strains were grown in any of the three sugars maltose, glucose or maltotriose (II, Fig. 3), *i.e.* maltose and maltotriose were, in addition to glucose, good inducers of the *PGK1* promoter. Conversely, the endogenous *AGT1* gene was observed to be expressed at a very weak but constant level in the host strain when grown in any of the three sugars (II, Fig. 3). It was also observed that integration of the cassette DNA did not alter the expression of the endogenous maltose transporter genes (*MTT1* and *MALx1*) or the maltase gene (*MALx2*) (II, Fig. 3). The *AGT1* gene was expressed more strongly in the stationary phase cells than in the exponential growth phase in shake flask cultivations (II, Fig. 3). However, during wort fermentations expression of *AGT1* in the integrant strains was observed to be strongest during the first three days of fermentation and after that remained at a low level until the end of the fermentation (Fig. 5).

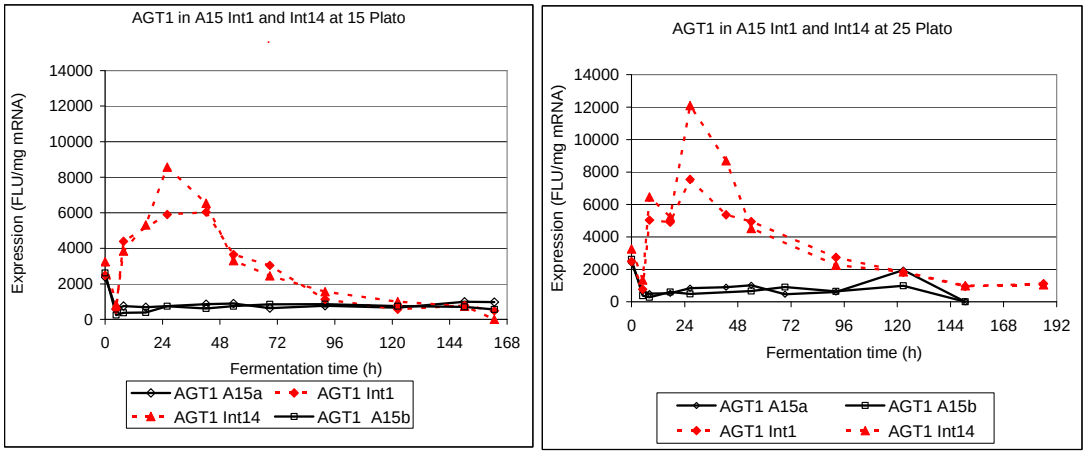

Figure 5. *AGT1* expression levels during the main fermentations with Integrant and host strains.

Maltose transport activity was observed to be higher in the integrant strains and this effect was clearly more pronounced when cells were grown on glucose, which represses the endogenous α-glucoside transporter genes and induces the *AGT1* under the control of *PGK1* promoter. After growth on glucose, an increase in maltose transport capacity (up to 6-fold) was observed in all the integrant

strains compared to the host strain (II). After growth on maltose, which induces the endogenous α-glucoside transporter genes, increase in the maltose transport capacity was smaller (10–20% compared to the A15 strain). Similarly, during the tall-tube fermentations (described in more detail in the next chapter) it was observed that when glucose concentration was high integrants had significantly better maltose transport capacity compared to the host, whereas after glucose had been used up and only maltose and maltotriose were present, a slight increase in the maltose transport capacity was observed. This could be due to limited capacity to functionally express membrane proteins in yeast cell membranes. When grown on maltose, endogenous maltose/maltotriose transporter genes are strongly expressed and this possibly limits the amount of Agt1 transporters that can be present on the plasma membrane.

Maltotriose uptake capability was observed to be increased 1.5–2.5-fold compared to the host strain when maltose was present and induction of the endogenous maltose/maltotriose transporters took place. This suggests that there is a certain amount of Agt1 transporters present in the plasma membrane when grown on maltose. Possibly Agt1- and Malx1-type transporters compete with each other for the yeast cell's capacity to functionally express them in the plasma membrane.

As a conclusion, maltotriose transport and utilization is observed to be intensified during the whole main fermentation process whereas maltose uptake is intensified mostly at the beginning of the fermentation when the glucose concentration is high.

3.9.3 Tall-tube fermentations with the integrant strains

Fermentation performance of some of the integrant strains and the host strain A15 were tested in static tall-tube fermentors that imitate industrial cylindroconical fermentation tanks. Fermentations were performed both in VHG (24°P) and HG wort (15°P). VHG wort was prepared by adding high-maltose syrup, which has been shown to result in more balanced fermentations than addition of, for example, glucose-rich syrup to the wort (Piddocke *et al.*, 2009). HG wort was prepared from the resultant VHG wort by diluting with water. A temperature of 14°C was used in HG fermentations but for VHG fermentations temperature was raised to 18°C to increase the efficiency of fermentation as a positive effect of temperature rise on high-gravity fermentation performance has been shown (Dragone *et al.*, 2003).

All the integrants fermented both 15°P wort and 24°P wort faster and more completely than did the parent, A15, lager strain (II, Fig. 4). The apparent attenuation limits of these worts were close to 86%. Brewers would like to reach an apparent attenuation as close as possible to this limit, but in practise some fermentable sugars always remain in the final beer. Time savings were estimated as the difference between the time required for host strain A15 to reach its highest apparent attenuation (AA) level (80–83% AA were reached in these experiments) compared to the time needed for the integrants to reach the same AA level.

For the 15°P wort, the differences in final apparent extract were small and increases in ethanol yield were close to experimental error (II, Table 5). Time savings were also small (2 to 8 h) at an apparent attenuation of 80% but more significant (13 to 32 h) at 83% apparent attenuation. The 13–32 hours time savings mean 8–20% decrease in the total fermentation time.

In the 24°P fermentations larger differences between integrants and host strain were observed. Differences in the final apparent extracts were 0.25 to 0.53°P whereas in HG fermentations corresponding values were 0.14 to 0.19°P (II, Table 5). Also, 2% more ethanol was produced with the integrant strains compared to the parent strain. Residual maltose and maltotriose in the final beers made by integrant strains were markedly decreased. All three integrants utilized maltose and maltotriose faster than did the parent strain. There were differences between the integrants in the utilization of maltotriose. Integrant 1 was observed to be fastest and it used all the maltotriose present in the VHG wort. In the 24°P fermentations, time savings on reaching the apparent attenuation of 80% were 13–57 hours (fermentation series A) meaning 10–30% decreases in the fermentation time. Greater proportional decreases in fermentation time (14–37%) were observed in the B fermentation series, performed 10 months later. These are significant time savings since the main fermentation, which usually lasts for seven days, could, based on these results, be reduced to only five days. This represents a marked increase in annual output from the same size brewhouse and fermentation facilities.

These results confirm the earlier findings (Kodama *et al*., 1995; Rautio and Londesborough 2003; Alves *et al*., 2007) that the rate of α-glucoside uptake is a major factor limiting the rate of wort fermentations. The extent of the time savings obtained in 24°P wort fermentations with these integrant strains were approximately similar to those observed by Kodama *et al*., (1995) in 24°P fermentations carried out with *MAL61* over-expression strain. Thus, it is seen that introduction of the integration cassette in the genome, where it is present probably

at a maximum of two or three copies, is enough to confer similar time savings as obtained in the strain where the α-glucoside transporter gene is over-expressed from a multicopy plasmid and thus present in several copies. This suggests that probably there are some constraints in the amount of transporter proteins, which can be localized to the yeast cell membrane. Possible reasons for reduced ability to functionally express a transporter are discussed in more detail in chapter 3.15.

When industrial production strains are engineered it is important that only the target properties are changed and other important characteristics of the yeast like, for example, growth characteristics and viability remain unaltered. In the case of brewing yeasts also flocculation and sedimentation properties are important and change in these properties can deteriorate the performance of yeast significantly. In the integrant strains, growth and flocculation/sedimentation properties were observed to be unchanged compared to the host strain (II, Fig. 5). Also, the viability after the propagation and the viability of the yeast collected after the main fermentations (cropped yeast viability) were unaltered in the host and the integrants (II, Table 5). Another important characteristic for each beer type is its flavour. Yeast metabolites, which contribute to beer flavour most include higher alcohols, esters and carbonyls. Higher alcohols, most important of which is 3-methylbutanol, intensify the beer taste and aroma. Esters impart floral and fruity flavours and aromas to beers and are regarded to be among the most important desirable flavour compounds. Low ester concentrations result in an empty and characterless taste. Aldehydes almost without exception have unpleasant flavour (Boulton and Quain, 2001) like, for example, acetaldehyde, which produces an unpleasant “grassy” flavour (Meilgaard, 1975). The most important yeast-derived volatile aroma compounds were analysed in beers collected at the end of the 24°P wort fermentations (II, Fig. 7). The greatest difference between fermentations performed with the integrant and A15 strains was increased acetaldehyde production in main fermentations by Integrant 14 strain. It was observed to produce 2.5-fold as much acetaldehyde as strain A15 and acetaldehyde at this level would be undesirable. All the three integrant strains produced also slightly more ester ethyl acetate (fruity flavour) and integrants 1 and 2 produced more ester 3-methylbutyl acetate (banana-like flavour). These flavours are generally regarded as beneficial and these beers would most probably meet the quality criteria.

3.9.4 Commercial applicability

Integrant strains constructed as described in paper II can be regarded as strains generated by self-cloning. The definition of self-cloning is that it refers to targeted genetic modifications generating an organism which contains DNA exclusively from species closely related to the host organism. That is, any foreign DNA temporarily used as a cloning tool, *e.g.* vector DNA and genetically selectable markers, has been removed (Nevoigt, 2008). For the exact definition of the European Legislature see Council Directive 98/81/EC (http://rod.eionet.europa.eu/instruments/569). After the selection plasmid has been removed only *S. cerevisiae* and *S. pastorianus* DNA remain in the integrant strains constructed. *S. cerevisiae* (ale) and *S. pastorinaus* (lager) strains are regarded as closely related species so that the criteria in the definition of self-cloning are met. There is a patent covering the use of constructs with intact genes in improving the sugar uptake of *Saccharomyces* genus (IPC A21D8/04;C12N1/19;A21D8/02). However, constructs made in the present study do not have interfering intellectual property rights (IPR) in regard to the patent because transforming DNA is not an intact gene and does not itself encode a functional protein.

The integration cassette constructed can be introduced (integrated) to the genomes of other brewer's yeast strains in addition to lager strain A15, as verified by the sequencing of promoter regions of different ale and lager strains. *AGT1* promoters of lager strains A15 and A24 as well as ale strains A60 and A179 were sequenced by chromosome walking (Paper IV). Sequences between ale and lager strains were observed to be very homologous in the whole region up to 1900 bp upstream from the *AGT1* gene (except one 22 bp deletion and two ~95 bp insertions between ale and lager promoters as described in paper IV) and practically identical in regions included in the cassette. Based on this, the same cassette can be used for integration in ale or lager strains. The benefits of constructing corresponding ale integrant strains would be that glucose repression of the *AGT1* gene would be avoided. It was observed that under the control of the *PGK1* promoter, strong expression of *AGT1* was observed already 6 h from the start of the fermentation (Fig. 5). Changed amino acids found in some ale strains decreasing the maltotriose transport ability should however be taken into consideration and the cassette possibly modified to repair these changes.

There are great differences between countries in relation to how GMOs are approved, classified and labelled (Nevoigt, 2008). There is very low acceptance by consumers of GMO in the food and beverage industry, especially within the

European Union (EU). European legislation on GMO is particularly strict. Although many advantageous brewer's, baker's and wine yeasts have been engineered during recent years (Donalies *et al.* 2008; Nevoigt, 2008) and some of them have obtained approval in respective countries for their commercial use (Akada 2002; Husnik *et al*., 2006) none of them has entered larger scale commercial use in Europe. The commercial application of genetically modified micro-organisms has been problematic due to public concerns (Sybesma *et al*., 2006). For example, two recombinant yeast strains received official approval from the British Government for commercial use already in 1990 and 1994. One was baker's yeast that displays a shorter rising time (Aldhous, 1990) and another was brewer's yeast designed for the production of diet beers (Hammond, 1995). However, due to negative public acceptance these strains have not been used on a larger scale. There is also a modified sake yeast strain FAS2-1250S that has received official approval for its industrial use in 2001 by the Japanese Government as a self-cloning yeast so that the yeast does not need to be treated as GM yeast (Akada, 2002). With this strain a higher amount of ethyl caproate, an apple-like flavour compound, was produced in the modified strains (Akada *et al*., 1999). The situation is approximately similar with the wine yeasts. At the moment there is one genetically modified wine yeast worldwide that has been approved for commercial use. This is a modified wine yeast strain where the formation of toxic bioamines, which are detrimental to health, is prevented. It has been approved for commercial use in USA and in Canada (and also Moldova) (Husnik *et al*., 2006).

3.10 Temperature-dependence of maltose uptake in ale and lager strains (Paper III)

Reactions catalysed by integral membrane proteins usually exhibit non-linear Arrhenius plots with increased temperature-dependence at low temperatures. This is usually interpreted as reflecting the change of state of the plasma membrane (freezing of the membrane). As temperature decreases, membranes become increasingly viscous with decreasing membrane fluidity and at some point will undergo a phase change to a gel (rigid) phase and biological function is impaired (Nedwell, 1999). However, it has been shown, at least for yeast cells, that there is no sharp phase transition but rather a continuous change of membrane fluidity when temperature decreases (Beney and Gervais 2001). Sugar transporter molecules are suggested to change shape during each transport cycle

between inward and outward conformations (Abramson *et al.*, 2003). This change of shape requires that the transporter moves against the surrounding lipid, *i.e.* the transporter-lipid-complex moves. When temperature decreases and the plasma membrane becomes more viscous the conformational change occurring between inward and outward conformations is probably more tedious.

Temperature-dependence of maltose transport in an ale strain was shown to follow the typical pattern described for integral membrane proteins (Rautio and Londesborough, 2003). Maltose transport activity decreased four-fold when the temperature was decreased from 20°C to 10°C and even a 20-fold decrease was observed when the temperature was decreased further from 10°C to 0°C. There was practically no maltose transport at 0°C in the ale strain studied (Rautio and Londesborough, 2003). However, Guimarães *et al.* (2006) observed that a lager strain was still able to carry maltose at 0°C but at low velocity. Takahashi *et al.* (1997) observed that when the temperature was decreased from 14°C to 8°C, a larger decrease in the utilization rate of maltose and maltotriose was observed in an ale than in a lager strain. In the ale strain, 10% and 30% decreases in the utilization rate of maltose and maltotriose, respectively, were observed whereas in the lager strain utilization rate of both sugars decreased only by 5%. Interestingly, it was also shown that a temperature decrease from 14°C to 8°C didn't have any effect on glucose utilization in either of the strains. Different maltose (maltotriose) utilization patterns observed between ale and lager strains when temperature decreases could be due to differential temperature-dependence of maltose (maltotriose) uptake in ale and lager strains.

As described in the paper III, four brewer's yeast strains, two ale and two lager strains, were studied for temperature-dependence of maltose transport. Brewer's yeasts were harvested during growth on maltose at 24°C and their maltose transport activities were assayed at a temperature range of 20–0°C. At 20°C all four strains had similar maltose transport activities (III, Fig. 2). However, at 15°C there was already difference observed between ale and lager strains in their temperature-dependence and when temperature was reduced further to 10°C this difference was even more pronounced. Below 10°C the change in the Arrhenius plot was steeper for ale than for lager strains and at 0°C the lager strains had about five-fold greater activity than the ale strains. With temperature decrease, a continuous decrease in maltose transport capacity, even if more pronounced below 10°C, was observed (III, Fig. 2). This is in accordance with observations of Beney and Gervais (2001) that there is no sharp phase transition but rather a

continuous change in the membrane fluidity where transporters loose gradually their activity.

Fermentations with ale strains are usually performed near 20°C and thus these temperature-dependence effects described have only minor significance for ale fermentations. Lager strain fermentations are, however, usually carried out at temperatures between 6°C and 14°C. A decrease in maltose uptake rate observed at this temperature range can have relatively large effects on maltose transport capacity during the main fermentation. For maltose (and maltotriose) uptake it would be beneficial if the process temperature during the lager main fermentation is raised from the conventional temperature.

When temperature decreases also affinity for substrate can be affected. However, when K_m and V_{max} changes in maltose transport were followed in an ale strain in respect to temperature decrease (Rautio and Londesborough, 2003) it was observed that K_m remained approximately the same in all temperatures tested and it was V_{max} which changed dramatically (more than 100-fold) when temperature was decreased. Similar temperature-dependence of V_{max} was observed for a lager strain (Rautio and Londesborough, 2003). Other authors report similar results, for example Nedwell (1999) has shown that low temperature diminishes specific affinity (V_{max}/K_m) for substrate uptake. Most probably velocity of transport decreases because conformational change between inward-facing and outward-facing structures is slower when the membrane becomes more viscous.

3.11 Effect of different dominant maltose/maltotriose transporters of ale and lager strains on the temperature-dependence of maltose transport (Paper III)

It has been shown that there are strain-dependent differences among *Saccharomyces* species in the fluidity of membranes (Torija *et al*., 2003). These differences can be due to adaptation of the organism to its environment. In organisms adapted to low temperature environments there tends to be an increased proportion of unsaturated membrane lipids, and a decreased proportion of branched chain lipids compared to species adapted to moderate or high ranges of temperature (Nedwell, 1999). Ale and lager strains have been used in different process temperatures for hundreds of years. Thus, it is possible that the plasma membranes of ale and lager strains have adapted to the different process temperatures

to the extent that also the lipid composition of their membranes has been modified accordingly.

The effect of possible differences between ale and lager strain plasma membrane composition on the temperature-dependency of maltose transport was studied with Integrant 1 strain (III). In this strain the ale type *AGT1* gene is expressed under the control of the *PGK1* promoter in the lager strain background, *i.e.* present in the lager strain plasma membrane. When Integrant 1 strain was grown on glucose it expressed practically only the pure Agt1-type introduced transporter. It has been observed that the Agt1 transporter is the dominant transporter type in ale strains and that ale strains are more temperature-dependent in their maltose transport. Thus, when ale type Agt1 transporter was functionally expressed in the lager strain plasma membrane in Integrant 1 strain, this strain was expected to be as temperature-dependent as ale strains, assuming temperature-dependence is an intrinsic property of the transporter molecule itself (and not a property of the plasma membrane). Very strong temperature-dependence of maltose uptake for the Integrant 1 strain was observed. Its temperature-dependence was even slightly stronger than those observed for ale strains (III, Fig. 2). This implies that differences in temperature-dependence of maltose uptake are not due to the possible plasma membrane differences between ale and lager strains but are instead intrinsic characteristics of the Agt1 transporter itself. Thus, different temperature-dependences of maltose transport by these ale and lager yeasts seem to result from different maltose transporter types present in these strains.

3.12 Temperature-dependence of maltose transport by Mtt1 and Malx1 transporters (Paper III)

The Agt1 protein seems to be particularly sensitive to the physical state of the surrounding lipid, resulting in very low activity at low temperatures. Temperature-dependence of two other maltose transporters Mtt1 and Malx1 were studied separately, with constructs over-expressing either the *MALx1* (99% identical to *MAL31* in the SGD) or *MTT1* genes under the control of the *PGK1* promoter. *MALx1* transformants exhibited strong temperature-dependence. The rates of maltose uptake at 0°C were approximately 2–3% of those observed at 20°C (III, Fig. 3). The Mtt1 transporter instead was observed to exhibit relatively little temperature-dependence. There was even 7.5–9% of the relative activity left when the uptake rates were compared at 0°C and 20°C. Taken together the tem-

perature-dependence of the three transporters was observed to decrease in the order Agt1≥Malx1>Mtt1. These results further confirmed that high temperature-dependence is a property of the transporter protein itself rather than due to an hypothetical difference in the lipid membranes of the strains.

In brewery fermentations when *AGT1* integrant strains are used it must be taken into consideration that the Agt1 transporter is particularly temperature-dependent. In the present study it was observed that the main fermentation temperature of 18°C is enough to confer the positive effect of the integrant strains.

3.13 Effect of energetic status of the yeast cells and glucose stimulation on maltose uptake (Paper III)

Electrochemical proton potential is significantly lowered in starved yeast cells compared to actively metabolizing cells. This is because ATP content and adenylate energy charge (EC) is relatively low in starved cells. When yeast cells growing on fermentable sugar are harvested, washed and suspended in a medium lacking a carbon source, their intracellular adenylate energy charge can decrease. The adenylate energy charge and active transport rates of sugars such as lactose and maltose of such cells can be increased by treatment with glucose for a few minutes immediately before the zero-*trans* sugar uptake assay (Guimarães *et al*., 2008). There might be differences in the energetic status between ale and lager strains and this could explain different temperature-dependences observed. To test this hypothesis, yeast suspensions were treated with glucose immediately before the maltose transport assays. Temperature-dependence of maltose transport was observed to be similarly relieved in both ale and lager stains when glucose stimulation was performed before the uptake rate measurement (III, Fig. 1). Thus, there was a difference between the temperature sensitivities of maltose transport by the lager and ale strains that was not explained by differences in adenylate energy charge.

3.14 Possible reasons for different temperature-dependences between Agt1, Malx1 and Mtt1 transporters (Paper III)

Several possible reasons can be suggested for observed different temperature-dependences between Agt1, Malx1 and Mtt1 transporters. Amino acid sequences

of these transporters differ from each other. In particular, the Agt1 transporter differs from the other two significantly. Agt1p is only 54% identical to Mtt1p and Malx1p, whereas identity between Mtt1p and Malx1p is 90%. Differences in the amino acid sequences can, for example, have effect on α-helix formation leading to different topology of the protein. It is possible that due to different topology, Agt1 and Malx1 transporters exhibit greater shape changes than Mtt1 during the catalytic cycle. Thus, these transporters need to move against the surrounding lipid more and because of this can't work efficiently when the membrane becomes more viscous at low temperatures.

All three transporters, Agt1, Mal31 and Mtt1 are thought to be members of the major facilitator superfamily (MFS) characterized by 12 transmembrane-spanning domains (Nelissen *et al*., 1995; Salema-Oom *et al*., 2005). However, different results have been obtained when the number and location of the transmembrane domains is predicted by different software and computational programs. There are also data that contradict the 12-spanner analysis. For the Agt1 protein, 12 transmembrane domains have usually been suggested (Han *et al*., 1995; Nelissen *et al*., 1995; Smit *et al*., 2008) but in the SGD database only 10 transmembrane domains are predicted for the Agt1p. Also, Malx1 protein has been reported to possess 12 transmembrane domains (Cheng and Michels, 1989; Nelissen *et al*., 1995) but Smit *et al*. (2008) predicted Mal31p would possess 13 transmembrane domains, whereas in the SGD database only 8 transmembrane domains are predicted for this protein. Mtt1p has been suggested to possess 12 transmembrane domains (Salema-Oom *et al*., 2005). It is possible that transmembrane domain number actually differs between these transporters and possibly results in differential temperature-dependence. However, as long as there are no three dimensional models available for these maltose transporters nothing can be confirmed of their transmembrane domain number.

In studies by Han *et al*. (1995) and Salema-Oom *et al*. (2005) (where 12 transmembrane domains for each of these transporters were predicted) it has been shown that amino acid changes between Agt1, Mtt1 and Malx1 proteins are predominantly located in certain transmembrane domains. Transmembrane domains 2, 9 and 11 were found to be the least similar regions between Mtt1 and Mal31 (Salema-Oom *et al*., 1995) as well as between Mal61 and Agt1 transporters (Han *et al*., 1995) (Mal31p and Mal61p have practically identical amino acid sequences). Interactions between certain amino acids of integral membrane proteins and membrane lipids have been shown to be important for stabilization and functionality of membrane proteins. It is possible that these particular trans-

membrane domains, 2, 9 and 11, have specific roles in protein-lipid interactions between the transporter protein and surrounding lipid. Thus, a particular effect on temperature-dependence might be observed when these domains are affected.

Some amino acid residues have been reported to be particularly important in interactions between integral membrane proteins and surrounding lipid. These are amino acids possessing aromatic amphipathic side chains, *i.e.* tryptophan and tyrosine. Another group of important interactions is formed between basic side chains of lysine and arginine and with phosphate groups of lipids (Deol *et al.*, 2004). Amino acid residue analysis was performed on Agt1, Malx1 and Mtt1 proteins (http://www.expasy.ch/tools/protparam.htmlit) and it was observed that in Agt1p there is less of both tryptophan and tyrosine compared to the Mal31 and Mtt1 proteins (6,1% compared to 6,8% and 6,5%, respectively). A similar observation was made when arginine and lysine residue presence was compared between Agt1, Mtt1 and Malx1 proteins (8,4% in Agt1 compared to 9,1% and 9,3% in Mtt1 and Mal31, respectively). This possibly suggests that there are fewer interactions between the Agt1 protein and lipid surrounding it than there is between Malx1 and Mtt1 type proteins and lipids around them. Possibly the less interactions there are between transporter and surrounding lipid the more prone the transporter is to work less well at lower temperatures. Another explanation could be linked to the Agt1 transporter having broadest substrate specificity. It is possible that the central cavity of the Agt1 protein is largest and shape change during the transport cycle is greater than is needed for other transporters with more restricted substrate range.

3.15 Yeast cells have limited capacity to functionally express transporters in their cell membranes (Paper II, III)

Several studies have shown that capacity of the yeast cell to functionally express transporter proteins in the cell membrane is limited (Hopkins *et al.*, 1988; van der Rest *et al.*, 1995b; Opekarová *et al.*, 1993). It was also observed in the present study that the positive effect of over-expressed *AGT1* on maltose/maltotriose uptake is limited. There probably is limitation in the amount of transporters able to localize and/or be functionally active on the plasma membrane. This restriction could be due to several factors. First, the secretory pathway's capacity to deliver the transporters to the plasma membrane may be limited. Secondly, the space in the plasma membrane where transporters are inserted may be lim-

ited. Thirdly, since protein–lipid interactions are important for proper function of transporters, the availability of specific lipids might be the limiting factor.

Because of their hydrophobicity, the membrane proteins are trafficked in a complex with specific lipids within the vesicle membrane (Opekarová *et al*., 1993). Lipid rafts have been shown to function in biosynthetic delivery of proteins to the cell surface. Proteins that are delivered to the plasma membrane in lipid rafts also remain in these rafts after delivery (Hearn *et al*., 2003; Malínská *et al*., 2003; Umebayashi and Nakano, 2003). For this, the presence of an adequate amount of ergosterol and/or sphingolipids (main components of lipid rafts) in the yeast cell is necessary for efficient delivery of transporters through the secretory pathway to the cell surface and stable location there. It has been shown, for example, that Can1p (arginine H^+ symport), Fur4 (uracil permease), Tat2 (high affinity tryptophan permease) and Pma1 (ATPase) delivery is impaired if there is a deficiency in ergosterol and/or sphingolipids in the cell (Hearn *et al*., 2003; Malínská *et al*., 2003; Umebayashi and Nakano, 2003).

In addition to targeting to the plasma membrane, lipid composition has been reported to be important also for determining the correct topological organization of membrane proteins, either during the initial membrane assembly or dynamically after membrane insertion has occurred (Bogdanov *et al*., 2002). Stabilization of the transporters in the membrane after delivery has been shown to be dependent on lipids (Bagnat *et al*., 2000). Moreover, post-assembly reorganization has been observed in the response to lipid environment, *i.e*., transmembrane domains can reorganize in response to the lipid environment (Bogdanov *et al*., 2002). Maltose transporters have been shown to need an adequate amount of ergosterol for their proper function (Guimarães *et al*., 2006). At least catalytic function of maltose transporter molecules in the plasma membrane was shown to be dependent on ergosterol content. Guimarães *et al*. (2006) suggest that ergosterol also possibly has a role in delivery of the maltose transporters to the cell surface.

The capacity of the secretory pathway to deliver transporter proteins to the plasma membrane seems to be mostly affected by the availability of specific lipids, especially ergosterol and sphingolipids. Thus, the secretory pathway may be a limiting factor, particularly at the later phases of fermentations, when there is a deficiency of lipids in the yeast cell. Yeast cells are not able to synthesize unsaturated fatty acids or sterols in anaerobic fermentation conditions after oxygen depletion. During the second half of brewery fermentations, maltose and maltotriose uptake rates are low (II, Table 5). At the late phases of fermentation, in addition to difficulties in delivery of transporters to the surface, also trans-

porters that already exist in the plasma membrane probably work suboptimally because the membranes do not contain sufficient sterols. In particular, the daughter cells formed in the last rounds of cell division may be deficient in sterols because no new sterols can be made (Guimarães, 2008). Thus, during the second half of the fermentation, maltose transport is severely affected. A similar effect is observed also at the early phase of the fermentation if the fresh wort is pitched with sterol-deficient cropped yeast. In this case, yeast cells need time to synthesize lipids needed before the maltose uptake can be initiated.

Precise localization of maltose transporters in the plasma membrane has not been confirmed, *i.e*. whether they are present in lipid rafts. However, since maltose transporters have been shown to be dependent on ergosterol for their efficient function (Guimarães *et al*., 2006), like other transporters localized in the lipid rafts it would seem plausible that they also are located in these microdomains. In addition, H^+ symporters generally seem to be localized to the lipid rafts (Hearn *et al*., 2003; Umebayashi and Nakano, 2003; Malínská *et al*., 2003).

Lipid rafts have been shown to regulate the turnover of transporter proteins in yeast. Grossmann *et al*. (2008) suggest that exclusion of transporter protein from the lipid raft to surrounding membrane leads to endocytosis of the transporter. Thus lipid rafts represent a protective area within the plasma membrane to control turnover of transporters (Grossmann *et al*., 2008). Lipid rafts are small (10–200 nm) microdomains (Pike, 2009) and thus probably constitute only a minor part of the plasma membrane. Space in lipid rafts can be therefore limited. It can be that excess proteins are removed from lipid rafts and exposed to endocytosis. Possibly limited space in the lipid rafts restricts the capacity to functionally express transporter proteins. It is known that maltose transporters are removed from the plasma membrane (by endocytosis) when they are not required, whereas the cytosolic maltase enzyme is not the target for such inactivation (Görts, 1969). Limited space in the plasma membrane could be one possible reason for the phenomenon observed.

3.16 Benefits of non-functional Agt1 transporters for lager strains (Paper III)

Frame shift mutation in the *AGT1* gene leading to the formation of a truncated non-functional protein product is found in all lager strains studied so far. Possibly this change was present already in the original *S. cerevisiae* component forming the lager strain hybrid. Otherwise, frame-shift mutation should have

occurred soon after the hybridization event since it is present in all lager strains studied. This point mutation (that can be regarded as a quite easily reversible mutation) has not been selected against during the evolution of lager strains but instead is nowadays a common feature in these strains. There is probably some reason for preservation of this non-functional form during the evolution. It is possible that "loss" of the Agt1 protein from lager yeast may have been beneficial for lager strains. This might be because the capacity of the yeast cell to functionally express transporters in the plasma membrane is limited (van der Rest *et al.*, 1995b; Hopkins *et al.*, 1988; Opekarová *et al.*, 1993) and thus putative loss of the Agt1 transporters from the plasma membrane would have provided more capacity, for example, for functional expression of Mtt1-type transporters, which have higher activity at low temperatures. Fermentations by lager strains have been traditionally carried out at approximately 10°C and thus strains functionally expressing Mtt1 transporters would have a clear selective advantage in the low temperature conditions where lager yeasts evolved. It is not clear why the apparently defective *AGT1* genes are still present in all tested lager strains. It is possible that although truncated Agt1 cannot transport maltose it has some other important function and the mutated gene has been retained in the genome for this reason. Based on amino acid sequence analysis, truncated Agt1 possess six or seven transmembrane helices (www.ch.embnet.org/software/TMPRED_form .html). However, it has not been shown experimentally whether the truncated Agt1 transporter is able to localize to the plasma membrane.

3.17 Identification of regulatory elements in the *AGT1* promoters of ale and lager strains (Paper IV)

Significant differences in the *AGT1* gene expression levels in ale and lager strains were observed (I). The possibility that *AGT1* genes are differently regulated in these strains due to different structures of their promoters was investigated in study IV. Generally very little is known about the composition of promoters in industrial strains as most of the promoter region studies have been performed with laboratory strains.

AGT1 upstream regions of one ale (A60), two lager (A15 and A24) and one laboratory yeast strain CEN.PK2-1D were sequenced by chromosome walking (IV). It was observed that the upstream sequence of laboratory strain CEN.PK.2-1D was identical to the *AGT1* promoter of S288C strain of *Sccharomyces* Genome Database whereas the upstream sequences of ale and lager strains diverged markedly

from the laboratory strains, *i.e.* the first 315 bp of the *AGT1* upstream sequence (–315 to –1 from the start codon) were highly identical (99%) to the corresponding promoter of the laboratory strains S288C and CEN.PK2-1D, after which the sequences diverged totally from that of the laboratory strains (IV, Fig. 2).

In the *AGT1-MAL12* promoter region of laboratory strains there are three MAL-activator (Levine *et al*., 1992; Sirenko *et al*., 1995) and one Mig1 binding element (Hu *et al*., 1995) present (IV, Fig. 2). This is one Mig1 binding element less than in the conventional *MALx1- MALx2* intergenic region (Levine *et al*., 1992; Sirenko *et al*., 1995; Hu *et al*., 1995) (IV, Fig. 1). Although the first 315 bp upstream from the *AGT1* gene are practically identical between ale, lager and laboratory strains only TATA box is located in this region but not any of the MAL-activator or Mig1 elements (IV, Fig. 2).

The *S. bayanus*-derived *AGT1* upstream sequence of WS34/70 diverged markedly from corresbonding *S. cerevisiae*-derived sequences (only 43% identity) (IV, Table 2). Thus, it is highly unlikely that the primers used in the chromosome walking of the present study would have annealed to the promoter region of the *S. bayanus AGT1* gene. Since lager strains are polyploid (possessing more than one copy of each *S. cerevisiae*- and *S. bayanus*-derived chromosome) (Querol and Bond, 2009) and sister chromosomes probably have diverged during evolution, different forms of *S. cerevisiae*-derived *AGT1*-bearing loci may exist. However, only a single type of *AGT1* promoter sequence was identified in the chromosome walking performed, indicating that there is no such variation between the *S. cerevisiae*-derived sister chromosomes in the *AGT1* loci in the lager strains studied.

To find similar promoter regions for the ale and lager strains in the present study, SGD Fungal Genome Database (http://www.yeastgenome.org/cgi-bin/blast-fungal.pl) and NCBI database were searched using BLASTN limited to different *Saccharomyces* strains. Highly identical *AGT1* upstream sequences were found in three different natural isolates of *S. cerevisiae*; M22, RM11-1a and YPS163 and in *Saccharomyces pastorianus* Weihenstephan 34/70. Less conserved matches found were upstream sequences of the putative *AGT1* genes in *S. paradoxus* NRRL Y-17217 and *S. mikatae* IFO 1815 (IV, Table 2). Multiple sequence alignment of the *AGT1* upstream sequences of the ale and lager of the present study, *S. cerevisiae* strains S288C, RM11-1a, YPS163, and M22, *S. paradoxus* NRRL Y-17217, *S. mikatae* IFO 1815 and *S. pastorianus* WS34/70 strains was constructed (IV, Supplementary Fig. 1). Generally, all promoters showed high levels of similarity in various, rather long, segments but some in-

sertion/deletion changes between the strains were also observed (IV, Table 3). All the upstream sequences included in the analysis contained a TATA-box and several Mig1p and MAL-activator binding sites.

Among the brewer's yeast strains the *AGT1* promoter regions of lager strains A15 and A24 were identical to each other and 97% identical to WS34/70 *AGT1* upstream sequences (*S. cerevisiae*-derived). Moreover promoter regions in ale and lager strains were observed to be otherwise highly identical to each other except for one 22 bp deletion and two 94 and 95 bp insertions in the ale strain compared to the lager strains (IV, Fig. 2). Computational analysis of promoter elements revealed that the promoter regions of ale and lager strains contained several Mig1p and MAL-activator binding sites, *i.e.* ale strain contained seven Mig1p and five MAL-activator binding sites, whereas the corresponding number in the lager strain were two and four (IV, Fig. 2; IV, Supplementary Fig. 1). The extra MAL-activator site in the ale strain promoter may explain the stronger expression of *AGT1* observed in the ale strains compared to the lager strains.

A remarkably long distance between both the MAL-activator and Mig1 binding elements and the *AGT1* coding sequence was observed. The most proximal MAL-activator site was found to be located approximately 830 bps from the start codon of the *AGT1* gene and the distance observed between the Mig1 binding sites and the *AGT1* start codon was approximately 1100 bp. However, the *AGT1* expression was observed to be strongly repressed in these strains when glucose was present (I). This suggests that the Mig1 transcription factors binding to the promoter region of *AGT1* at a considerable distance are functioning as repressors. Furthermore, the earlier studies revealed strong maltose induction of *AGT1* expression in the ale strains (I), indicating that MAL-activators, located more proximal to *AGT1* gene than the Mig1 elements, are also functional. However, MAL-activator sites located further upstream in the ale and lager *AGT1* promoters (>1500 from the start codon) possibly have only minor contribution to the induction. Because of the extra MAL-activator binding site in the ale strains compared to the two binding sites in the lager strain, the promoter of the ale strain has in the 1500 bp promoter region proximal to the *AGT1* gene the same number (three) of MAL-activator binding sites as the well-studied promoter of *AGT1* in the laboratory strain S288C. It is possible that for the strong expression of *AGT1* all three MAL-activator elements are necessary.

Another explanation for the stronger expression of the *AGT1* gene in the ale strain may be linked to telomeric silencing. The role of chromatin remodelling in the regulation of expression of *MAL* genes has been reported (Houghton-Larsen

and Brandt, 2006; Dietvorst and Brandt, 2008). The *MAL* genes were found to be strongly silenced in some strains, whereas in other strains the non-functional COMPASS complex did not cause any changes in the expression of *MAL* genes (Houghton-Larsen and Brandt, 2006). It has also been observed that in a single strain some chromosome ends are more prone to the telomeric silencing than others (Loney *et al*., 2009). It is possible that the different *MAL* loci located at different chromosome ends are not uniformly regulated by telomeric silencing. The earlier expression studies (I) showed that the *MALx1* genes were strongly but the *AGT1* weakly expressed in the lager strains. This suggests that if telomeric silencing is the reason for the low expression of *AGT1* genes in the lager strains, chromosomes possessing other *MAL* loci, however, are not regulated by telomeric silencing.

3.18 Comparison of *AGT1*-bearing loci in *S. cerevisiae*, *S. paradoxus* and *S. mikatae* (Paper IV)

Among the similar sequences found in gene banks ale and lager strain *AGT1* promoter regions were found to be more similar to corresponding regions in natural isolates of *S. cerevisiae*, *i.e.* strains M22, YPS163 and RM11-1a than to *AGT1* promoter regions of laboratory strains. Ale and lager strain *AGT1* promoter sequences were also more similar to the *S. paradoxus* and *S. mikatae AGT1* upstream regions than those of the laboratory strains. The strong similarity between the *AGT1* upstream sequences of *S. paradoxus*, *S. mikatae*, ale and lager yeasts, and the three natural isolates of *S. cerevisiae* prompted us to compare the structures of the *MAL* loci containing *AGT1* in these strains using sequences from gene banks. RM11-1a strain was chosen as a representative of natural isolates of *S. cerevisiae*. The *AGT1*-containing *MAL* loci from *S. cerevisiae* RM11-1a, *S. paradoxus* NRRL Y-17217 and *S. mikatae* IFO 1815 were found to be much more extensive (IV, Fig. 3) than the classical *MAL* locus, with its single copies of the *MALx1*, *MALx2* and *MALx3* genes. At least 9 putative *MAL* loci genes were spread throughout the 32–34 kbp contig regions from *S. paradoxus* and *S. mikatae*, and there are probably more *MAL* genes beyond the borders of the contigs since there were *MAL* ORFs at the borders. There were also multiple putative *MAL* genes in the 22 kbp RM11-1a contig, at least 7, including one at the border of the contig. The laboratory strain S288C contains a putative isomaltase-encoding gene, *YGR287c* (73% identical to *MALx2*) in its *AGT1* bearing locus in addition to the

AGT1, *MAL12* and *MAL13* genes. *YGR287c* was found also in *S. cerevisiae* RM11-1a and the *S. mikatae* and *S. paradoxus* contigs.

The overall sequences of *S. mikatae* contig 789 and *S. paradoxus* contig 294 *MAL* loci were observed to be very similar to each other. There were even three different putative maltose transporter-encoding genes in the same *MAL* locus in the *S. paradoxus* and *S. mikatae* strains. These genes (*MALx1*, *AGT1* and a gene most similar to *MTT1*), relate to the three major maltose/maltotriose transporters known in *S. cerevisiae* (Malx1p, Agt1p and Mtt1p (Salema-Oom *et al.*, 2005; Alves *et al*., 2007)). Evidently, the *MAL* genes had already been multiplied in the locus of the ancestral strain from which the *Saccharomyces sensu stricto* strains derive. Divergence of *S. cerevisiae* from this ancestor and further divergence of laboratory strains from natural *S. cerevisiae* seems to be accompanied by deletion of some of the repeats. The *AGT1*-containing *MAL* locus of *S. cerevisiae* RM11-1a could have derived from the *S. mikatae* and S. *paradoxus* loci by deletion of the sequence between the first and third *MALx3* ORFs (IV, Fig. 3). This deletion would remove only extra copies of the maltose transporter and MAL-activator genes. The *MAL1* locus of *S. cerevisiae* S288C could be derived by a further deletion, this time of the sequence between *AGT1* and the first *MALx2* seen in the RM11-1a contig (IV, Fig. 3). Sequence data from S288C shows that compared to RM11-1a there is a deletion from 317 bp upstream of the *AGT1* ORF until approximately 450 bp upstream of the *MALx2* ORF.

4. Conclusions

Maltose and maltotriose are the two most abundant sugars of wort. Thus, efficiency of their utlization is an important factor defining the overall fermentation efficiency. It has been earlier shown that sugar transport into the yeast cells is the rate limiting step in their utilization and this was further confirmed in the present sudy. Over the last years, efficient uptake of maltose and maltotriose into the yeast cells has become an important issue because of brewer's tendency to move to a greater extent to the utilization of high and very-high-gravity worts where the sugar concentrations have been increased. Yeast cells are usually not able to utilize efficiently and fast such high amounts of sugars and often there is residual maltose and especially maltotriose left after (very) high-gravity wort fermentations. This lowers the efficiency of the process and also impairs the beer flavour. Improved maltose and maltotriose uptake into the yeast cells would make fermentation process more efficient.

Ale and lager strains were shown to differ markedly in maltose and maltotriose transporter types they functionally express in their plasma membranes. In the lager strains Malx1-type transporters were observed to be the dominant transporter type at 20°C, whereas the relatively high activity of maltose transport by lager strains at low temperatures suggests that the Mtt1 transporters, with low temperature-dependence, dominate at low temperatures. In the ale strains Agt1-type transporters were observed to predominate. A common characteristic for the lager strains was that the *AGT1* gene they possessed was defective. This was because of a frame shift mutations leading to premature stop and formation of truncated non-functional protein product. Thus, maltotriose must be carried in the lager strains either by Mphx, Mtt1 or putative SbAgt1 transporters.

It was shown that sugar utilization efficiency during the main fermentation can be improved significantly by using a modified lager strain where the *AGT1* gene has been repaired and put under the control of a strong *PGK1* promoter not

susceptible to glucose repression. The integrant strains constructed fermented wort faster and more completely compared to the host strain. They also produced beer containing more ethanol and less residual maltose and maltotriose. Importantly there was no change in yeast handling performance, *i.e*. propagation, growth and sedimentation behaviour and with little or no change in the level of yeast-derived volatile aroma compounds. Improvement of fermentation performance was more pronounced in the VHG (24°P) wort fermentations compared to those performed in HG (15°P) wort. Time savings in the running time of the main fermentation were significant with the integrant strains. In 15°P wort fermentations, time to reach the highest apparent attenuation level was decreased by 8–20% with integrant strains compared to the host and in VHG wort fermentations even more significant, 11–37%, time savings were obtained. These are economically significant changes, *i.e*. marked increase in annual output from the same size brew house and fermentor facilities can be obtained. This means in practise that the main fermentation, which usually lasts approximately seven days could be reduced to even five days.

There is also an environmental aspect since beer brewed with these integrant strains can be regarded as more environmentally friendly produced beer. This is because brewhouse fermentor facilities needed are decreased, which leads to a decrease in the energy needed for the process, *i.e*. less facilities to be cooled, cleaned, *etc*.

Maltose transport in the ale strains was observed to be more sensitive to decreasing temperature compared to the lager strains. It was observed that the reason for the difference in temperature-dependence between ale and lager strains was not the hypothetical differences between the ale and lager strains plasma membrane compositions but instead was related to different dominant transporter types present in plasma membranes between ale and lager strains. Temperature-dependence of each transporter was observed to decrease in the order Agt1≥Malx1>Mtt1. Different sensitivity to low temperatures between ale and lager strains is most probably due the different environments in which they have evolved, *i.e*. their utilization at different process temperatures for centuries, which possibly lead to development of different dominant roles for different maltose/maltotriose transporters between ale and lager strains. Lager fermentations are traditionally performed at a temperature range of 6–14°C. In lager strains in the present study maltose uptake was observed to be highly temperature-dependent at this temperature range and significantly lower compared to that at 20°C. Thus, rising the lager fermentation temperature from the traditional

temperature near 20°C would have a positive effect on maltose and maltotriose uptake during the fermentation. The Agt1 transporter was observed to be especially temperature-dependent and thus when the integrant strains constructed are used the process temperature must be particularly considered. It was observed that 18°C was high enough to confer the advantages of integrant strains.

Mutation in the *AGT1* gene, leading to the formation of a non-functional product, seems not to be selected against during evolution of lager strains but instead is found in all lager strains studied. This would suggest that non-functional Agt1 transporters must have some beneficial features for lager strains. It has been earlier shown that yeast cells have limited capacity to functionally express transporters in plasma membrane. It is possible that membrane space or the secretion capacity of transporters to the plasma membrane is limited. Thus, “loss” of Agt1 transporters from lager yeast may have increased capacity for functional expression of other maltose/maltotriose transporters. Mtt1 transporter was found to be lager strain-specific among brewer’s yeast strains as well as the least temperature-dependent of the transporters studied. Lager strains expressing Mtt1 type transporters would have clear selective advantage in the low temperature conditions where lager yeast evolved. Possibly rendering the Agt1 non-functional has released either secretory capacity or plasma membrane space or both for Mtt1 transporters.

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Maltose and maltotriose transport into ale and lager brewer´s yeast strains

Abstract

Maltose and maltotriose are the two most abundant sugars in brewer's wort, and thus brewer's yeast's ability to utilize them efficiently is of major importance in the brewing process. The increasing tendency to utilize high and very-high-gravity worts containing increased concentrations of maltose and maltotriose renders the need for efficient transport of these sugars even more pronounced. Residual maltose and especially maltotriose are quite often present especially after high and very-high-gravity fermentations. Sugar uptake capacity has been shown to be the rate limiting factor for maltose and maltotriose utilization. The main aim of the present study was to find novel ways to improve maltose and maltotriose utilization during the main fermentation. Maltose and maltotriose uptake characteristics of several ale and lager strains were studied. Genotype determination of the genes needed for maltose and maltotriose utilization was performed. Maltose uptake inhibition studies were performed to reveal the dominant transporter types actually functioning in each of the strains. Temperature-dependence of maltose transport was studied for ale and for lager strains as well as for each of the single sugar transporter proteins Agt1p, Malx1p and Mtt1p. The *AGT1* promoter regions of one ale and two lager strains were sequenced by chromosome walking and the promoter elements were searched for using computational methods.

The results showed that ale and lager strains predominantly use different maltose and maltotriose transporter types for maltose and maltotriose uptake. Agt1 transporter was found to be the dominant maltose/maltotriose transporter in the ale strains whereas Malx1 and Mtt- type transporters dominated in the lager strains. All lager strains studied were found to possess a non-functional Agt1 transporter. The ale strains were observed to be more sensitive to temperature decrease in their maltose uptake compared to the lager strains. Single transporters were observed to differ in their sensitivity to temperature decrease and their temperature-dependence was shown to decrease in the order Agt1≥Malx1>Mtt1. The different temperature-dependence between the ale and lager strains was observed to be due to the different dominant maltose/maltotriose transporters ale and lager strains possessed. The *AGT1* promoter regions of ale and lager strains were found to differ markedly from the corresponding regions of laboratory strains. The ale strain was found to possess an extra MAL-activator binding site compared to the lager strains. Improved maltose and maltotriose uptake capacity was obtained with a modified lager strain where the *AGT1* gene was repaired and put under the control of a strong promoter. Modified strains fermented wort faster and more completely, producing beers containing more ethanol and less residual maltose and maltotriose. Significant savings in the main fermentation time were obtained when modified strains were used. In high-gravity wort fermentations 8–20% and in very-high-gravity wort fermentations even 11–37% time savings were obtained. These are economically significant changes and would cause a marked increase in annual output from the same-size of brewhouse and fermentor facilities.

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