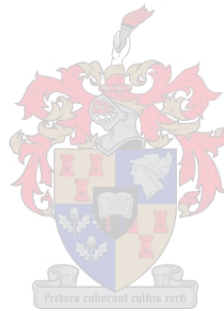


Fructophilic yeasts to cure stuck fermentations in alcoholic beverages

by

Klaus A Sütterlin



Dissertation presented for the degree of
Doctor of Philosophy (Agricultural Science)

at

Stellenbosch University

Institute of Wine Biotechnology, Faculty of AgriSciences

Promoter: Prof Florian Bauer
Co-promoter: Prof Jürg Gafner

March 2010

Declaration

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Date: 20.12.2009

Summary

Stuck alcoholic fermentations are a major enological problem for the international winemaking industry. Incomplete wine fermentations are frequently characterized by high residual fructose concentrations and the near-absence of residual glucose, a fact that is due to the glucophilic character of the wine yeast *Saccharomyces cerevisiae*. Wines with high contents of post fermentation sugar are very susceptible for microbial spoilage since residual fructose and/or glucose can be metabolized by bacteria and yeast to undesired by-products such as volatile acid and off-flavours, resulting in wine spoilage and considerable economic losses. It has been reported that stuck fermentations are usually caused by several synergistically acting inhibition factors, and the glucose to fructose ratio (GFR) is thought to play an important role in this context. This study is aimed at contributing towards a better understanding of this industrial problem, and at finding industrially applicable solutions.

In a first part, this study describes the isolation of two appropriate strains of the fructophilic yeast *Zygosaccharomyces bailii* from the natural microflora of grapevine, followed by trials in small scale test fermentations using stuck industrial fermentations as model media. These experiments were expanded to also investigate large scale industrial fermentations. As a result, a strategy for the treatment of stuck fermentations was developed and successfully applied in several wineries with fermentation problems. This methodology represents an entirely novel and industrially applicable solution to high residual fructose levels.

In a second part, the data contributes to elucidating the molecular nature of the fructophilic phenotype of *Z. bailii* by characterizing some of the genes and proteins that may be responsible for the fructophilic character. In particular, the investigation focused on the first two steps of hexose metabolism, the transport of sugar into the cell by permeases and sugar phosphorylation by hexokinases, which combined are thought to be primarily responsible for sugar preference.

One result of this study was Fructoferm W3©, a dry yeast product which is commercially available. Fructoferm W3 was awarded with the innovation medal for enological products at Intervitis/Interfructa, Stuttgart, Germany in 2007.

Opsomming

Die voorkoms van steek alkoholiese fermentasies is 'n ernstige probleem in die internasionale wyn industrie. Onvolledige fermentasies word dikwels gekenmerk deur hoë residuele fruktose konsentrasies en die veitlike afwesigheid van residuele glukose. Die kenmerke kan meestal toegeskryf word aan die glukofilliese karakter van die wyngis *Saccharomyces cerevisiae*. Wyne met 'n hoë suiker inhoud na die afloop van fermentasie is vatbaar vir mikrobiële bederf aangesien residuele fruktose en/of glukose gemetaboliseer kan word deur bakteriële en gis om ongewenste byprodukte soos vlugtige sure en bygeure te vorm wat kan lei tot wyn bederf en aansienlike ekonomiese verlies. Dit is vasgestel dat steek fermentasies gewoonlik veroorsaak word deur verskeie sinergistiese werkende inhibisie faktore, waartoe die glukose/fruktose verhouding 'n noemenswaardige bydrae lewer. Die mikpunt van hierdie studie was om 'n bydrae te lewer tot die begrip van steek fermentasies en die daarstelling van moontlike industriële oplossings.

Die eerste deel van die werk beskryf die isolasie van twee rasse van die gis *Zygosaccharomyces baillie* uit die natuurlike wingerd mikroflora, gevolg deur steekproewe in die vorm van kelinskaalse fermentasies met steek industriële fermentasies gebruik as model media. Hierdie eksperimente is vervolgens uitgebrei om grootskaalse industriële steek fermentasies te bestudeer. Die uitkoms van hierdie werk het gelei tot die ontwikkeling van 'n strategie vir die behandeling van steek fermentasies wat suksesvol toegepas is in verskeie wynmakerye. Die metodiek bring 'n nuwe en industrieel toepasbare oplossing vir hoë residuele fruktose vlakke.

Die data aangebied in die tweede afdeling dra by tot die verheldering van die molekulêre natuur van die fruktofilliese fenotipe van *Z. baillie* deur die tipering van gene en proteïene wat moontlik verantwoordelik is vir die fruktofilliese karakter van die gis. Die ondersoek het spesifiek op die eerste twee stappe van heksose metabolisme, naamlik die invoer van suiker in die sel deur permeases en suiker fosforilering deur heksokinases, gekonsentreer. Die kombinasie van die twee prosesse is vermoedelik verantwoordelik vir die regulering van suiker voorkeur.

'n Gevolg van die studie was die ontwikkeling van 'n droë gisprodukt, Fructferm W3©, wat kommersieel beskikbaar gestel is. Fructoferm W3 is in 2007 toegeken met die innovasie medalje vir wynkundige produkte by Intervittis/Interfructa in Stuttgart, Duitsland.

This dissertation is dedicated to my parents

Biographical sketch

Klaus Sütterlin was born in Müllheim, Germany on June, 27, 1971. After completing school at “Technisches Gymnasium Müllheim” Academic High School, he made a professional education as winemaker. Afterwards he studied Biology at the Albert-Ludwigs-Universität in Freiburg, Germany with the completion of a Diploma in Microbiology. The external Diploma Thesis “Regulation of Glycerol Production of *Saccharomyces cerevisiae*” was done in Wädenswil, Switzerland under the supervision of Profs Georg Fuchs and Jürg Gafner. Subsequent he was a scientific employee at Agroscope ACW Changins- Wädenswil, where he worked in the department of beverage microbiology, resulting in the first contacts with the matter of stuck and struggling wine fermentations. He registered as a PhD student at Stellenbosch University in 2006 to start a PhD on this topic under the supervision of Profs Florian Bauer and Jürg Gafner.

Acknowledgements

I wish to express my sincere gratitude and appreciation to the following persons and institutions:

- I thank Prof. Jürg Gafner, my external promoter and supervisor in Wädenswil for all the help and the patience with me. Jürg, you always provide encouragement and I am very grateful for the establishment of a special relationship with the industry.
- My sincere thanks to Prof. Florian Bauer, my promoter, for the opportunity to do my Ph.D. at Stellenbosch University. Florian, with your knowledge and your casual manner, you are always an ideal to me.
- Thanks to my love Claudia Keller, who always stands behind me, in good as well as in bad times. Thank you for your love and trustfulness; it was a great support for me.
- Thanks to all friends at Stellenbosch University, I had wonderful moments in the last years. Thank you Michael, Patrick, Jaco, Anita, Hamilton, Gustav, Sulette, Adriaan, Maret, Heidi, Desiree, Adri, Philip, John, Benoit, Vasu, Marius, Alex, Stephany, Linda, Lynn, Talitha, Judy, Egon, Wessel, Pierre, Edmund, Chidi, Siew and Melané, for the fun, the outings and the support. Very special thank to Karin, for all your tireless help in every situation.
- Thanks to my brother Armin and his family, who always provided deep insight in the winemaking world. Thanks to my sister Rita for always being there for me.
- I also would like to say thank you to all the friends at ACW in Switzerland, namely: Petra, Andrea, Daniel, Naomi, Frank, Rolf, Jürg, Bea, Jörg, Markus, Cosima, Ernst, Hans, Theo, Thierry and the cellar crew, Hans-Peter, Anneliese, Carole, Franz, Anita, Fabio, Walter, Peter, Stephan, Toni, Manuela, Monika, Katharina and Uta. A very special thank to Heidi Horsch, for the companionship all the way as doctoral students in Stellenbosch and Wädenswil, which was a particular and important stage of my life.
- Many thanks to Lallemand Inc., and Karl Burger for the support of the research in Wädenswil, also thanks to KTI-Iwood and KTI-Detektionssysteme. For funding in Stellenbosch, thanks to the South African Wine Industry.

Preface

This dissertation is presented as a compilation of 5 chapters, each chapter is introduced separately.

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Chapter 2 **Literature review**

Discrimination between glucose and fructose utilisation in wine yeasts

Chapter 3 **Research results**

Microbiological experiments: Isolation of fructophilic yeasts from natural habitats and the development of a novel methodology for the cure of stuck fermentations

Chapter 4 **Research results**

Molecular experiments: Isolation and characterization of transport systems and a hexokinase from *Zygosaccharomyces bailii*

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Chapter 1

**Introduction and
project aims**

1.1 Stuck fermentations

Stuck alcoholic fermentations are a major and persistent problem for the international wine industry. As results of a survey in France indicate (Association for the Development of Wine Biotechnology, 1996), more than 60% of responding winemakers admitted to having experienced this problem. The economic loss for the enological industry can be very significant. Firstly, such wines do not usually present the organoleptic profile desired by the winemaker, and will in particular display an undesirable sweetness. Secondly, wines with high post fermentation sugar content are very susceptible to microbial spoilage during the further process of vinification and bottling. Residual fructose and/or glucose can be metabolized to undesired by-products like volatile acid and off-flavours which may lead to spoilage of the product. Moreover, microbial and chemical stability is an important prerequisite to the bottling of the product. A complete or “dry” fermentation is typically indicated by a residual sugar level of less than approximately 4 g/l. Incomplete or stuck fermentations are defined as those leaving a higher than desired residual sugar content at the end of alcoholic fermentation. Slow or sluggish fermentations are characterized by a low rate of sugar utilisation (Bisson, 1999).

Many causes of stuck fermentations are well known and have been described in the literature (Bisson, 1999; Bisson and Butzke, 2000; Blateyron and Sablayrolles, 2001; Charoenchai *et al.*, 1998; Kudo *et al.*, 1998; Schütz and Gafner, 1993a and 1995). With regard to metabolic aspects, the decrease in rate of sugar consumption by wine yeast strains is correlated with a decrease in sugar uptake capacity. Fructose and glucose consumption are reduced in response to various stress conditions which have an impact on hexose transporter expression and activity (Alexandre and Charpentier, 1998). These stress conditions can be broadly classified into three groups, nutritional limitations, inhibitory substances and physical factors. Deficiencies in nitrogen, oxygen, mineral nutrients or vitamins are principal reasons for nutritional limitations. Examples for inhibitory substances include ethanol, fungicides, killer toxins, medium chain fatty acids, sulphite or other cell toxic compounds. Physical changes such as temperature shifts and excessive must clarification also contribute to the onset of stuck fermentation. However, empirical observations suggest that stuck fermentations are rarely due to a single factor in isolation but may rather be the result of complex synergistic effects amongst several of these factors (Alexandre and Charpentier, 1998; Bisson, 1999; for review see: Malherbe *et al.*, 2007).

1.2 Hexose preference of yeasts

Due to the glucophilic phenotype of the regular wine yeast *Saccharomyces cerevisiae*, the residual sugar in incomplete fermented must is mainly fructose. This was confirmed by analysis of stuck wines in the vintages 2003-2007 from Switzerland, Austria, Germany and Italy

(unpublished data, this dissertation). More than 90% of all investigated samples were characterized by a glucose to fructose ratio ≤ 0.1 , or a tenfold higher fructose than glucose concentration. This fact can clearly be a contributing factor to stuck fermentation, since fructose utilisation in *S. cerevisiae* is less efficient than glucose utilisation.

In the fermentative pathway of yeasts, the reason for a sugar preference appears to be linked to the transport and/or the phosphorylation steps, since from the point of fructose-6-phosphate the metabolism of fructose and glucose are the same. Regarding sugar transport, more than 20 genes that encode proteins with significant structural and sequence homologies to hexose transporters have been identified in *S. cerevisiae* and are referred to as hexose transporter (*HXT*) genes (Özcan and Johnston, 1999). These genes encode for permeases that transport hexoses through facilitated diffusion. While not all of these genes have thus far been biochemically characterized as functional hexose transporters, the transport active proteins during fermentation have been shown to be restricted to a smaller group of seven transporters (Hxt1-7p) that truly contribute to hexose transport, presenting different affinities and are active at different stages of fermentation (Luyten *et al.*, 2002). The kinetic characterization of these hexose transport systems in *S. cerevisiae* indicates that the K_m , while variable between the different transporters, is always higher for fructose than for glucose (Reifenberger *et al.*, 1997). However, transport systems with a preference for fructose were recently isolated from the yeasts *Saccharomyces carlsbergensis* (Gonçalves *et al.*, 2000), *Kluyveromyces lactis* (Diezemann and Boles, 2003) and *Z. bailii* (Pina *et al.*, 2004). The two closely related genes *FSY1* and *FRT1* encode specific H^+ -fructose symporters, while *FFZ1* encodes for a facilitated diffusion system specific for fructose with a poor homology to other facilitated diffusion systems like the HXT family. In *S. cerevisiae*, no specific transport proteins for the different hexoses exist (Reifenberger *et al.*, 1995).

Once the sugars have been imported into the cell, they are phosphorylated by one of three sugar kinases Hxk1, Hxk2 and Glk1 (Entian and Barnett, 1992). All hexose phosphorylating enzymes of *S. cerevisiae* prefer glucose as substrate, compared to fructose. Furthermore, hexokinases are also involved in a complex network of sugar mediated signal transduction not completely understood at present. Fructose specific hexokinases, called fructokinases or ketohexokinases exist mainly in prokaryotes and plants. In the fission yeast *Schizosaccharomyces pombe*, a hexokinase with affinity for fructose has previously been characterized (Petit *et al.*, 1996).

1.3 Methodology and aims

Since glucose preference and problem fermentations have been shown to be correlated, this dissertation aimed at providing solutions that could be applied in the wine industry, and to provide insights into the molecular nature of a fructophilic phenotype. For this purpose, a new

approach to reduce the fructose residues of incompletely fermented wines was investigated. This approach focused on the use of fructophilic yeast species that were isolated from the natural microflora of grapevine. The indigenous microflora of grapevine usually contains only two prominent species of fructophilic yeasts, *Z. bailii* and *Candida stellata* (Schütz and Gafner, 1993b; Torija *et al.*, 2001). Both non-*Saccharomyces* yeasts usually display relatively low fermentation rates and are nearly always present during the first two days of spontaneous alcoholic fermentations of grape must (Torija *et al.*, 2001; Zott *et al.*, 2008). Strains with an adequate ethanol tolerance have been shown to exist within both species (Jolly *et al.*, 2006); however, due to their weak fermentation activity they will be displaced by the strong fermenting yeast *S. cerevisiae* (Torija *et al.*, 2001; Combina *et al.*, 2005; Zott *et al.*, 2008). However, we here show that when inoculated into incomplete fermented wine, some strains of the fructophilic species are able to reduce the concentration of fructose residues, which results in an increase of the glucose to fructose ratio GFR.

Furthermore, little is known about the molecular systems governing the fructophilic phenotype in these species. To increase the understanding of this character, genes that may be responsible for this trait were then characterized from the isolated species and strains. These genes were cloned through heterologous complementation of the corresponding *S. cerevisiae* mutants.

The aims of this thesis were therefore twofold:

The first aim was the development of a methodology to cure stuck fermentations in large scale vinifications. This implied the isolation of suitable yeasts, mainly focusing on the ability to ferment fructose residues in incomplete fermented wines or musts without the production of undesired by-products. Following the first trials with small scale test fermentations in stuck wines, a further aim was to expand the method to the point of industrial scale winemaking, to cure stuck fermentations in commercial wineries.

The second aim was to contribute to the understanding of fructophilic wine yeast species, which are present in relatively small numbers in the natural environment of grapevine. This task involved the molecular characterization of proteins which are thought to be involved in determining a preference for sugar, namely the hexose transport systems and hexokinases.

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Chapter 2

Literature review

**Discrimination between glucose and fructose
utilisation in wine yeasts**

2. Literature review:

This chapter reviews sugar utilisation by *Saccharomyces cerevisiae*, and focuses in particular on the differences in the utilisation of glucose and fructose, as well as associated signal transduction pathways. In addition, the known fructose transport systems of yeasts and, more particularly, the hexose transporters of fructophilic yeasts are discussed.

2.1. Transport

2.1.1 Hexose uptake in *Saccharomyces cerevisiae*

Hexose import into *S. cerevisiae* is mediated by a group of membrane-spanning transport proteins, called hexose transporters (HXT). They are members of the diverse major facilitator superfamily, which includes over 10'000 sequenced members (Pao *et al.*, 1998). In *S. cerevisiae*, at least 20 members of the major facilitator superfamily (MFS) can be found (for review see: Özcan and Johnston, 1999). *HXT1* to *HXT17*, *SNF3*, *RGT2* and *GAL2*, have been identified by genetic studies based on sequence homology. Hxt proteins transport their substrate by passive, energy-independent facilitated diffusion down a hexose concentration gradient. Two members of the gene family *SNF3* and *RGT2* seem to encode proteins that function as sensors of extracellular glucose, generating an intracellular signal for glucose-induced transcription of the *HXT* genes (Özcan *et al.*, 1996; Özcan *et al.*, 1998). However, more proteins involved in hexose transport seem to exist, since deletion of the *SNF3* sensor gene in a strain deleted for all known hexose transporter genes restored growth on glucose (Wieczorke *et al.*, 1999). *S. cerevisiae* has the largest number of MFS transporters of any investigated organism (Pao *et al.*, 1998). With the exception of Hxt12p, all *HXT* gene products are able to restore growth on glucose when expressed individually in a strain deleted for all other established transporter genes (Wieczorke *et al.*, 1999). *HXT1-4* and *HXT6-7* are the best characterized members of the HXT family and are classified as the major hexose transporters in yeast. All Hxt proteins are also able to transport glucose, fructose and mannose, while *HXT9*, *10*, *11* and *14* have also been shown to transport galactose (Wieczorke *et al.*, 1999). Generally the K_m values seem to be higher for fructose, mannose and galactose than for glucose. There are no specific transport proteins for the different hexoses as had been postulated by Bisson *et al.*, (1993). However, for disaccharide uptake, proton coupled symport systems which belong to the maltose permease family exist in *S. cerevisiae* (Day *et al.*, 2002).

Proteins of the MFS family are characterized by structural similarities and conserved amino acid residues, possess mostly 12, 14 or 24 putative transmembrane α -helical domains and average 400-600 amino acid residues in length. The MFS catalyze uniport, symport or antiport (Pao *et al.*, 1998). The mechanistic principles applicable to all MFS carriers have been

summarized by Law *et al.* (2008). The number of transmembrane domains of many members of the MFS carrier family, including the yeast Hxt's, has not yet been confirmed. Most publications assume 12 transmembrane domains (TMD), whereas some recent predictions suggest 11 TMD for at least some of the yeast hexose transporters such as Hxt1 and Hxt9 (SGD: *Saccharomyces* Genome Database; TMHMM Server v. 2.0, Prediction of transmembrane helices in proteins). However, recent work shows that perhaps all members of the MFS share the same three-dimensional structure, consisting of two domains that surround a substrate-translocation pore (Law *et al.*, 2008). Furthermore, they seem to operate via a single substrate-binding site, alternating-access mechanism which involves a rocker-switch type movement of the two halves of the protein (Law *et al.*, 2008).

2.1.1.1 *S. cerevisiae* HXT null mutant

Hexose uptake experiments have frequently used mutant strains that are defective in hexose uptake. A significant number of publications describe the *Hxt* null mutant, deleted in *Hxt1-Hxt7*, as unable to grow on glucose, fructose or mannose (Boles and Hollenberg, 1997; Özcan and Johnston, 1999; Wieczorke *et al.*, 1999; Gonçalves *et al.*, 2000; Pina *et al.*, 2004). Furthermore, no glycolytic flux is detectable in this mutant (Boles and Hollenberg, 1997). Based on these findings the remaining HXT genes, HXT8-17, are postulated to encode proteins that either are unable to transport glucose or are not expressed under the conditions tested. The observation of very low expression levels of the genes HXT8-HXT17 supports this argument (Boles and Hollenberg, 1997; Özcan and Johnston, 1999). In contrast, an *Hxt1-7* deletion mutant in the CEN.PK strain background is still able to grow on glucose, although slowly. To block hexose uptake in this strain, a concurrent knock-out of all 20 transporter genes is required (Wieczorke *et al.*, 1999). Most kinetic data of the different glucose transport proteins was determined by their expression in the *Hxt* null mutant. Due to the possibility of post transcriptional modulation by interaction with other transporters, a single protein might behave differently in this mutant than in a wild-type strain. It is therefore possible that results of the protein expression in the *Hxt* null mutant do not necessarily reflect the *in vivo* function of these transporters.

2.1.1.2 HXT Transport Kinetics

Cells of *S. cerevisiae* grow well on a broad range of glucose concentrations, from a few μM to more than 2M. When taking this fact into consideration, the presence of multiple hexose transporters with different affinities is not surprising, especially since hexose uptake into the cell

is considered to be the rate limiting step of glycolytic flux (Cortassa *et al.*, 1995). This unusual multiplicity of hexose transporters provides the cell with kinetically optimal transporters for different environmental conditions. The hexose uptake systems were formerly described in two systems, namely a constitutive, low affinity uptake system with high K_m above 20 mM and a glucose repressed, high affinity uptake system with low K_m between 1 and 2 mM (Bisson and Fraenkel, 1983). However, it was confirmed that the high-affinity and the low-affinity uptake systems represents the sum of several transporters rather than the result of individual transporters (Wieczorke *et al.*, 1999). Serving as an environmental stimulus, it appears that it is mainly the glucose concentration that regulates much of the type, the quantity and the activity of the transport system, both at the transcriptional and the post-translational levels. The transcription of the low-affinity transporter gene *HXT1* (encoding a transporter with a K_m of 100mM) is induced only by high levels of glucose, whereas the expression of *HXT3* (encoding a transporter with a K_m of 60mM) is induced by both, high and low concentrations of glucose. Hxt1p and Hxt3p are responsible for transporting glucose in cells growing on high glucose levels due to the fact that both transporters confer low affinity glucose transport when expressed in a Hxt null mutant (Reifenberger *et al.*, 1997). On the other hand, *HXT2*, *HXT6* and *HXT7* expression is induced only by low glucose concentrations, suggesting that they encode high-affinity transporters. Hxt6p and Hxt7p show a K_m between 1 and 2 mM when expressed in the Hxt null mutant, while Hxt2p expression results in a biphasic uptake kinetic with K_m values of 1.5 mM and 60 mM respectively (Reifenberger *et al.*, 1997). It is possible that the affinity of the Hxt2p transporter is post-translationally modulated in response to different glucose concentrations; alternatively Hxt2 could play a regulatory role in activating the expression of other transporters with different affinities. Hxt2p, 6p and 7p seem to be responsible for glucose transport when glucose is scarce. *HXT4* is induced by low levels of glucose and encodes for a protein with an intermediate affinity ($K_m = 9$ mM). It is still unclear why yeasts need several transporters with nearly identical affinities (Özcan and Johnston, 1999). Potentially, this balance of affinities of different transporters could provide an advantage in glucose or hexose uptake and conversion, compared to competing yeast species in natural wine fermentations.

Generally, the characteristics of the main Hxt carriers can be summarized and simplified as follows: Hxt1p is a low-affinity, high capacity transporter and is required when glucose or fructose is abundant; Hxt2p is a high-affinity, low capacity transporter which is necessary when the sugars are scarce. The other hexose transporters have evolved for dealing with different concentrations of sugar under different conditions. Most of the *HXT* genes are expressed only under the appropriate conditions; Hxt3p, a transporter with intermediate affinity is expressed under both, high sugar levels and low sugar levels (Johnston and Kim, 2005).

In contrast to the major hexose transporters, little is known about *HXT5* and *HXT8-17*. The expression of some of these transporters remains an enigma, except for Hxt5 and Hxt13, which are expressed at very low levels, approximately 30-300 fold less than Hxt1 (Özcan and

Johnston, 1999). Up to now it was not possible to functionally express Hxt12, supporting the view that *HXT12* is a pseudogene (Kruckeberg *et al.*, 1996; Wieczorke *et al.*, 1999). All other genes of this group are able to complement the growth defect of a mutant strain which is deleted for all known hexose transporters. Nevertheless, some of these genes might have only regulatory functions or might be involved in the transport of different energy sources. For example, *HXT5*, *HXT13* and *HXT15* are all induced in the presence of non-fermentable carbon sources, whereas *HXT17* promoter activity is regulated in response to different pH-values in media containing raffinose and galactose (Greatrix and van Vuuren, 2006).

2.1.1.3 Regulation of HXT genes

The major carbon and energy source for most organisms is glucose; hence, most studies to characterize hexose transport in yeasts usually refer to glucose as the substrate. Glucose is metabolized through glycolysis to pyruvate, which can follow two pathways: in the presence of oxygen, most organisms convert pyruvate to carbon dioxide and water, generating up to 36 units of ATP per molecule of glucose via oxidative phosphorylation. When oxygen becomes rare, most cells resort to fermentation, yielding only 2 ATPs per molecule of glucose via substrate-level phosphorylation of ADP. *S. cerevisiae* is one of the few organisms that prefer to ferment glucose, even when oxygen is abundant. On the basis of this moderate rate of yield of ATP production, the metabolic rate must be adequate and exceedingly economical (Van den Brink *et al.*, 2008). It is not surprising that glucose can regulate the expression of genes required for its own efficient utilisation acting like a growth hormone to regulate several aspects of metabolism and growth. This includes regulation at transcriptional, post-transcriptional, translational and post-translational levels (Johnston and Kim, 2005). For these adaptations to occur, the cell must sense the energy source and transmit a signal to the associated effectors. Up to now, the presence of three glucose or hexose sensing systems have been established for *S. cerevisiae* (Kruckeberg *et al.*, 1996). This review focuses on the various mechanisms, including those that operate through the Snf3 and Rgt2 glucose sensors to induce expression of genes encoding hexose transporters by the ultimate target of this pathway, the transcription factor Rgt1. Another mechanism works through the Snf1 protein kinase, which results in the repression of gene expression when glucose levels are high (Bisson, 1988). A third glucose sensing mechanism employs the G-protein-coupled receptor, Gpr1 and cyclic AMP as a second messenger (Lemaire *et al.*, 2004).

The induction of the expression of *HXT* genes is one of the first responses of yeast cells to the presence of glucose (Kim *et al.*, 2006). Central components of the glucose induction pathway are two members of the *HXT* family that are located in the plasma membrane, the glucose sensors Snf3 and Rgt2. These proteins sense the presence of extracellular glucose by

binding the molecule on the outside of the cell and generate an intracellular signal for induction of *HXT* gene expression. Both sensor proteins are about 60% identical to each other and exhibit a homology of 26 to 30% to other members of the HXT gene family (Özcan *et al.*, 1998). However, they are not able to transport hexoses through the cell membrane. Expression of *SNF3* or *RGT2* in the *hxt* null mutant does not complement the inability of glucose uptake (Liang and Gaber, 1996). The observations of various studies indicate that the two proteins act like a receptor with a conformational change occurring after glucose binding (Celenza *et al.*, 1988; Marshall-Carlson *et al.*, 1990; Bisson *et al.*, 1993; Özcan *et al.*, 1996). This is clearly demonstrated by studies using a single missense mutation in each of the glucose sensors. The Snf3p (R229K) and Rgt2p (R231K) mutations cause constitutive expression of HXT genes, probably because these mutations convert the sensors into their glucose-bound forms, signaling conformation (Özcan *et al.*, 1996). Both proteins have 12 predicted transmembrane domains, similar to those of the actual hexose transporters. On the other hand, unusually long C-terminal tails which are composed of 341 (Snf3) or 218 (Rgt2) amino acids are structurally characteristic of Snf3p and Rgt2p, compared to other HXT members. These cytoplasmic tails are similar to one another for a stretch of 25 amino acids, the so-called “glucose sensor domain”. The presence of this sequence is essential for the generation of an intracellular signal, since deletion of this sequence while leaving the rest of the tail intact abolishes signaling (Vagnoli *et al.*, 1997). Snf3p contains two of these 25 amino acid sequences, Rgt2p contains only one. The Snf3 tail is required for the induction of low affinity transporter genes *HXT2* and *HXT4*, and the Rgt2p tail is required for high glucose induction of *HXT1* expression (Özcan *et al.*, 1998). The yeast glucose sensors may have evolved from a glucose transporter that changed the transporter domain into a glucose-binding domain, thereby gaining the ability to transmit information about extracellular glucose concentrations to the interior of the cell (Lafuente *et al.*, 2000).

2.1.1.4 The significant role of Rgt1p in glucose induction of HXT genes

The signal generated by the sensor proteins in response to glucose activated casein kinase I (YckI), a protein kinase which is anchored via palmitate moieties to the membrane in the environment of the sensor proteins (Moriya and Johnston, 2004). Subsequently, the activated casein kinase catalyzes the phosphorylation of the paralogous proteins Mth1 and Std1, which are bound to the C-terminal tails of the glucose sensors. The phosphorylated Mth1 and Std1 can then be recognized by the multienzyme complex SCF^{Grr1} which catalyses the ligation of ubiquitin to Mth1/Std1. This ensuing ubiquitination targets them to the 26S proteasome for degradation (Flick *et al.*, 2003). The degradation of Mth1/Std1 leads to the phosphorylation of Rgt1, a bifunctional transcription factor that displays three different transcriptional modes in response to glucose (Özcan *et al.*, 1996). In case of phosphorylated Rgt1, the binding to the

HXT promoter is inhibited, resulting in the derepression of *HXT* gene expression. The phosphorylation of Rgt1 can be mediated by a low glucose signal, activated by the Snf3 glucose sensor. In this first case, Rgt1 neither represses nor activates transcription. Second, at high concentrations of glucose, Rgt1 becomes hyperphosphorylated, mediated by the Rgt2 glucose sensor, which leads to a dissociation from the repressor complex. This converts the transcription factor Rgt1 from a repressor to an activator, which then may stimulate the expression of a transcriptional activator that is required for maximal expression of the low affinity and high capacity *HXT1* transporter gene (Figure 2.1). Thirdly, in the absence of glucose, Rgt1 agglomerates with Mth1 and Std1 and binds to *HXT* promoters, which represses the expression of the transporter genes (Figure 2.1). The transcription factor Rgt1 contains an amino-terminal C6 zinc cluster motif (Cys₆ Zn₂) and binds to the promoters of *HXT* genes by recognition of the sequence 5'-CGGANNA-3' (Schmidt *et al.*, 1999; Johnston and Kim, 2005; Polish *et al.*, 2005).

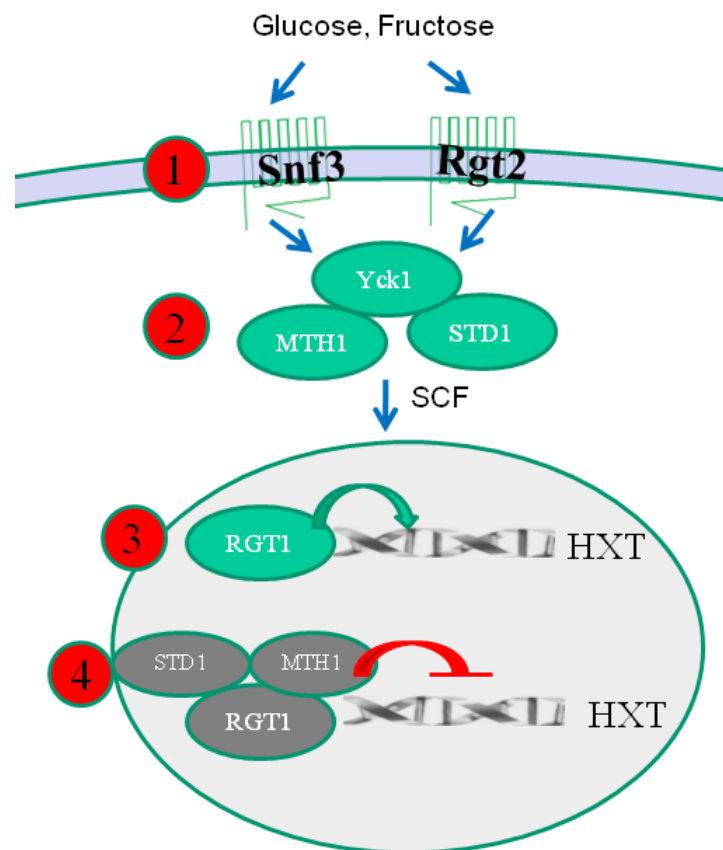


Figure 2.1: Snf3/Rgt2 signaling pathway: Rgt1 can act as activator or suppressor of *HXT* hexose transporter genes, depending on hexose concentration. Snf3 and Rgt2 sensor proteins generate a signal in response to glucose and fructose (1), casein kinase I (Yck1) (2) transfers the signal to Mth1 and Std1. The bifunctional transcription factor Rgt1 activates (3) or represses *HXT* expression (4) under agglomeration with Std1 and Mth1, due to Rgt1 phosphorylation status. The multienzyme complex SCF acts as mediator of the Rgt1 phosphorylation.

The regulation of *HXT1* expression shows some specific features when compared to the regulation of the other *HXT* genes. *HXT1* expression is induced only at high glucose

concentrations and requires Hxk2p and Reg1p. The same genes are, however, not required for induction of Hxt3 expression at high levels of glucose (Özcan and Johnston, 1995). The mechanism of this regulation has not yet been identified. Additionally, *HXT1* expression is induced by high osmolarity via the HOG signal transduction pathway (Tomás-Cobos *et al.*, 2004). Furthermore, *HXK1* expression cannot be effectively repressed by the repressor Rgt1 (Özcan *et al.*, 1996). A deletion of *rgt1* can completely abolish the repression of *HXT2* and *HXT4* in the absence of glucose, but *HXT1* is still repressed. Interestingly, different *HXT* promoters have different numbers of Rgt1 binding sites: the *HXT1* promoter region contains 8 Rgt1 binding sites, the *HXT2* and *HXT4* promoters only 3, whereas the *HXT3* promoter contains 10 Rgt1 binding sites (Kim, 2009). Rgt1-dependent repression appears to be more effective in the *HXT2* and *HXT4* promoters with fewer Rgt1-binding sites than in the *HXT1* promoter. In opposition to these observations, Hxt3p expression is constitutive and glucose-independent in a Δ rgt1 mutant and consequently highly Rgt1-dependent, in spite of the 10 Rgt1 binding sites in the *HXT3* promoter region. Consequently, Rgt1 functions differently at different promoters, perhaps due to different architecture of the Rgt1-binding sites in the promoters (Kim, 2009). These results suggest that the intervening sequences between the Rgt1-binding sites in the *HXT* promoters likely play a role in expression of the promoter, and that a yet unidentified regulatory mechanism seems to be involved in binding to the intervening sequences to influence the activity of Rgt1. This view is supported by the finding that Rgt1 is efficiently recruited to multiple copies of the Rgt1-binding sites without intervening sequences and mediates synergistic repression of transcription (Kim, 2009).

Glucose can repress the expression of many genes, for instance some enzymes for metabolism of alternative carbon sources, gluconeogenesis enzymes or proteins of the respiratory pathway (Carlson, 1999). The main player of this glucose induced repression is the Snf1 protein kinase. If glucose is scarce, Snf1 is activated by its phosphorylation catalyzed by one of three protein kinases. This activated Snf1 catalyses the phosphorylation of the Mig1 transcriptional repressor, as well as the phosphorylation of other gene repressors and activators (Carlson, 1999; Kuchin *et al.*, 2002). The paralogues of Mig1, Mig2 and Mig3, which have essentially the same binding sites, might also play a role in glucose repression of the expression of some genes (Lutfiyya *et al.*, 1998). The promoters of the high-affinity hexose transporters *HXT2* and *HXT4* have binding sites for the Mig1 glucose repressor, which mediate the repression of transcription when glucose levels are high (Özcan and Johnston, 1995; Özcan and Johnston, 1996). The addition of glucose mediates the dephosphorylation of the Snf1 kinase, resulting in a deactivation. The transmission of the glucose signal to Snf1 involves hexokinase 2 (Hxk2), a hexokinase that catalyzes the first catalytic step of glucose phosphorylation (Rolland *et al.*, 2002). This Snf1-Mig1 glucose repression pathway affects the expression of many genes (Young *et al.*, 2003) and will be further mentioned in the chapter concerning the roles of the hexokinases (Chapter 2.5).

2.1.1.4.1 Rgt1 targets

Only six genes are known to be fundamental targets of the Snf3/Rgt2-Rgt1 glucose induction pathway, and all of them are HXT hexose transporter genes (Boles and Hollenberg, 1997). Seven further genes were recently validated as Rgt1-targets, but the roles of Rgt1 in regulating the expression of these genes are modest at best (Kaniak *et al.*, 2004). Currently, the possible functions of these few newly discovered Rgt1 targets can only be speculated about. However, glucose repression and glucose induction pathways are interlocked in an elaborate network of autoregulatory and cross-pathway-regulatory circuits (Kaniak *et al.*, 2004). In conjunction with the glucose repression and the glucose induction pathways, the opposing transcriptional regulation by Mth1 and Std1, which are paralogous proteins that regulate the function of the Rgt1 transcription factor, also plays a role, Mth1 and Std1 transcription is regulated via feedback and feed forward regulatory mechanisms that operate through two different glucose signal transduction pathways. Std1 expression is induced by glucose via the Rgt2/Snf3-Rgt1 signal transduction pathway and, additionally, the degradation of Std1 is dampened via the same pathway. In contrast, Mth1 expression is repressed by glucose via the Snf1-Mig1 glucose repression pathway which also reinforces Mth1 degradation (Kim *et al.*, 2006). This converse transcriptional regulation of Mth1 and Std1 expression provides rapid induction of HXT gene expression in response to glucose and efficient repression of HXT gene expression when the available glucose has been depleted. This intricate and highly evolved regulatory network ensures stringent regulation of hexose utilisation. However, none of the hexose facilitators has been directly implicated in the repression mechanism and repression signalling was found to be independent of the plasma membrane sensors Snf3 and Rgt2 (Belinchón and Gancedo, 2007; Reifemberger *et al.*, 1997).

2.1.1.4.2 Post- translational mechanisms

In addition to these described regulatory pathways, the function of several hexose transporters is regulated by further post-translational mechanisms. Transporters of alternative sugar sources like galactose (Gal2) or maltose (Mal62) can be degraded if glucose is available, which helps to ensure that yeast cells utilize these two sugars only if glucose is absent (Ramos and Cirillo, 1989). Ubiquitination seems to mediate the glucose induced inactivation of Gal2, which targets it to the vacuole where it is degraded. The degradation of the maltose permease involves two signaling pathways, one dependent on glucose transport which requires the function of HXT proteins and the other independent with the assistance of Rgt2p, Snf3p, Grr1p and Rgt1p (Jiang *et al.*, 1997). In contrast to these examples, little is known about posttranslational regulation of glucose transport. In nitrogen starved cells, glucose inactivation

of both, high and low affinity glucose transport was observed (Busturia and Lagunas, 1986). Also, most of the Hxt proteins contain consensus sequences for N-linked glycosylation and phosphorylation by protein kinase A and casein kinase II, but it is not known if any of them are indeed modified (Boles and Hollenberg, 1997; Moriya and Johnston, 2004).

A further possibility of post-translational regulation might be an oligomerisation of hexose transporters as suggested by the presence of a leucin zipper motif which is known to mediate protein-protein interactions (Mueckler, 1994). As a further post-translational mechanism of hexose transport, the stabilization of the mRNA transcript of the low affinity transporter *HXT1* was observed by Greatrix and van Vuuren (2006). Under conditions of osmotic stress, they detected a fourfold mRNA increase 30 minutes after transcriptional arrest in osmotically stressed versus non-stressed yeast cells, indicating that mRNA stabilization seems not to be gene specific. The mechanism of mRNA stabilization remains to be characterized. This complexity of hexose sensing and the regulation of energy transport might confer a crucial advantage to *S. cerevisiae* to prevail over competing microorganisms in complex media like wine must.

2.1.1.5 HXT expression during alcoholic fermentation

In synthetic wine must MS300, containing 100 g/l glucose and 100 g/l fructose, the genes of the hexose transporters *HXT1-3*, *HXT6* and *HXT7* are expressed during alcoholic fermentation. Expressed individually in the *hxt* null mutant under winemaking conditions, the low affinity carriers *HXT1* and *HXT3* are the only transporters ensuring near-complete fermentation of sugars, indicating that these carriers play a predominant role in wine fermentation (Luyten *et al.*, 2002). However, these two transporters are thought to play different roles in hexose uptake during the fermentative metabolism. When expressed individually, the Hxt3 transporter was the only carrier that ensured an almost normal fermentation profile and has the highest capacity to support fermentation. *HXT3* is, therefore, thought to play a major role in the hexose uptake under winemaking conditions (Luyten *et al.*, 2002). At the promoter and protein levels, *HXT3* and Hxt3p are the only transporter gene and protein to be expressed throughout all phases of fermentation. The Hxt1 carrier was much less effective during the stationary phase and its role is thought to be restricted to the beginning of fermentation. Also required for a normal ongoing fermentation are the high-affinity carriers Hxt2, Hxt6 and/or Hxt7, but with different functions. Hxt2p is involved in growth initiation and Hxt6 and/or Hxt7 are required at the end of alcoholic fermentation (Perez *et al.*, 2005). The successful alcoholic fermentation to dryness of wine therefore involves at least four or five hexose carriers, playing different roles at various stages in the fermentation cycle. The apparent kinetic parameters of yeast cells, reflecting the combined activity of the various transporters present at different stages, changes throughout wine fermentation. At the beginning of fermentation, growing yeast cells exhibited a low-affinity

apparent K_m of about 112 mM for glucose, which decreases to a lower K_m of 47 mM at the beginning of stationary phase. In stationary phase, two transport components could be identified, one corresponding to a low affinity transport with a K_m of 70 mM and one with a much higher affinity for glucose with a K_m of about 1.9 mM (Perez *et al.*, 2005). This balance of expression and repression of high- and low- affinity transporters requires a sophisticated sensing system with the ability to distinguish the change in the substrate levels and transduce the signal to different *HXT* target genes. Recent models are simplified by investigating the presence of low and high glucose or the absence of glucose, which only addresses a limited number of scenarios. In addition, for research concerning stuck fermentations, it would be of particular interest to investigate the apparent K_m values for the *HXT*'s with fructose under the same wine making conditions.

2.1.1.6 Fructose transport in *S. cerevisiae*

All Hxt proteins investigated so far mediate facilitated diffusion of glucose and fructose. However, most studies concerning hexose transport are focused on the substrate glucose. It has been shown that, for all hexose transport proteins tested, K_m is higher for fructose than for glucose (Reifenberger *et al.*, 1997). This means that both, high- and low-affinity carriers have a higher affinity for glucose than for fructose. The K_m value for the low affinity transporter Hxt3p is measured at approximately 65 mM for glucose versus 125 mM for fructose. In addition, the low affinity transporter HXT7 has been characterised with K_m values of 2.1 mM for glucose versus 4.6 mM for fructose (Reifenberger *et al.*, 1997). Several classic studies have reported for a three- to five fold higher K_m for fructose compared to glucose for both high and low affinity transport systems (Cirillo, 1968; Wendell and Bisson, 1994; Reifenberger *et al.*, 1997). Such differences in the affinity may affect the rates of utilisation of the two sugars and could be one principal reason for the glucophilic phenotype of *S. cerevisiae*. The lower fructose affinity is thought to be due to the fact that glucose is transported preferentially in the pyranose form, whereas in the case of fructose the furanose form is preferred for transport (Heredia *et al.*, 1968). However, only about 30% of the fructose present in solutions is in the furanose form, so the transport competent concentration of fructose is below the total concentration (Cirillo, 1968). This theory was already proposed in 1931 and validated by a study conducted in 1950, which indicated that only the furanose form of fructose is fermented by yeast (Slein *et al.*, 1950). To draw a parallel between Hxts and the mammalian GLUT transporters, experiments suggest that GLUT2 recognizes fructose in the furanose form, allowing alignment with the same residues within the binding pocket as for the glucose pyranose structures. Thus, C2 and C3 of the furanose ring would form the hydrogen bonds, whereas C6 may still provide the possible hydrophobic interaction (Colville *et al.*, 1993).

All present information suggest that both the glucose induction pathway and the glucose repression pathway can also be mediated by fructose, since it was shown that the expression of the high affinity transporter Hxt2p is repressed 15- to 20-fold in high concentrations of glucose or fructose (Wendell and Bisson, 1994). There is no evidence for a separate fructose sensing mechanism in *S. cerevisiae*. Both binding sites of the glucose sensor proteins Rgt2 and Snf3 share a high degree of homology to the binding sites of the hexose transporter proteins, which accept both glucose and fructose as a substrate (Marshall-Carlson *et al.*, 1990). The putative 12 transmembrane domains, which are common features of the transporter proteins as well as the sensor proteins, constitute the hexose transporting or binding domains in the transporters or sensors respectively. This allows speculating that these sensor domains, considered as mutated transport domains (Neigeborn and Carlson, 1984), could bind the same hexoses that are transported by the carriers. Accordingly, Rgt2p and Snf3p could act as sensor for all of the sugars transported by the hexose transport proteins and may also act as trigger for the Rgt2-dependent gene induction pathway.

Glucose and fructose seem to induce the expression of the same *HXT* genes, as observed by Greatrix and Van Vuuren (2006), under non-fermentative conditions. Yeast cells transformed with *HXT* promoter-lacZ fusions indicate no difference between glucose and fructose growth on X-gal plates with 0.2 to 40% sugar. However, the only *HXT* promoters that exhibited activity under these conditions were HXT1-5 and HXT13. It is possible that the sensor proteins trigger different signaling events with fructose than with glucose under fermentative conditions, especially at later growth phases when fructose is dominant. Hence, consequences regarding the gene induction rate of the transporter genes during the different stages of wine fermentation may be affected by the glucose/fructose ratio and require further investigations.

Limited data is available concerning the molecular nature of substrate specificity of the HXT carriers. Two aromatic amino acid residues within transmembrane domain 10 might be important for substrate recognition in the Hxt2p and the Gal2p carriers of *S. cerevisiae*, since replacement of Phe⁴³¹ in Hxt2p with any other amino acid drastically changes substrate specificity (Kasahara and Maeda, 1998). Glucose transport was only supported by the aromatic amino acids Phe, Tyr and Trp. A similar function seems evident for the residues Tyr⁴⁴⁶ and Trp⁴⁵⁵ in galactose recognition of the Gal2p transporter of the budding yeast (Kasahara and Maeda, 1998).

S. cerevisiae is the preferred and main yeast species for winemaking. Various wineries all over the world favor the inoculation of industrial yeast starter cultures of *S. cerevisiae* for the conduction of alcoholic fermentation. There are countless industrial strains of *S. cerevisiae* available, distinguishable in many fermentation-related properties, including flavour production, formation of by-products and preference of different fermentation temperatures (Sütterlin *et al.*, 2001; Lopandic *et al.*, 2007). As a consequence, special yeast strains can produce specific wine styles, and primarily for red or white grape musts, but also for grape varietal specific aroma

profiles. However, all known *S. cerevisiae* strains are identical in their favoritism for glucose compared to fructose due to the preference for glucose of the hexose transport proteins and the consequential higher utilisation rate of glucose (Berthels *et al*, 2004). At the beginning of fermentation, grape juice contains nearly identical amounts of glucose and fructose, resulting in a glucose to fructose ratio (GFR) of approximately 1. Fructose consequently becomes the main sugar present during the late stages of fermentation and the glucophilic *S. cerevisiae* has to ferment this non preferred sugar in the presence of large amounts of ethanol and periods of starvation (Schütz and Gafner, 1995). The difference between the amount of glucose consumed and the amount of fructose consumed increases rapidly at the beginning of the fermentation and declines only in the last phase of the process. Most of the different industrial strains of *S. cerevisiae* vary only slightly in the pattern of hexose utilisation profile (Berthels *et al*, 2004), except for the industrial wine yeast Fermichamp (DSM), which displays divergence in its sugar utilisation pattern (Guillaume *et al.*, 2007). For Fermichamp, the difference in the rates of consumption of glucose and fructose is much smaller, compared to the difference observed with other wine yeast strains, probably due to a higher capacity for fructose fermentation. Investigations of the low affinity carriers Hxt1 and Hxt3 of this yeast revealed a mutated allele of the *HXT3* transporter gene, which enhances the fructose fermentation capacity. Compared with the *HXT3* sequence of yeasts with a usual sugar utilisation pattern, the *HXT3*-Fermichamp sequence has 38 mutations in the coding region, 10 of which resulted in amino acid substitutions. The mutations were found to be clustered in a region that included transmembrane domain 9 and an external loop between transmembrane domain 9 and 10. Also mentionable is the six changes in the nucleotide sequence of the *HXT*-Fermichamp promoter. The mutations in *HXT3* and its promoter may therefore result in a protein with enhanced transport properties for fructose. Interestingly, since the preferred hexose of this industrial yeast is still glucose, the mutated genotype does not result in a fructophilic phenotype for this *S. cerevisiae* strain. However, the enhanced fructose utilisation rate is a useful property to avoid stuck alcoholic fermentations with large amounts of residual fructose, which can evolve to a further inhibitory factor of alcoholic fermentations for standard *S. cerevisiae* wine yeast strains.

2.1.2 Fructose uptake in non- *Saccharomyces cerevisiae* yeasts

As per description, the Hxt carriers of *S. cerevisiae* are the main transport system for the hexoses glucose, fructose and mannose. However, the specialization of this highly complex uptake system might sacrifice the efficiency for fructose and/or mannose uptake (Özcan and Johnston, 1999). To utilize all natural niches efficiently, different yeast species evolved transport mechanisms with specificities for diverse sugars. A special uptake system for fructose was discovered in *Saccharomyces pastorianus* (synonym: *Saccharomyces carlsbergensis*) who is closely related to *S. cerevisiae*. Both species, together with *Saccharomyces bayanus* and

Saccharomyces paradoxus, made up the former *Saccharomyces sensu stricto* group (Van der Walt, 1970). Described by the modern phylogenetic concept of *Saccharomyces*, *S. pastorianus* and *S. bayanus* are thought to be hybrid species, since their genomes have contributions from both *S. cerevisiae* and *Saccharomyces uvarum* (formerly designated *S. bayanus var. uvarum*) (Nguyen and Gaillardin, 2005). A special fructose/H⁺ symport system (Fsy1) was found in *S. carlsbergensis*, co-existing with the well-characterized facilitated diffusion system (Gonçalves *et al.*, 2000). These energy-dependent H⁺ symport systems are mainly found in Crabtree-negative yeasts (van Urk *et al.*, 1989). The characterization of active fructose transport revealed that it mediates high-affinity fructose transport with a K_m of approximately 0.2 mM and it does not accept glucose as a substrate. The Fsy1 protein is predicted to consist of 570 amino acids with 12 membrane spanning domains. This permease seems to be only distantly related to the Hxt proteins, presenting a low level of homology with transporter proteins belonging to the major facilitator superfamily. Information about regions of this carrier which determines substrate specificity is scarce. Fsy1 does share some features of transmembrane domain 10 with the Hxt proteins. However, the region surrounding Phe⁴³¹ in Hxt2p, which might be responsible for hexose recognition by this permease, is considerably different in Fsy1p. The expression of Fsy1p is strongly regulated by both the carbon source and its concentration in the growth medium. Low concentrations of either fructose or glucose induce expression but higher sugar concentrations prevent transcription of the gene. Glucose was shown to be considerably more effective than fructose in repressing *FSY1* expression. Analysis of *FSY1* expression in *S. cerevisiae* mutants, under control of the complete *FSY1* promoter, shows that repression is mainly dependent on Mig1p, the dominant transcription factor of the main glucose repression pathway. Interestingly, Mig1p also seems to mediate repression of *FSY1* expression by high maltose concentrations (Gonçalves *et al.*, 2000; Rodrigues de Sousa *et al.*, 2004).

A separate fructose carrier with similar properties was cloned from the genome of the predominantly aerobic milk yeast *Kluyveromyces lactis* (Diezemann and Boles, 2003). This gene, referred to as *FRT1*, is closely related to the *FSY1* transporter of *S. pastorianus*, based on the significant amino acid sequence similarity (71%) between the two fructose transporters. Similar to Fsy1, the Frt1 profile suggests 12 membrane-spanning domains with the N- and C-termini of both proteins residing in the cytoplasm. The yeast *K. lactis* is also closely related to *S. cerevisiae*, but with distinctive physiological properties (Breunig *et al.*, 2000). The redundancy of genes involved in hexose uptake is not found in *K. lactis*, since genetic studies revealed a maximum of three genes encoding hexose transporters. The Frt1 open reading frame of 1698 bp encodes for a protein of 566 amino acids. Expressed in a *S. cerevisiae* mutant strain, deleted for all known hexose transporters (Wieczorke *et al.*, 1999), Frt1 restores growth on media containing low amounts of fructose. This result indicates that *FRT1* encodes for a high affinity fructose transporter. Kinetic experiments exhibited fructose uptake with a K_m value of 0.16 mM. Frt1 also demonstrated a capacity to transport glucose in low amounts. Proton

symport activity was assessed by measuring the alkalization of aqueous cell suspension after the addition of fructose.

Both described fructose symporters, Fsy1 as well as Frt1, are more closely related to energy-dependent proton symporters like GalP from *Escherichia coli*, Hup1 from *Chlorella kessleri* or pentose proton symporters from bacteria, than to facilitated diffusion transporters. This might indicate that the structural specifications for a symport mechanism are more conserved than those for sugar specificity (Diezemann and Boles, 2003). Moreover, the active transport mechanism with proton symport and the high affinity for fructose supports uptake even at very low concentrations and is typical for Crabtree-negative yeasts (van Urk *et al.*, 1989).

An additional fructose H⁺ symport system was isolated from the gray mould fungus *Botrytis cinerea* with a high similarity on the amino acid level (53% and 55%) to the Frt1 and Fsy1 fructose symporters (Doehlemann *et al.*, 2005).

Aside from these transport systems specific for fructose, a yeast permease with higher affinity to fructose than other sugars was isolated from *Schizosaccharomyces pombe*. Within the family of six hexose transporter genes in this fission yeast, the transporter Ght6p was identified to prefer fructose compared to glucose, as indicated by a slightly higher affinity to fructose than to glucose (Heiland *et al.*, 2000). The Ght1-Ght6 transporters support transport of both glucose and fructose. Sequence homology to *S. cerevisiae* and mammalian hexose transporters and secondary-structure predictions of 12 transmembrane domains for each of the Ght proteins place them into the sugar porter subfamily within the major facilitator superfamily. Within this sugar porter family, the emerging *S. pombe* hexose transporter family clusters separately from the described monosaccharide transporters of other yeasts and of humans, suggesting that these proteins form a distinct structural family of hexose transporters.

2.1.2.1 Deletion of hexose sensors results in fructose specific phenotype

In terms of fructose signaling, the hexose sensors from Crabtree-negative yeasts, Hxs1 from the methylotrophic yeast *Hansenula polymorpha* (Stasyk *et al.*, 2008) and the Hgt4 glucose sensor from the human opportunistic pathogen *Candida albicans* (Brown *et al.*, 2006), are interesting proteins. HpHxs1 and CaHgt4 are orthologous to the Snf3 and Rgt2 glucose sensors of *S. cerevisiae* and are more closely related to each other than to the *S. cerevisiae* glucose sensors. A notable characteristic in both proteins is the absence of the “glucose-sensor domain”, which is essential for the sensing function in Snf3 and Rgt2. The sensor proteins of both yeasts are able to sense glucose, fructose and mannose, similar to the orthologous proteins in *S. cerevisiae*. However, the *C. albicans* $\Delta hgt4$ mutant as well as the *H. polymorpha* $\Delta hxs1$ mutant displays a more severe growth defect on fructose, compared to glucose and mannose. These fructose specific phenotypes are recognized in both high- and low- affinity fructose transport, whereas only low level sensing of glucose might be affected in the mutants.

A plausible explanation of these phenotypes is that Hgt4 and Hxs1 may affect different targets (hexose transporters), specific for fructose transport. In addition, the next step in hexose metabolism which is the phosphorylation catalyzed by hexokinases might be affected by the deletion of sensors, due to the fact that hexokinases also being regulated by these proteins. For example, *C. albicans* possesses two hexokinases and four glucokinases, only one of which is regulated by CaHgt4. Therefore, it is possible that fructose phosphorylation may require the other hexokinase (Brown *et al.*, 2006).

2.1.3 Fructose uptake by fructophilic yeasts

In nature, fructophilic yeast species are by far outnumbered by yeasts with a glucophilic phenotype. Fructophilic microorganisms can be isolated from different ecological niches characterized by adequate fructose levels, and include species such as *Candida magnoliae*, which was recently isolated from honeycomb (Yu *et al.*, 2006). Grapes or grape must are one of these environments in which the occurrence of fructophilic yeasts could be detected. However, a diversity of at least 20-25 different yeast species can be found on grapes or in grape juice (Schütz and Gafner, 1993b; Torija *et al.*, 2001; Mills *et al.*, 2002; Zott *et al.*, 2008) and only two of them have been described as presenting fructophilic properties. These two yeast species, *Candida stellata* (or *C. zemplinina*) and *Zygosaccharomyces bailii* are both yeasts with relatively weakly fermentative metabolisms when compared to *S. cerevisiae*. In natural fermentations, their growth is generally limited to approximately the first two days of fermentation after which the stronger fermenting and more ethanol tolerant strains of *S. cerevisiae* take over the fermentation. *Candida* and *Zygosaccharomyces* are genera in the family of *Saccharomycetaceae*. *Zygosaccharomyces* was first described under the *Saccharomyces* genus, but in 1983 it was reclassified to the current name in the work by Barnett *et al.* (1983). *Zygosaccharomyces* sp. has osmophilic properties and can be isolated from environments with high sugar content like dehydrated or mummified fruit as well as fruit tree exudates. To date, 12 species of *Zygosaccharomyces* have been identified (Kurtzman, 1990; Kurtzman *et al.*, 2001). Of the recognized species, *Z. bailii*, *Z. bisporous*, *Z. rouxii* and *Z. florentinus* have been isolated from grape must, concentrate and sweetened wines. In some publications, *Z. bailii* is characterized as a food spoilage yeast due to the abnormal resistance to common preservatives like sulfur dioxide, sorbic acid, benzoic acid, ethanol and acetic acid (Fugelsang, 1998) and also for its potential for the synthesis of undesired by-products and off-flavours like acetic acid or acetaldehyde. In published works about fermentation dynamics in natural wine fermentations there is no reference for a persistence of this yeast throughout fermentation (Torija *et al.*, 2001; Mills *et al.*, 2002; Zott *et al.*, 2008), which is in absolute accordance with the conclusions presented in this thesis.

Another common member of early yeast populations in grape must is the fructophilic yeast *C. stellata*. In general, *Candida* sp. are a heterogeneous genus of yeasts, containing endosymbionts of animal hosts, commensals of the skin, the gastrointestinal and the genitourinary tracts, plant pathogens as well as species utilized in the refinement of food and beverages. Currently, more than 150 species are included within this genus (Barnett *et al.*, 2001).

In the work of Yarrow and Meyer (1978) the fructophilic yeast *C. stellata* was reclassified to its current name. This yeast species was initially described by Kroemer and Krumbholz in 1931 as *Saccharomyces stellatus*. In contrast to *Z. bailii*, this yeast with fructophilic properties may remain active throughout most phases of wine fermentation and is thought to contribute to a more complex aroma because of the production of specific aroma compounds (Soden *et al.*, 2000) and high glycerol levels (Ciani *et al.*, 2000). At normal fermentation temperatures its growth rate is significantly lower than that of *S. cerevisiae* (Ciani *et al.*, 2000), whereas lower fermentation temperatures at approximately 10°C have been shown to encourage growth and persistence of *Candida* species, most likely due to the increased ethanol tolerance of this yeast at lower temperatures (Charoenchai *et al.*, 1998). Some *C. stellata* strains, isolated from fermenting botrytized grape musts were recently taxonomically separated and reclassified as the novel strain *C. zemplinina*, on account of morphological and physiological differences (Sipiczki, 2003). This taxonomic separation was confirmed by sequence and phylogenetic analysis of the D1/D2 domains of the 26S rDNA, which differs in 8.1% of the sequence. The molecular taxonomic examination of 41 strains described as *C. stellata* revealed that most of those isolated from grapes or wines belonged to *C. zemplinina* species (Csoma and Sipiczki, 2008).

Publications concerning *C. stellata* or *C. zemplinina*, aside from fermentation dynamics and nomenclature, are limited and characterization of the specific attributes of hexose metabolism and fructophilic properties still needs to be clarified.

There is evidence that the genotypic fundamentals for the fructophilic phenotype might reside in the performance of transport systems for hexose, since the relocation across the plasma membrane is the first and frequently considered limiting step of hexose metabolism (Berthels *et al.*, 2004). The kinetics of hexose uptake observed in *Z. bailii* presumes the presence of a high capacity, low affinity transporter specific for fructose, due to the fact that no other sugar inhibits fructose uptake. A second transporter system is predicted to take up fructose and glucose in a low capacity and high affinity manner (Sousa-Dias *et al.*, 1996). However, the fructophilic behaviour of fructose preferring yeasts has not yet been fully elucidated. A further likely genotypic target for fructose preference is the second step of sugar metabolism, the mechanism of hexose phosphorylation, whose regulation is intimately connected with the regulation of transport. Recently, a novel fructose facilitator was cloned from *Z. bailii* (Pina *et al.*, 2004) by the use of functional complementation of an *hxt* null mutant strain

of *S. cerevisiae*. The identified Ffz1 permease was the first known facilitated diffusion system specific for fructose. The homology with other facilitated diffusion systems as well as with the prior described fructose specific symport systems is very poor, suggesting that fructose specificity does not have a close phylogenetic link with glucose uptake systems. The predicted Ffz1 protein consists of 616 amino acids, which exhibit the typical characteristics of a membrane transporter with 12 membrane-spanning domains. Kinetic measurements indicate a low affinity transport with a K_m value of 80, 4 mM for fructose uptake. By contrast, the H^+ -fructose symporters displayed a much higher affinity. In environments with low sugar concentrations the yeasts invest energy through active maintenance of the proton motive force in transport, whereas the concentration gradient across the plasma membrane is enough to maintain an active catabolism when sugar concentration is high. The isolation of this low affinity carrier from *Z. bailii* corresponds to the observation that this yeast is most frequently isolated from environments with high sugar concentrations.

In order to analyze the molecular basis of the fructophilic properties of the osmotolerant and erythritol producing yeast *C. magnolia* a different strategy was described by Yu *et al* (2008). An efficient preparation of membrane proteins explored sugar delivery systems by proteomic techniques including two-dimensional electrophoresis, mass spectrometry and homology searching. One hexose transporter, up-regulated under fructose supplemented conditions, was found to be highly homologous to Ght6p in *Schizosaccharomyces pombe*, which is known as a predominant transporter for fructose uptake due to the higher affinity for fructose than glucose. Due to the limited sequence information of the genome of *C. magnolia*, the investigators decided to explore the fructophilic mechanism at a protein level (Yu *et al.*, 2008). Further data, especially the kinetic parameters of hexose uptake by the isolated permease will shed light on the fructophilic properties of *C. magnolia*.

In the glycolytic pathway there are only two steps which could form the basis of a fructophilic phenotype, namely hexose transport and phosphorylation, since the next step, the conversion of glucose-6-phosphate to fructose-6-phosphate by phosphoglucisomerase consolidates the metabolism of glucose and fructose (Figure 2.2). In the next part of this review, all known metabolic processes in which the sugar kinases are involved will be discussed, with specific focus on differences between fructose and glucose utilisation.

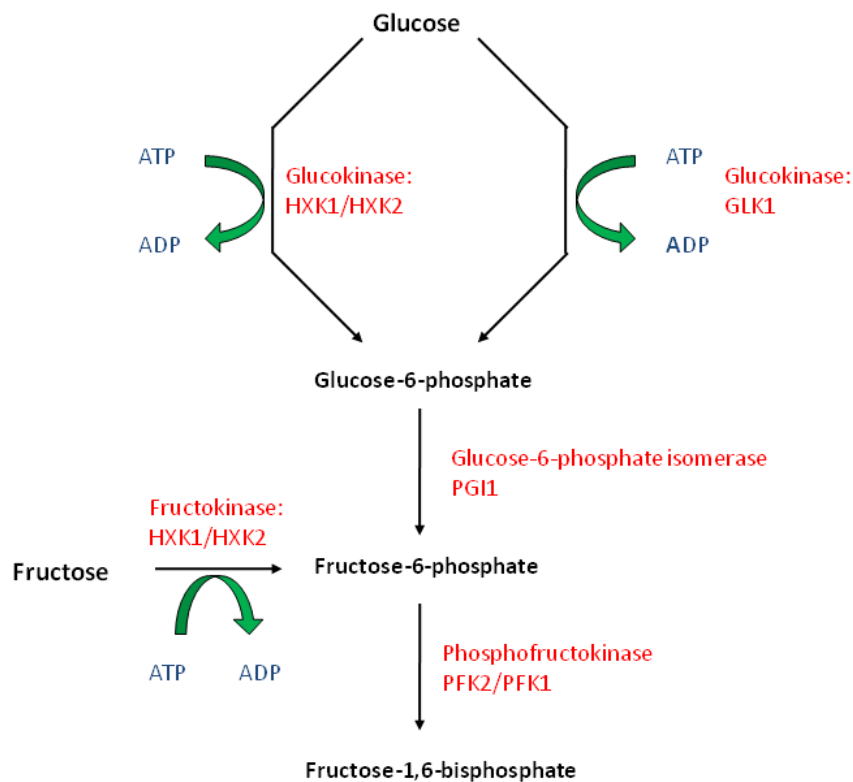


Figure 2.2: Direct transfer of glucose and fructose to the glycolytic pathway of the yeast *Saccharomyces cerevisiae*.

2.2 Phosphorylation - the first step of hexose metabolism

Apart from the transport, the phosphorylation of hexose to a hexose phosphate at C6 is the first step of hexose metabolism and is present in nearly all organisms. Consequently it seems to be one of the key steps associated with the regulation of the fermentative metabolism of yeast. The hexokinases that catalyze the phosphorylation of the keto- and aldohexoses, glucose, fructose and mannose to hexose-6-phosphate use MgATP as phosphoryl donor. The chemical equilibrium of this reaction is strongly shifted towards the product, since it is immediately metabolized. Hexokinases have been found in every organism investigated (Ureta *et al.*, 1987). In general, there are four types of hexokinases, hexokinase type I-III and the glucokinase, which is classified as hexokinase type IV. With the exception of the glucokinase, hexokinases are inhibited by their products. As a further characteristic, the glucokinases have an up to 50 times lower affinity to glucose, compared to hexokinases type I-III. All hexokinases are categorized as actin fold proteins which contain a structural motive involved in binding of ATP to an otherwise diverse family of proteins. With a few exceptions, the molecular mass of a variety of hexokinases may be either 25 kDa, 50 kDa or 100 kDa. The smaller hexokinases have been found in prokaryotic microorganisms, whereas the 50 kDa enzymes are found in most invertebrates as well as in yeast and in a particular isozyme from vertebrates (hexokinase D). The 100 kDa enzymes are restricted to vertebrates. This information has led to the hypothesis

that gene duplication events have played a decisive role in the evolutionary development of the hexokinases from existent organisms (Ureta *et al.*, 1987).

In the yeast *S. cerevisiae* there are three different isozymes, hexokinase I (Hxk1p), hexokinase II (Hxk2p) and glucokinase (Glk1p) (Lobo and Maitra, 1977). All three proteins are involved in the uptake of glucose, however, Hxk2p appears to play the main role during hexose phosphorylation in a fermenting environment, since it is the predominant isoenzyme during growth on glucose or fructose. Glucokinase specifically phosphorylates glucose and the two hexokinases have a preference for this sugar. A main characteristic of both *S. cerevisiae* hexokinase enzymes is the higher affinity for glucose than for fructose, which may play a critical role in the development of the glucophilic phenotype of *S. cerevisiae* (Berthels *et al.*, 2008). The affinity of Hxk1 for fructose has a K_m of 1.5 mM, while for the K_m for glucose is 0.12 mM, and Hxk2 has a K_m of 0.25 mM for glucose and of 1.5 mM for fructose. In summary, the affinity for fructose is similar with both hexose kinases, while the affinity for glucose is slightly higher for Hxk1 (Bernard, 1975; Entian and Mecke, 1982). Interestingly, Hxk1 has a threefold higher V_{max} with fructose as substrate than with glucose, whereas the difference with Hxk2 is only marginal. Different strains of the wine yeast *S. cerevisiae* display different rates of consumption of both glucose and fructose sugars, which can be described as a discrepancy in the glucose to fructose utilisation rate (G/F discrepancy). The K_m values of hexose phosphorylation correlates with the G/F discrepancy, whereas the V_{max} values of the different yeast strains shows no correlation (Berthels *et al.*, 2008).

Fructose specific hexokinases have been characterized from various plants. In tomatoes, for instance there are also two divergent fructokinase genes that are differentially expressed and which also have different enzymatic properties. Both fructokinase enzymes show different affinities for fructose as well as diverse inhibition properties (Odanaka *et al.*, 2002). Fructokinases are also present in many prokaryotes. In *Rhizobium meliloti*, a common gram negative soil bacterium, fructokinase is also used in the metabolism of mannitol and sorbitol, in addition to the metabolism of fructose (Gardiol *et al.*, 1980).

2.2.1 Substrate affinity of Hxk2 catalytic function

There are several lines of evidence suggesting that Hxk2 binds glucose and fructose via distinct mechanisms (Hohmann *et al.*, 1999). As previously mentioned, *S. cerevisiae* hexokinase seems to act on fructose in the furanose form (with a free hydroxyl on carbon 6), whereas in the case of the substrate glucose it is the pyranose form which is acted upon by the yeast enzyme (Slein *et al.*, 1950).

Different alleles of the yeast Hxk2 enzyme, with indicated single amino acid substitutions, affect glucose and fructose phosphorylation to a different extent (Hohmann *et al.*, 1999). The change of Pro-160 to Ala converts the enzyme from a bifunctional glucose/fructose kinase to a

glucokinase. Pro-160 is the last in a motive of highly conserved amino acids in the hexokinase gene family which has previously been proposed to function in sugar binding. This mutation has no impact on glucose binding affinity, whereas fructose-binding affinity is severely affected. In a further study, differences in Hxk2 substrate binding were observed after chemical treatment of the enzyme with S-nitrosoglutathione (GSNO). The homolytic and heterolytic cleavage of this molecule may result in the formation of nitric oxide as well as other reactive radicals, which may modify the primary structure of hexokinase. After GSNO exposure, the V_{max} with glucose was significantly different. No difference was found with regards to the apparent K_m for ATP or glucose or the apparent K_m and V_{max} with fructose. These results can be explained by the weaker binding of fructose compared to glucose at the same active center. The modifications introduced by GSNO might affect the tight binding of glucose to the enzyme, but not that of fructose (Miller *et al.*, 2007).

2.2.2 Involvement of hexokinase in the regulation of hexose metabolism

The enzymatic phosphorylation of sugar has been proposed as one of the two possible triggers for the development of phenotypical hexose preference in yeasts (Berthels *et al.*, 2004). An evident reason is the higher affinity of all three *S. cerevisiae* sugar kinases for glucose than fructose (Bernard, 1975; Entian and Mecke, 1982). However, it has been observed that the function of the sugar kinases is not restricted to the phosphorylation of the hexose substrate. One of the first reports which may affirm the involvement of the hexose kinase enzymes in regulatory sequences concerning hexose uptake and glycolytic metabolism was the work of Entian and Mecke (1982), which provided the genetic evidence for a role of Hxk2 in carbon catabolite repression. Bisson and Fraenkel (1983) described an involvement of hexose kinases in glucose and fructose uptake by performing hexose uptake experiments in yeast mutants lacking the two hexokinases, which are able to grow on glucose but not on fructose, and mutants lacking glucokinase which also do not grow on glucose. The results of these studies suggested that mutants lacking all three kinases for glucose (or two for fructose) only express the low affinity uptake system. On the other hand, strains with kinase activity for glucose or fructose also seem to express the high affinity system for their uptake. Thus, inability of hexose phosphorylation seemed to be associated with loss of high affinity (low K_m) uptake. Genetic studies with a cross between a wild type and a $\Delta h x k$ double mutant confirmed the kinetic results. At this time little was known about yeast hexose uptake systems. The first hypotheses and the preferred explanation of these early results was a model with the hexokinase as a functional part of the hexose uptake system.

Northern analysis has indicated that of all three phosphorylating enzymes in the yeast *S. cerevisiae* only Hxk2 is highly expressed in the presence of the fermentable carbon sources

glucose, fructose and mannose. Both *HXK1* and *GLK1* are repressed upon addition of glucose or fructose, with fructose repression of HXK1 being only transient (De Winde *et al.*, 1996). In addition, mutations of Hxk2, but not of Hxk1 or Glk1 were identified in searches for genes impacting on glucose repression. Therefore, the Hxk2p enzyme appears to play the main role during glucose and fructose phosphorylation, and the fact that Hxk1p and glucokinase cannot completely substitute for loss of Hxk2p regardless of the actual cellular level of glucose phosphorylation suggests that hexokinase PII has a specific regulatory role in a fermenting environment *in vivo* (Entian and Mecke, 1982). Conversely, the expression of *HXK1* and *GLK1* genes only takes place when the culture medium contains non-fermentable carbon sources or galactose. After shifting the cells to one of these carbon sources, the *HXK2* gene is repressed and the *HXK1* and *GLK* genes are rapidly de-repressed (Herrero *et al.*, 1995). The functions of hexokinase 2 are assumed to be twofold: cytoplasmic Hxk2p is a key enzyme in glycolysis, and nuclear hexokinase 2 is a factor involved in signaling hexose repression and induction of several genes (for review see: Santangelo, 2006). Furthermore, the protein acts as a key factor controlling the gene expression of the hexose phosphorylating enzymes of *S. cerevisiae* via the Snf1 protein kinase complex.

A separate metabolic enzyme with an additional function as a transcriptional regulator is the galactokinase Gal1. This enzyme is involved in both galactose phosphorylation and also appears to activate the transcription factor Gal4 by binding to the Gal4 inhibitor Gal80 (Zenke *et al.*, 1996).

2.2.3 Sugar kinase single, double and triple mutants

Successful and important tools for experiments regarding hexose phosphorylation, the activity and regulation of the glycolytic pathway or other distinct roles of Hxkp are the *S. cerevisiae* mutants carrying deletions of the hexokinase genes. Kinase deficient mutations have been investigated as deletions of single genes ($\Delta h x k 1$ or $\Delta h x k 2$ or $\Delta g l c 1$) or combinations of two or all three genes ($\Delta h x k 1 h x k 2 g l c 1$) (Hohmann *et al.*, 1993; De Winde *et al.*, 1996). All single deleted mutants show no specific growth phenotype on glucose or fructose. Due to the presence of glucokinase, the *h x k 1* and *h x k 2* double deleted mutant does not grow on fructose but on media containing glucose. The triple deleted hexokinase mutant is not able to grow on either glucose or fructose.

2.3 Gene repression and induction

2.3.1 Glucose and fructose induced gene repression and induction

S. cerevisiae and many other yeast species are able to use several carbon sources, but glucose and sometimes fructose are in many species the preferred growth substrates. In these cases, the synthesis of enzymes required for the utilisation of alternative carbon sources is usually taking place at low rates or is entirely inhibited when glucose or fructose are present. This phenomenon is known as carbon catabolite repression or glucose repression (for review see: Gancedo, 1998). There may be some bias in these data since many studies have focused on glucose as a repressing fermentable carbon source. Although fructose is probably as important as glucose for many yeast species in their natural environment, research on fructose repression has been limited.

While the mechanism of fructose and glucose utilisation is metabolically very similar, some important differences exist between glucose and fructose induced repression. General regulation by glucose and fructose appears similar in that a very large number of genes whose products are responsible for the metabolism of several other carbon sources like sucrose, maltose or galactose, as well as numerous other genes are repressed. Differences in the regulatory impact of the two sugars however have been shown to occur and appear at least in part related to Hxk function (Entian and Mecke, 1982). The deletion of *HXK2* impedes glucose repression, whereas deletion of *HXK1* or *GLK1* has no effect (Rose *et al.*, 1991). If grown on fructose media, the *HXK2* deletion however has a different effect: Whereas the specific activities of invertase (*SUC2*) and hexokinase1 on glucose containing medium are clearly derepressed in this mutant, this is not the case with fructose as carbon source. The addition of fructose to a strain only expressing *HXK1* results in a repression response similar to that observed in a wild type strain, indicating that both *HXK1* and *HXK2* can function in establishing fructose induced repression. Accordingly, the triggering of fructose repression may differ from triggering of glucose repression. Moreover, *HXK1* efficiently mediates fructose repression but not glucose repression of its own gene (De Winde *et al.*, 1996). A further evidence for a putative difference between fructose and glucose repression was published by Hohman *et al.* (1999), suggesting that a Hxk2 allele affected in its catalytic affinity for fructose but not for glucose phosphorylation (change of Pro-160 to Ala), was unable to mediate fructose repression, while glucose repression was normal. However, this correlation between the catalytic activity of Hxk2 and its ability to mediate catabolite repression could not be confirmed by the study of further mutant alleles, which implies that catabolite repression may be more complex than simply catalytic.

Hexoses are also able to induce the expression of some genes by an Rgt1-dependent pathway. Hxk2p seems also to be involved in induction of *HXT* genes. Derepression of the *HXT1-4* genes is reduced about three to six fold in a Δ h_{xk2}-mutant (Özcan and Johnston, 1995), indicating that Hxk2p is required for full induction of the transporter genes by glucose.

Deletion of Hxk2 also results in an abnormally active Snf1 protein kinase which inhibits Hxt expression (Tomas-Cobos and Sanz, 2002), which led to the assumption that Hxk2p functions as a negative regulator of Snf1.

2.3.2 Cyclic AMP dependent pathway

Beside the described hexose induced signaling pathway, mediated by the sensors Snf3 and Rgt2, there is another signaling cascade system which acts through a G-protein coupled receptor controlled signaling pathway and is responsible for the hexose induced repression of several genes (Figure 2.3, page 33). Evidence for this system was first proposed based on the observation of a transient increase of the cAMP level after glucose addition to derepressed (grown on a non-fermentable carbon source) *S. cerevisiae* cells (Beullens *et al.*, 1988). This increase of cAMP is transient and lasts for about 1-2 minutes. The fructose induced cAMP level is always lower than glucose induced cAMP levels, while the increase in glucose-6-phosphate is very similar in both cases (Rolland *et al.*, 2001). Cyclic AMP is a second messenger for intracellular signal transduction and is synthesized from ATP by the adenylate cyclase Cyr1, which is located at the cell membranes. This sugar induced cAMP signal triggers a protein phosphorylation cascade in glucose derepressed cells. The target of this second messenger, a cAMP dependent protein kinase (PKA) is activated by a defined concentration of cAMP (Toda *et al.*, 1987). In the absence of cAMP, PKA consists of two catalytic and two regulatory subunits. After increases in the cAMP level, the holoenzyme dissociates into the subunits and becomes active.

Glucose repressed cells (grown on glucose) act different to de-repressed yeasts grown on non-fermentable carbon sources. When glucose is added to glucose-repressed cells, the cAMP signal is much smaller or in many cases practically absent compared to derepressed cells. This indicates that one of the components of the induction mechanism is repressible by glucose (Beullens *et al.*, 1988). Experimental cAMP determination in yeast mutants which are deleted in one or several genes encoding for glycolytic enzymes gave further insight in the mystery of this transduction chain. A *S. cerevisiae* phosphoglucose isomerase deleted mutant (Δpgi) shows a similar cAMP answer to glucose addition than wildtype cells, therefore metabolization of glucose beyond the initial phosphorylation step seems not to be required for the generation of a cAMP signal. Mutants who are blocked further down in the glycolytic pathway display similar behaviour. Interestingly, the addition of fructose to de-repressed cells of this mutant also induces a cAMP response but with a significant delay compared to the addition of glucose. Glucose addition causes the accumulation of glucose-6-phosphate, while the addition of fructose generates an accumulation of fructose-6-phosphate and fructose 1.6-bisphosphate (Beullens *et al.*, 1988).

Addition of glucose or fructose to the hexokinase triple mutant $\Delta h x k 1 h x k 2 g l c 1$ causes no increase of intracellular cAMP, similar to the addition of fructose in the double mutant ($\Delta h x k 1 h x k 2$). However, rapid cAMP synthesis is still possible in these strains as demonstrated by the addition of 2,4 dinitrophenol, which is known to cause an increase in the cAMP level within 15 seconds. The absence of a cAMP signal in the triple mutant (for both glucose and fructose addition) or double mutant (fructose addition) indicates that the presence of extracellular or intracellular sugar alone does not trigger the signal. The presence of one sugar phosphorylating enzyme is necessary and each of the three kinases are able to cause induction of the cAMP signal. A question raised by this observation was whether one of the kinase products can trigger the signal. This eventuality was excluded, since there was no correlation between the increase in the level of glucose-6-phosphate, fructose-6-phosphate or ATP after hexose addition and the increase in the cAMP level (Beullens *et al.*, 1988). An increase of the ATP level, which occurs after addition of glucose or fructose would be a trivial explanation for hexose induced increase of cAMP. To further exclude ATP as signal transducer for the activation of the cAMP pathway, the cells were treated with iodoacetate. This compound inhibits glycolysis upstream of the ATP generating steps (Campbell-Burk *et al.*, 1987). Since the hexose- induced cAMP signals were comparable for both treated and non-treated cells although the ATP content was almost half in the iodoacetate-treated cells at the moment that sugar was added, ATP seems to be required only as substrate for adenylate cyclase and not as signal transducer for the activation of the RascAMP pathway (Rolland *et al.*, 2001).

2.3.3 cAMP synthesis is dependent by two hexose sensing mechanisms

Glucose induced cAMP synthesis relies on the effect of two independent sugar sensing processes (Figure 2.3, p.33). First, the activity of adenylate cyclase is controlled by a G-protein coupled receptor system with extracellular glucose detection, the so-called GPCR system (Xue *et al.*, 1998). The G protein activity is regulated through a guanidine nucleotide exchange cycle wherein the ligand-bound or active receptor stimulates the exchange of GDP for GTP on the α -subunit of the complex. Involved in this mechanism is the G-protein coupled receptor Gpr1 and the α -subunit of this protein complex, Gpa2. It has been indicated by different research groups in two-hybrid-screens that the membrane-bound sensor protein Gpr1 interacts with Gpa2 (Kraakman *et al.*, 1999b). Disruption of the Gpr1 as well as the Gpa2 gene has no effects on growth, but eliminates glucose induced cAMP signaling (Yun *et al.*, 1997; Xue *et al.*, 1998; Kraakman *et al.*, 1999a). The second process is described as an intracellular mechanism with a further G-protein coupled system, the Ras proteins (Colombo *et al.*, 2004).

Gpr1 displays structural and functional homology to mammalian G-protein-coupled receptors, indicating that it may function as a receptor. The gene for Gpr1 encodes a 961 amino acid protein with the characteristic seven transmembrane helices of GPCRs and a large

intracellular loop between transmembrane helices five and six (Yun *et al.*, 1997). Interestingly, the Gpr1 receptor displays very low affinity for glucose with a K_m value of about 75 mM (Rolland *et al.*, 2000). Furthermore, it responds specifically to extracellular D-glucose and sucrose, but not to fructose and mannose. This may explain why low glucose levels or high fructose concentrations only result in a moderate increase of cAMP. The requirement for the sensor Gpr1 can be bypassed by a constitutively active Gpa2^{val132} allele which increases the fructose or the low-glucose-induced marginal cAMP signal to the cAMP intensity associated with a high glucose concentration (Rolland *et al.*, 2000). In summary, one reason for the low cAMP increase in the presence of fructose or low levels of glucose might be the low affinity and specificity of the Gpr1-Gpa2 system. The independence of fructose sensing from the GPCR system was also verified by the comparison of the cAMP increases obtained by addition of 100 mM fructose to a wild-type strain, a $\Delta gpr1$ deletion strain or a $\Delta gpa2$ deletion strain; no difference was observed in the cAMP levels (Rolland *et al.*, 2001).

The second G-protein system which is involved in intracellular glucose sensing is the Ras protein system. Ras proteins are membrane-bound, monomeric GTPases that function as switches, where GTP bound equals active and GDP bound equals inactive (For review see: Lowy and Willumsen, 1993). Change from GTP to GDP is stimulated by GTPase activating proteins, called GAPs which hydrolyzes one phosphate from GTP. Guanine nucleotide exchange factors (GEFs or GNRPs= Guanine nucleotide releasing proteins) catalyze the reverse reaction that results in a change from GDT to GTP and thereby result in activation. *S. cerevisiae* contains two Ras proteins with approximately 70% similarity (Powers *et al.*, 1984). Growth on glucose is unaffected by the absence of either Ras1 or Ras2, whereas loss of both causes arrest in the G1 phase of the cell cycle (Toda *et al.*, 1985). Important effector molecules modulate Ras activity during the glucose response. Ras stimulation by the GEFs stimulates production of cAMP and the inactivation of Ras is facilitated by the GAPs Ira1 and Ira2, resulting in a lower cAMP level (Tanaka *et al.*, 1990).

The presence of glucose thus generates signals that converge on membrane-bound Ras and its modifiers (GEFs and GAPs). The result of the generated signal is a spike in cAMP levels of about 5- to 50-fold within 1 to 2 min after glucose addition, which returns to basal levels within 20 min.

It seems to be clear, that nothing beyond the hexose phosphorylation step is necessary for generating the PKA response, since repression also occurs even when the metabolic steps after sugar phosphorylation are deleted (Rose *et al.*, 1991). Furthermore, the described extracellular sensing mechanism by the Gpr1/Gpa2-system does not participate in the activation of the second adenylate cyclase regulating protein complex, the Ras system, since the deletion of Gpr1 or Gpa2 does not prevent the glucose-induced increase in Ras2-GTP levels. So, it could be concluded that the hexose phosphorylation step must generate the signal through the G-proteins or a possible alternative pathway. In a hexokinase-less yeast mutant, the glucose

induced increase of Ras-GTP is absent. If one of the Gap's, Ira1 or Ira2, is deleted additionally a high basal level of Ras-GTP without increasing after glucose addition can be observed. These data suggests that the phosphorylation step somehow increases Ras-GTP levels, possibly by inhibiting the Ras GTPase activating proteins Ira (Colombo *et al.*, 2004).

It has been clearly established that down-regulation of the Ras protein system lowers cAMP levels as a result of reduction of the adenylate cyclase activity. Decreased cAMP levels in turn lead to reduced activity of protein kinase A (Bond and Forgac, 2008). As a deduction it could be suggested that fructose addition may mediate a lower PKA activity comparable to the one observed for low glucose addition. Due to the discrepancies in the signaling as well as utilisation pathways of both sugars, fructose triggers, in general, a lower cAMP response compared to glucose. It can be assumed that this characteristic also contributes to the glucophilic phenotype and therefore a lower fructose utilisation rate in *S. cerevisiae*.

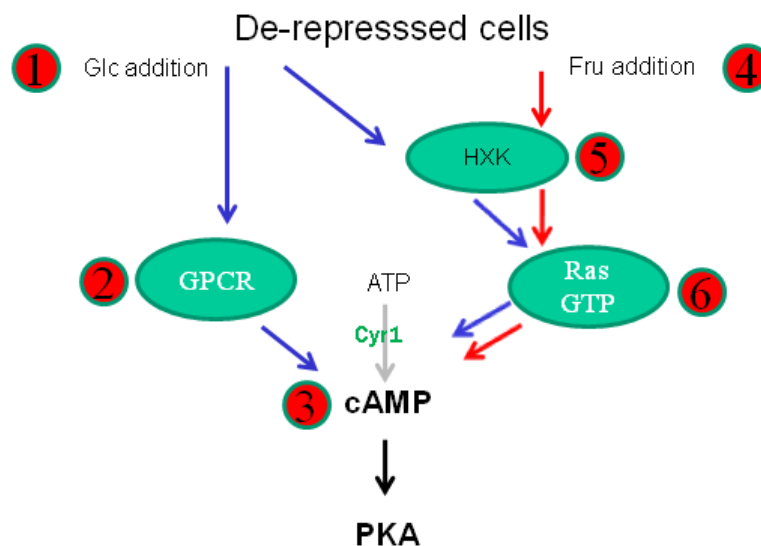


Figure 2.3 Glucose induced cAMP response is mediated by the extracellular GPCR-system dependent process (1-3) and an intracellular sensing (5-6), involving a sugar kinase Hxk (5) and the Ras-GTP system (6). Fructose induced cAMP response (4-6) is only triggered by Hxk/Ras-GTP, resulting in a lower cAMP level.

2.3.4 PKA and sugar kinase expression

It seems likely that the sole purpose of the glucose induced spike in cAMP levels is to block the inhibitory effect of the *BCY1*-encoded regulatory subunit on the catalytic subunits of protein kinase A (PKA), encoded by *TPK1-3*. Overexpression of any of this genes or deletion of *BCY1* restores growth of a $\Delta ras1 \Delta ras2$ strain (Toda *et al.*, 1987). PKA is a heterotetramer with two catalytic (Tpk) and two regulatory (Bcy1) subunits. In the absence of glucose Bcy1 is present in both the cytoplasm and the nucleus, but in the presence of glucose Bcy1 is only located in the nucleus (Griffioen and Thevelein, 2002). Active PKA is thought to phosphorylate proteins

involved in transcription, energy metabolism and cell cycle progression. A significant correlation exists with the regulation of the expression of the sugar kinase genes. In wild type cells, there is a modest induction of Hxk2 by glucose. Unregulated high PKA activity has no effect on Hxk2 expression, but the reduction of PKA activity significantly reduces glucose induced HXK2 induction (De Winde *et al.*, 1996). In general, glucose addition caused rapid and strong repression of both the *HXK1* and *GLK1* genes. However, in the case of reduced PKA activity, this repression of *HXK1* and *GLK1* is also reduced to a weak and transient effect. Furthermore, mRNA levels of both kinases were drastically reduced under both de-repressing and repressing conditions.

2.3.5 Bypass of the GPCR extracellular and intracellular sensing mechanism

Heterologous induction of activated alleles of Ras2 or Gpa2 can bypass the comprehensive glucose response of the GPCR system without addition of the sugar (Wang *et al.*, 2004). In the absence of glucose almost all of the changes in transcript levels after glucose addition were also observed after activation of Ras2 or Gpa2. It was also demonstrated that all of the resulting changes in transcript levels are mediated by protein kinase A.

2.3.6 Function of hexose carriers in cAMP signaling

To clarify the role of the Hxt carriers within the cAMP signaling pathway, the levels of cAMP and glucose-6-phosphate were quantified after glucose and fructose addition to the *hxt*-null mutant ($\Delta hxt1-7$). This mutant still accumulates glucose-6-phosphate after glucose addition, but at very low levels. The formation of this intermediate after fructose addition was not detectable. In both cases, hexose uptake is too low to support growth or trigger the cAMP signaling. A similar observation was made in the null mutant after constitutive expression of the low affinity carrier Hxt1, although able to grow there is nearly no glucose-6-phosphate or cAMP accumulation (Reifenberger *et al.*, 1997). Strains expressing one of the other Hxt carriers are able to trigger a signal resulting in a clear cAMP increase, but never with a signal comparable to the wild type. Furthermore, experiments with single and double deleted mutants of the Hxt homologous sensors Snf3 and Rgt2 could exclude an influence of these transmembrane proteins within the regulation of cAMP production (Reifenberger *et al.*, 1997). All these data support the conclusion that the hexose carriers do not have a regulatory function in cAMP signaling and their role might be confined to maintain a critical level of intracellular hexose for a second trigger of cAMP synthesis. Overexpression of the galactose carrier, Gal2, under the control of an *ADH1* promoter in the null mutant confirmed these observations, since it has been shown that the Gal2 permease is able to transport glucose and fructose (Reifenberger *et al.*, 1997). Therefore it is not important which transporter delivers the intracellular hexose since the

pADH-Gal2 constructs in *hxt* null mutants generates a wild type like glucose-6-phosphate and cAMP signal after addition of glucose or fructose. A very simple and elegant experiment supports this insight, since the *hxt* null mutant is able to transport maltose using the maltose specific permease, the intracellular glucose requirement can be provided by maltose addition which is hydrolyzed intracellularly to two molecules of glucose. With this method it is possible to separate the GPCR-glucose sensing from the intracellular requirement (Rolland *et al.*, 2001). Maltose addition does not increase cAMP levels, but glucose-6-phosphate level, which is probably due to the need of glucose for the extracellular Gpr1 sensing mechanism. This requirement can be fulfilled by subsequent glucose addition, hence this model can restore glucose induced cAMP signaling. As a further finding, this system shows the need of Gpr1 as extracellular glucose detector. Since this sensor is specific for glucose, deletion of Gpr1 or Gpa2 has no effect on the cAMP increase observed after addition of 100 mM fructose, compared to the wild type yeast after fructose addition. As mentioned above, this sugar cannot induce the cAMP increase by the extracellular cAMP triggering system. The $\Delta gpr1$ *GPA2*^{val132} strain, which possesses a constitutively activated Gpa2, can bypass the *gpr1* deletion and accordingly trigger the cAMP response of fructose or low glucose to a similar magnitude than high glucose. This confirms that the fructose induced cAMP increase is only activated by the intracellular sugar-dependent process, while the high glucose induced process is additionally supported by Gpr1-Gpa2. Fructose and low glucose are phosphorylated but not detected by the Gpr1-Gpa2 system. In consideration of these results it seems likely, that sugar induced activation of cAMP synthesis requires two systems, namely, an extracellular detection system dependent on Gpr1 and an intracellular sensing process requiring at least one of the hexose kinases (Beullens *et al.*, 1988). Several lines of evidence suggest that the flux through the enzymatic phosphorylation step, but not the levels of substrates, cofactors or products is the critical process detected by downstream sensing machinery (Bisson and Kunathigan, 2003). This would imply that the sugar affinity of the catalytic function of the sugar kinases also interferes with different level of signal transduction.

2.4 Roles of Hxk2p

2.4.1 HXK2 regulates the expression of all sugar phosphorylating enzymes

HXK2 point and null mutations have been shown to cause failure of glucose repression (Moreno and Herrero, 2002). An useful tool in this regard has been a *HXK2* mutant gene with a 30 bp deletion between nucleotide +19 and +48 in which glucose repression is absent but Hxk2 catalytic activity is still present (Rodriguez *et al.*, 2001). The absence of these nucleotides generates a truncated protein with similar specific activity to the wildtype Hxk2p, but it affects its nuclear localization which is required for glucose repression. Approximately 14% of Hxk2p is

nuclear in glucose grown cells of wildtype yeasts (Moreno and Herrero, 2002). In order to analyze the role of Hxk2p in the regulation of Hxk1 and Glk1, a $\Delta h x k 2$ mutant strain was transformed with two different plasmids, one with the native *HXK2* gene as insert and another which contains the described truncated mutant gene. Deletion of the *HXK2* gene resulted in a 10-fold increase of Hxk1 mRNA transcript levels in the glucose medium, whereas the level of Glk1 mRNA only increased 4-fold under the same conditions. Transformation of the *HXK2* deleted mutant with the native gene under the control of its own promoter restored the wildtype phenotype, whereas transformation with the truncated version was not able to recover the Hxk1 mRNA to a wildtype level. When using the double deleted mutant $\Delta h x k 1 \Delta h x k 2$, the *GLK1* mRNA level was observed to behave in a similar way. To underline the major role of Hxk2p in regulation of the hexose phosphorylating enzymes, the wildtype phenotype can be restored in this mutant after transformation with a *HXK2* containing plasmid, but not with the truncated version of this gene (Rodriguez *et al.*, 2001). These results confirm the need for nuclear localization of Hxk2p and the conclusion that glucose-controlled expression of the Hxk1 and Glk1 genes requires Hxk2p, however, the *in vivo* function of Hxk1p and Glk1p remains to be elucidated. The simplest interpretation of these data is that Hxk2p is involved in a positive feedback which may serve to amplify its own expression and in a negative feedback that represses *HXK1* and *GLK1* expression in response to high glucose. Work on the function of Hxp1p has indicated that this protein seems to reduce Hxk2p mediated regulatory functions (Rodriguez *et al.*, 2001). It has also been reported that overexpression of Hxk1p partially restores the glucose repression ability of a $\Delta h x k 2$ mutant.

As previously mentioned, Hxk2p is required for full induction of *HXT* expression by both high and low levels of glucose. Expression of *HXT2* and *HXT4* in $\Delta h x k 2$ mutants is about fivefold higher on high levels of glucose and the induction of expression of *HXT2*, *HXT3*, and *HXT4* by low levels of glucose is significantly reduced in the same mutant. *HXT1* expression on high glucose is reduced about fivefold in the $\Delta r e g 1$ glucose repression mutant, suggesting that Reg1p, the targeting subunit of the Glc7 phosphatase and Hxk2p proteins are both required for the high- glucose-induction pathway (Özcan and Johnston, 1995). As expected, glucose repression of *HXT2* and *HXT4* is relieved by the $\Delta r e g 1$ mutation. Surprisingly, induction of the *HXT3* gene at high glucose concentrations is independent of Reg1p and Hxk2p function, suggesting that *HXT3* expression and *HXT1* expression may be regulated differently (Özcan and Johnston, 1995).

2.4.2 Regulatory elements

Since about 14% of Hxk2 is nuclear in glucose grown cells (Moreno and Herrero, 2002), the focus of this review must be expanded to include the involvement of further regulatory elements. The negatively acting DNA-bound glucose regulator Mig1 appears to mediate nuclear

localization of the Hxk2p. Hxk2 and Mig1 were shown to interact via the yeast two hybrid system, immunoprecipitation and glutathione S-transferase pull down assays. An interaction between Hxk2 and the mediator component Med8 has also been described. Med8 (subunit of the mediator CTD complex of RNA polymerase II) appears to bind directly to both positive and negative acting glucose regulatory elements in DNA (Chaves *et al.*, 1999). Med8p was shown to bind to the *HXK2* and *SUC2* regulatory regions and also to the promoters of *HXK1*, *GLK1* and *HXT1* through a heptameric motif. Among these genes, *HXT1* and *HXK2* are induced, while *HXK1*, *GLK1* and *SUC2* are repressed by high glucose levels (de la Cera *et al.*, 2002).

2.4.3 HXK phosphorylation

Intracellular Hxk2p as well as Hxk1p is known to occur *in vivo* in a monomeric-/dimeric equilibrium which is caused by phosphorylation through an unknown process so far (Figure 2.4). The monomeric form seems to be phosphorylated at a higher level and is characterized by a higher affinity to the substrates than the dimeric form (Golbik *et al.*, 2001). Although a PKA consensus phosphorylation sequence was identified and the residue serine-15 is phosphorylated *in vitro* by protein kinase A, this cAMP dependent phosphorylation system seems to act to reduce *in vivo* phosphorylation of yeast hexokinases. Increasing the extracellular glucose concentration or hyperactivation of PKA via deletion of the regulatory subunit *bcy1* results in a reduced HXK2 phosphorylation, whereas attenuation of PKA activity increases the phosphorylation state, as analyzed with the ³²P-labeling method (Vojtek and Fraenkel, 1990). It remains possible that *in vivo* phosphorylation of hexokinase could be the result of substrate-induced autophosphorylation, which has been reported previously (Fernández *et al.*, 1988). This reversible phosphorylation is carbon source dependent and more extensive on poor carbon sources like galactose, raffinose or ethanol, whereas addition of fermentable carbon sources promotes the dephosphorylation of Hxk2p. The influence of fructose fermentation on Hxk2p phosphorylation status was not verified in this study (Randez-Gil *et al.*, 1998). However, compared to glucose, fructose mediates a lower cAMP response (Rolland *et al.*, 2001) which results in a lower PKA activity (Bond and Forgac, 2008). Consequently the Hxk2p phosphorylation status in response to fructose would be a further interesting question with reference to divergences with the utilisation pathways of the main hexoses.

The role of HXK2 subcellular localization and phosphorylation state in glucose response represents one of the several potential connections between Ras/cAMP/PKA-dependent events that have a direct impact on target gene expression. The consistent model of explanation in this case describes an Hxk2 dephosphorylation with the result of a conversion from a monomer to a dimer, which somehow transduces the glucose signal to downstream effectors and eventually

results in repression or induction of glucose regulated genes. Alternatively, the signal mediated by nuclear Hxk2 could be received by two additional phosphoproteins, namely, the Reg1-targeting subunit of the Glc7 phosphatase and the Snf1 kinase.

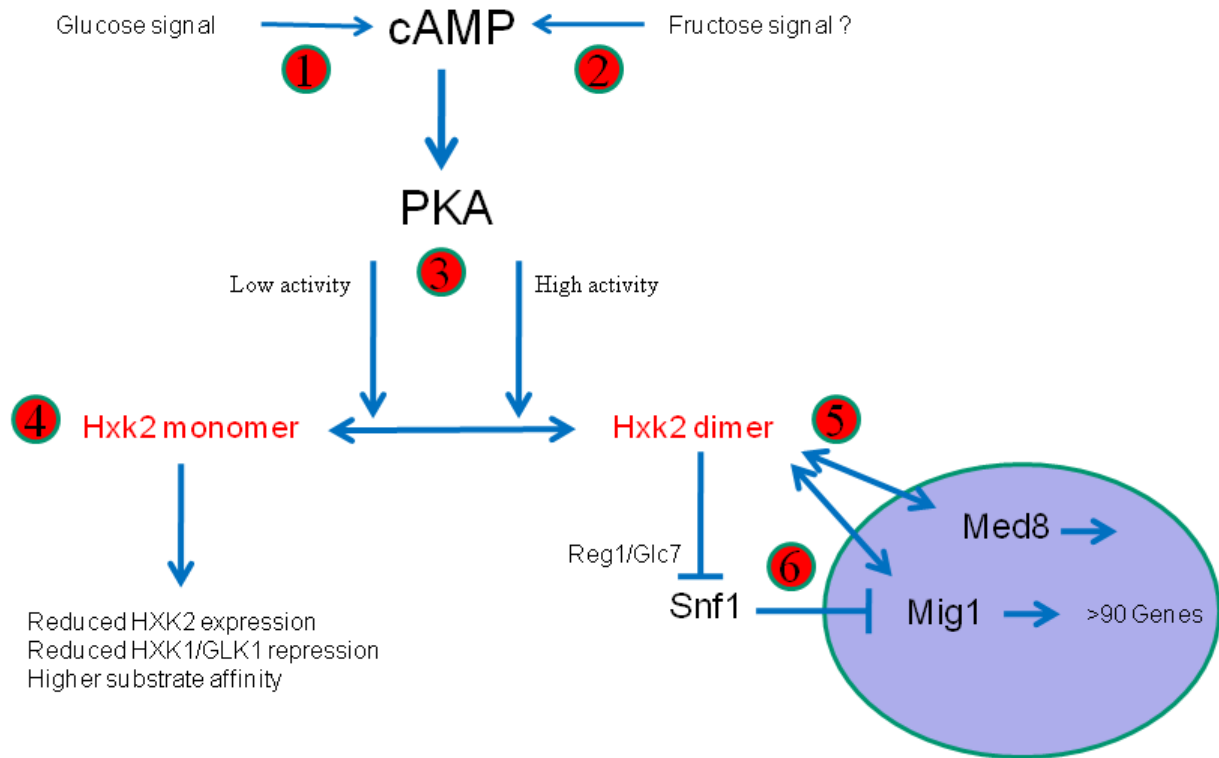


Figure 2.4: Hxk2p monomeric/dimeric equilibrium is carbon source dependent, but the Hxk2p phosphorylation status in response to fructose is unknown so far (2). Glucose signaling (1) triggers a PKA response (3) in which high active PKA results in a shift towards the dimeric form (5). Contrarily, low PKA activity results in a shift towards the monomer Hxk2p (4), which is phosphorylated at higher level than the dimer. The Hxk2 phosphorylation state seems to have a direct impact on Hxk subcellular localization and target gene expression, mediated by Snf1, Mig1 and Med8 (6).

2.5 The Snf1 protein kinase complex

2.5.1 The Snf1 protein kinase complex and association with PKA signaling

The SNF1 protein complex is described as a serine-threonine protein kinase that is involved in the mediation of glucose derepression. Snf1 consists of a catalytic N-terminal kinase domain and a regulatory C-terminal domain (Celenza and Carlson, 1986). At high glucose concentrations, the regulatory subunit remains bound to the catalytic domain, maintaining Snf1 in an auto-inhibited and therefore inactive conformation. Low glucose mediates phosphorylation of Snf1 by the upstream kinase Sak1, followed by the release of the catalytic domain from the

autoinhibited state. Phosphorylation of a conserved threonine T210 in Snf1 by the upstream kinase Sak1 is essential for activation and is required for full Snf1 kinase activity. Since Snf4 co-immunoprecipitates with Snf1 and is required for maximal Snf1 kinase activity, it is a physically associated activator of the Snf1 kinase (Estruch *et al.*, 1992). Following the binding of Snf4 to the regulatory domain which opens and activates the complex, the Reg1/Glc7 phosphatase compound binds to Snf1 and Reg1 is phosphorylated by Snf1. This phosphorylated phosphatase complex Reg1/Glc7 facilitates the transition back to the auto-inhibited form by dephosphorylating Snf1 (Sanz *et al.*, 2000). Snf4 is the γ -subunit of the Snf1 kinase complex which appears to be influenced by PKA signaling. The counterpart of Snf1 is known as protein phosphatase Glc7, which is also influenced by PKA signaling. This regulatory element is needed for glucose repression, carbohydrate accumulation, normal progression through the G2/M phase of the cell cycle, actin organization, translation and sporulation and other functions in *S. cerevisiae* (Stark, 1996). The protein phosphatase exhibits little substrate specificity and requires regulatory subunits that alter the conformation and/or targets it to its substrates (Feng *et al.*, 1991). All of the different regulatory binding proteins seem to be specific for different functions. The regulatory subunit Reg1 is specific to glucose repression functions and is the only regulatory subunit known to participate in glucose repression. Other regulators have been reported to have ulterior responsibilities (Tu *et al.*, 1996). Some of these regulatory subunits appear to compete for Glc7 binding *in vivo*. The dimerized form of Hxk2p seems to be a link between the dephosphorylated status and the phosphorylated status of the Reg1/Glc7 phosphatase compound of Snf1. Stimulated by a cAMP increase in hexose signaling events, PKA inhibits phosphorylation of Hxk2 which results in a shift in the balance towards the dimerized Hxk2 form. The dimer is targeted to Reg1/Glc7 and blocks dephosphorylation of Reg1 by Glc7 or stimulates the binding and/or phosphorylation of Reg1. The result is a switch in Glc7 phosphatase substrate selection from Reg1 to Snf1 and an inactivation of the Snf1 kinase, which inhibits the phosphorylation of its DNA-bound substrates, resulting in an altered expression of the target genes (Sanz *et al.*, 2000).

Glc7 and Snf1 are interconnected components of the glucose signaling pathway due to the fact that Reg1 deletion results in a derepression of glucose-repressed genes. In the same context it was observed that the deletion of Snf1 results in a failure to derepress many or all of the same genes. In summary, Reg1 targets Glc7 phosphatase and Snf4 activates Snf1 kinase (Erickson and Johnston, 1994).

2.5.2 β -subunits of the Snf1 complex, subcellular localization and further subunits

Numerous additional participants of the Snf1 complex have been discovered, e.g. Sip1 Sip2 and Gal83. Each of these three proteins contains a Snf1 binding internal region and a Snf4

binding C-terminal region. Through analogy with the heterotrimeric mammalian Snf1 homolog, the AMP-activated protein kinase encodes the yeast α subunit, Snf4 encodes the γ subunit and SIP1, SIP2 and GAL83 encode the β subunit. With respect to Snf1 substrate specificity, all three β subunits play distinct roles *in vivo*. Like Glc7 and its regulatory subunits, the Snf1 kinase appears to be localized by its β subunits to distinct cellular subcompartments, thereby targeting it to different substrates. In glucose grown cells, Snf1 is found exclusively in the cytoplasm. Whereas, after shifting to nonfermentable carbon sources, Snf1 is targeted by Gal83 to the nucleus (Vincent *et al.*, 2001). In addition, no other subunits are associated with Snf4 in the Snf1 kinase complex with glucose as carbon source. It has not been established what the consequences of different fructose levels would be on Snf1 localization and substrate targeting.

The cytoplasmic roles of Snf1 are distinct from its mediation of glucose derepression and are independent of Snf4 activity. The conversion of the kinase complex from the active to the autoinhibited state is presumably mediated by dephosphorylation of Snf1. The active kinase complex is phosphorylated at T210 and associates with its counterpart, Glc7 of the Reg1/Glc7 phosphatase complex. The Sip5 protein binds to both Reg1/Glc7 and the Snf1 kinase complex and may stabilize the interaction between them. Only the phosphorylated form of Reg1 promotes dissociation of Snf4 from Snf1 which results in a deactivation. Hence, the PKA mediated association from monomeric to dimeric Hxk2 (Figure 2.4) is potentially a direct glucose trigger that stabilizes the repressing form of Reg1/Glc7 and thereby inactivates Snf1 (Jiang and Carlson, 1996).

A novel group of recently identified proteins may serve as mediators of Reg1 function. For instance, Bmh1 and Bmh2 are the yeast homologs of the mysterious 14-3-3 proteins which are known as a family of highly conserved eukaryotic proteins involved in a wide variety of cellular processes including signaling, apoptosis, cell-cycle control and transcriptional regulation (Bridges and Moorhead, 2004). Although the exact function of the 14-3-3 proteins is still not completely understood, three main mechanisms appear to be important. First, 14-3-3 proteins positively or negatively regulate the activity of enzymes; secondly, 14-3-3 proteins may act as localization anchors, controlling the subcellular localization of proteins and thirdly, these proteins can function as adaptor molecules or scaffolds, thus stimulating protein–protein interactions. Binding motifs have been identified in a number of proteins that bind to the 14-3-3 proteins (Ichimura *et al.*, 2004). Additionally, they are known to respond to Ras/PKA signaling (Mayordomo *et al.*, 2003).

Recently, more than 50 different proteins could be identified as associated with the Snf1 kinase complex (Elbing *et al.*, 2006). This group of peptides includes Snf1 and Snf4, one α and one γ subunits respectively, three β -subunits, upstream kinases, several members of the Hsp70 class of chaperones, ribosomal proteins, the two described 14-3-3 peptides, several synthases and factors of transcription and elongation. This reflects the complexity and diversity of the

regulation mechanisms of one protein complex, which acts as mediator and transmitter of several signals from the sensing machinery to the target genes.

2.5.3 Transmission of the Ras/cAMP-dependent glucose signal to Reg1/Glc7 and Snf1/Snf4

A potential intermediary between PKA and Snf1 is Sak1, a recently identified upstream activator of Snf1 that regulates Snf1 kinase activity and is localized in the nucleus. Sak1 was identified as the most important regulator of Snf1-dependent glucose regulation (Hedbacker *et al.*, 2004a). If glucose is depleted from the media, Sak1 phosphorylates Snf1 which enters the nucleus and becomes active by releasing the catalytic domain (Hedbacker *et al.*, 2004b). Sak1 is able to associate with several identified peptides of the Snf-complex. Regarding the mechanisms of signal transduction, there is still a gap between the earliest events in glucose signaling and Sak1, but there are evidence that PKA and Snf1 act antagonistically in affecting a similar set of cellular functions (Hubbard *et al.*, 1992; Ashe *et al.*, 2000).

2.5.4 Downstream of Snf1

Several repressors downstream of the Snf1 kinase are known to be phosphorylated and inactivated by Snf1 in the absence of glucose. The first discovered example is the multicopy inhibitor Mig1, a Cys₂His₂ zinc finger protein, whose deletion abolishes glucose repression of numerous target genes (Johnston *et al.*, 1994). The Mig1 gene product is known to interact with the intergenic regions of at least 90 different genes *in vivo* (Mukherjee *et al.*, 2004). Mig1 and the related Mig3 can act as both a repressor and an activator, depending on the chromosomal and cellular context (Levine and Manley, 1989). However, the primary physiological role of Mig1 seems to be that of a negative regulator of glucose repressed genes. This may explain the phenomenon of repression of the low affinity carrier Hxt1 and the derepression of the high affinity carriers Hxt2, 3 and 4 by active Snf1 as a result of low glucose levels (Özcan and Johnston, 1995).

Deletion of either *reg1* or *hpk2* results in Mig1 hyperphosphorylation under repressing or low glucose conditions. The simplest hypothesis, based on to this data, is that phosphorylation of Mig1 by Snf1 is at least partly responsible for inactivation of Mig1 repressor function. Further data suggests that this phosphorylation inactivates Mig1 by changing its subcellular localization. (DeVit and Johnston, 1999). In contrast, *Hpk2* and *Reg1/Glc7* dependent inactivation of Snf1 in the presence of glucose results in hypophosphorylation of Mig1 which causes it to relocate rapidly to the nucleus (DeVit *et al.*, 1997).

2.5.5 Reverse recruitment

Most of the models that have been recommended to explain the action of hexokinase 2 in sugar signal transduction have been built on classic concepts of soluble factors and diffusion. Currently, an alternative paradigm of signal transduction has been suggested that takes into account the physical background of living cells and provides an architectural perspective of cellular regulation. This model allows that many cellular processes are organized along cytoskeletal interconnections as insoluble molecular networks. A key feature of the Hxk2/Mig1/Snf1 dependent glucose repression pathway could be explained with this reverse recruitment model (Sarma *et al.*, 2007). The reverse recruitment model of transcriptional control postulates that eukaryotic genes become active by moving into contact with transcription factories that are localized to nuclear substructures and that at least some of these factories are tethered to nuclear pore complexes (NPCs) (Menon *et al.*, 2005). All three subunits of the Snf1 kinase are perinuclear when they are needed to counteract glucose repression by Hxk2/Mig1. Importantly, the canonical target gene SUC2 is highly mobile and randomly positioned in the yeast nucleus when repressed, but associates tightly with NPCs when derepressed.

2.6 Summary: Sugar preference and association of sugar sensing with stuck fermentations

The biochemical pathways underlying sugar fermentation by yeast are well established. There are only two steps in the fermentative pathway in which differences could occur that may explain the preference for either glucose or fructose, i.e the transport of the sugar into the cell and hexose phosphorylation. The next step is a reversible conversion of glucose-6-phosphate to fructose-6-phosphate by phosphoglucosomerase, which unifies the pathways of the two hexoses. However, these first two steps are deeply involved in a sensing and gene regulation network, which is not entirely understood at present (Figure 2.5).

The main reason for a preference of either glucose or fructose seems to be the affinity of the transport systems. In the case of the main wine yeast, *S. cerevisiae*, all of the Hxt hexose transporters indicate a higher affinity to glucose than to fructose, which is thought to be due to the fact that glucose is transported preferentially in the pyranose form and fructose, on the contrary, in the furanose form (Heredia *et al.*, 1968; Lagunas, 1993). Indeed, only less than 30% of the fructose present in solutions is in the furanose form, so the transport competent concentration of fructose is below the total concentration (Cirillo, 1968). Hxt proteins transport their substrate by passive, energy-independent facilitated diffusion down a hexose concentration gradient. The specialization of this highly complex glucose preferred uptake system might sacrifice the efficiency for fructose or alternative sugars. On the other hand, this

might consequently create a niche for other yeast species which are better equipped for the utilisation of these energy sources. Three fructose preferential uptake systems of non-*S. cerevisiae* yeasts were recently characterized. Two of these systems are specific fructose/H⁺symport systems for high affinity fructose symport and one specifically facilitates fructose diffusion into the fructophilic yeast *Z. bailii*. These permeases seem to be only distantly related to the HXT proteins due to their low level of homology with transporter proteins belonging to the major facilitator superfamily.

The putative second reason seems to be dependent on the enzyme hexokinase. All of three hexokinases of *S. cerevisiae* are characterized to have a higher affinity to glucose than fructose; still, the matter is much more complicated since the sugar kinases are involved in gene expression as well as repression and intracellular sugar sensing processes in addition to the catalytic activity. However, as is also the case in hexose transport, the yeast hexokinases seems to prefer to act on fructose in the furanose form, which is underrepresented in solutions compared to the fructopyranose form.

Hexokinases with a preference to fructose are widespread in a large variety of organisms, including plants, several vertebrate tissues and prokaryotes. Fructose specific fructokinases (or ketohexokinases) have not been characterized in yeasts, however, the genome sequence of *Schizosaccharomyces japonicas* is assumed to encode for a fructokinase.

First of all, Hxk2 mediates the expression of *HXT* genes by an Rgt1 dependent pathway. This pathway is based on the extracellular sensing of glucose or fructose, but it is not known if this process contributes to the preference of one sugar in a fermentative environment. Since both sugars are transported via the same permeases and all of them have a higher glucose affinity, it seems unlikely that the gene repression or induction of the carriers contributes to a transport advantage of one hexose.

The second known signaling pathway is dependent on the production of the second messenger cAMP, which influences the activity of protein kinase A. Fructose is not able to mediate the same signal level as glucose due to the specificity of the extracellular sensor for glucose and the higher affinity of the intracellular sensing mechanism to glucose, which is mediated by Hxk2. As a deduction, fructose induces a lower cAMP response which results in lower PKA activity. This, in turn, seems to result in a change in the balance of monomer-dimerization of Hxk2. As a further consequence, Snf1 activation changes the phosphorylation state of the Mig1 repressor with a associated change in the transcriptional regulation of various genes. Furthermore, modifications of PKA activity also mediate changes in the sugar induced expression of the *HXK1*, *HXK2* and *GLK1* hexokinase genes.

A further absorbing discrepancy in the signal transduction pathway, triggered by either glucose or fructose, is the fact that only Hxk2 is important for glucose activated catabolite repression, but either Hxk1 or Hxk2 can signal fructose catabolite repression. In conclusion, the triggering of fructose repression may differ from triggering of glucose repression.

The hexokinases, mainly Hxk2, involves a widespread network of hexose sensing and regulatory expression of target genes, as well as the catalytic function of glycolytic utilisation. Several questions about the involvement remain unanswered and it cannot be ruled out that something aside from the catalytic activity of the hexokinases has an influence on the hexose preferring phenotype of yeasts. Further studies are required, especially concerning the discrepancies between the outcome of fructose addition compared to glucose addition on the expression patterns of target genes.

Grape must provides both of the sugars and wine fermentation follows a specific sugar utilisation pattern with a discrepancy in the utilisation rate of glucose and fructose. Due to the glucophilic phenotype of *S. cerevisiae*, glucose is always consumed faster than fructose. In late stages of alcoholic fermentations, when fermentation problems may occur, the environment has changed in the sense that glucose is scarce and fructose may be present in concentrations more than 10 times higher than glucose. However, fructose always seems to trigger a reduced signal, similar to low glucose levels. The yeast cells have to adapt continuously to the changing environment and it seems evident that these conditions lead changes in transcriptional regulation which could contribute to an impeded fermentation, although some residual fructose and glucose are still available.

A further piece of the puzzle of “hexose preferring phenotypes in putative connection with stuck fermentations” is the finding that depletion of sugar from the growth media is known to cause an almost complete inhibition of translation (Ashe *et al.*, 2000). This inhibition is observed for both glucose and fructose as carbon source and occurs rapidly after removal of the sugar. This effect can be readily reversed upon re-addition of one of the sugars. This inhibition and its reversal do not require transcription of new mRNA, since the level of mRNA does not decrease when the sugar is removed. However, the withdrawal of the carbon source does result in a loss of polyribosomes, which is indicative of the existence of a previously undescribed pathway that can lead to the rapid inhibition of protein synthesis in response to environmental changes. Interestingly, the Δ hxk2 deleted mutant is resistant to translational inhibition induced by either glucose or fructose withdrawal, whereas protein synthesis in the Δ hxk1 glk1 double deleted mutant was still inhibited. This finding illustrates that resistance to the inhibition of translation follows the same hexokinase specificity than the glucose repression pathway. Several further mutants in the glucose repression, in hexose transporter induction and cAMP-dependent protein kinase pathways are resistant to the inhibitory effects of glucose withdrawal on translation. Due to these results, it seems likely that the translational inhibition is interconnected with the main glucose repression pathway in which the response to fructose seems to differ in intensity compared to the response to glucose.

Taken together, it cannot be ruled out, that fructose levels less than 15 gram per liter together with glucose levels less than 1 gram per liter in the environment of a late fermentation phase are below a possible threshold for a positive signal concerning hexose sensing. The

observation that glucose addition can restart stuck fermentations (see next chapter; Schütz and Gafner, 1993a) supports this idea of decreased hexose signaling as a possible contribution to the cause of stuck fermentations.

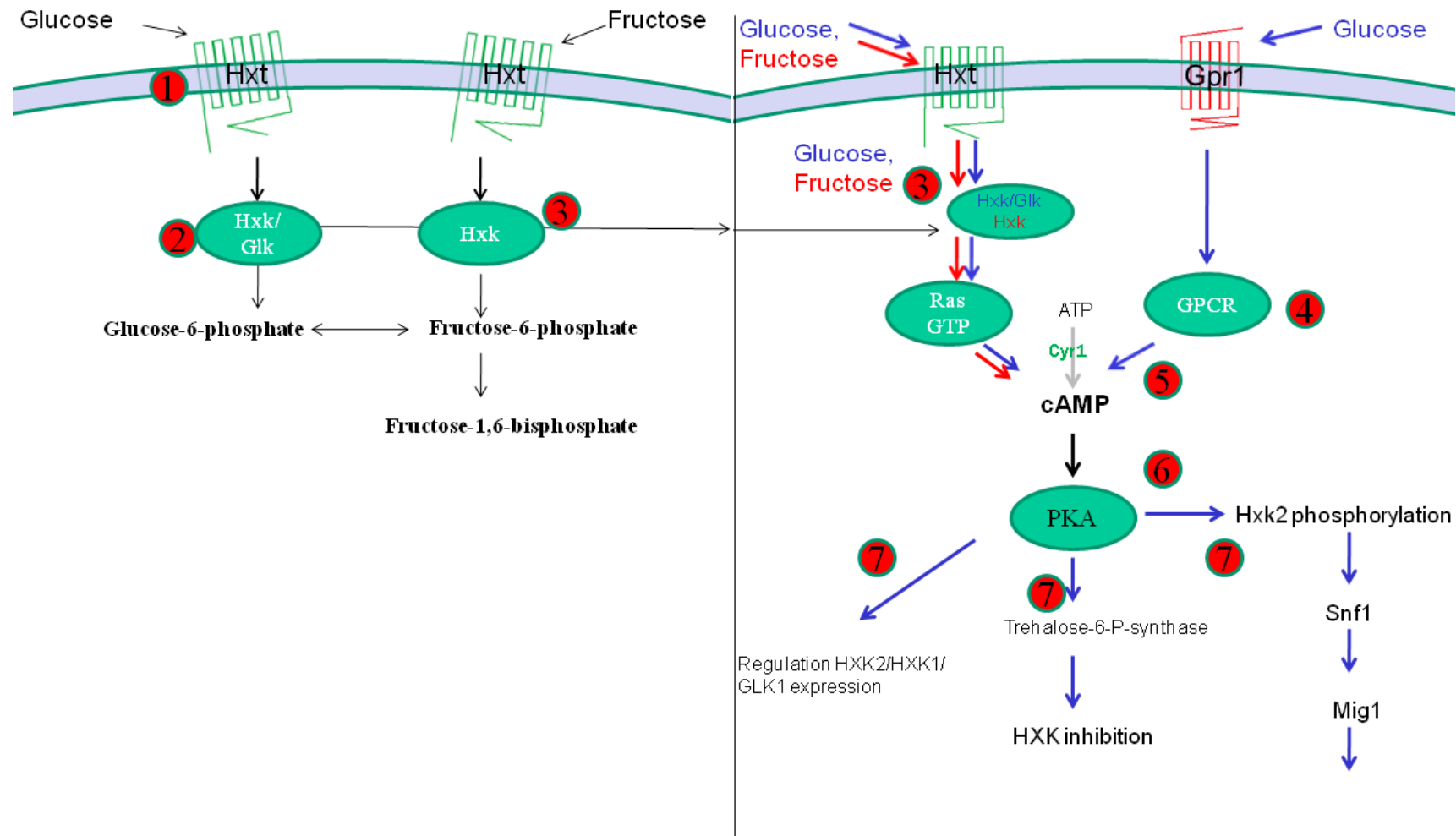


Figure 2.5: Possible basis for hexose preference of *S. cerevisiae*. Hxt hexose transporters (1) and Hxk sugarkinases (2) show a higher affinity for glucose than fructose. The metabolic rate is affected by the transport protein and/or sugarkinase substrate affinities. Furthermore, Hxk is involved in intracellular sensing process (3) which mediates activation of Ras-GTP. Glucose and fructose can trigger pathway (3), whereas activation of the GPCR system is glucose specific (4). Both pathways resulting in a spike of cAMP (5), which is lower with fructose compared to glucose. cAMP generates a protein kinase A response (6), resulting in a widespread effect on gene regulation by affecting several regulatory elements like Snf1 and Mig1 (7).

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Chapter 3

Research results

Isolation of fructophilic yeasts from natural habitats and the development of a novel methodology for the cure of stuck fermentations

Parts of this chapter will be submitted for publication in wine-related journals in two separate articles

3.1 Introduction

3.1.1 Definition of stuck and sluggish fermentations

Incomplete or stuck fermentations are defined as fermentations that leave a higher than desired residual sugar content at the end of alcoholic fermentation, while slow or sluggish fermentations are characterized by a low rate of sugar utilisation (Bisson, 1999). Such fermentations rank as the second most important enological concern to winemakers, according to an American Vineyard Foundation survey in (May/June 2003). A similar result was obtained from the Association for the Development of Wine Biotechnology in France (1996). It can therefore be seen as a chronic problem for the wine industry, and more than 60% of responding winemakers admitted to having experienced this problem. Incomplete fermentation is associated with important economic losses because it frequently results in a decreased quality of the final product and leads to a rise in the costs of wine making. However, there are no official statistics about the number and quantity of wines which are affected by stuck fermentations. According to the vintage, anecdotal evidence would suggest that up to 10% of a harvest can be affected in any given year. Wines with a high content of residual post-fermentation sugar are very sensitive to microbial spoilage and are therefore at increased risk of experiencing off-flavour formation (Butzke and Dukes, 1996). Such wines therefore cannot be bottled until it has been established that they are microbially stable. Spoilage bacteria such as acetic acid bacteria or lactic acid bacteria can metabolize sugar to increase volatile acidity and also favour the formation of unusual and sometimes unpleasant esters. They may also alter the pattern of diacetyl formation (O'Connor-Cox and Ingledew, 1991). Therefore, residual post fermenting sugar increases the risk of the growth of undesired microorganism and the formation of off-flavours. A large spectrum of spoilage microorganisms and the corresponding undesired by-products of these organisms could be detected by the department of beverage microbiology from Agroscope Research Station Changins- Wädenswil ACW in incomplete fermented musts. Most isolated spoiling organisms found in stuck musts belong to the species *Pediococcus*, *Lactobacillus* and *Leuconostoc* (own observations). For instance, *Leuconostoc mesenteroides* is able to produce mannitol, lactate and/or acetic acid (Sponholz, 1993). *Pediococcus damnosus*, *Pediococcus parvulus* and *Pediococcus pentosaceus* are able to decarboxylate free amino acids, which results in biogenic amines such as the allergenic histamine (Landete *et al.*, 2005). Lactic acid bacteria, like *Lactobacillus brevis* were also found to spoil stuck musts with biogenic amines, acetic acid, mannitol or acetylpyrrolin, which is known to be involved in the "mousy taint" (Weiller and Radler, 1970; Landete *et al.*, 2005; Snowdon *et al.*, 2006).

Members of acetic acid bacteria, principally *Acetobacter* are also present in the grape microflora. However, members of these genera are strict aerobes, so once the juice becomes anaerobic further growth and metabolism is inhibited, resulting in a minor risk for spoilage (Sponholz, 1993).

3.1.1.1 Fermentation to dryness

In a typical unproblematic fermentation, residual sugar concentration is usually less than 2 g/l (Bisson, 2001). Dryness is defined as a residual sugar concentration not exceeding 4 grams per liter or 9 grams per liter, provided that the total acidity per liter is not more than 2 grams below the residual sugar content. Fermentations are generally complete within two to three weeks under typical vinification conditions (Bisson, 2001).

3.1.2 Causes of stuck fermentation

Many causes of stuck or struggling fermentations are described in the literature (Lafon-Lafourcade *et al.*, 1984; Kudo *et al.*, 1988; Bely *et al.*, 1990; Henschke and Jiranek, 1993; Schütz and Gafner, 1993a; Bisson and Butzke, 2000; for review see: Malherbe *et al.*, 2007). However, in most cases it is very difficult to determine the explicit physiological reason which leads to a decrease in fermentation rate in any given fermentation because of the possibility of synergistic effects. The complexity of grape must and the numerous parameters of winemaking complicate a clear definition of the causes. It is complicated to perform reliable and repeatable experiments concerning these topics in stuck grape must, since each stuck must defines a unique environment. Therefore, it seems to be difficult to draw an analogy between studies and the appearance of problematic fermentations in industry. In fact, standard culture conditions are very different from winemaking conditions, where multiple fermentation inhibiting factors occur simultaneously and sequentially throughout the fermentation. Nevertheless, the factors which lead to an incomplete fermentation can be summarized as falling into three groups, nutritional deficiencies, inhibitory substances and physical factors. While there is a large body of literature concerning these topics, but a lack of reports which describes the correlation or relationship of different factors.

3.1.2.1 Nutritional deficiencies

3.1.2.1.1 Nitrogen

Most of the studies about stuck fermentations have focussed on nutritional deficiencies, which are mainly concerned with assimilable nitrogen sources, oxygen, vitamins and minerals (for reviews see: Bisson, 2000; Malherbe *et al.*, 2007). Of all the nutrients that are important for yeasts to conduct alcoholic fermentation of musts, one of the most important is nitrogen. Hence, nitrogen is also the primary nutrient associated with these fermentation problems (Agenbach, 1977; Kunkee, 1991; Butzke and Dukes, 1996). Yeasts can assimilate nitrogen from organic sources such as free amino acids or peptides and proteins via hydrolysis as well as from

inorganic sources such as ammonia salts and urea. Depending on many parameters like grape variety, the viticultural region or winemaking practices, the nitrogen content in grape juice has been shown to range from 60 to 2400 mg nitrogen per liter (Henschke and Jiranek, 1993). Various studies have shown that a minimum of about 150 mg per liter yeast assimilable nitrogen (YAN) appears required to be on the safe side for an uncomplicated fermentation rate in a standard dry white wine (Bely *et al.*, 1990; Henschke and Jiranek, 1993; Bisson and Butzke, 2000). However, the nitrogen utilisation of wine yeasts is influenced by multiple factors, including the presence of oxygen, the initial sugar content, the temperature and the occurrence of competing microorganisms in the grape juice (Agenbach, 1977; Monk, 1982; Kunkee, 1991; Butzke and Dukes, 1996). When all other factors are in an acceptable range, fermentation duration is a function of the initial YAN concentration (Bell and Henschke, 2005). If the initial YAN is low, the risk of a slow or stuck fermentation is increased. The impacts of nitrogen on alcoholic fermentation are as varied as they are complex. Nitrogen compounds, due to the requirement as components of structural and functional proteins during growth and metabolism, have a direct relevance for biomass production, the fermentation rate and the time to fermentation completion. Nitrogen composition is also important for the production of secondary by-products responsible for fermentation flavours and consequently has a direct influence in wine quality. For example, specific amino acids can be converted into specific higher alcohols and their corresponding esters (Lilly *et al.*, 2006). Deficiency in assimilable nitrogen may result in an inhibition of protein and enzyme synthesis which decreases the fermentation rate and therefore the growth and reproduction of the yeast cells. In this context, an irreversible inhibition of the synthesis of hexose transporters was observed under nitrogen starvation conditions in fermentations with synthetic grape must (Salmon, 1989). The essential roles of amino acids are not restricted to use as a nitrogen source in polypeptide formation, since amino acids may also be used as redox agents for the balance of the oxidation-reduction potential under certain conditions (Albers *et al.*, 1996; Mauricio *et al.*, 2001).

3.1.2.1.2 Oxygen

Although yeasts exhibit growth under close-to anaerobic conditions, they require low amounts of oxygen for sustained viability. A lack of oxygen in grape must was already described in 1985 as one of the major causes of stuck fermentations (Ingledew and Kunkee, 1985). Oxygen is essential for the synthesis and/or function of membrane compounds like sterols and unsaturated fatty acids which are necessary for membrane permeability (Andreasen and Stier, 1953; Lafon-Lafourcade *et al.*, 1979). The addition of oxygen and/or ergosterol, referred to as an oxygen substitute, may act as survival factors that decrease the inhibitory effects of ethanol (Lafon-Lafourcade *et al.*, 1984). Consequently, a lack of oxygen induces a deficit in ergosterol

which decreases the growth rate during alcoholic fermentation. Ergosterol addition can partially restore the growth rate, but may not restore the ability to complete fermentation to dryness. Various practical experiments came to the conclusion that an addition of not more than 2 mg/l oxygen at an early state of alcoholic fermentation can stimulate production of ergosterol and unsaturated fatty acids and therefore help avoid incomplete sugar consumption (von Meyenburg, 2004).

3.1.2.1.3 Phosphate, Minerals and Vitamins

Saccharomyces cerevisiae contains up to 5% phosphate on a dry weight basis (Walker, 1998). Phosphate is incorporated into nucleic acids, phospholipids, adenosine-phosphates and some other compounds. A limitation of phosphate can directly affect fermentation rate and biomass production (Gancedo and Serrano, 1989). Since the mineral potassium is necessary for phosphate uptake, a potassium deficiency may also be linked to stuck or sluggish fermentations (Kudo *et al.*, 1988). Potassium contributes up to 2.5% of yeast dry weight (Monk, 1994). Other essential minerals required by yeasts during fermentation are magnesium, sulfur, calcium, chlorine, copper, iron, zinc, nickel, molybdenum and manganese (Monk, 1994; Walker, 1998). These minerals have a variety of functions but are used primarily as enzyme activators.

Besides minerals, yeast requires various vitamins such as thiamin, riboflavin, pantothenic acid, pyridoxine, nicotinamide, biotin and inositol, depending on species or strain and the condition during growth and fermentation. Biotin and pantothenic acid are essential for all *Saccharomyces*-strains, whereas inositol and thiamine are required by some strains (Walker, 1998). An insufficient availability of these vitamins has been associated with sluggish fermentations (Ough *et al.*, 1989), since these vitamins are required for sugar and lipid metabolism, NAD and NADP synthesis and cell division. The growth and fermentation rate can be highly stimulated by the presence of extracellular vitamins, even by those that can be synthesized by the cells (Ough *et al.*, 1989; Fleet and Heard, 1993).

3.1.2.2 Inhibitory substances

A second contributing factor to the onset of problematic fermentations is the occurrence of fermentation inhibitory substances or toxins. Substances that inhibit growth or lead to cell death will also impact fermentation rate and can lead to sluggish or stuck fermentations. Toxins can arise from several sources during wine production. Such substances can be synthesized as products or by-products of metabolism by either the yeasts itself or by other microorganisms in

the environment of grapes or grape juice. A further alternative is the addition of toxic compounds as a result of the winemaking process.

3.1.2.2.1 Ethanol

Ethanol, the end-product of alcoholic fermentation, is a strong inhibitor of growth and fermentation rate of microorganisms and adversely affects cell viability (Alexandre and Charpentier, 1998). The strength of inhibition is dependent on the concentration and the species and strain specific ethanol tolerance. At ethanol concentrations of about 5 vol% this generates a critical advantage for *S. cerevisiae* compared to other fungal, yeast and bacterial species (Fleet and Heard, 1993). In conjunction with the superior growth rate in the anaerobic environment, this factor ensures the predominance of the species in the environment of fermenting grape juice. Other *Saccharomyces* spp are also able to adapt to increasing ethanol levels up to a content of 16-18 vol%. Ethanol is known to damage mitochondrial DNA (Ibeas and Jimenez, 1997), it causes inactivation of some important enzymes (Augustin *et al.*, 1965; Nagodawithana and Steinkraus, 1976) and it has an influence on proton flux (Cartwright *et al.*, 1986). Furthermore, it inhibits several transport systems such as the general amino acid permease and the hexose transporters (Alexandre and Charpentier, 1998) and also affects yeast plasma membrane composition (Jones and Greenfield, 1987). However, the primary targets of ethanol stress are the cell membrane compounds, since it is known that ethanol influences membrane fluidity (van Uden, 1985). A relationship between the fatty acid composition of lipid membranes and alcohol stress tolerance has been suggested by various authors (Mishra and Prasad, 1989; Sajbidor *et al.*, 1995). It has, additionally, been confirmed that the specific ethanol tolerance is dependent on the composition of unsaturated fatty acid in the lipid membranes. The incorporation of oleic acid into lipid membranes, which affects a compensatory decrease in membrane fluidity that counteracts the effects of ethanol, is considered to be of specific importance (You *et al.*, 2003). However, a recent study supports the assumption that ethanol tolerance is under polygenic control as a typical quantitative trait in which more than 250 genes are involved (Hu *et al.*, 2007).

When considering the fermentation process and possible problems, it is important to mention that physical factors such as temperature influence ethanol tolerance, with lower temperatures ensuring greater ethanol tolerance (Henschke, 1997). This impact can probably be directly linked to membrane fluidity.

3.1.2.2.2 Acetic acid

Acetic acid toxicity has been suggested as a cause of stuck fermentation, since it is known to inhibit the growth and fermentation activity of *S. cerevisiae* (Kreger-Van Rij, 1984). The impact of acetate is particularly prominent at the end of fermentation when the concentration of acetic acid and other toxic compounds, such as ethanol, are at their highest (Casey and Ingledew 1986; Rasmussen *et al.*, 1995). The minimal inhibitory concentration (MIC) of acetic acid for preventing yeast growth is strain dependent and varies widely from 4.5 g/l to 7.5 g/l (Edwards *et al.*, 1999). However, in the presence of 8 vol% ethanol the MIC ranges from 1.5 to 4.0 g/l (Egliton and Henschke, 2001).

The mechanism of this inhibition is diverse and it is noteworthy that both fermentation and respiration are affected to the same extent by the presence of acetic acid (Fernandes *et al.*, 1997). This suggests that the response is not a simple inhibition of glycolysis, but that mitochondrial activity is also disturbed. Further results indicate that acetic acid is able to stimulate apoptosis in yeasts (Ludovico *et al.*, 2001; Madeo *et al.*, 2002) by activating the metacaspase Yca1p, similar to hydrogen peroxide or aging. However, the mechanism of acetic acid inhibition seems to have several targets, as it was shown to reduce the ability of *Saccharomyces* to transport and store the vitamin thiamine (Iwashima *et al.*, 1973) and can also result in intracellular acidification, which leads to an increased glucose consumption rate with a reduced ATP yield (Pampulha and Loureiro-Dias, 2000).

Various microorganisms that occur in association with grapes, grape juice and must are able to produce acetic acid, since it is a normal by-product of sugar metabolism in these organisms. Acetic acid can appear already in the vineyard, on mouldy, botrytized or injured berries and throughout most steps of the winemaking process (Drysdale and Fleet, 1988). The highest risk persists during must holding times and alcoholic and malolactic fermentation, particularly in the case of spontaneous fermentations. Incomplete fermentations in which the residual sugar may be metabolized by spoilage microorganisms can also result in the unwanted formation of high acetate levels (Edwards *et al.*, 1999). Accordingly, and as with many of the other factors discussed here, it is sometimes ambiguous if the occurrence of a high level of acetic acid causes stuck fermentation or if the elevated acetic acid concentration is a consequence of the stuck fermentation.

3.1.2.2.3 SO₂

Another major fermentation inhibition factor is the wine preservative sulphur dioxide. Intracellular SO₂ results in a rapid decrease in the ATP levels, which results in cell death (Hinze and Holzer, 1986). High levels of SO₂ are toxic to all microorganisms and therefore may cause

problems ranging from a decreased fermentation rate to a nonreversible, stuck fermentation (Alexandre and Charpentier 1998). In contrast, most yeast strains release marginal amounts of SO₂ during alcoholic fermentation as an intermediate of sulfurous amino acid synthesis and the reduction of sulfate to sulfite (Lonvaud-Funel *et al.*, 1988; Henick-Kling and Park, 1994). Depending on the strain and the fermentation conditions, *Saccharomyces* strains produce an average of 5-10 mg/l, but in exceptional cases up to 100 mg/l of sulphur dioxide. For commercial wine yeasts, sulfur dioxide tolerance is a desirable trait. *Saccharomyces* detoxifies SO₂ via the formation of acetaldehyde adjuncts. Sulfur dioxide tolerant strains are viable in fairly high concentrations of SO₂ of about 200-500 mg/l (Bisson, 2001).

3.1.2.2.4 Other fermentation inhibitors

Further confirmed inhibitors of alcoholic fermentation are medium chain fatty acids, which increase membrane fluidity and permeability and can impede hexose transport systems (Lafon-Lafourcade *et al.*, 1984). The appearance and function of yeast killer toxins has also been the focus of several interesting studies. So-called *Saccharomyces* killer strains secrete a proteinaceous extracellular toxin that kills sensitive yeast strains (Woods and Bevan, 1968). This toxin inhibits fermentation by sensitive yeasts through multiple targets and has, for instance, been shown to interrupt the coupled transport of protons and amino acids and causes a cellular loss of small metabolites like ATP, glucose and amino acids (Van Vuuren and Wingfield, 1986). In addition, Non-*Saccharomyces* yeasts such as *Hansenula* and *Kluyveromyces* may produce killer factors which are active against *Saccharomyces*. Other toxins may be produced by moulds or plants. Most *Saccharomyces* strains are resistant to a variety of mycotoxins, although an inhibitory impact on some members of the must or juice flora cannot be excluded, particularly in co-operation with additional factors encountered during winemaking conditions (Bisson, 1999).

During the seasonal work in the vineyards, the treatment with fungicides or pesticides is essential. Since *Saccharomyces* is a member of the kingdom of fungi, fungicides used in the vineyard to inhibit mould infestation may also inhibit yeast if present in high enough concentrations at the time of harvest (Bisson, 1991; Viviani-Nauer *et al.*, 1997; Specht, 2003). Several herbicides may also have an influence on yeast growth (Echeverrigaray *et al.*, 1991). In years of late-season drought, high concentrations of pesticide or fungicide residues may remain on the grapes, resulting in an additional inhibition factor which may result in higher rates of stuck and sluggish fermentations.

An inhibiting function has also been suggested for the second major product of alcoholic fermentation, carbon dioxide. It has been suggested that high viscosity media may prevent the

emission of CO₂ which could elevate the carbon dioxide pressure of the must to an inhibitory level (Thomas *et al.*, 1994).

3.1.2.3 Stuck or sluggish fermentation as a result of physical factors

3.1.2.3.1 Fermentation temperature management

Temperature extremes and temperature shocks during the fermentation can cause stress conditions for the yeasts. The temperature of wine fermentations range from 10°C to 35°C, and is an object of the winemakers philosophy because the choice of fermentation temperature significantly influences the wines aromatic and sensory attributes (Sütterlin *et al.*, 2001). Some white wines are fermented at temperatures as low as 10-12°C, and many red wines, in order to facilitate extraction, at temperatures as high as the yeast can tolerate (35°C). Yeasts are particularly heat sensitive in the growth phase of alcoholic fermentation and become stressed when the temperature is greater than approximately 30°C. This affects viability and results in an increased production of volatile acidity during fermentation, increasing the risk of a stuck fermentation (Ribéreau-Gayon *et al.*, 2000). Temperature directly affects membrane fluidity, with higher temperatures resulting in increased membrane fluidity. This effect could also explain yeast's enhanced ethanol sensitivity at higher temperatures since ethanol concentration also has a directly proportional effect on the fluidity of cell membranes. Furthermore, an increased temperature of fermentation also increases yeast consumption of nitrogen (Ribéreau-Gayon *et al.*, 2000).

Lower fermentation temperatures may enhance ethanol resistance by increasing levels of oleic acid in yeast cell membranes (You *et al.*, 2003). In "cold fermentations" at about 10-15°C the fermentation rate of *S. cerevisiae* is strongly decreased. This increases the risk of growth of undesired microorganisms which may be better adapted to these temperatures and could be responsible for the completely modified sensory attributes of "cold" fermented wines. Holding the must at low temperatures is inhibitory to the bacteria, but the undesired yeast *Hanseniaspora uvarum* (anamorph *Kloeckera apiculata*) tolerates low temperatures well and will be dominant in musts at temperatures between 10°-15°C (Schütz and Gafner, 1993b). This unwanted yeast has been associated with ester taints and high levels of acetic acid (du Toit and Pretorius, 2000).

Temperature shocks refer to a dramatic change in the fermentation temperature during a short time-period. Changes in both directions can generate stress, which may result in a stuck fermentation (Bisson, 2001).

3.1.2.3.2 Excessive must clarification

Extreme clarification of must is an additional factor which reduces the fermentation rate. Clarification can reduce the musts content of nitrogen, vitamins and minerals as well as sterols and unsaturated fatty acids and increase the probability of stuck or sluggish fermentations. This appears to be due to the removal of solids, cell particles or microbes that have sequestered these components (Bisson, 1999). Additionally, the degradation of solids also affects ethanol tolerance in a negative way (Henschke, 1997).

3.1.2.3.3 Microbial rivalry

High populations of non-*Saccharomyces* yeast and bacteria increase the risk of problematic fermentations (Drysdale and Fleet, 1989; Bisson, 1999). According to current literature, the reasons are twofold, namely, the competition for nutrients (Edwards *et al.*, 1998) and the production of metabolic products which may be toxic (Drysdale and Fleet, 1989) can cause growth inhibition for *S. cerevisiae*. An important aspect concerning this rivalry is the appearance of incompatible pairings of wine yeasts and malolactic bacteria (Bisson, 1999). Some yeast strains are very susceptible to inhibition by some bacteria strains. In certain cases a reversible or a non-reversible interruption of the alcoholic fermentation can be observed due to the initiation of malolactic fermentation. It is known that several *Lactobacillus* strains have antifungal properties which may trigger this inhibitory effect (Edwards *et al.*, 1998; Schwenninger *et al.*, 2005). Nevertheless, further study is required to identify the connection between lactic acid bacteria and stuck fermentations and the exact mechanism involved.

3.1.2.4 Fructose

3.1.2.4.1 Fructose utilisation vs. glucose utilisation during alcoholic fermentation

Grape juice contains approximately equal amounts of the two hexoses glucose and fructose. During alcoholic fermentation by the wine yeast *S. cerevisiae* the consumption of both sugars follows a predetermined pattern. Although both sugars are metabolized concomitantly, glucose decreases faster than fructose from the grape must (Schütz and Gafner, 1993a; Berthels *et al.*, 2004; Jolly, 2008). The balance of sugars can be characterized as the glucose to fructose ratio (GFR). The GFR at the beginning of fermentation will be of a value of about 1, and will decrease during on-going fermentation due to the glucophilic character of the regular wine yeast. In addition, the discrepancy between the actual amounts of utilized sugar among two

time points can be described by the term $\Delta\text{glucose} - \Delta\text{fructose}/\Delta\text{glucose}$, referred to as G/F discrepancy (Berthels *et al.*, 2004). In most cases of stuck fermentation in practice there is a lack of reliable data concerning the separate utilisation of both sugars, since standard fermentation controls are restricted to an indirect measurement of total sugar. Hence, in most instances of incomplete fermented musts only the GFR at this stage can be ascertained. In contrast, controlled conditions with measurements at many different time points illustrate hexose consumption patterns during the varying phases of fermentation.

Some possible reasons for the glucophilic phenotype were mentioned in the literature review, including the higher affinity for glucose of hexose transport facilitators as well as the hexose phosphorylating enzymes (Entian and Mecke, 1982; Reifemberger *et al.*, 1997). Consequently, fructose becomes the main sugar during the late stages of alcoholic fermentation and wine yeasts have to ferment this non-preferred sugar after periods of starvation and in the presence of large amounts of ethanol. The stress associated with these conditions seems to be amplified by several additional inhibition factors of alcoholic fermentation, resulting in sluggish or stuck fermentations (Bisson, 1999). In such situations, the lower fructose utilisation capacity of *S. cerevisiae* is thought to contribute to the low fermentation rate, which can result in a the termination of the fermentation (Schütz and Gafner, 1993a). The ability of wine yeasts to metabolize fructose is therefore critically important for the maintenance of a high rate of fermentation at the end of the process and for fermentation of the must to dryness. Based on these facts and observations, we conclude that a high fructose contingent in the available sugar is an inhibition factor for fermentation by glucophile wine yeast strains. At this difficult stage of fermentation fructose utilisation seems to be the final contributing factor for the occurrence of a stuck fermentation.

3.1.2.4.2 Discrepancy of the hexose utilisation rate

All known *S. cerevisiae* strains seem to show preference to glucose, but to varying degrees. The discrepancy in the utilisation rate of glucose and fructose decreases during the course of a fermentation in a strain dependent manner. Berthels *et al.* (2004) observed that all seventeen of *Saccharomyces* strains that were analyzed utilized glucose more rapidly than fructose. Interestingly, this discrepancy could be intensified by the addition of ethanol to both natural and synthetic grape must as media. Fructose utilisation seems to be inhibited more than glucose utilisation under high ethanol conditions, with a difference in inhibition ranging from 56% to 196% between the strains. In aqueous solutions, it is known that ethanol causes a shift in the tautomeric equilibrium from fructopyranose to fructofuranose (Flood *et al.*, 1996), and the yeast hexose transport facilitators may prefer the transport of the pyranose form of glucose (Cirillo, 1968), but the furanose form of fructose (Heredia *et al.*, 1968; Lagunas, 1993).

Consequently, the amount of transport-competent fructose increases with increasing ethanol concentration. These results are in contradiction to the findings that fructose uptake is more inhibited under high ethanol conditions than glucose, and needs further investigation to be clarified.

Yeast assimilable nitrogen was identified as a further influencing factor on the variance of the hexose utilisation, since fructose utilisation after supplementation of yeast assimilable nitrogen to the fermenting media increases more than glucose utilisation after nitrogen addition., Fructose utilisation also seems to be more susceptible to different types of stress which may occur during fermentation, regardless of the type of media and the strain background.

3.1.2.4.3 Glucose to fructose-ratio (GFR)

In plants, there is a large variance of the GFR in fruits; apples and pears contain more than twice as much free fructose as glucose, while apricots contain less than half the amount of fructose compared to glucose (Park and Yetley, 1993). According to literature, the GFR of ripe grapes can vary between 0.80 and 1.2 and is dependent on the cultivar, the viticultural region and the ripeness of the grape, but it is generally accepted to be close to one (Amerine and Thoukis, 1958; Kliewer, 1967; Soulis and Avgerinos, 1984). In phase III of berry growth, which represents the transition from berry growth to berry ripening (Veraison; Change of color of the grape berries) the berry becomes a sugar accumulating organ. Sucrose is transported from the leaves, trunk, branches and shoots to the berry where it is hydrolysed to glucose and fructose, resulting in equilibrium of both sugars. At the end of this phase, a slightly decrease in the GFR is observed, probably because glucose can be converted to fructose using sorbitol as an intermediary product (Kliewer, 1965; Esteban *et al.*, 1999; Snyman, 2006). Further observations indicate that the occurrence of this effect is intensified during warm and dry seasons (Snyman, 2006). Due to the discrepancy in the utilisation rate mentioned above, the GFR of grape must continually decreases during alcoholic fermentation and is usually found to be between 0.3 and 0.2 during the late yeast growth phase of fermentation when around 50 % of sugar has been consumed. This value tends to decrease below 0.1 when more than 90% of fermentation is completed (Wucherpfennig *et al.*, 1986). To assess the impact on fermentation efficiency, GFR has to be evaluated in relationship to the total amount of residual sugar. A low GFR early in fermentation may indicate a strong risk of incomplete fermentation. Data from the vintages 2003-2005 in some European viticultural regions indicate that 49 out of 55 randomly selected cases of incomplete fermented grape must had a GFR of less than 0.1 (Table 3.1; ACW Changins-Wädenswil, 2003-2005). Documentation extending over several years indicate that the glucose to fructose ratio in approximately 90% of all cases of stuck fermentation is below 0.1. Similar observations were communicated in several personal discussions (Neil Jolly,

Stellenbosch 2008; Netzwerk Mikrobiologie, Germany, 2005/2006). This additional inhibition factor minimizes the possibility of a restart of fermentation, even after a re-inoculation with *S. cerevisiae*.

Table 3.1: Stuck wines according to the definition of Chapter 3.1.1 from different European regions (Data ACW Changins-Wädenswil from vintages 2003-2005).

Vintage	Variety	Region	Glucose g/l	Fructose g/l	GFR
2003	Pinot Noir	East Switzerland	1.7	21.8	0.078
2003	Chardonnay	East Switzerland	1.5	23.1	0.065
2003	Pinot Noir	East Switzerland	1.5	28	0.054
2003	Chardonell	East Switzerland	3.5	47.5	0.074
2003	Pinot Noir	East Switzerland	1.3	26.3	0.049
2003	Pinot Noir	East Switzerland	1.1	24.6	0.045
2003	Müller-Thurgau	East Switzerland	1.4	27.6	0.051
2003	Pinot Noir	East Switzerland	0.7	14.4	0.049
2003	Pinot Noir	East Switzerland	0.5	12.6	0.040
2003	Pinot Grey	East Switzerland	1.6	23.7	0.068
2003	Pinot Noir	Liechtenstein	1.7	23.5	0.072
2003	Pinot Noir	Liechtenstein	1.8	22.5	0.080
2003	Pinot Noir	Liechtenstein	1.0	19.4	0.052
2003	Pinot Noir	Liechtenstein	0.6	17.5	0.034
2003	Pinot Noir	Liechtenstein	1.7	28.8	0.059
2003	Sauvignon Blanc	East Switzerland	0.7	9.2	0.076
2003	Chardonnay	East Switzerland	0.5	11.9	0.042
2003	Pinot Noir	East Switzerland	0.4	11.3	0.035
2003	Pinot Noir: Oeil de Perdrix	East Switzerland	0.4	15.8	0.025

Vintage	Variety	Region	Glucose g/l	Fructose g/l	GFR
2004	Rosé	East Switzerland	0.4	16.7	0.024
2004	Chardonnay	East Switzerland	1.5	18.9	0.079
2004	Pinot Blanc	East Switzerland	0.8	14.8	0.054
2004	Chardonnay	East Switzerland	0.5	13.6	0.037
2004	Rosé	East Switzerland	1.2	10.0	0.120
2004	Pinot Noir	East Switzerland	2.0	19.2	0.100
2004	Chardonnay	East Switzerland	2.6	26.7	0.097
2004	Pinot Noir: Oeil de Perdrix	East Switzerland	1.2	16.6	0.072
2004	Chardonnay	East Switzerland	6.4	35.5	0.180
2004	Chardonnay	East Switzerland	4.1	31.3	0.131
2004	Schillerwein	East Switzerland	0.9	15.4	0.058
2004	Pinot Blanc	East Switzerland	n.d.	10.4	<0.1
2004	Pinot Blanc	South Germany	0.4	9.6	0.041
2004	Pinot Blanc	South Germany	n.d.	7.5	<0.1
2004	Pinot Blanc	South Germany	0.6	10.2	0.059
2004	Riesling	South Germany	0.8	10.7	0.075
2004	Pinot Blanc	South Germany	2.5	21.8	0.115
2004	Garanoir	East Switzerland	0.4	16.7	0.024
2004	Pinot Blanc de Noir	Ticino	1.2	22.4	0.054

Vintage	Variety	Region	Glucose g/l	Fructose g/l	GFR
2005	Chardonnay	East Switzerland	0.5	9.4	0.053
2005	Pinot Noir	South Tyrol	0.7	5.5	0.127
2005	Chardonnay	South Tyrol	n.d.	13.1	<0.1
2005	Pinot Noir	East Switzerland	1.5	19.0	0.079
2005	Pinot Gris	East Switzerland	1.4	21.7	0.065
2005	Pinot Noir: Oeil de Perdrix	East Switzerland	2.3	29.1	0.079
2005	Auxerois	South Germany	1.0	14.5	0.069
2005	Auxerois	South Germany	2.2	22.1	0.100
2005	Chardonnay	East Switzerland	1.0	24.0	0.042
2005	Pinot Noir	Valais	0.9	14.6	0.062
2005	Pinot Noir	Valais	0.5	10.5	0.048
2005	Pinot Gris	East Switzerland	4.8	33.0	0.145
2005	Pinot Noir	East Switzerland	1.7	20.3	0.084
2005	Pinot Noir	East Switzerland	1.6	18.3	0.087
2005	Pinot Gris	East Switzerland	0.5	20.4	0.025
2005	Chasselas	East Switzerland	n.d.	7.7	<0.1
2005	Pinot Noir	East Switzerland	3.1	30.6	0.100

n.d.= not detectable.

The first indication that a low GFR could be an inhibition factor for fermentation and therefore a possible cause of stuck fermentations was mentioned in the publication of Schütz and Gafner in 1993 (1993a). The generation of a stuck or sluggish fermentation from a normal ongoing fermentation by reducing the GFR was a key experiment, which supports the theoretical framework of this thesis. In this experiment, glucose was enzymatic converted by glucose oxidase into the non-fermentable substrate gluconic acid at different phases of small scale fermentations. This resulted in an artificial decrease of the GFR and was accompanied by a significant decrease of the fermentation rate compared to the untreated negative controls. This effect was observed at three different time points during fermentation in both synthetic media and grape must. However, the described alteration of the GFR by enzymatic glucose conversion would not occur in normal alcoholic fermentations, where the most likely cause for abnormal glucose conversion would be the occurrence of competing microorganisms. Several heterofermentative lactic acid bacteria strains are known to utilize measurable amounts of glucose when present in high numbers. These bacteria metabolize glucose via the pentose cycle at a much higher rate than fructose (Henick-Kling, 1988). It has been shown that inoculation of high numbers of several *Oenococcus oenus* strains can cause alteration of the GFR. Whereas the mean GFR in normal, untreated fermentations after 90% completion was at 0.16 for synthetic media and 0.15 for grape must, the GFR values of all samples treated with bacteria and glucose oxidase was clearly below 0.1 (Schütz and Gafner, 1993a). This value can be used as an indication whether the GFR shifts into a problematic range, and is corroborated by observations from incompletely fermented wines, since most of the stuck musts investigated had a GFR of below 0.1 (Schütz and Gafner, 1993a; Snyman, 2006; Jolly, 2008; Table 3.1).

These observations suggested that increasing the GFR by addition of glucose could be a very simple and rapid method to reactivate the alcoholic fermentation process. Tests on large scale fermentation under actual winery conditions confirmed this hypothesis, also indicating that the GFR is indeed a major causative factor of stuck fermentation (ACW Changins-Wädenswil, Figure 3.1). However, the addition of glucose to wine is prohibited by legislation worldwide. Based on these facts the first aim of this study was to investigate the potential of fructophilic wine yeasts to specifically increase the GFR and to restart stuck fermentations.

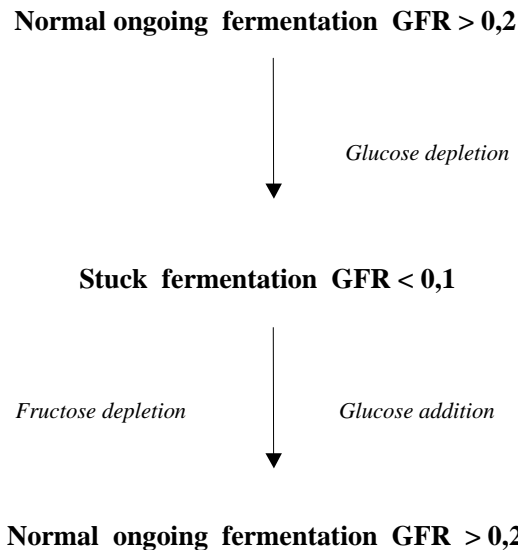


Figure 3.1: Observations about the connection between the GFR and fermentation problems in grape must.

3.1.3 Non-*Saccharomyces* and fructophilic wine yeasts

Fructophilic yeast species are defined as yeasts which prefer the utilisation of fructose, compared to glucose. The natural microflora of grapes and grape juice contains many characterized yeast species, but only two of them have been described as displaying a fructophilic phenotype, *Candida stellata (zemplinina)* and *Zygosaccharomyces bailii* (Schütz and Gafner, 1993a; Mills *et al.*, 2002).

Wines which are fermented without inoculation are the result of a combined action of several yeast species and strains. Natural fermentations are initiated by the growth of various genera such as *Candida*, *Debaryomyces*, *Hanseniaspora*, *Kloeckera*, *Metschnikowia*, *Pichia*, *Schizosaccharomyces*, *Torulasporea*, and *Zygosaccharomyces* (Esteve-Zarzoso *et al.*, 1998). Further species can be isolated from the environment of grapevine, grapes or wine (Table 3.1. 2). Their growth is generally limited to the first phase of fermentation, after which they die off and the stronger fermenting and more ethanol tolerant species of *Saccharomyces* take over the fermentation (Fleet and Heard, 1993; Combina *et al.*, 2005). Because of its almost invariable

dominance of wine fermentations, *S. cerevisiae* has become universally accepted as the principal wine yeast and is often simply referred to as “the wine yeast”. At best, the presence of the non-*Sacchaomyces* species can contribute to the final taste and flavour of wines by the production of secondary metabolites which are able to increase the complexity of the resulting wine. However, some members of these species are also able to spoil the wine by the formation of high amounts of undesired metabolites or off-flavours like acetic acid, abnormal esters, diacetyl or ethyl acetate (Bisson, 1999).

Table 3.1.2: A list of yeast genera according to Kurtzman & Fell (1998) that can be encountered in vineyards, on winery surfaces, in grape musts and/or in wine.

Teleomorphic ascomycetous genera (Ascomycotina)	Anamorphic ascomycetous genera (Deuteromycotina)	Anamorphic heterobasidio-mycetous genera (Basidiomycotina)
<i>Citeromyces</i>	<i>Brettanomyces</i>	<i>Cryptococcus</i>
<i>Debaryomyces</i>	<i>Candida</i>	<i>Rhodotorula</i>
<i>Dekkera</i>	<i>Kloeckera</i>	
<i>Hanseniaspora</i>		
<i>Issatchenkia</i>		
<i>Kluyveromyces</i>		
<i>Lodderomyces</i>		
<i>Metschnikowia</i>		
<i>Pichia</i>		
<i>Saccharomyces</i>		
<i>Saccharomyces</i>		
<i>Schizosaccharomyces</i>		
<i>Torulasporea</i>		
<i>Zygoascus</i>		
<i>Zygosaccharomyces</i>		

Strains of the fructophilic yeasts *C. stellata (zemplanina)* or *Z. bailii* are not able to dominate any phase of a natural alcoholic fermentation under normal winemaking conditions, although the cell numbers of *Candida* sp. in studies about fermentations kinetics can reach 10^5 cfu/ml in the first two days. However, after rapid growth of *S. cerevisiae*, no cfu from *Candida* sp. can be detected after 6 days of fermentation (Combina *et al.*, 2005). These cells might still be present throughout the fermentation, but in low numbers compared to *S. cerevisiae*, (Jolly *et al.*, 2006). To survive until the end of fermentation, the yeasts have to tolerate up to 12 vol% ethanol. Beside *S. cerevisiae*, only three species have been reported to fulfill this condition, namely both fructophilic species (Peynaud and Domercq, 1959; Combina *et al.*, 2005) and *Pichia* sp. (Bisson and Kunkee, 1991).

As mentioned above the fructophilic wine yeasts display poor fermentation efficiency and *S. cerevisiae* is always required to complete a wine fermentation. *Candida* sp. and *Zygosaccharomyces* sp. are therefore not suitable for a monocultural alcoholic fermentation

(Soden *et al.*, 1998; Soden *et al.*, 2000; Ferraro *et al.*, 2000). However, before fermentation stopped, *Candida* sp. was able to deplete the fructose from the medium, but not the glucose. The same behaviour could be observed in our own studies with several different strains of *Candida* sp. and *Z. bailii* (unpublished observations).

3.1.4 Microbiological aim and experimental strategy

The first intention of this study was the isolation of several strains from the fructophilic yeast species *Z. bailii* and the comparison thereof with another fructophilic yeast from the natural environment of grapevine, *C. stellata*. The aim was to ascertain the best representatives for the depletion of residual fructose in small scale stuck wine fermentations. This yeast should have the following characteristics:

1. the ability to metabolize fructose to ethanol and CO₂ under the difficult conditions of stuck wines which contain different and unknown inhibition factors;
2. no or very low production of wine spoiling secondary metabolites and off-flavours with a main focus on low acetic acid, and;
3. an ethanol tolerance similar to *S. cerevisiae*.

Z. bailii can be isolated mainly from grape juice and musts in the early stages of alcoholic fermentations and from musts of some unusual or problematic fermentation, but they can also be found on grapes and leaves or other parts of the grapevine (Schütz and Gafner, 1993b; Martini *et al.*, 1996; Esteva-Zarzoso *et al.*, 1998).

3.1.5 Isolation of different strains of the fructophilic yeast *Candida stellata/zemplinina*

C. stellata is an imperfect yeast with a taxonomic history characterized by numerous changes of names and definition. There are several taxonomic synonyms used for this yeast, namely *Torulopsis stellata*, *Torulopsis bacillaris*, *Brettanomyces italicus*, *Cryptococcus bacillaris* or *stellatus* and *Saccharomyces bacillaris* or *stellatus* (Kurtzman and Fell, 1998). These taxa were united in a single species named *C. stellata* for which the strain originally described as *S. stellatus* was chosen as type strain (CBS 157). In a recent publication the consequence of some controversial data about metabolic characteristics, e.g. low or high acetic acid production and low or high glycerol production during fermentation was investigated. It results in the assumption that most *C. stellata* strains isolated from grape must and described in these publications or preserved in Centraalbureau voor Schimmelcultures culture collection (CBS) belong to the new species *C. zemplinina*. This recently identified new species is closely related and can easily be

confused with *C. stellata* when conventional taxonomic tests or routine restriction fragment length polymorphism analysis of the internal transcribed spacer region are used for identification (Csoma and Sipiczki, 2008). The strain used in this study (FAW 3) and classified by us as *C. stellata* was also identified by this group as *C. zemplinina*. At the time of isolation, the species *C. zemplinina* was unknown as well as the taxonomic status of some other *C. stellata* strains which were used in a preceding study. On this account, the name *C. stellata* will be maintained in this thesis, knowing well the taxonomic reclassification. In previous publications, *C. stellata* is always mentioned in connection with a fructophilic and osmotolerant phenotype. Generally, there is no information about the metabolic phenotypes and possible fructophilic properties of the reclassified strain *C. zemplinina*.

An initial study from our group describes the comparison of 13 strains of the natural fructophilic yeast which were classified as *C. stellata*. Six of these strains have been isolated by Agroscope Research Station Changins-Wädenswil from the grapevine microflora or musts, four strains were found in Canadian must from the Niagara region (Holloway *et al.*, 1992) and three of the strains originated from the CBS collection. In initial experiments the fermentation behaviour was investigated in a Müller-Thurgau grape must from Wädenswil, Switzerland. Fructose was always metabolized faster than glucose, therefore, all of these strains indicated fructophilic behaviour to varying degrees. Due to the results of sensory evaluations and HPLC analysis, three of these strains were selected for the application in stuck fermented musts mainly because of low acetic acid production. In further vinification experiments the fructose decreasing ability was tested in 25 Liter wine fermentations under the condition of stuck musts. These medium scale fermentations were carried out with stuck musts of the varieties Gutedel (Chasselas) and Pinot Noir (Oil de Perdrix) (Porret *et al.*, 2003). The most suitable strain from this preliminary study, which gave best results for the criteria of ethanol tolerance, low acetic acid production and also in the sensory evaluation was *C. stellata* FAW3. In continuation of this work, the characteristics of this yeast was compared to the traits of several strains of the fructophilic yeast *Z. bailii* for the evaluation of yeast that would be best suited to cure stuck fermentations in alcoholic beverages. In the study of Csoma and Sipiczki (2008), *C. stellata* FAW3 was reclassified to the new species *C. zemplinina*, together with two of the 13 strains that were initially identified. One of these strains was confirmed as *C. stellata*, whereas the taxonomic status of the other nine strains was not investigated. Interestingly, the strains which displayed the strongest fructophilic properties in this study all belong to the new species *C. zemplinina* (FAW3, CBS1713 and CBS2649).

3.2 Materials and Methods

3.2.1 Isolation of strains of the fructophilic yeast *Z. bailii* from the indigenous microflora of the vine, naturally fermented wine and problem fermentations

Samples of grapes in different state of maturity and vine leaves, from the *Vitis vinifera* varieties Mueller-Thurgau, Pinot Noir and an unknown wild type were washed with sterile water supplemented with 0,9% NaCl and 1% Proteoseptone in a laboratory shaker with gentle shaking. All plant samples and grapes were collected in Wädenswil. Several dilutions of the washing liquid were spread onto YPF plates (1% yeast extract, 2% peptone, 2% fructose, 2% agar), supplemented with 5 % ethanol after autoclaving to avoid the growth of ethanol intolerant yeasts. The incubation temperature was 25°C.

In the same manner dilution series of natural fermented wines and incomplete fermented wines with residual fructose were spread onto similar plates. The samples were diluted with sterile water. The wine and must samples originated from different wineries from the Eastern region of Switzerland and included the varieties Pinot Noir, Pinot blanc and Müller-Thurgau.

For propagation, the yeast cells were grown in liquid YPF media. Incubation was carried out with vigorous agitation at 25°C.

3.2.2 Species and strain discrimination

3.2.2.1 Isolation of yeast chromosomal DNA

Chromosomal DNA was isolated using mechanical disruption of cells by glass beads followed by phenol/chloroform extraction as described in current protocols, Unit 13.11 (Cryer *et al.*, 1975). After harvesting by centrifugation, the cells were resuspended in breaking buffer (2% (v/v) Triton X-100, 1% (v/v) sodium dodecyl sulfate (SDS), 100 mM NaCl, 10 mM TrisCl, pH 8.0, 1 mM EDTA, pH 8.0), followed by the addition of glass beads and phenol/chloroform/isoamyl alcohol (25:24:1) and were vortexed for 3 min. at maximum speed. This was followed by the addition of 200 µl TE buffer, centrifugation at 13 000 rpm and transfer of the aqueous layer to a clean microcentrifuge tube. DNA was precipitated by the addition of 2.5 volumes of 100% ethanol. The precipitated DNA was harvested by centrifugation at 13 000 rpm and the pellet was washed once with 100% ethanol followed by a final washing step using 70% ethanol. The samples were dried and the precipitated DNA resuspended in sterile water.

3.2.2.2 ITS- PCR and restriction fragment length polymorphism (RFLP)

PCR reactions were performed using DNA Amplimix (Millipore) or GoTaq (Promega), according to the suppliers instructions. Annealing temperature was chosen between 48°C and 54°C. The species were discriminated by restriction fragment length polymorphism of the internal transcribed spacer ITS, amplified using the ITS1 (5'- TCCGTAGGTGAACCTGCGG-3') and ITS4 primers (5'- TCCTCCGCTTATTGATATGC-3'). The amplified PCR-products were digested with restriction endonucleases from Roche according to the supplier's manuals. The enzyme *Tru9I* was used for strain discrimination and *TaqI* and *CfoI* for species discrimination. The restriction fragments were separated by gel electrophoresis using a 1.2% agarose gel. Species identification was validated by the Centraalbureau voor Schimmelcultures in Delft, Netherlands.

3.2.3 Stuck wines for small scale fermentations

Four samples of industrial stuck wine fermentations (Table 3.2.5) from the viticultural regions Eastern Switzerland and Liechtenstein were provided by wineries in the 2003 vintage. All wines were treated with 30-50 mg SO₂ per liter by the winemakers to avoid the growth of undesired microorganisms. The incompletely fermented wines were pasteurized at 72°C for 30 minutes before inoculation.

3.2.4 Fermentation conditions

The fermentations were carried out in 0.5 Liter glass bottles at 25°C. The inoculation rate in all experiments related to the comparison of yeasts was 1x10⁸ cells/ml. For the co-inoculation series 1x10⁷ cells/ml *Z. bailii* and 50 g/hl of the commercial dry yeast *S. cerevisiae* Lalvin W15 were used. The fermentations were performed in duplicate and repeated at least two times.

3.2.5 Wine analysis by HPLC

The HPLC analysis was done according to the method by Kaufmann (Kaufmann, 1993, modified by U. Hämann, Bataillard and Cie, and by P. Hoffmann, ACW). This method allows the determination of the most important wine components, amongst others glucose, fructose, tartaric acid, malic acid, lactic acid, acetic acid, glycerol and ethanol. Wine samples were centrifuged for 10 min at 13'000 rpm. The supernatant was diluted 1:10 with 10 mM H₂SO₄

(0.2 µm filter sterilized). The diluted sample was filter sterilized by a 0.2 µm filter and transferred to the sampling tubes fitting the auto sampler. A Bio-Rad Aminex HPX-87H column (300 mm x 7.8 mm; particle size: 9 µm) was used for the analysis. Substances to be analyzed were eluted off the column by an isocratic flow (0.6 ml/min) of 2.5 mM H₂SO₄.

Table 3.2.5: Stuck wine sample analyses before treatment, used in this study

Number	Variety	Fructose (g/l)	Glucose (g/l)	GFR	Ethanol (g/l)	Free SO ₂ (mg/l)
1	Pinot Noir Liechtenstein	21.0	1.6	0.08	98.0	32
2	Pinot Noir East Switzerland	28.0	1.5	0.05	101.6	36
3	Sauvignon blanc East Switzerland	9.3	0.7	0.08	104.3	50
4	Pinot blanc de Noir East Switzerland	21.8	1.7	0.08	110.3	41

3.2.6 Cure of a stuck fermentation by glucose addition

For confirmation of the observations by Schütz and Gafner (1993a), which forms an essential foundation of the GFR hypothesis (Figure 3.1), the experiments indicating a restart of stuck fermentations after glucose addition were replicated. To achieve this, the GFR of a stuck Pinot Noir must from Liechtenstein was increased by the supplementation with glucose in medium scale fermentations in glass bottles with a volume of 25 L. The residual fructose of this wine was 11.5 g/l and residual glucose was 0.5 gram per liter, resulting in a GFR of 0.043. Prior to this, the wine was unsuccessfully treated by using traditional winemaking methods (warming to 25°C, re-inoculation with Uvaferm 43, 0.5 gram per liter dry mass) in the original fermentation vessel in the winery. About three weeks after this procedure, the wine was sterile filtered by decision of the producer and 50 liter of a total of 1 600 liter was provided by the winemaker for experiments at ACW Wädenswil. For inoculation, 0.5 g per liter of the dry yeast product *S. cerevisiae* Lalvin W15 was used. After two weeks with no activity, the wine was supplemented with glucose to a concentration of 3 g/l, which results in a GFR of 0.260. A second batch in a 25 liter glass bottle was used as negative control. This control was treated identically with the exception of the glucose addition.

3.3 Results

3.3.1 Isolates of *Z. bailii*

For the identification of the isolates, restriction fragment length polymorphism of the 5.8 internal transcribed spacer ribosomal DNA (rDNA) region was used. The ITS1 and ITS4 primers were used to amplify this gene repeat unit, which includes two noncoding regions designated as the internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene. PCR products showed a high length variation. Several species of yeasts could be isolated from the indigenous microflora in the vineyards by the methodologies described from both the grapes and from the liquid media. Besides several unidentified isolates, a total of 10 isolates could be identified as belonging to the fructophilic yeast *Z. bailii* by PCR restriction fragment length polymorphism with the use of the ITS1 and ITS4 primers for PCR amplification (White *et al.*, 1990; Guillamón *et al.*, 1998). The amplification product has a length of about 790 bp. For species discrimination the amplified product was digested with the restriction endonucleases *CfoI* and *TaqI*. A *CfoI* digestion of *Z. bailii* yields fragments with sizes of approximately 320 bp, 270 bp and two fragments of 95 bp. *TaqI* restriction of the ITS product results in fragments of nearly 370 bp, 220 bp and 200 bp (Esteve-Zarzoso *et al.*, 2003). Examples of yeast species identification are shown in figures 3.3.1 (PCR-products digested with *CfoI*) and 3.3.2 (same products digested with *TaqI*).

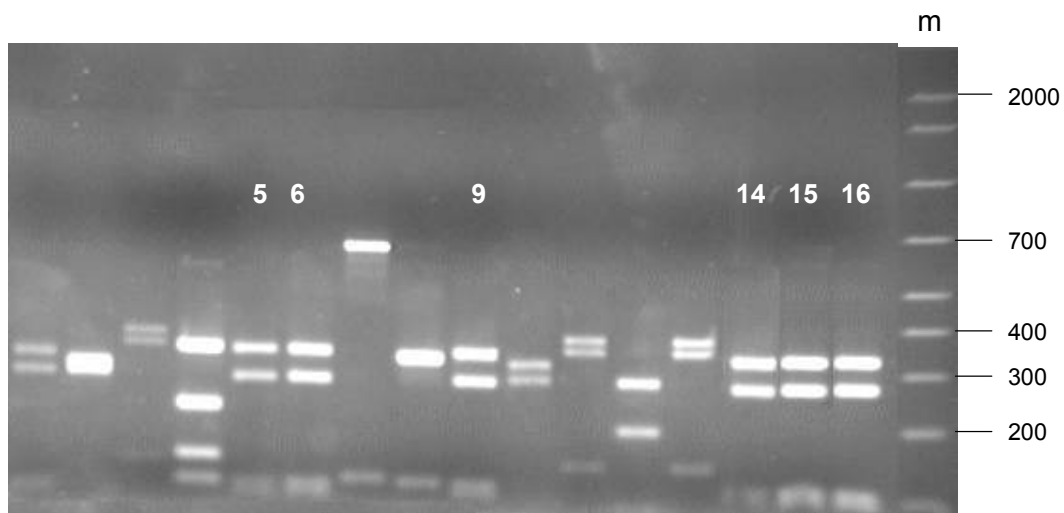


Fig. 3.3.1: ITS-RFLP of yeasts, isolated from the indigenous microflora in the vineyards or must, Lane 5, 6, 9, 14, 15 and 16 shows the pattern of the fructophilic yeast *Z. bailii*. Digested with *CfoI*. Lane m shows DNA size markers.

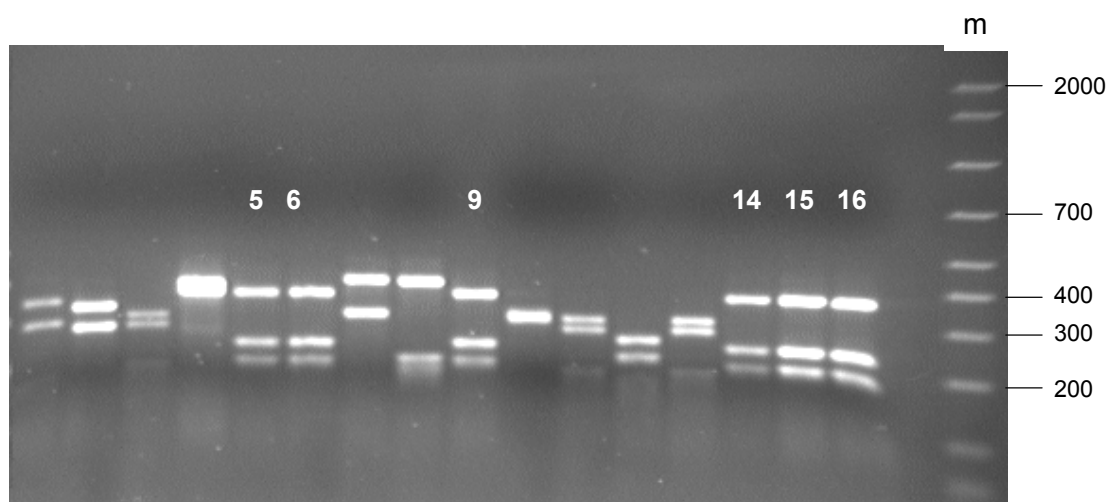


Fig. 3.3.2: ITS-RFLP of yeasts, isolated from the indigenous microflora in the vineyards or must, Lane 5, 6, 9, 14, 15 and 16 shows the pattern of the fructophilic yeast *Z. bailii*. Digested with *TaqI*. Lane m shows DNA size markers.

Five strains were isolated from grapes and parts of the grapevine plant and were named “the outside strains”. The remaining five strains were isolated from grape juice, musts and incomplete fermented musts and labeled as “the inside strains”.

It was possible to discriminate the outside strains from the inside strains with the same method, using the restriction enzyme *Tru9I*. After migration on an agarose gel, specific patterns for both types of strains could be found. The inside strains showed visible bands at approximately 250 bases, 270 bases, 130 and 100 bases, whereas the visible bands of the outside strains are at 400, 250 and 100 bases (Figure 3.3.3). In both cases there might be some invisible short fragments since the total length of the PCR product is of about 790 bases. However, it was not further verified if there are any genotypic differences within each group of strains, but all of the isolations were carried out at diverse time points and the substrate from which the yeasts could be isolated was derived from variable regions.

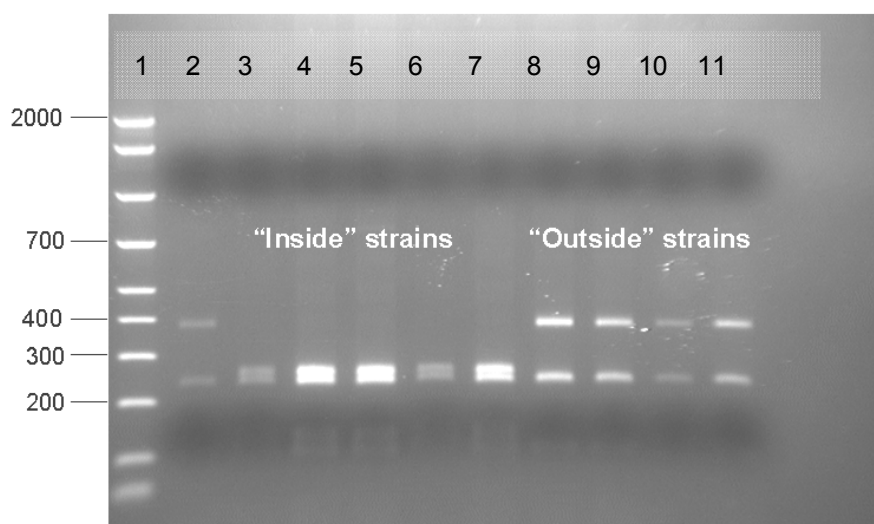


Fig. 3.3.3: Strain discrimination of the “outside” isolated strains of *Z. bailii* (lane 2, 8, 9, 10, 11) and the “inside” strains isolated from natural fermented wines (other lanes), detected by ITS-RFLP digested with the enzyme *Tru 9I*. Lane 1 shows DNA size markers.

3.3.2 Fermentation behaviour of fructophilic yeasts in incompletely fermented wines

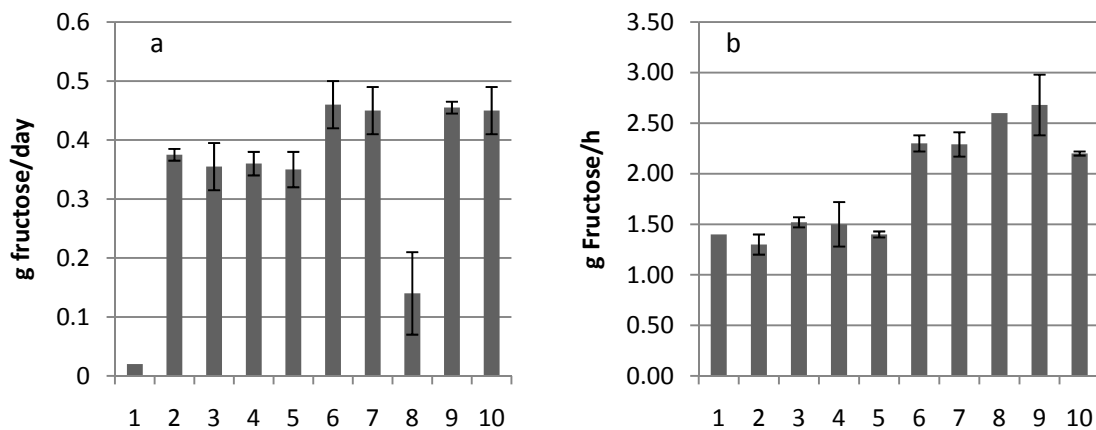
3.3.2.1 Differences within both groups of *Z. bailii*

Strains were screened in different musts that had ceased to ferment prematurely (stuck fermentation) to determine the ability for fructose depletion without production of acetic acid or further spoilage metabolic products. The first test series was completed in small scale fermentations with the objective of selecting the best suited yeast strains for use in incomplete industrial fermentations. All musts which were used in these preliminary trials were derived from stuck fermentations, according to our definition in chapter 2.1.1, which means that strains of the common wine yeast *S. cerevisiae* were unable to deplete the residual sugar even after reinoculation. In a first experimental series it was possible to assess differences between the isolated strains of *Z. bailii* when comparing the aerobic utilisation rate of fructose in synthetic YPF media and the fermentation rate in stuck wines. The fructose utilisation rate of *Z. bailii* was determined by inoculation to an initial cell density of 1×10^8 cells/ml in 2 % YPF (2 % fructose, 2 % peptone, 1 % yeast extract), incubation at 25 °C, shaking at 160 rpm for at least 4 hr. The fructose remaining after incubation was determined by HPLC.

The fermentation rate in stuck wine number three (Table 3.2.5) was determined in fermentation series at 25°C with the same inoculation rate of 1×10^8 cells per ml. Fructose depletion is indicated in gram fructose per day. It was not possible to observe any crucial differences within the two groups, neither in ITS-RFLP assays with several restriction enzymes (*CfoI*, *TaqI*, *Tru9I*, *Hinfi*, *HaeIII*; data not shown) nor in the rate of fermentative or aerobic

metabolism (Figures 3.3.2 a and b). Exceptions were strain 1 from the outside group and strain 8 from the inside group, which showed no (strain 1) or very low (strain 8) activity under fermentative conditions within stuck wines and were consequently excluded from further experiments. A clear difference between the remaining members of the inside and outside strains was detectable for both the fructose phenotype and the ITS genotype.

To confirm these results, one representative of the remaining members of each group was selected for further experiments. The member of the outside strains was labeled as *Z. bailii* 210 and the inside strain as *Z. bailii* 3a. Both strains were characterized by the Centraalbureau voor Schimmelcultures (CBS) and included in the yeast strain collection at Agroscope Research Station Changins-Wädenswil ACW.



Figures 3.3.2 a and b: Left hand diagram a shows fructose fermentation rate in stuck wine number 3 (see table 3.2.5) in gram fructose per day, right hand diagram b shows the aerobic fructose utilisation rate in YPF in gram fructose per hour; bars 1-5 for the outside strains and bars 6-10 for the inside strains.

3.3.2.2 Comparison of *Z. bailii* strain 210, 3a and *C. stellata* in small scale wine fermentation with pasteurized stuck wine samples

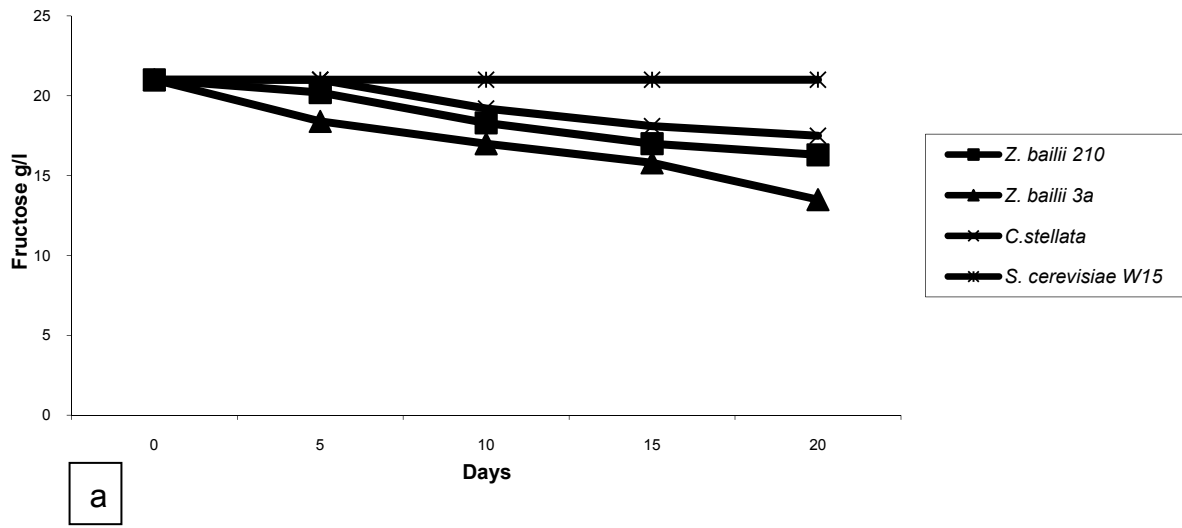
Microvinification experiments were carried out with the three selected yeasts. The yeasts were inoculated in 4 stuck wine samples, with two technical and at least two biological repetitions. These wine samples had been provided by wineries from Switzerland and Liechtenstein. *S. cerevisiae* W15 was used as a negative control to verify that *S. cerevisiae* was indeed unable to complete the fermentation. The inoculated cell density was 1×10^8 cells per milliliter, which was counted using a Neubauer counting cell chamber. As shown in Figure 3.3.2.2 a, b, c, and d, *Z. bailii* strain 3a (“inside strain”) showed the strongest fructose depletion rate in all investigated stuck wines, although to a different degree in each wine sample. *Z. bailii* strain 210 (“outside strain”) consistently showed a slightly lower fructose degrading activity in the stuck wine samples than strain 3a. However, both *Zygosaccharomyces* strains displayed

beneficial characteristics in the treatment of stuck wines when compared to *C. stellata* (*zemplinina*) strain FAW3. As expected, *S. cerevisiae* was not able to reduce the fructose content in the conditions tested. We also confirmed that the fructophilic yeasts did not metabolize the residual glucose content in any of the wine samples. This phenomenon was initially illustrated using mono-cultural and bi-cultural grape must fermentations carried out by some fructophilic yeast strains of *C. stellata* that depleted fructose and had stopped fermenting (Soden *et al.*, 1998; Soden *et al.*, 2000). Similar to the data generated in our study, only a reduction in fructose is observed resulting in an increase of the glucose/fructose ratio. However, as indicated on the graphs, none of the fructophilic yeast strains was able to ferment stuck wines to dryness, and each individual experiment was characterized by different fructose reduction kinetics.

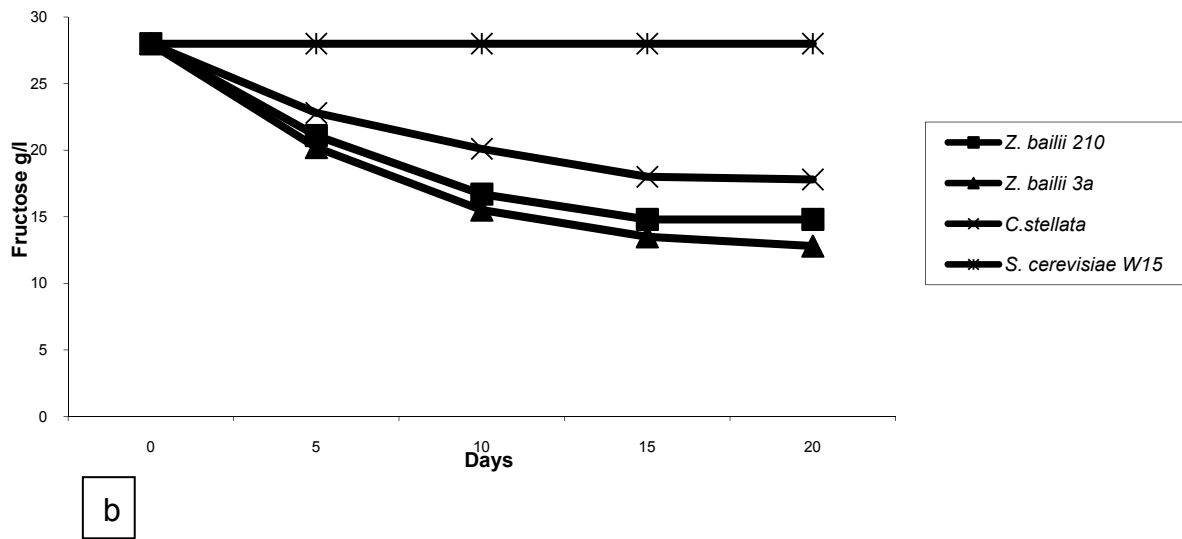
This may possibly be explained by the presence of many other yeast inhibitory factors in those samples, such as high ethanol levels (exceeding 10 vol%), a concentration of acetic acid between 0,6 and 1,05 g/l and the presence of between 35 and 50 mg/l of the preservation agent SO₂. The synergistic effects of these inhibition factors are difficult to assess, as is the case in most stuck fermentations in practice. When survival rates were assessed through measurement of colony forming units (cfu), the data show a continual decrease after inoculation of *Z. bailii*. Table 3.3.2.1 shows this decrease of living cells during the treatment of stuck wine 1 at different time points after an initial inoculation of 1x10⁸ cfu per ml. These data suggest that the observed decrease in fructose levels was due to uptake of the sugar by non-growing cells.

The concentrations of acetic acid as well as volatile acidity in all samples remained constant, with only slight increases of maximal 0.1- 0.2 g/l in some cases, as detected by HPLC. The strains therefore all appeared potentially of interest for the treatment of stuck fermentation. However, compared to the group of the outside strains of *Z. bailii* and the tested strains of *Candida sp.*, the strain 3a seems to have the most promise for the treatment of stuck fermentations in industrial winemaking environments.

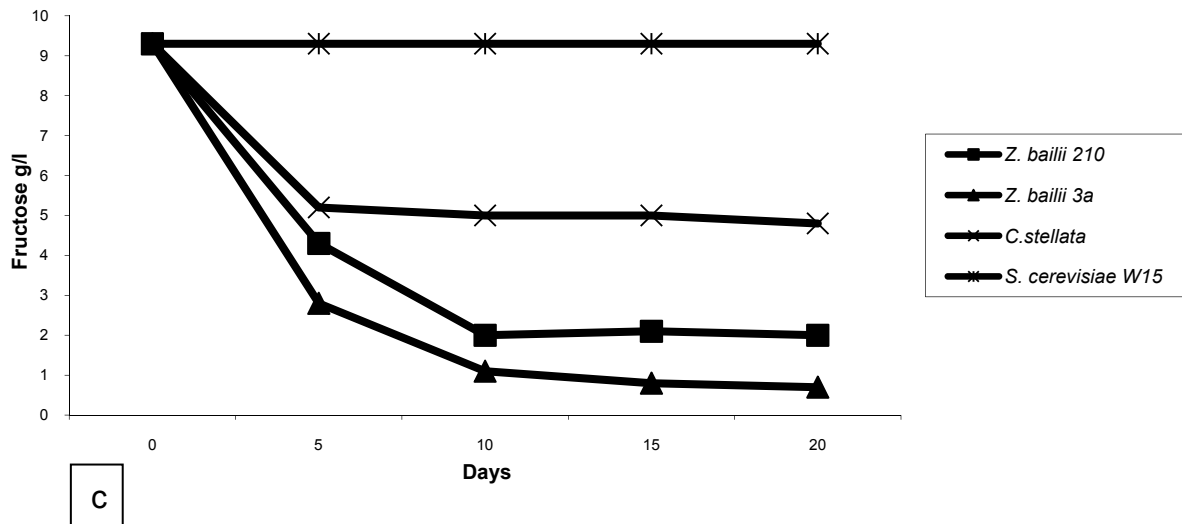
Wine 1

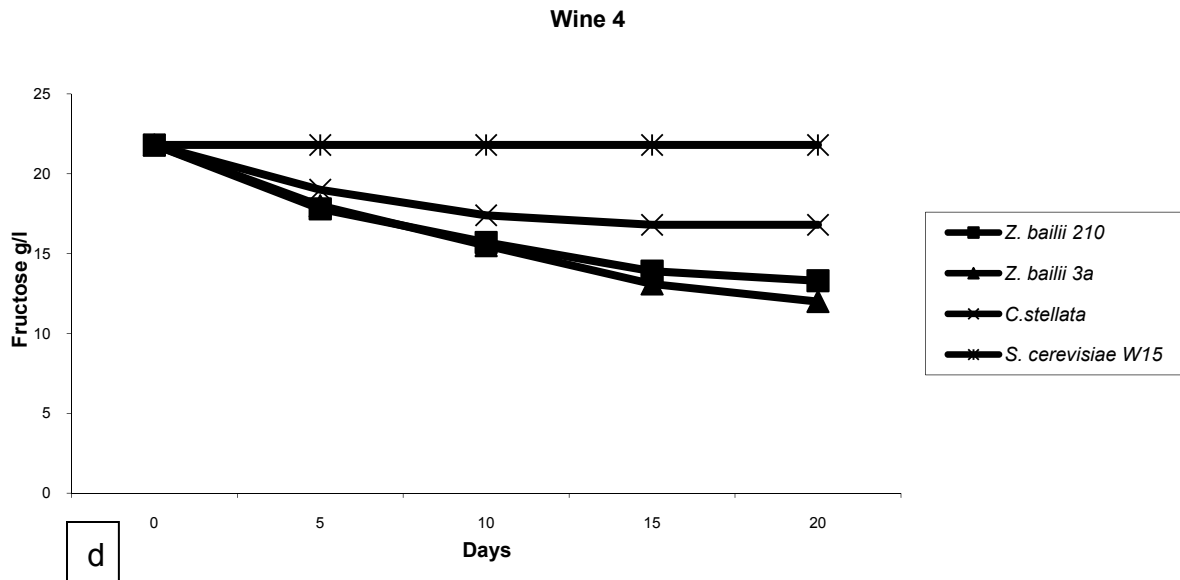


Wine 2



Wine 3





Figures 3.3.2.2: Comparison of three fructophilic yeasts and *S. cerevisiae* as negative control in four different samples of incompletely fermented wine. a) Pinot Noir, b) Pinot Noir, c) Sauvignon blanc, d) Pinot Noir (see table 3.2.5).

Table 3.3.2.2: Decrease of cfu/ml during treatment with *Z. bailii* 210 in wine 1

Days	Cfu/ml	Fructose g/l	GFR
0	1.0×10^8	21.0	0.076
10	3.1×10^4	18.3	0.087
20	0.7×10^3	15.8	0.1

3.3.2.3 Optimization of inoculation cell density

The fructose depletion rate of the treatments of incompletely fermented wines is likely dependent on several factors, including firstly the combination and strength of inhibiting parameters that led to the fermentation becoming stuck in the first place. These parameters will vary between each individual stuck fermentation samples. Due to the synergistic action of inhibiting parameters, the impact of such parameters is difficult to assess or predict. A second factor likely to impact on the fructose depletion rate is the inherent fermentation efficiency of the yeast strain chosen for treatment. This efficiency can be assessed in standard must or synthetic media. However, for the practical treatment of stuck wines this factor may also be strongly affected by the inhibiting factors characterizing the stuck fermentation. Due to the difficulty of reproducing data in such highly variable and rather hostile environments presented by stuck fermentations, a third factor to be considered is the inoculation cell density of the fructophilic yeast. Figure 3.3.2.3 indicates the correlation between the inoculated cell counts and the fructose depletion rate in stuck wine sample 1. This stuck wine was inoculated with four different amounts of cells from *Z. bailii* 210. The wine presented a GFR of 0.08 and contained 1.05 g/l

acetic acid and 12.4 vol% ethanol, which suggested that it would present a significant challenge for treatment. The data clearly show that the higher the inoculated cell concentration, the faster fructose is removed from the media, and that an inoculation density of more than 5×10^7 cells *Z. bailii* per milliliter of wine was necessary for achieving an increase in the GFR to above 0.1.

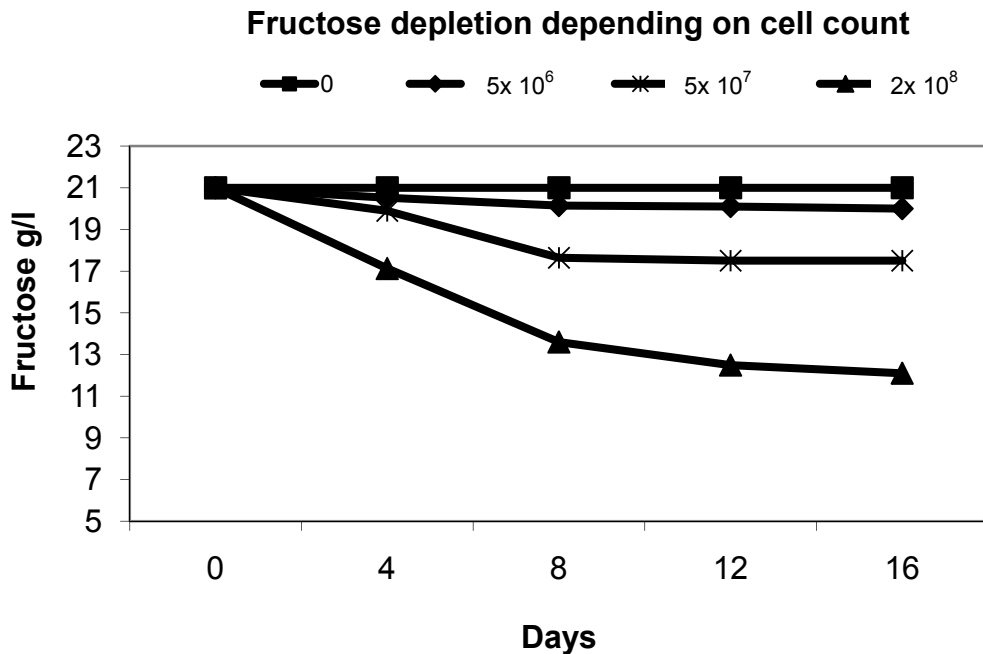


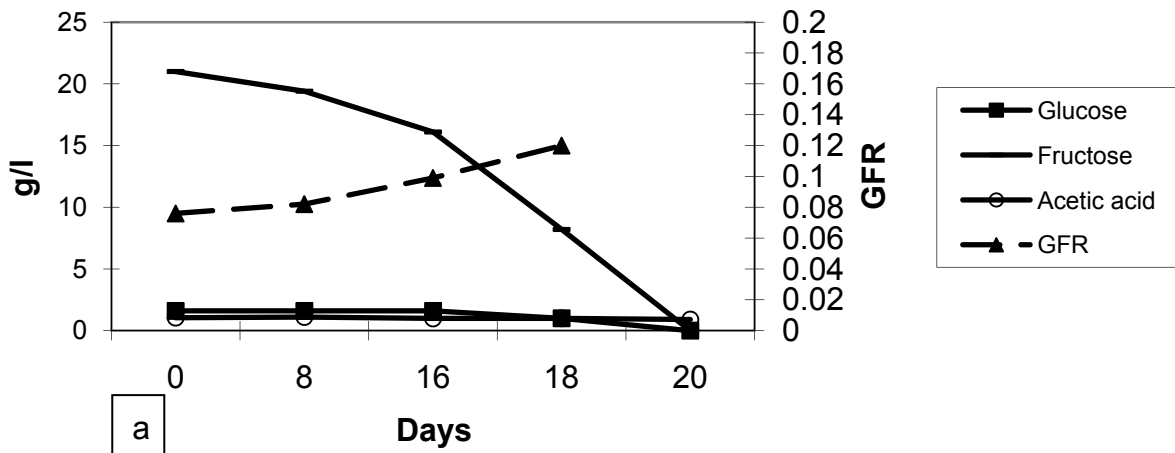
Figure 3.3.2.3: Fructose depletion rate for different inoculation densities in stuck wine 1, inoculated with *Z. bailii* 210.

3.3.2.4 Fermentation to dryness by co-inoculation of *Z. bailii* and *S. cerevisiae*

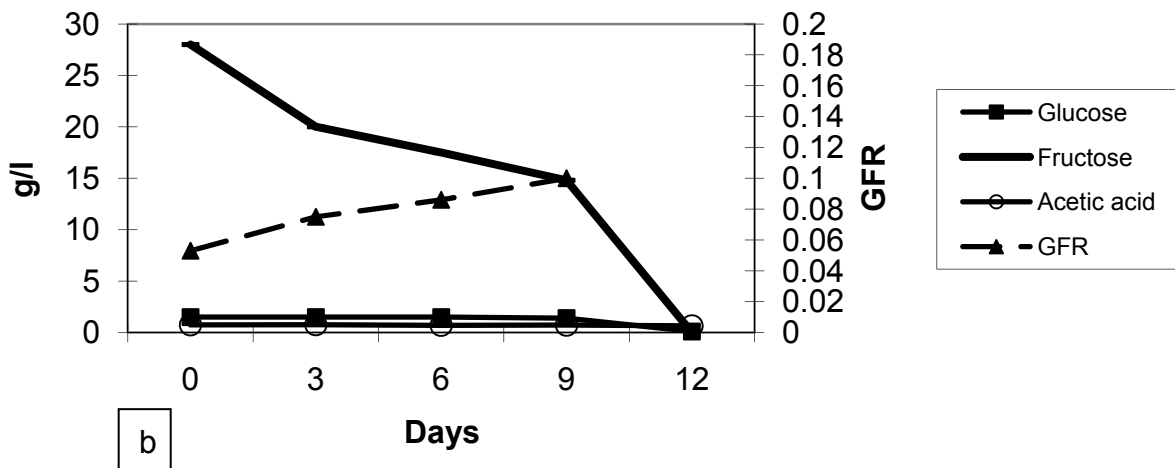
As mentioned above, the pure culture inoculation of any fructophilic yeast appears insufficient to ensure a complete treatment and fermentation to dryness of stuck wines within an adequate period of time. We observed in every case of treatment a slight decrease of the residual fructose, whereas the residual glucose content remained unaffected by the fructophilic yeasts resulting in an increase of the glucose to fructose ratio. Strains of the general wine yeast *S. cerevisiae* appear unable to reduce sugar (glucose and fructose) in a stuck fermentation. To assess whether stuck fermentation could be cured through a co-inoculation strategy, the four stuck wine fermentations were co-inoculated with the fructophilic yeast strain of *Z. bailii* together with a particularly ethanol tolerant wine yeast from the species *S. cerevisiae*. *Z. bailii* was inoculated at 1×10^7 cells/ml, while the commercial dry yeast *S. cerevisiae* W15 was used as recommended by the manufacturer at 0.5 g/L. The data, as determined by HPLC analyses, show that fermentation of the residual sugars follows a similar pattern in all cases (Fig. 3.3.2.4). A first phase is characterized by a decrease of residual fructose probably by the inoculated fructophilic yeast in a slow process which lasts from several days up to some weeks, dependent

on the amounts of residual sugars. This decrease ensures that the GFR increases. At some stage, normal fermentation appears to restart, and the process will be finished within a few days. Interestingly, fermentation appears to restart when the GFR reaches values of about 0.1. Based on these observations, we suggest that the fructophilic yeast may increase the GFR to a level which is high enough for *S. cerevisiae* to ferment the residual sugar.

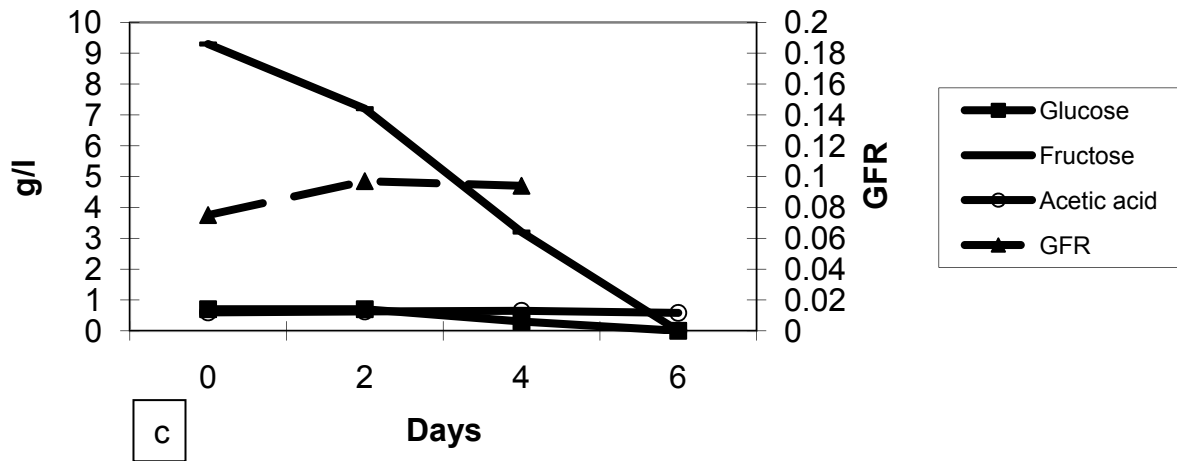
Co-inoculation wine 1



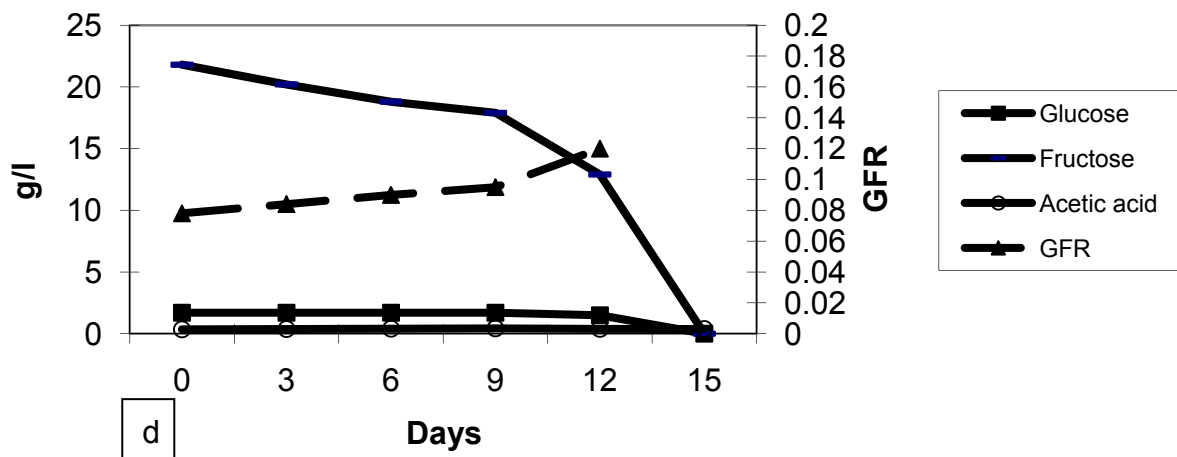
Co-inoculation wine 2



Co-inoculation wine 3



Co-inoculation wine 4



Figures 3.3.2.4: Stuck wines number 1-4 in a small scale treatment. *Z. bailii* 3a and *S. cerevisiae* W15 were co-inoculated for a successful cure of stuck fermentations. At the first phase the fructophilic yeast depletes the residual fructose without fermenting the glucose content with the result of an increasing GFR. Until the GFR achieve the value of about 0.1, both sugars start to be fermented, most likely by *S. cerevisiae*. a) Pinot Noir, b) Pinot Noir, c) Sauvignon blanc, d) Pinot Noir (see table 3.2.5).

3.3.3 Curing stuck fermentation by glucose addition

The publication of Schütz and Gafner (1993a) described a restart of fermentation by increasing the GFR triggered by glucose addition. To further assess whether the GFR may be the primary factor for restarting stuck fermentation in our conditions, *S. cerevisiae* W15 was inoculated into a stuck fermentation at an initial amount of 0.5 g/l dry mass. The fermentations were incubated at room temperature. As shown in Figure 3.3.3 during the first 21 days, no fermentation activity was observed. On day 21, glucose was supplemented to a concentration of

3 g/l. Fermentation activity could be observed approximately two days later and the fermentation was completed at day 26. The ethanol content increased from 103 g/l to 109 g/l, acetic acid content was stable during treatment at 1 g/l. A negative control was treated identically without supplementation of glucose. No change of the analysis values could be observed in the negative control to the point of 14 days after completion of the treated batch (data not shown).

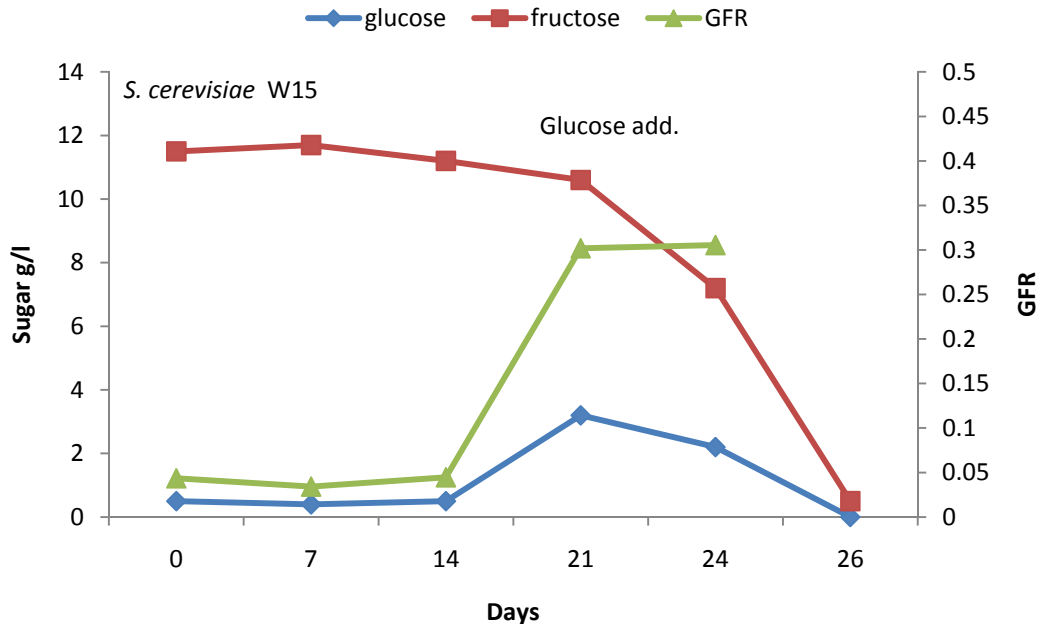


Figure 3.3.3: Cure of a stuck Pinot Noir by glucose addition in a 25 liter glass bottle. Yeast strain Lalvin W15 was inoculated into a stuck wine fermentation on day 0, and 3 g/l of glucose were added on day 21.

3.3.4 Conclusions

The conclusions drawn from this section of the work can be summarized in a hypothetical case of a stuck fermentation. The data indeed suggest that when must fermentation is at risk of ceasing prematurely at a late stage of the process due to the combination of several inhibitory factors, the residual sugar will be mainly fructose. During this late stage of the fermentation, the low GFR contributes significantly to the inability of glucophilic strains of *S. cerevisiae* to complete the fermentation resulting in the onset of a stuck fermentation. The inoculation of fructophilic yeasts into a stuck must results in a decrease of the GFR and finally in the completion of fermentation. The data also show that this completion requires the presence of *S. cerevisiae*, suggesting that the role of the fructophilic yeast is in fact limited to adjusting the GFR. *S. cerevisiae* appears able to restart the fermentation in such conditions. We identified the best suited candidate for the metabolism of excess fructose in these conditions, strain *Z. bailii* 3a, from an initial selection of 10 strains of the yeast *Z. bailii* and 13 strains of *C. stellata/zemplinina*. This choice was mainly focused on the absent (or very low) production of

wine spoilage metabolites and the highest fructose fermentation activity under the conditions tested.

This hypothesis is supported by the following findings:

1. The GFR of stuck musts is below 0.1 in the vast majority of all cases (own analyses 2003-2007, Table 2.1; Jolly, 2008).
2. Fructophilic yeasts can reduce fructose levels in most stuck musts, the glucophilic species *S. cerevisiae* not (This study).
3. The fermentation activity of *S. cerevisiae* in stuck fermentations with a low GFR can be restarted by glucose addition that are sufficient to increase the GFR above 0.1-0.2 (Schütz and Gafner, 1993a; Part 3.3.3 of this study).
4. We have shown that in about 90% of all cases of stuck fermentations the fermentation activity of *S. cerevisiae* can be restarted after the inoculation of fructophilic yeasts which are able to increase the GFR to above 0.1-0.2 by fructose depletion.
5. *Z. bailii* strains 3a as well as 210 are able to cure stuck fermentations.

3.4 Large scale fermentation: Curing stuck fermentations under conditions of commercial wine production

3.4.1 Introduction

The aim of the microbiological part of this thesis was to establish an effective tool for a serious problem associated with commercial wine production. The work in the following section focuses on the development of a methodology for the cure of large scale stuck fermentations under conditions of industrial winemaking. Based on the data from small scale fermentations we extended these results from laboratory scales to industrial scales with volumes of between 1'000 Liters up to 16'000 Liters of stuck must.

A general problem with conducting such work in an industrial environment is that in most cases of incompletely fermented musts, no negative or positive controls regarding the development of the fermentation can be integrated into the experimental design. Furthermore, our investigation in these conditions was restricted to after-the-fact interventions, following reports by winemakers on the occurrence of a fermentation problem. Fermentation monitoring

by wine producers is generally restricted to the indirect measurement of total sugar by the assessment of specific gravity or density of the fermentation. The measurement of the sugars fructose and glucose requires sophisticated analytical equipment like HPLC, FTIR or enzymatic assays. On account of this, the information about the reasons underlying the problems experienced in these fermentations is limited.

However, a significant, and in many ways unique advantage for our study was the fact that, as a contact point for vinification consulting, the department of beverage microbiology from Agroscope Research Station Changins- Wädenswil was able to investigate a large number of such problematic fermentations in many different commercial wineries. This significant number allows drawing of some significant conclusions even in the absence of controls or the lack of some supporting data.

The musts from problematic fermentations were mainly provided by wineries from Switzerland, Liechtenstein and Germany. The methodologies used to approach the problems were directly derived from the findings presented in the previous section. An important condition was that fermentations fulfilled the definition provided in part 3.4.4, that a struggling or sluggish fermentation is not stuck if it can be restarted by conventional winemaking techniques like re-inoculation with alcohol tolerant yeasts at high inoculation rate together with an increase of the fermentation temperature to 22° – 25°C. In stuck fermentations, these measures are insufficient to reduce the residual sugar content, and our methodology can be employed.

3.4.1.2 Development of a methodology for the cure of stuck fermentations

The experience gained by treating small scale fermentations and the close co-operation with concerned wine producers was instrumental in the successful development of the following methodology (Figure 3.4.1.2). The first step was the discrimination of sluggish fermentations and stuck fermentations. Sluggish or decelerated fermentation can be treated with normal winemaking procedures. The first activity after noticing a slowdown in fermentation activity involves the careful heating to a temperature of between 22°-25°C in order to support the activity of the wine yeast population. Additionally, this step results in a loss of CO₂ and therefore a reduction of one of the potentially inhibiting factors, carbon dioxide pressure (Bisson, 1999). On the other hand, increases in temperature will also increase the toxicity of ethanol (Henschke, 1997). Microbial monitoring is essential after the warming of wine due to the risk of accelerated growth of spoilage microorganisms. If there is any indication of bacterial or undesired yeast growth, an immediate reaction to ensure inhibition of microbial growth to avoid the production of undesired spoilage metabolites has to be implemented. Usually sterile filtration is recommended, however, the unwanted growth of bacteria can also be antagonized with a

lysozyme treatment. In some less serious cases of stuck fermentation, this method may lead to positive results, otherwise further treatment has to be considered. The second recommendation is re-inoculation with an ethanol tolerant strain of *S. cerevisiae* to a high cell number, usually 0.5 g of a dry yeast product per liter. The publication of Egliton and Henschke (2001) describes yeast strains, referred to as rescue yeasts, which seem to be particularly suitable for the restart of stuck fermentations. Generally, these yeast strains are characterized as tolerant against several known fermentation inhibiting factors, especially ethanol and acetic acid. After a period of about two weeks the combination of these conventional winemaking techniques will either result in a dry wine or in an absolute stuck fermentation with no measurable activity of the conventional wine yeast *S. cerevisiae*. It seems reasonable to assume that the inhibiting factors exceed a putative barrier which results in a complete prevention of fermentation activity. Only in such a case should the treatment with *Z. bailii* be considered, since fructophilic yeasts are weak fermenters and even a low activity of *S. cerevisiae* will result in a growth advantage. The inoculation rate of *Z. bailii* should be at least 1×10^7 cells per ml, since a higher inoculated cell number results in a reduced period of treatment. For optimal fermentation activity, the temperature has to be between 22°C and 25°C. Microbial monitoring must accompany the period of treatment, since microbial susceptibility of the fermentation is increased under conditions of residual fructose and elevated temperatures. In the majority of all experimental treatments, a slow decrease of the residual fructose was observed, continued by a rapid period of fast fermentation action, a restart of fermentation activity by the reinoculated *S. cerevisiae*.

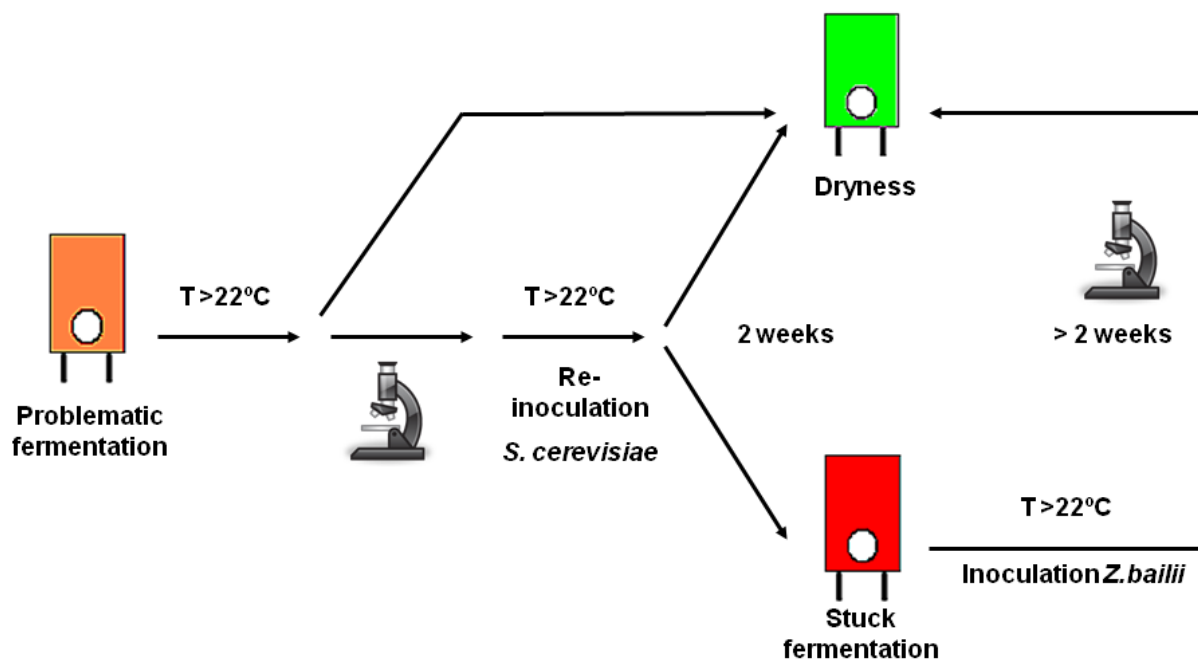


Figure 3.4.1.2: Methodology for the cure of stuck fermentations under commercial winemaking conditions.

Figure 3.4.1.3 outlines the hypothetical development of such a treatment strategy. In phase 1 of the example, at a late stage of alcoholic fermentation the continued faster consumption of glucose leads to a continued decrease of the GFR. Combined with other inhibition factors, this results in a deceleration of sugar consumption. Phase 2 is characterized by a stop of sugar consumption and no fermentation activity is measurable. After initiation of the described winemaking techniques, the inoculation of fructophilic yeasts takes place at phase 3 followed by a slow decrease in fructose. At one stage, fermentation will restart (phase 4).

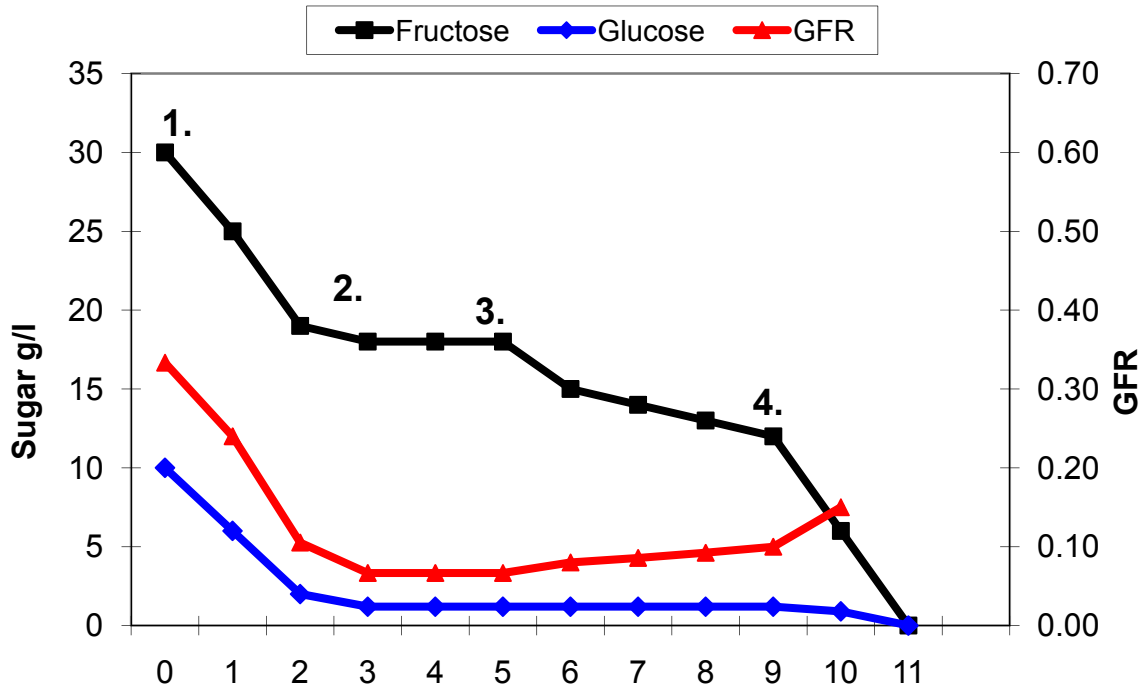


Figure 3.4.1.3: Putative example of a stuck fermentation, based on practical data from commercial wineries; A slowdown of fermentation activity is indicated at phase 1, GFR decreases due to the glucophilic phenotype; phase 2 shows a stuck of the fermentation activity, no sugar consumption can be observed; phase 3 means the start of the treatment with fructophilic yeasts, followed by a slow decrease of the residual fructose whereas glucose remains intact, consequently the GFR increases. At phase 4 the GFR is re-balanced onto an appropriate level for *S. cerevisiae*, the wine yeast restarts their fermentation activity and metabolizes the residual fructose and glucose.

3.4.2 Materials and Methods

3.4.2.1 Yeast strains

Based on the evaluation in the preliminary tests, *Z. bailii* strain 210 and *Z. bailii* strain 3a were used for the industrial trials. Both strains were isolated by Agroscope Research Station Changins- Wädenswil ACW in Wädenswil from the natural grapevine microflora and characterized by ACW and the Centraalbureau voor Schimmelcultures (CBS). In laboratory trials no production of wine spoilage metabolites nor off-flavours could be observed.

Lalvin W15 was used as co-inoculated *S. cerevisiae* strain for completion of the fermentations. Lalvin W15 is a commercially available wine yeast strain (Lallemand Inc.,) which was used as rehydrated dried yeast product. Rehydration was done according to the supplier's manual.

3.4.2.2 Propagation of *Z. bailii* strains

Enquiring winemakers that were confronted with the problem of incomplete fermented wines were supplied with the strains of *Z. bailii* in sufficient amounts to ensure a minimum inoculation number of 1×10^7 cells per ml.

Z. bailii strains 210 and 3a were maintained on 2 % YPF agar plates (2 % fructose (Fluka), 2 % peptone (DB Biosciences), 1 % yeast extract (DB Biosciences), 2 % agar (Acros Organics)). For the production of pre-cultures of both *Z. bailii* strains to be used for the treatment of stuck fermentations a single colony from the agar plate cultures was inoculated into 50 ml of 2 % liquid YPF (2 % fructose, 2 % peptone, 1 % yeast extract), incubated at 30 °C in a lab shaker under aerobic conditions until stationary phase of biomass production was reached.

For the first set of experiments, the propagation of *Z. bailii* 210 took place in several Erlenmeyer flasks with a nominal volume ranging between 2 and 5 liters under vigorous shaking in an incubator. For reproduction of higher volumes of *Z. bailii* 210, approximately 10 ml of the pre-culture was inoculated into 2 Liter autoclaved YPF media. After about 36 to 40 hours of cultivation, the cells were harvested by centrifugation.

For the propagation of *Z. bailii* 3a, a laboratory scale bioreactor (Infors AG) with a working volume of 5 L was used. The production batches were done in a 5 L scale in 5 % YPF (5 % fructose, 2 % peptone, 1 % yeast extract, pH 3.5). The medium was autoclaved in the bioreactor for 20 minutes at 121°C. After cooling down the medium approximately 10 ml of the pre-culture was inoculated into the bioreactor. As an alternative, a small volume of a previously produced culture population was used to inoculate the new batch. For aeration of the fermentation culture, filter sterilized air was used, and agitation was at 700 rpm. The

temperature was maintained at 30 °C. SIHA anti foam was used to avoid excessive foam formation in the culture vessel. Cells were harvested after a minimum of 36 hr of cultivation. The harvested culture was further used as wet biomass.

Due to the limited resources of manpower and equipment and the high demand on fructophilic yeasts in the viticultural region of Agroscope Research Station Changins- Wädenswil ACW, it was not always possible to deliver the targeted minimum amount of 1×10^7 cells per ml, especially for treatments with high volumes of affected must. In such cases the supply and the subsequent inoculation by the winemaker was divided into two batches. This was also applied to musts that were judged to present a higher level of other inhibition factors, when higher cell number than the recommended minimum amount of 1×10^7 cells per ml were thought to be required.

3.4.2.3 Treatment of stuck wines

The precondition for a treatment of problematic fermentations with *Z. bailii* was the prior application of the recommended traditional winemaking techniques by the winemaker. The department of beverage microbiology at Agroscope Research Station Changins- Wädenswil ACW supported this treatment by consultation and HPLC analysis of the affected wines. The microbial flora of the treated wines was observed by microscopy and/or molecular detection with RT-PCR. Weekly 100 ml samples for analysis were provided. However, the accurate enforcement of the treatment remained in all cases in the hands of the responsible winemaker.

3.4.2.4 Safety control

In several early publications, the genus *Zygosaccharomyces* sp. was mainly described as food spoilage yeast (Sousa *et al.* 1998; Prudencio *et al.*, 1998). Under high osmotic pressure, some strains seem to produce high amounts of acetic acid and *Zygosaccharomyces* sp. is also dreaded as a spoilage organism of bottled sweet wines. Criticism often refers to the osmotic tolerance, the relatively high resistance against preservatives like sulphur dioxide, sorbic acid, benzoic acid and the high resistance against acetic acid of some species and strains of *Zygosaccharomyces* sp. The main focus of the safety control therefore was to follow up on the inoculated strain to ensure absence of unwanted side effects.

- **Wine:**

To calculate a possible risk of a treatment with *Z. bailii*, we analyzed some of the treated wines at two time points, first after completion of the treatment and second after bottling.

Therefore the wines were plated out in different dilutions on 2% YPF respectively 2% YPD plates, followed by an incubation at 30°C for at up to 96 hours. After growth, colony morphotypes were differentiated visually and counted. The genomic DNA of random colonies was isolated for the identification of the yeast species by RFLP of the ITS-fragment.

- **Winery equipment:**

In the winery which hosted the first attempt, we investigated several samples taken from the winery equipment including tanks, barrels, filters, gaskets and tubes. Sampling equipment was done by random smear tests with sterile cotton buds followed by streaking on 2% YPD plates and incubation in liquid YPD media. Plates were incubated at 30°C, and liquid media maintained in a laboratory shaker at the same temperature. After first signs of microbial growth the liquid media was plated out in appropriate dilutions for separation of the cells. After colony growth, identification of the species was handled by ITS RFLP as described in the previous sections.

3.4.3 Results

3.4.3.1 Successful treatments of stuck wines in wineries

This methodology for the cure of stuck fermentations was tested in large scale stuck wine fermentations in wineries from Switzerland, Liechtenstein, Germany, Austria and South Tyrol. We attend to treatments of up to 16'000 Litres with the fructophilic yeasts strains *Z. bailii* 210 and 3a. The success rate of this new method was above 90% when used in winemaking scale fermentations on condition that the fermentation temperature of 22°-25°C and the recommended inoculation cell density was adhered to.

3.4.3.1.1 Example of treatments

Subsequently, some examples of successful treatments in commercial wineries will be presented. All treatments were done by the responsible winemakers of the concerned wineries. The wineries were invited for weekly sampling during the treatment, which were immediately analyzed per HPLC by Agroscope Research Station Changins- Wädenswil ACW. Microbial monitoring was included with the analysis.

The Figures 3.4.3.1.1 to 3.4.3.1.6 show the development of the sugar consumption after the analysis had established that the fermentation was stuck according to the definition of chapter 3.4.4, which illustrate up to the time of first inoculation with fructophilic yeasts. The inoculation cell density was 1×10^7 cells per ml, unless otherwise noted.

- **Wine 1:**

This example describes the first treatment of a stuck fermentation in a commercial winery with the fructophilic yeast species *Z. bailii*. The chemical characteristics of the wine before the treatment are described. The total volume of the treated wine was 16'000 liters of the grape variety Pinot Noir from a winery in Liechtenstein. The wine was a blend of six separately fermented Pinot Noirs which all were characterized as being affected by stuck fermentation. One part of this blend was also used in the small scale experiments and is described in part 3.2.5 as wine 1. The blend contained 18.9 g/l fructose and 1.2 g/l glucose as residual sugar corresponding to a GFR of 0.066. The ethanol concentration was 101 g/l and the acetic acid content was 0.77 g/l. A notably high glycerol concentration of 11.3 g/l was also a characteristic of this blend. This wine was co-inoculated with *Z. bailii* 210 and Lalvin W15 (Fig. 3.4.3.1.1). After co-inoculation a slow decrease of the residual fructose was observed presumably due to the activity of the fructophilic yeast. Consequently, the GFR increased and fermentation restarted, presumably by *S. cerevisiae*, until completion. The

residual sugar after the treatment was 1 g/l fructose and no glucose was detectable. Ethanol content increased during the treatment from 101 gram per Liter to 108.7 gram per Liter. A slight decrease of the concentration of acetic acid was observed, from 0.77 g/l to 0.65 g/l. This effect might be due to the emerging CO₂ production, which may result in the partial loss of this volatile acid. At the end of fermentation mainly viable cells of *S. cerevisiae* could be detected by ITS RFLP (Data shown in part 3.4.3.3), and no cfu of *Z. bailii* were identifiable, supporting the suggestion that it is indeed *S. cerevisiae* that is responsible for the final fermentation activity in the GFR adjusted must.

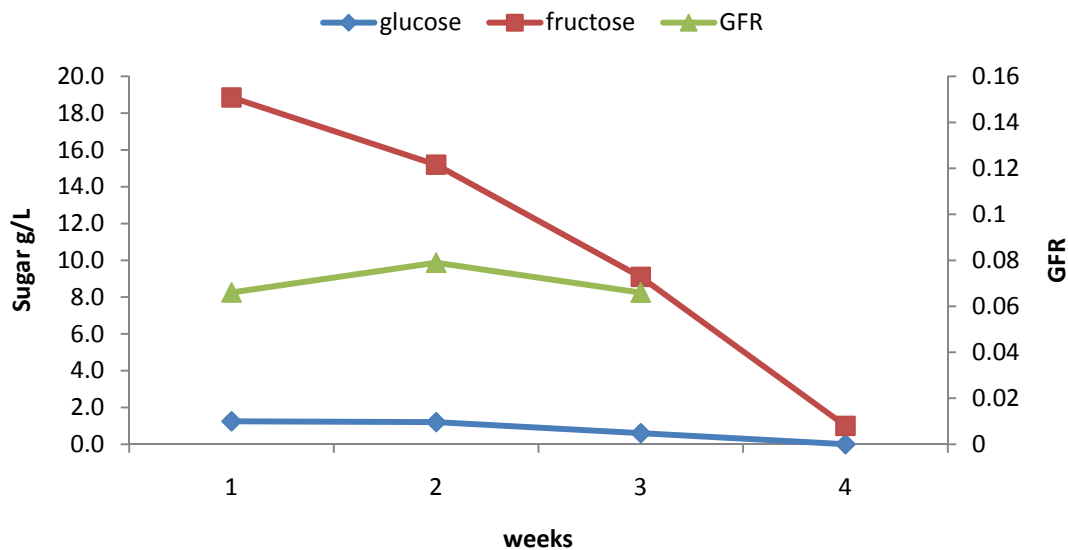


Figure 3.4.3.1.1: Wine 1, Blend of Pinot Noir, 16'000 Liters. On the left hand side of the figure sugar is indicated in gram per liter, the right axis shows the development of the GFR.

- Wine 2:

The total volume of the wine treated was 2'600 liters of the grape variety Pinot Noir from the viticultural region East Switzerland, vinified as a white wine ("Blanc de Noir"). The data are shown in Figure 3.4.3.1.2. The residual sugar content when treatment started was 21 g/l fructose and 1.7 g/l glucose corresponding to a GFR of 0.08. The ethanol volume was 13.5 vol% and the acetic acid content was 0.5 g/l. This wine was co-inoculated with *Z. bailii* 210 and Lalvin W15, followed by two single inoculations with *Z. bailii* 210 after 14 days and 28 days of treatment. The residual fructose slightly decreased during the treatment, but an acceptable GFR value was only reached by the application of a 3rd dose of the fructophilic yeast. For a restart of fermentation activity by *S. cerevisiae* an inoculation density of more than 1×10^8 cells per ml was used for this wine.

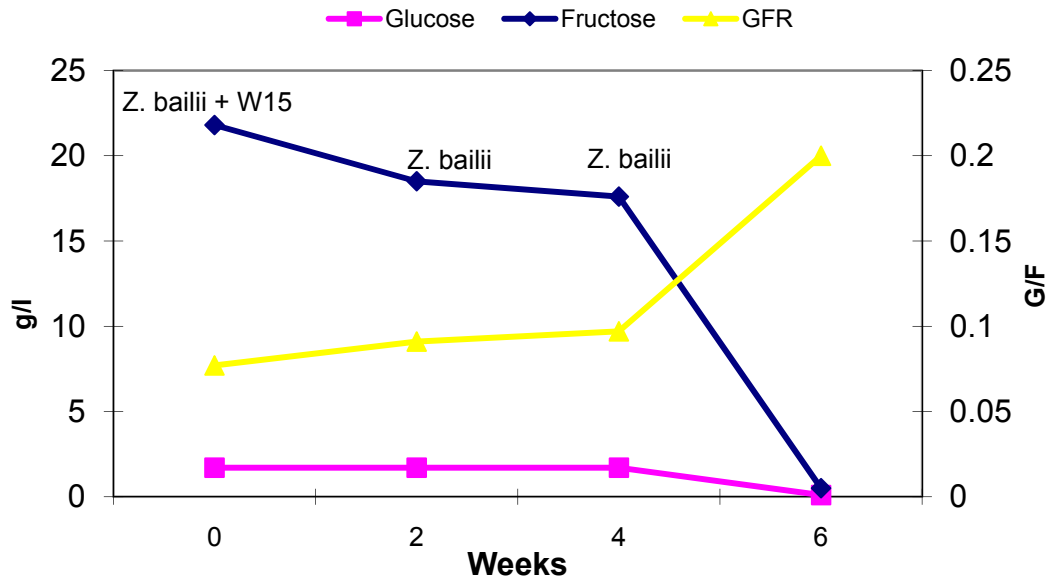


Figure 3.4.3.1.2: Wine 2, Pinot Noir “Blanc de Noir”, 2’600 Liters. On the left hand side of the figure sugar is indicated in gram per liter, the right axis shows the development of the GFR.

- **Wine 3:**

The characteristics of the wine before treatment are described. The total volume of the treated wine was 5’000 liters of the grape variety Pinot Noir from the viticultural region East Switzerland. The data are shown in Figure 3.4.3.1.3. The wine had 20 g/l fructose and 1.2 g/l glucose as residual sugar with a GFR of 0.06. The ethanol volume was 13% and the acetic acid content was 1.1 g/l. This wine was also co-inoculated once with *Z. bailii* 210 and Lalvin W15 followed by a single inoculation with *Z. bailii* 210 after 21 days due to the insufficient fructose utilisation rate after the first inoculation. The utilisation of residual glucose occurred after the GFR had reached a value of above 0.1. The fermentation was completed after another 20 days. The entire treatment took six weeks. At the end of fermentation, only viable yeast cells of Lalvin W15 could be detected and the acetic acid concentration was decreased from 1.0 g/l to 0.9 g/l probably due to fermentation activities.

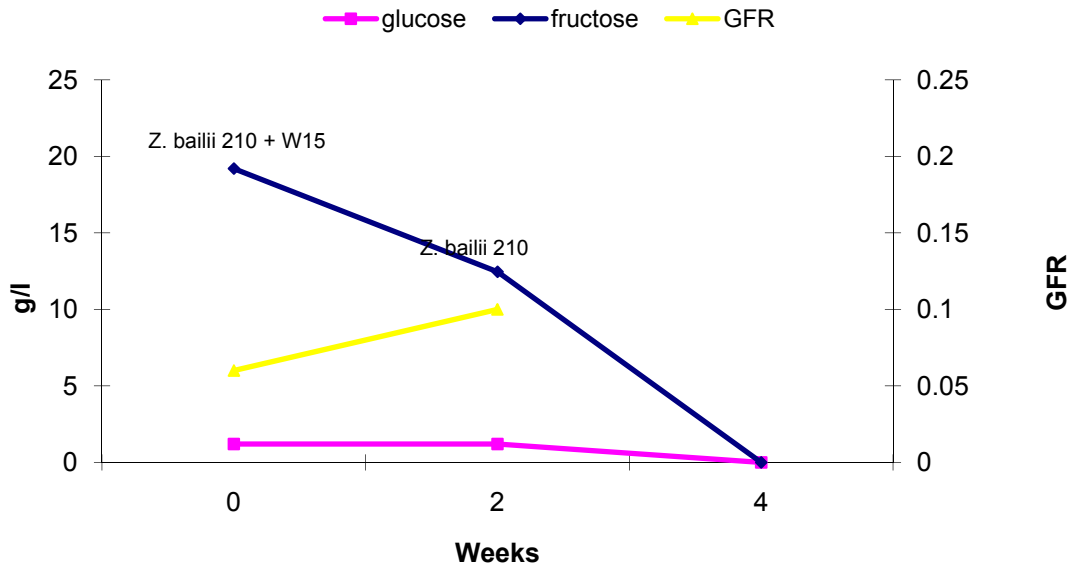


Figure 3.4.3.1.3: Wine 3, Pinot Noir, 5'000 Liters. On the left hand side of the figure sugar is indicated in gram per liter, the right axis shows the development of the GFR.

- Wine 4:

The total volume of the wine treated was 1'000 liters of the grape variety Garanoir from the viticultural region Eastern Switzerland, vinified as rosé wine. As shown in Figure 3.4.3.1.5, the residual sugar content was 16.8 g/l fructose and 0.5 g/l glucose corresponding to a GFR of 0.03. The ethanol content was 80 g/l and the acetic acid content was 1.2 g/l and slightly increased after treatment to 1.36 g/l. This wine was co-inoculated with *Z. bailii* 3a and Lalvin W15, followed by a single inoculation with *Z. bailii* 3a after 30 days of treatment to attain the minimum inoculation number of 1×10^7 cells per ml. Since a marginal amount of residual sugar was desired by the winemaker, the fermentation was stopped at 5 g/l fructose.

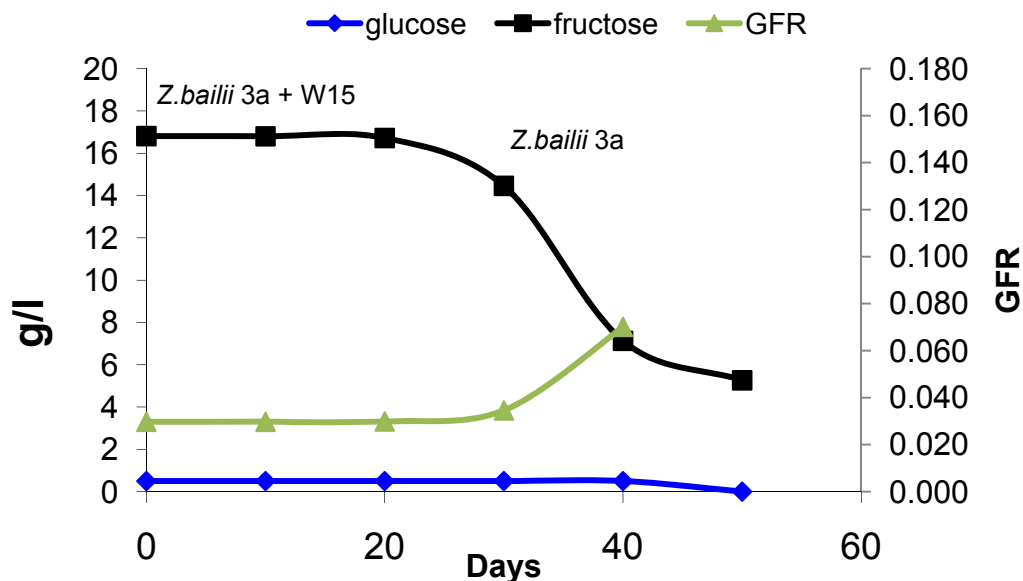


Figure 3.4.3.1.4: Wine 4, Garanoir rosé, 1'000 Liter. On the left hand side of the figure sugar is indicated in gram per liter, the right axis shows the development of the GFR.

- **Wine 5:**

The total volume of the wine to be treated was 2'000 liters of the grape variety Chardonnay from the viticultural region East Switzerland. Residual fructose was at 24 g/l, residual glucose at 2.5 g/l with a corresponding GFR of 0.104. The ethanol content was 89.6 g/l, acetic acid level was below 0.4 g/l. This wine was also co-inoculated once with *Zygosaccharomyces bailii* 210 and Lalvin W15. Fermentation was completed after 20 days with an ethanol content of 103.6 g/l and the same acetic acid level as before treatment.

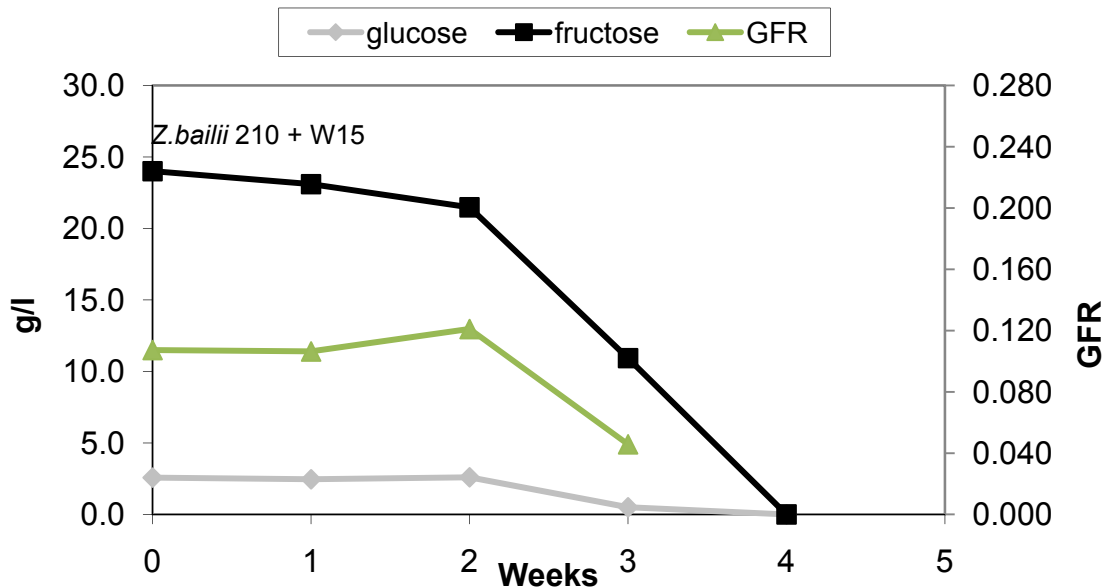


Figure 3.4.3.1.5: Wine 5, Chardonnay, 2'000 Liter. On the left hand side of the figure sugar is indicated in gram per liter, the right axis shows the development of the GFR.

- **Wine 6:**

This example describes the cure of 2'000 liter stuck must of the grape variety Chardonnay from Switzerland. The residual sugar content was 16.7 g/l fructose and 1.2 g/l glucose with a GFR of 0.072. The ethanol level was 98 g/l and the acetic acid content was 0.5 g/l. This wine was co-inoculated with *Z. bailii* 210 and Lalvin W15, followed by a single inoculation with *Z. bailii* 3a after 6 weeks of treatment. The first inoculation was done with a cryopreserved dry yeast product of *Z. bailii* 210. The residual fructose slightly decreased during the treatment, however, an acceptable GFR value was not reached until the second dose of fructophilic yeasts. The first batch of the cryopreserved *Z. bailii* may not have the same activity as fresh yeast; therefore the second inoculation was done with fresh *Z. bailii* strain 3a. The fermentation after treatment stopped at 4 g/l residual fructose and no glucose, no further treatment was desired by the winemaker. Acetic acid level was stable during the period of treatment.

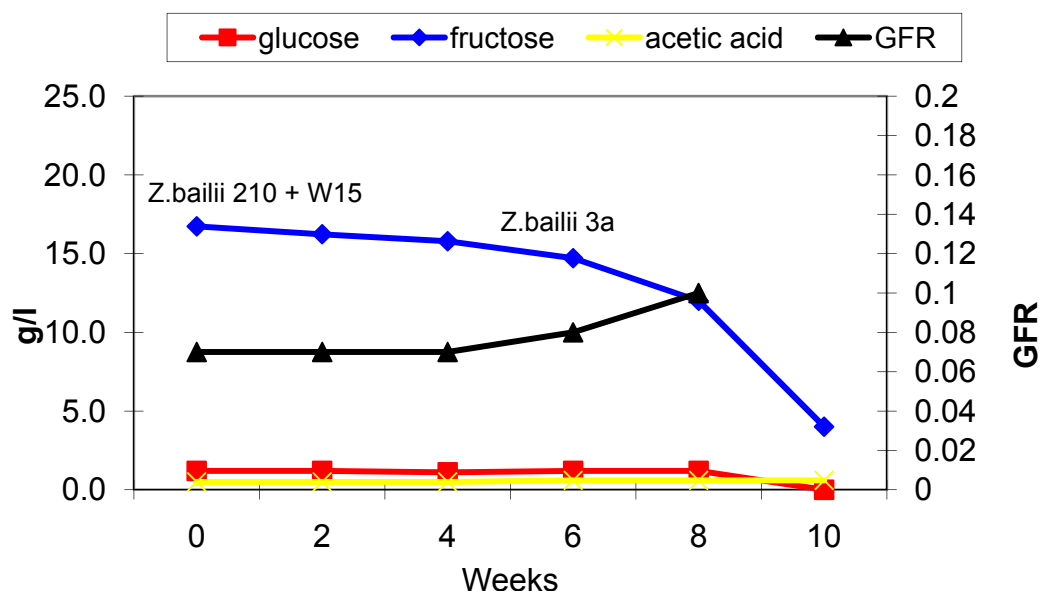


Figure 3.4.3.1.6: Wine 6, Chardonnay, 2'000 Liter. On the left hand side of the figure sugar and acetic acid is indicated in gram per liter, the right axis shows the development of the GFR.

3.4.3.1.2 Summary of further treatments

A number of other wines were successfully treated, and the results are shown in Table 3.4.3.1.2. As can be seen from these data, these treatment represent several grape varieties and wine making practices, such as barrel and tank fermentations. Taken together, these data clearly demonstrate the general applicability of the proposed methodology over a wide range of stuck fermentations.

Table 3.4.3.1.2: Additional successful treatments of stuck musts in wineries, located in Eastern Switzerland. All wines were additionally co- inoculated with 50 g/hl Lalvin W15.

Wine Nr.	Variety	Volume L	Before treatment			Treatment with		After treatment	
			Fru g/l	Glc g/l	GFR	<i>Z. bailii</i> 210	<i>Z. bailii</i> 3a	Fru g/l	Glc g/l
7	Sauvignon blanc	1'600	9,3	0,7	0,08	1x10 ⁷ cells/ml		2,5	0
8	Chardonnay	1'000	13,9	0	n.d.		1x10 ⁷ cells/ml	4,3	0
9a	Chardonnay Barrique	225	8.2	0	n.d.		1x10 ⁷ cells/ml	1,5	0
9b	Chardonnay Barrique	225	12.8	0	n.d.		1x10 ⁷ cells/ml	2,2	0
9c	Chardonnay Barrique	225	16.8	0.6	0.036		1x10 ⁷ cells/ml	0,2	0
9d	Chardonnay Barrique	225	26.7	2.6	0.097		1x10 ⁷ cells/ml	0	0
9e	Chardonnay Barrique	225	31.3	3.8	0.121		1x10 ⁷ cells/ml	0,5	0
10	Chardonnay	1'800	13,2	0	n.d.		>1x10 ⁸ cells/ml	1,2	0

11	Pinot Noir	2'600	19,6	2	0,102	1x10 ⁷ cells/ml	1	0
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n.d.= not detectable.

3.4.3.2 Unsuccessful treatments

During the vintage 2004, the treatment of about 6'075 liter of stuck must was not successful. In the case of 4'075 liter it was not possible for the responsible winemakers to increase the temperature of the affected fermentation (Wines U1, U2a, U2b, U2c and U4; see table 3.4.3.2), as recommended in the standard methodology. Furthermore, wine U1 contained mannit, which can be seen as a marker substance for the heterofermentative metabolism of unwanted lactic acid bacteria, whereas wine U4 was spoiled with a high concentration of acetic acid. Both of the spoiling metabolites were present before treatment of these wines with *Z. bailii*.

Interestingly, and with the exception of wine U1, all unsuccessful treatments are characterized by a GFR of between 0.15 and 0.36 which would normally not be considered problematic. The GFR as therefore probably no reason for the problems experienced in these fermentations, and the failure of our methodology is therefore not unexpected. In fact, these data further confirm that the reason other treatments were successful is indeed directly linked to the low GFR in these cases. The data also suggest that these problem fermentations are the result of single and strong inhibiting factors that probably appeared at an early stage of fermentation.

Only in the case of U3 was there no evident other reason for the failure, and in this case, the treatment was canceled due to the decision of the responsible winemaker.

Table 3.4.3.2: Unsuccessful treatments of stuck wines with *Z. bailii* in vintage 2004.

Wine Nr.	Variety	Volume L	Fru g/l	Glc g/l	GFR	Remarks
U1	Pinot Gris	2600	13,7	0,5	0,036	Wine contains Mannit; Temperature < 18° during treatment
U2a	Pinot Noir Rosé Barrique	225	18,9	5,7	0,30	Temperature 16°C during treatment
U2b	Pinot Noir Rosé Barrique	225	22,6	7,1	0,31	Temperature 16°C during treatment
U2c	Pinot Noir Rosé Barrique	225	21,6	6,8	0,31	Temperature 16°C during treatment
U3	Sauvignon blanc	2000	22,7	3,5	0,15	

U4	Pinot Noir	800	33,4	8,1	0,24	Acetic acid 1,03 g/l; Temperature 17°C during treatment
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3.4.3.3 Safety controls

Most skepticism encountered during our experiments with fructophilic yeast was due to the perception that yeast from the genus *Zygosaccharomyces* can act as major spoilage organisms in industrial food production processes (Sousa *et al.* 1998, Prudencio *et al.*, 1998). The methodology presented here is, indeed, very unusual as a treatment in beverage processing. However, the strains presented in this publication indicate no negative attributes during the treatment in stuck wines. In no case could we detect a substantial increase of acetic acid. Secondly, no negative sensory impact could be detected during the treatment, as was confirmed by the winemakers of the affected wineries. For treatment in large scale stuck fermentation, an accurate filtration after the usage will minimize a possible risk of undesired spoilage post fermentation.

Z. bailii and other non-*Saccharomyces* yeast species can be isolated from the grapevine microflora, grape juice and natural fermentations, but in all reports about growth kinetics in wine fermentations they die off and the stronger fermenting species *S. cerevisiae* take over the fermentation (Fleet and Heard, 1993; Schütz and Gafner, 1993b; Esteve-Zarzose *et al.*, 1998; Combina *et al.*, 2005). In our experiments, although inoculated at a late stage of fermentation and in a high cell number, *Z. bailii* was also overgrown by the wine yeast after they take over fermentation.

3.4.3.3.1 Wines

- Wine 1 after treatment:

The sample after completion of the fermentation was used to plate out 100 µl of a 10⁻⁴ dilution on YPD. After incubation for 36h at 30°C, about 50 colonies were analysed. Visual differentiation of the colony morphotypes showed only one morphologically different colony out of approximately 250 colonies screened visually. The results of an ITS-RFLP of seven different and randomly picked colonies are shown in Figure 3.4.3.3a. The expected sizes of the PCR products is 880 bp for *S. cerevisiae*, 470 or 500 bp for *Pichia sp.* and 775 bp for *Z. bailii* (Guillamón *et al.*, 1998). Figure 3.4.3.3b shows the specific *HhaI* fragment pattern, which indicates that the seven colonies can be identified as *S. cerevisiae*, while the single morphologically different colony can be identified as belonging to *Pichia sp.*

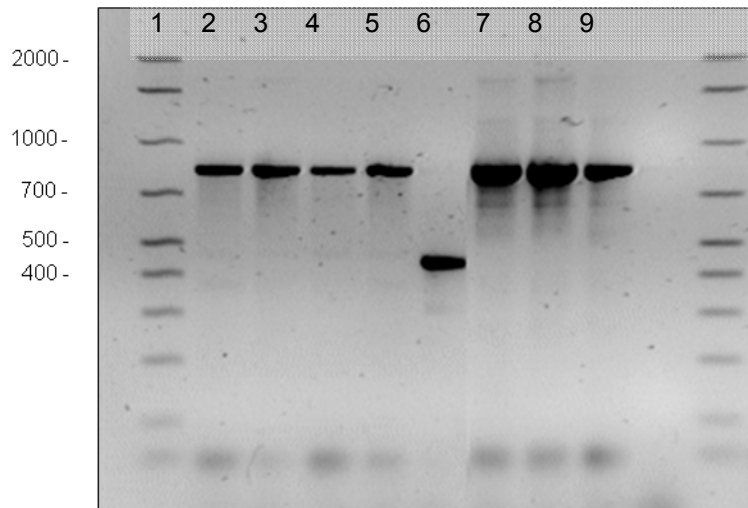


Figure 3.4.3.3 a: PCR-product of ITS fragment of seven randomly (lanes 2-5 and 7-9) and one selective (lane 6) picked colonies, grown on YPD plates spread out with wine 1 directly after treatment. Lane 1 shows DNA size markers. No cfu of *Z. bailii* could be identified.

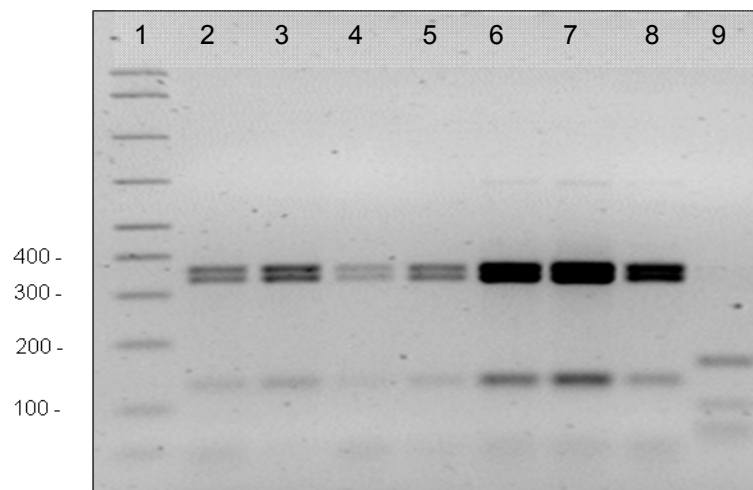


Figure 3.4.3.3 b: RFLP with *Hha* I of ITS fragments from figure 2.4.3.3 a. Seven colonies of *S. cerevisiae* (Lanes 2-8) and one *Pichia* sp. (Lane 9) could be identified. Lane 1 shows DNA size markers.

Before bottling, the treated wine was sterilized by cross-flow filtration before filling and no yeast colonies grew after plating of the post-bottled wine on YPD plates.

The various post-bottling microbiological analyses are summarized in Table 3.4.3.3.1. The data clearly suggest that there were no microbiological contamination problems in any of the treated wines.

Table 3.4.3.3.1: Summary of security checks; colony forming units of yeasts after treatment and after bottling of different cured wines from commercial wineries.

Wine	Colonies after treatment	Identification	Colonies after bottling
Wine 1	5000/ μ L	1 <i>Pichia</i> sp. <i>S. cerevisiae</i> >99%	n.d.
Wine 2	8800/ μ l	<i>S. cerevisiae</i> 100%	n.d.
Wine 3	2300/ μ l	<i>S. cerevisiae</i> 100%	n.d.
Wine 5	1100/ μ l	<i>S. cerevisiae</i> 100%	n.d.
Wine 7	7100/ μ l	<i>S. cerevisiae</i> 100%	n.d.

n.d.= not detectable.

3.4.3.3.2 Safety control of winery equipment:

The main focus of this test was to screen for the inoculated *Z. bailii* strain to disprove the argument of a contamination of the winery's equipment for vinification. Bacterial and mould colonies were ignored in this procedure. The results showed the growth of several yeast colonies after inoculation and are indicated in percent of the total amount of grown colonies in table 3.4.3.3.2. While various yeast species were detected on the equipment, the data show that no *Z. bailii* could be identified, suggesting a very low or non-existing risk for such cross contamination.

Table 3.4.3.3.2: Yeast species isolated from cellar equipment of the winery from treated wine 1, identified by ITS-RFLP.

Yeast	Percentage
<i>S. cerevisiae</i>	38%
<i>Kloeckera</i>	21%
<i>Candida stellata</i>	5%
<i>Candida pulcherima</i>	9%
<i>Pichia</i> sp.	25%
Colored yeast colonies	2%
<i>Zygosaccharomyces</i>	0%

3.4.4 Conclusions

Incomplete or stuck fermentations are defined as those leaving a higher than desired residual sugar content at the end of alcoholic fermentation, while slow or sluggish fermentations are characterized by a low rate of sugar utilisation. This definition by Bisson (1999) became in the course of this work expanded to include an additional requirement:

We indeed defined a struggling or sluggish fermentation as a fermentation that can be restarted by conventional winemaking techniques such as:

1. Re- inoculation with an alcohol tolerant yeast at high inoculation rate and
2. Increase of temperature to 22° – 25°C

In our definition, a fermentation only is declared as stuck if the measures listed above are insufficient to reduce the residual sugar content. By this definition, a stuck fermentation indicates no fermentation activity even after a re-inoculation with any *S. cerevisiae* strain or any other conventional winemaking technique.

The industrial experiments in commercial wineries were done during two vintages. In the vintage 2003/2004 approximately 50'000 liter of stuck must was cured with the presented methodology that utilizes the fructophilic yeast *Z. bailii* strain 210. Approximately 5'000 liter was treated with conventional enological methods.

A volume of about 50'000 liter of stuck must was successfully treated in the vintage 2004/2005 with *Z. bailii* strain 3a and 210. The initial experiments were done with cryopreserved *Z. bailii* 210, which seems to have less activity compared to fresh yeasts, highlighting that the procedure and handling of cryopreservation of *Z. bailii* requires further testing (data not shown). The treatment of about 6'000 liter of stuck must failed. The majority of problematic fermentations of this vintage, a total about 74'000 liter, was cured using the conventional winemaking methods. A representation of the treatments from the vintage 2004/2005 is given in figure 3.4.4.1.

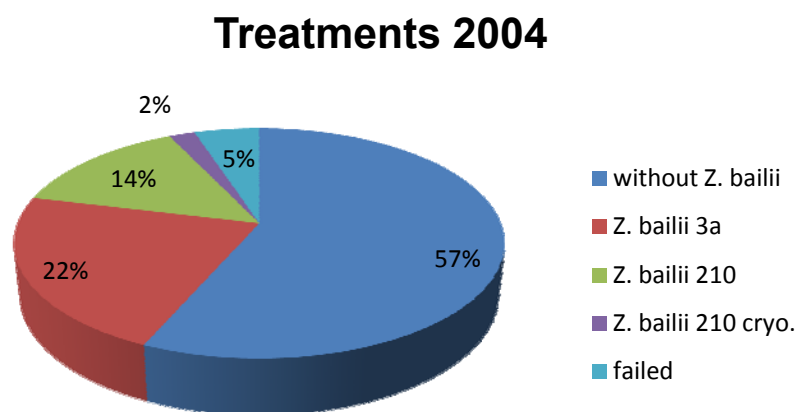


Figure 3.4.4.1: Statistic of treatments of stuck and sluggish fermentation by Agroscope Research Station Changins- Wädenswil ACW.

Since the vintage 2005, a dry yeast product of *Z. bailii*, produced by Lallemand Inc., is commercially available in some European countries under the name Fructoferm W3. Due to the commercial availability of Fructoferm W3, treatments are no longer under the control of Agroscope Research Station Changins- Wädenswil ACW and the methodology is the individual

responsibility of the concerned wine producers. Fructoferm W3 was awarded with the innovation medal for enological products on Intervitis/Interfructa in Stuttgart, 2007 (Figure 3.4.4.2).

We could not detect any risk in the use of the fructophilic yeast *Z. bailii* for the treatment of stuck wine musts. Furthermore, the data illustrate that spoilage with undesired metabolic by-products released by *Z. bailii* can be excluded. In no condition could an increase of the major spoilage product acetic acid be detected. Furthermore, post bottling spoilage of the treated wines was also never encountered. In all investigated cases the inoculated fructophilic yeasts was overgrown by the co-inoculated wine yeast *S. cerevisiae*, which took over the fermentation in the last stage of the treatment. A common sterile filtration before filling resulted in no detectable yeast growth after plating out the cured bottled wines and appears efficient in avoiding microbial spoilage.

The microbial flora of the cellar and the vinification equipment from the winery in which the first treatment were done was within a normal range. As expected, the major representative yeast in the cellar environment was *S. cerevisiae*, followed by *Pichia* sp. and species of *Kloeckera*. An isolation of *Z. bailii* from the cellar environment was not possible, so we presume that there is no risk for spoilage or undesired infection, for both of the *Z. bailii* strains used.

Several panels and discussions with the concerned winemakers and the sensory evaluation of the wines by both the winemakers and researchers of Agroscope Research Station Changins- Wädenswil ACW has generated a productive feed-back. No negative sensory impressions were described during repeated wine tastings (data not shown). As mentioned above, in all cases the acetic acid concentration was stable. Finally, after completion of the residual sugar fermentation the fructophilic yeasts are outcompeted by the co-inoculated *S. cerevisiae* population.

A large disadvantage of this method is time. In some cases the treatments drag on for up to three months until the completion of fermentation. The time requirement is probably due to the facts that *Zygosaccharomyces* generally exhibit a slow fermentation rate and may also be linked to other inhibition factors of alcoholic fermentation in stuck wines. It may be possible that this methodology can be optimized by the isolation of better adapted fructophilic yeasts. However, it seems likely considering existing evidence that a fructose preferring yeast cannot reach the fermentation rates associated with that of glucophilic strains of *S. cerevisiae*. A possible remedy would be the use of a *S. cerevisiae* strain with fructophilic properties. Firstly, a fructophilic *S. cerevisiae* strain, inoculated for a standard wine fermentation could avoid a low GFR. Therefore, a fructophilic *S. cerevisiae* could prevent the occurrence of stuck fermentations in probably many cases. And secondly, such a potential fructophilic *S. cerevisiae* strain could also be used for the cure of stuck fermentations in the same manner as *Z. bailii*.

A potential screening for fructophilic wine yeast strains could be based on the molecular level. A specific genotypical definition of the fructophilic phenotype would be a helpful approach. For this reason, the next part of this thesis specifically investigates the molecular origin of the fructophilic phenotype of *Z. bailii*.



Fructoferm W3

Fructophile Weinhefe, *aus der Natur selektioniert*



Figure 3.4.4.2: *Zygosaccharomyces bailii* strain 3a as product Fructoferm W3©

3.4.5 References

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Chapter 4

Research results

Isolation and characterization of transport systems and a hexokinase from *Zygosaccharomyces bailii*

A modified version of this chapter will be submitted for publication in
Current Genetics

4.1 Introduction

The second part of the results section of this thesis presents a molecular investigation of the fructophilic yeast strains isolated in the previous chapter and focuses on the two enzymatic activities that are most likely responsible for sugar preference. Indeed, data suggest that the molecular basis for the fructophilic phenotype is likely to be in large part restricted to the first two steps of sugar metabolism, namely the hexose transport into the cell and the phosphorylation of the sugars by a hexokinase (Guillaume *et al.*, 2007, Berthels *et al.*, 2008). However, since the hexokinases of *Saccharomyces cerevisiae* are involved in the widespread network of hexose sensing and signaling, it cannot be ruled out that additional activities of the hexokinase also have a consequence on hexose preference of yeasts.

Publications about the genetic configuration of fructophilic yeasts are rare and there are only speculations about the molecular reasons for the fructophilic behaviour of such species. Most data have been generated regarding the sugar uptake systems of such yeast. Three fructose specific transport systems have been characterized (Gonçalves *et al.*, 2000; Diezemann and Boles, 2003; Pina *et al.*, 2004), and in addition one with a higher affinity for fructose than glucose (Heiland *et al.*, 2000). Two of the described carriers transport their substrate by facilitated diffusion and two by an energy-dependent proton symport. The fructose specific transporters are not closely related to the Hxt facilitators of *S. cerevisiae*, which have a higher affinity for glucose. Considering that the known fructose specific proton symport systems of yeasts are more closely related to bacterial proton symporters than to yeast facilitated diffusion systems with a preference to fructose, it seems probable that the structural requirements for transport mechanisms are more conserved than those for sugar specificity (Diezemann and Boles, 2003).

All of the *S. cerevisiae* hexose carriers have evolved a higher affinity to glucose than to fructose and other sugars (Reifenberger *et al.*, 1997). Modifications of the substrate affinity and/or specificity of sugar transporters has shown to be caused by the replacement of only a few amino acids. It was postulated that a segment of transmembrane domain 10 of the Hxtp's appears to be at least partially responsible for the mechanism of substrate recognition in *S. cerevisiae*, since it was identified to be involved in the differential recognition of glucose and galactose (Nishizawa *et al.*, 1995; Kasahara *et al.*, 1996). The replacement of only two aromatic amino acids within this segment allowed Hxt2p to transport galactose, suggesting that the two amino acid residues play a role in galactose recognition.

Data providing some insight into the substrate recognition of fructose or comparing fructose and glucose recognition in yeast transport systems were presented by Guillaume *et al.* (2007). It has been demonstrated that a specific allele of *HXT3* isolated from the industrial wine yeast strain Fermichamp was able to significantly improve fructose fermentation. Surprisingly, the mutated allele appeared to produce a protein with similar transports characteristics (K_m and

V_{max}) than the reference allele. Furthermore, the general preference of *S. cerevisiae* for glucose was still obvious in the Fermichamp strains as well as in strains expressing this allele, thereby confirming data by Berthels *et al.* (2004) indicating that all wine yeast tested thus far show this general sugar utilisation pattern. The authors also did not analyze which of the numerous mutations found in this allele was responsible for the improved fructose fermentation phenotype. In addition, it still remains unclear if the *HXT3* mutation was the only genetic determinant of the high fructose utilisation capacity in this wine yeast strain.

The reasons for the general observation that all *S. cerevisiae* Hxts appear to show preference for glucose are not fully understood. One likely explanation is that *S. cerevisiae* carriers may prefer to transport glucose in the pyranose form and fructose in the furanose form (Heredia *et al.*, 1968). In solutions, 99% of the aldose glucose is present in the pyranose form, whereas only less than 30% of the ketose fructose is present in the furanose form. With this in mind, the transport adapted concentration of fructose would be significantly below the total available concentration (Cirollo, 1968). However, the presence of ethanol seems to result in a slight shift in the tautomeric equilibrium to the fructofuranose form (Hyvönen *et al.*, 1977; Flood *et al.*, 1996).

The yeast transporter family Hxt has a clear similarity to the mammalian hexose transporter family, Glut (Mueckler, 1994), and the transmembrane topology of *S. cerevisiae* Hxtp has been modeled using the structure of Glut1. Within this protein family, Glut5 transports exclusively fructose and Glut2 is able to transport both of the sugars (Inukai *et al.*, 1995). Several studies of both naturally occurring and engineered point mutations in Glut1 suggests that there are a number of residues within the predicted transmembrane helix 7 that are important for functional activity of the transporter. In particular, analysis of Glut1–5 indicated that a conserved three amino acid motif (“QLS”) is involved in substrate recognition. These studies suggest that Glut1, 3, and 4, which transport glucose but not fructose, are similar but differ in this sequence when compared to Glut2 and 5, which both are able to transport fructose (Arbuckle *et al.*, 1996). Replacing the conserved “QLS” motive of the glucose specific Glut3 transporter with the “HVA” motive of Glut2 converts the protein to a glucose/fructose transporter, whereas a HVA to QLS replacement in Glut2 results in a strong reduction of the ability to transport fructose. However, it was not possible to change the K_m values of the native proteins with a simple replacement of the conserved three amino acid motive. This probably indicates that other regions of the transporters also contribute to substrate selection. It was additionally proposed that the “QLS” motif acts as a molecular filter that discriminates between hexoses at the exofacial (outer membrane) binding site (Seatter *et al.*, 1998). Additional competition experiments suggest that Glut2 recognizes fructose in the furanose form, allowing alignment with the same residues within the binding pocket as for glucose in the pyranose structures. Thus C2 and C3 of the furanose ring would form the hydrogen bonds, whereas C6 may still provide for hydrophobic interaction. It is also likely that, for both glucose and fructose, Glut2 does not

form a conventional hydrogen bond where the protein is the proton donor to the hydroxyl on C3 (glucose) or C2 (fructose), but instead the hexose appears to donate the proton (Colville *et al.*, 1993). Although all of these results indicate subtle differences in how hexoses bind to each Glut isoform, it appears that in all cases the hexose needs to enter the exofacial vestibule with C1 at the front (Kane *et al.*, 1997). In spite of the fact that no similar motives exist in the Hxt protein sequences, these findings impressively indicate the stereochemical interaction between small amino acid motives of transport proteins and the three-dimensional structure of substrates. Furthermore, it was postulated that yeast Hxt carriers as well as mammalian Glut transporters seems to recognize the substrate fructose in the furanose form (Heredia *et al.*, 1968; Colville *et al.*, 1993).

The protein structure of hexose transporters for any eukaryotic member of the Multiple Facilitator Superfamily has not been fully resolved yet and there is clearly a need for investigation, possibly by X-ray diffraction analysis, which also may aid in the clarification of differences between glucose and fructose transport. An additional step towards this goal would be further investigations of as yet unknown transporters, like the transport systems of fructophilic yeasts and the sequence alignment with known transport systems.

Some further evidence for the mechanism underlying substrate recognition of glucose and fructose was provided through the investigation of the transport systems of *Pichia stipitis*, which reveal intriguing differences between glucose and fructose transport (Weierstall *et al.*, 1999). The yeast *P. stipitis* is of great economic interest, since it is able to ferment not only glucose and fructose but also xylose, thereby producing ethanol. Three genes, *SUT1-3* were identified and shown to encode hexose transporters. The derived protein sequences are closely related to one another and show distinct sequence similarities to the *S. cerevisiae* hexose transporter family. Sut1 and Sut3, but not Sut2, are able to mediate low-affinity fructose transport. This is remarkable since Sut2 and Sut3 differ in only one conserved amino acid at position 277. Moreover, only Sut3 is able to transport galactose. It can be concluded from these results that amino acid 277 is involved in determining substrate specificity of *P. stipitis* Sut hexose transporters (Weierstall *et al.*, 1999). Those findings support the hypothesis that structural requirements of transport proteins for sugar specificity are not highly conserved. However, currently there is no evidence for a similar mechanism of substrate discrimination in *S. cerevisiae* hexose carriers.

The fructose recognition mechanisms of fructose transporters of yeasts are not as clearly understood as the mechanisms underlying substrate translocation. The investigation of substrate recognition and transport through the plasma membrane is taking into consideration the stereochemical molecular structure of the substrates and its binding to the transport protein, the tertiary three-dimensional structure of this protein and possible conformation changes during

the process. Functional models of *S. cerevisiae* Hxt facilitators are based on the related and better understood mammalian Glut transporters. However, understanding of how these transporters achieve substrate translocation has only progressed in a limited way from the models proposed 50 years ago (Manolescu *et al.*, 2007).

The first catalytic step of the degradation of glucose and fructose for energy production is the activation by phosphorylation of these substrates, a step catalyzed by a family of proteins referred to as hexokinases. This step has been suggested to also play an important role in glucose preference (Berthels *et al.*, 2008). In *S. cerevisiae*, the Hxk family is represented by two enzymes, Hxk1 and Hxk2, that phosphorylate both fructose and glucose, and a third enzyme, glucokinase that is specific for glucose. The two hexose kinases, Hxk1 and 2, both display a higher affinity for glucose. The affinity of Hxk1 for fructose has a K_m of 1.5 mM, while for the K_m for glucose is 0.12 mM, and Hxk2 has a K_m of 0.25 mM for glucose and of 1.5 mM for fructose (Entian and Mecke, 1982). However, Hxk1 has a threefold higher V_{max} with fructose than with glucose, whereas the difference with Hxk2 is only marginal (Bernard, 1975; Entian and Mecke, 1982). During alcoholic fermentation, in the early stage of active growth Hxk2 expression is the highest. This level of expression drops during fermentation, while Hxk1 and Glk1 expression increases (Varela *et al.*, 2005).

Several studies have focused on the specific interactions of sugars with these kinases. Substrate binding to hexokinase differs in the in case of glucose and fructose. Indeed, the theory, originally proposed by Hopkins (1931), suggested that yeast ferments the furanose form of fructose. This assumption was confirmed by the work of Slein *et al.* (1950), which indicated that hexokinase has, indeed, a higher substrate affinity to fructofuranose.

Hohmann *et al.* (1999) found that different mutated alleles of Hxk2 with a single amino acid substitution can affect the catalytic activity of the two sugar substrates fructose and glucose to different extents. For instance, the substitution of proline at position 160 to alanine largely decreases the affinity for fructose, while glucose affinity seems to be unaffected. Pro-160 is the last of a block of highly conserved amino acids in the hexokinase gene family. This 152-PLGFTFSFP-160 motif has previously been proposed to function in sugar binding (Schirch and Wilson, 1987), and Ser-158 was identified as an important determinant of sugar binding affinity (Xu *et al.*, 1995). This serine residue seems to be the site of hexokinase autophosphorylation and may play a central role in the phosphoryl transfer reaction (Heidrich *et al.*, 1997). Substitution of Pro-160 to Ala may turn Ser-158 out of position, resulting in an alteration of the fructose binding affinity without a significant impact on glucose binding affinity. This discrepancy is indicative of different substrate recognition requirements of Hxk2 for glucose and fructose. Furthermore, it appears to be reflected in the extent of catabolite repression with the same mutant, which is normal with glucose, but partially deficient with fructose. Previously, it was shown by Entian and Mecke (1982) that deletion of Hxk2 causes a clear derepression of the

specific activities of invertase and Hxk1p on glucose but not on fructose medium, which gave the first indication that triggering of fructose repression may differ from triggering of glucose repression.

The proteins involved in the first two steps of hexose metabolism of *S. cerevisiae* have evolved a preference to glucose. Naturally the fructose preferring counterparts of these proteins, or at least one of them, may have evolved in fructophilic yeasts. The aim of this work was the isolation of members of these two protein families, which are presumably responsible for hexose preference, from the isolated fructophilic yeasts *Zygosaccharomyces bailii* and *Candida zemplina/stellata*.

To achieve this aim, the experimental strategy was laid out as follows: Transporter and hexokinase deleted mutants of *S. cerevisiae* with a growth deficiency on fructose were transformed with genomic libraries of *Z. bailii* and *C. stellata/zemplinina*. Transformants growing on fructose, a phenotype suggesting functional complementation of the mutants, were selected. The characterization of the vector inserts was done by DNA sequencing followed by sequence analysis. Subsequently, the proteins encoded by the selected genes were characterized by kinetic assays. Finally, lab strains were transformed with the genes of interest, and the development of the GFR during fermentation of synthetic grape must MS300 was measured.

4.2 Materials and Methods

4.2.1 Strains and growth conditions

Z. bailii strain 210 and strain 3a were isolated from natural habitats as described in chapter 3 of this thesis. *C. stellata (zemplinina)* FAW3 was derived from the Agroscope Research Station Changins- Wädenswil ACW culture collection, and had been isolated previously from the grapevine microflora.

S. cerevisiae hxt null mutant strain JT5330 (=VWC700 CEN.PK2-1C, *MATa ura3 leu2 trp1 his3 hxt1-7Δ*) and the hxx deleted mutant YSH327 (W303-1A, *MATa leu2-3, 112 ura3-1 trp1-1 his3-11, 15 ade2-1 can1-100 GAL SUC2 hxx1Δ::HIS3 hxx2Δ::LEU2*) (Hohmann *et al.*, 1993) and the relevant parental strains JT5200 and JT9019 were donated by Johan Thevelein, Katholieke Universiteit Leuven (Leuven, Belgium).

Lab fermentation experiments were performed with *S. cerevisiae* FY2 (*MATα ura3Δ*) and BY4742 (*MATα his3 leu2 lys2 ura3*; Euroscarf). Both strains are isogenic to *S. cerevisiae* standard strain 288C.

Bacterial transformation and preparation of recombinant plasmid DNA were performed in *Escherichia coli* DH5α (fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17). Strains of *Z. bailii* and *C. stellata (zemplinina)* FAW3 were grown in

complete YPF media, containing 2% or 4% fructose, 2% peptone and 1% yeast extract. Strain JT5330 was routinely kept on solid yeast nitrogen base (YNB) medium with 20 g, 10 g or 5 g maltose, supplemented with uracil, leucine, tryptophan and histidine as required. The constitution of liquid media was similar except for the absence of agar. Strain YSH327 was grown on YNB medium with 20 g, 10 g or 5 g glucose, supplemented with uracil and the required amino acids adenine and tryptophan.

For plating out after yeast transformation, the media was supplemented with 1M Sorbitol as osmotic preservative.

E. coli DH5 α cells were grown in Luria–Bertani (LB) medium and, when required, ampicillin (100 mg ml⁻¹), X-Gal, (4 mg ml⁻¹) and IPTG, (4 mg ml⁻¹) were used to establish selective pressure and indicate the presence of the LacZ reporter gene.

4.2.2 Preparation of electro-competent cells

A single culture of *E. coli* was grown in LB medium to an OD of 0.6, washed three times with 10% glycerol and resuspended in GYT (10% glycerol, 0.125% yeast extract and 0.25% tryptone) for preparation of electro-competent cells.

Yeast electro-competent cells were prepared as follows: A single colony was grown overnight in appropriate medium (see chapter 4.2.1) for pre-culture. An appropriate amount of the pre-culture was used for inoculation of 500 ml media for growth with vigorous shaking to an OD₆₀₀ of 1.3-1.5. Cells were harvested and washed, followed by a treatment with 10 x lithium acetate and 1 M DTT (Ausubel *et al.* 1994). Three further washing and concentration steps with H₂O were carried out before electroporation. Excess cells, resuspended in freezing buffer (0.6 M Sorbitol, 10 mM CaCl₂, 10mM HEPES, pH 7.5), were cryo-preserved by freezing at -80°C. After thawing, cells were washed twice with 1 M Sorbitol.

4.2.3 Plasmids used in this study

The multicopy plasmid YEplac195 was used for the construction of the genomic libraries and for the main cloning experiments. The YEplac series of yeast- *E. coli* shuttle vectors are derived from the plasmid pUC19 and contains a multiple cloning site inside of the convenient *lacZ* reporter gene to screen for inserts. Furthermore, the plasmid contains the pMB1 replicon responsible for the replication of plasmid with *E. coli*, and the *bla* gene, coding for beta-lactamase that confers resistance to ampicillin. The plasmid also comprises a 2 μ DNA replication origin and the *URA3* gene for complementation of the auxotrophic marker (Gietz and

Sugino, 1988). For subcloning experiments, YEplac 112 (TRP1), YEplac 181 (*LEU2*), pUC19 (Parent *et al.*, 1985) and pGEM-T (Promega corp.) were used.

For kinetic assays and lab strain transformation the pCEL13 plasmid was used, containing *URA3*, *bla*, pMB1 and 2 μ replicons and the PGK1 (phosphoglycerate kinase) promoter and terminator sequences, which flank the *EcoRI*, *BglII* and *XhoI* restriction sites.

4.2.4 Genomic library construction

Genomic libraries of *Z. bailii* and *C. stellata/zemplinina* were constructed. The size of a library of random fragments of genomic DNA that is necessary to ensure representation of a particular sequence of interest is dictated by the size of the cloned fragments and the size of the genome. The likelihood that a sequence of interest is present in such a random library can be estimated by simple statistics based on Poisson distribution (Clarke and Carbon, 1976). Specifically, the number of independent clones, N, that must be screened to isolate a particular sequence with probability P is given by

$$N = \ln(1 - P) / \ln[1 - (I/G)],$$

where I is the size of the average cloned fragment and G is the size of the target genome in base pairs.

The type strain of the species *Z. bailii*, IGC 5167, was reported to have a genome size of 7.7 Mb (Török *et al.*, 1992). However, in the same study large intraspecies deviations were detected by electrophoretic karyotyping in the number and size of chromosomes as well as in the whole genome size. Accordingly, a second strain of *Z. bailii* was reported to have a genome size of about 10.5 Mb. Rodrigues *et al.* (2003) confirmed these intraspecies deviations by flow cytometry in 2003 and published a genome size of an additional strain, ISA 1307, of about 13.3 Mb, which is comparable with *S. cerevisiae* W303 with a genome size of 13.1 Mb. Based on these published results, approximately 12'000 independent clones of a genomic library with an average insert size of 5 kb should represent a 99% chance of isolating an individual sequence from a *Z. bailii* strain with an assumed genome size of 13.3 Mb.

The genome size of *C. stellata/zemplinina* strains were estimated at about 10 Mb by karyotyping (Sipiczki, 2004).

Genomic libraries were constructed as follows (Rodriguez and Tait, 1983; Ausubel *et al.*, 1994): genomic DNA of strain 210 was isolated using the large scale yeast genomic DNA preparation method, based on the protocol by Philippsen *et al.* (1991). Accordingly, the DNA was partially digested with *Sau3A*, and the resulting DNA fragments were separated in 10 to 40% (wt/vol) discontinuous sucrose gradients and centrifuged for 24h at 150'000 x g

(Beckmann SW28 rotor). Fractions containing fragments in the ranges of 4 to 18 kb were pooled and used in ligations. The yeast- *E. coli* shuttle vector YEplac195 was digested with *Bam*HI, dephosphorylated, and ligated to the purified genomic DNA fragments. *Escherichia coli* DH5 α competent cells were transformed by electroporation with the constructs and plated onto Luria-Bertani medium supplemented with ampicillin (final concentration 40 μ g/ml), X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside, 40 mg/ml) and IPTG (isopropyl- β -D-thiogalactopyranoside, 40 mg/ml). Approximately 12'000 white colonies were picked from the plates. Restriction analysis of plasmid DNAs isolated from 24 white colonies revealed inserts ranging from 4 kb to 18 kb with an average insert size of 10.7 kb. Library transformants were grown for two generations, glycerol stocks were prepared and plasmid DNA was isolated using a large scale plasmid preparation kit (Qiagen Plasmid Mega Kit).

The size of the library was calculated with the predicted maximum genome size of *Z. bailii* and the average insert size of 10.7 kb, resulting in coverage of theoretically > 99%.

A second genomic library was constructed with the genomic DNA of *C. stellata* (*zemplanina*) FAW3, following a similar library construction procedure as mentioned above. The number of independent clones derived from this library was about 8'000, with an estimated average insert size of about 5.1 kb. This corresponds to a theoretical genome coverage of 97%.

4.2.5 Transformations

4.2.5.1 Electroporation of *E. coli*

Electroporation was done under the following conditions: purified plasmid DNA and competent cells were added to chilled electroporation cuvettes and pulsed with 1100V, 200 Ω and 25 μ F, followed by an immediate addition of SOC media (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 10 mM MgSO₄, 10 mM MgCl₂, 0.4% glucose) and incubation at 37°C for 1 h. Afterwards the cells were spread out on selective plates and incubated overnight. Plasmid isolation was done by the miniprep method, as described by Birnboim and Doly (1979).

4.2.5.2 Electroporation of yeasts

Yeasts were transformed by electroporation. Purified plasmid DNA and competent yeast cells were added to prechilled cuvettes and pulsed at 1.5 kV, 200 Ω and 25 μ F. Ice-cold 1 M sorbitol was added, afterwards the cells were regenerated for 4h with 50% vol/vol full medium containing the appropriate sugar (Maltose for JT5330; Glucose for YSH 327). Cells were spread out on selective agar plates, supplemented with 1M Sorbitol and incubated at 30°C for

approximately 7 days. The re-isolation of plasmids from yeast was as described by Hoffman and Winston (1987).

4.2.5.3 Library screening

Electro-competent cells of strains JT5330 and YSH 327 were transformed with library plasmid DNA using the described electroporation method. The transformation mixture was first plated onto solid YNB medium with 10 g maltose (JT5330) or glucose (YSH327) per liter as sole carbon source, supplemented with leucine, tryptophan and histidine in case of JT5330 and tryptophan plus adenine for YSH327. After 7 days at 30°C, colonies were replica-plated onto the same medium containing 10 g per liter of fructose, instead of maltose or glucose. Growing colonies were checked for plasmids by colony PCR with primers homologous to the plasmid sequence. Plasmid DNA was isolated from yeast colonies and subsequently transformed into *E. coli* with isolated yeast DNA (Birnboim and Doly, 1979). Plasmids were proliferated, harvested and re-transformed into the corresponding mutants for confirmation of the complementing activity.

4.2.6 Sequencing

Inserts of positive isolated plasmids were sequenced by primer walking, with the starting sequence derived from the plasmid sequence as Yeplac F 5'-GGCACCCCAGGCTTTACT-3' and Yeplac R 5'-CAGGGTTTTCCCAGTCACGA-3'.

Linear amplification was done using a 45°C annealing temperature with between 20-30 ng DNA template. Sequencing was carried out with ABI3730xl DNA Analyzer (Stellenbosch University) or AB3130xl Genetic Analyzer (Agroscope Research Station Changins- Wädenswil).

Data analysis was done using the Sequencher (Gene codes) and Vector NTI (Invitrogen) software packages.

4.2.6.1 Internet database search

The sequencing results of the confirmed plasmid inserts were compared by BLAST search with the *Saccharomyces* genome Database (<http://www.yeastgenome.org/>) and the NCBI genome database (<http://www.ncbi.nlm.nih.gov/>). Protein structures were predicted with Protein Homology/analogy Recognition Engine Phyre (Kelley and Sternberg, 2009) (<http://www.sbg.bio.ic.ac.uk/servers/phyre/>), the predict protein database (Rost *et al.*, 2004)

(<http://www.predictprotein.org/>) and the Swiss model server (Arnold *et al.*, 2006) (<http://swissmodel.expasy.org>).

4.2.7 Subcloning

The putative ORF's contained within the isolated inserts were subcloned and checked for functional complementation of the corresponding fructose utilisation defective mutants. For overexpression, the genes were subcloned into pCEL13 under the regulation of the strong yeast PGK1 phosphoglycerate kinase promoter (Constructs are displayed at appendix 4.2, page 173). The ORF's were amplified by PCR with Ex Taq (Takara Bio Inc.), using primers which are modified to contain a restriction site of *Bgl*II or *Xho*I, allowing directional cloning with high efficiency into linearized vectors carrying appropriate ends.

4.2.8 Kinetic characterization of proteins, derived from the isolated sequences

4.2.8.1 Hexose transport assays

Hxt null mutants JT5330 were transformed with putative ORF's of *Z. bailii* permeases, ligated in the vector pCEL13 and the empty vector, respectively. Cells were grown in liquid YNB with 20g fructose or glucose and the required amino acids. Cells were harvested at an OD₆₀₀ of 0.5–0.8 by centrifugation (5000 g for 5 min), washed twice in an equal volume of 0.1 M potassium phosphate pH 6.5 and resuspended in an appropriate volume of phosphate buffer. After 3 min. incubation in a water bath at 30°C with aeration, uptake was initiated by the addition of 50 µl of cells to 12.5 µl aliquots of D-[U-¹⁴C]fructose (295 mCi/mmol; 10.9 GBq/mmol; Amersham) or D-[U-¹⁴C]glucose (3 mCi/mmol; 0.111 GBq/mmol; Amersham) at the appropriate concentrations. After 5 seconds, incorporation was stopped by the addition of 10 ml cold quenching solution (0.1 M potassium phosphate, 0.5 M D-glucose, pH 6.5). After briefly vortexing, the quenched cells were harvested under vacuum on GF/C glass fibre filters (Whatman, GF/C filter 25mm) and washed with another 10 ml quenching solution. Filters were transferred immediately into specific scintillation vials (OptiPhase HiSafe II; Amersham). The radioactivity remaining on the filters was measured with a Beckman Instruments LS 5000 TD liquid scintillation counter.

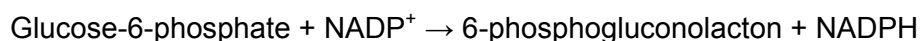
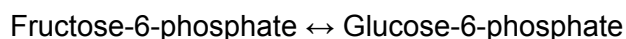
For blanks, labeled sugars were added to cold quenching solution before addition of yeast cells. The values obtained for the blank samples were subtracted from the measurements. All determinations were performed in triplicate, experiments were performed twice (Bisson and Fraenkel, 1983; Cirillo, 1989; Diderich *et al.*, 1999). Total cell protein determinations were

performed by the Lowry method (Lowry *et al.*, 1951) on duplicate samples from the stock cell suspension, and from each aliquot after it has been used for assay points. The transport assays were measured threefold and the whole experiment was repeated once.

4.2.8.2 Hexokinase activity assay

For determination of *Z. bailii* hexokinase activity, cells of the *S. cerevisiae* hexokinase deleted mutant, YSH327, were transformed with pCEL13 containing the putative *Z. bailii* hexokinase gene. Transformants were grown in YP medium supplemented with 2% of either glucose or fructose and harvested in the late exponential growth phase. Hexokinase activity was measured spectrophotometrically using crude cell extracts (Fernández *et al.*, 1985), prepared by washing and resuspension of cultures in sonication buffer (100mM Potassium phosphate buffer, pH 7.5 + 2 mM MgCl₂), supplemented with DTT (1mM final concentration). Glass beads were added to the cells, followed by a burst in the Fastprep machine. Centrifugation for 20 minutes at 47'000 x g at 4°C separated the cell extract from the insoluble cell material. A maximum of 200 µl of the extract was used for the determination of hexokinase activity (approximately 1 µg protein µl⁻¹). The appropriate amount of cell extract was mixed with buffer to obtain a volume of 300 µl and this final volume was transferred to a microtiter plate. For 24 assays, the following mixture was prepared: 900 µl imidazole buffer (0.5M), 720 µl ATP 10 mM, 9 µl glucose-6-P dehydrogenase (1.5 U), 225 µl NADP⁺ 40 mM, 9 µl phosphoglucosomerase (2 U), MgCl₂ 180 µl (0.5M) and 6390 µl water. A volume of 285 µl of this mixture was added to 5 µl extract sample. The microtiter plate was put in a Biomek 2000 robot (Beckman). Glucose or fructose solution was added in the appropriate concentration. The kinetic parameters were evaluated for concentrations ranging from 0.1, 0.2, 0.5, 1.0, 2.0, 3.0, 5.0 and 10.0 mM.

The *in vitro* reactions are:



The changes in the NADPH absorbance (OD340 nm) were measured automatically at 30 second intervals for 3 minutes after the addition of glucose or fructose. Protein concentrations in the crude extracts were determined using the method described by Lowry *et al.* (1951) with bovine serum albumin as a standard. Hexokinase activity was calculated as nmol NADPH min⁻¹ (mg protein)⁻¹. The kinetic parameters V_{max} and K_m were determined by nonlinear regression analysis using the Graph pad Prism software.

Experiments were done in three biological and three technical repeats.

4.2.8.3 Protein determination by Lowry

Protein determination of the cells or the cell extracts were performed according to the Lowry method (Lowry *et al.*, 1951), using bovine serum albumin as standard protein.

4.2.9 Laboratory strain transformation and fermentation experiments

The *S. cerevisiae* laboratory strain, FY2 (-ura), was transformed with the isolated *Z. bailii* ORF's, ligated into pCEL13 vector under the control of the PGK promoter, and the empty vector as control. For fermentation, the standard synthetic must MS300 (Bely *et al.*, 1990) was slightly modified in sugar content to contain 80 g/l fructose and 80 g/l glucose and was kept at room temperature. All fermentations were carried out in 500 ml bottles, containing 400 ml of the medium, provided with a bubbling CO₂ outlet. Inocula were prepared as follows: one colony from an YNB (-ura) plate was inoculated into liquid YNB broth and grown at 30°C until a cell density of approximately 10⁹ cfu/ ml was reached. The cells were counted and an equal amount of cells per strain was resuspended in the same medium as used for the fermentation. Each bottle was then inoculated with this cell suspension to a final cell density of 10⁶ cfu/ ml. Fructose and glucose content was measured continually by HPLC. Fermentations were done in two biological and three technical repeats.

4.3 Results

4.3.1 Screening for fructose transport genes

The first set of transformations of *S. cerevisiae* mutants JT5330 and YSH327 with the genomic library of *C. stellata* (*zemplanina*) FAW3 failed since no growing *S. cerevisiae* colonies could be obtained on fructose medium after transformation. Considering the size of the library and the transformation efficiency achieved (a total of more than 10 000 transformants were screened), this negative result was unexpected. Possible explanations range from differences in promoter sequences between the two yeast species, which may lead to non-expression of the *C. stellata* genes in *S. cerevisiae*, or unrecognized bias in the library. Since the primary focus of this dissertation was on *Z. bailii*, no further efforts were invested in the isolation and characterization of putative *C. stellata* (*zemplanina*) fructose preferring proteins.

The JT5330 strain, which is unable to use fructose and glucose as sole carbon sources, was transformed with the genomic library of *Z. bailii* strain 210. Approximately 1.75×10^6 URA⁺ transformants were selected on maltose-containing medium and were subsequently replica-plated onto YNB fructose medium. Three transformants were identified that were able to grow on fructose, and referred to as Z9, Z11 and Z12. After plating out on glucose medium, all transformants were also able to grow on glucose. The plasmids present in transformants ZT9, ZT11 and ZT12 were rescued and propagated in *E. coli*. All three transformants were found to contain only one library plasmid each, as assessed by the restriction pattern of the purified plasmids.

Plasmid ZT9 was found to include an insert of 9051 bases. The entire length of the insert was sequenced (Appendix 4.3.1, page 175) and used in BLAST searches on gene databases. The fragment was found to contain two complete ORF's of putative permeases and an additional incomplete ORF, encoding for another putative permease, arranged in a hypothetical *Z. bailii* permease gene cassette. For further experiments, the truncated gene sequence with a length of about 1000 bases was ignored. The two complete ORF's were named permease B, with a length of 787 amino acids, and permease S, which was composed of 634 amino acids.

Plasmid ZT11, containing an insert of 9827 bases, was also able to complement the function of hexose transport on fructose. Sequencing revealed that this fragment contains a truncated or an interrupted ORF. Blast screening on internet databases indicated a low identity (less than 30%) similarity to putative plasma membrane permeases. This putative transporter sequence was not further analyzed in this study.

Similar to Plasmid ZT11, the ZT12 plasmid mediates a very slow growth rate on both hexoses. Sequencing of the ZT12 insert indicates a sequence of 8136 bases, and a BLAST search results in no similarity to transport proteins. Amongst others, it was found to include a transcriptional repressor sequence (77% identity) that recruits the Cyc8p-Tup1p complex to

promoters. This repressor is known to mediate glucose repression and negatively regulates a variety of processes in hexose metabolism. It is uncertain if this putative regulatory element would indeed be able to influence hexose transport and an investigation into the possible effects thereof does, however, falls outside the immediate aims of this work. Since clear positive results were obtained with the two putative *Z. bailii* permeases of plasmid ZT9, both fragments from the plasmids ZT11 and ZT12 were not further analyzed and present attractive targets for future studies.

4.3.1.1 Permease S description

When expressed in *S. cerevisiae*, this permease was able to complement the function of hxt1-7 deleted transporter mutant and re-establish growth on fructose and glucose. The ORF consists of 1902 bases and can therefore be translated into 634 amino acids (see Appendix 4.3, page 182). Permease S is predicted to have 9 transmembrane domains (www.PredictProtein.org; Rost, 1996). A similarity search with internet databases using the predicted amino acid sequence, revealed a putative permease which is member of the major facilitator superfamily. The homology to several known or putative yeast permeases is below 60%. The closest relative of ZbpermeaseS appears to be a hitherto uncharacterized protein of *Zygosaccharomyces rouxii* (ZYRO0E08228p) with an identity of 64%. A high degree of identity (60%) was found with the putative permease encoded by *SEO1*, a member of the allantoin transporter subfamily of the major facilitator superfamily of *S. cerevisiae* (Appendix 4.3.1.1, transport protein alignments, page 165). The molecular weight was calculated with 72161 Da and the theoretical isoelectrical point pI with 5.45.

A motif search (PROSITE database) revealed several assumed phosphorylation sites of protein kinase C, casein kinase II and tyrosine kinase and additional cAMP and cGMP dependent phosphorylation sites. Bairoch (1993) and Kruckeberg (1996) published a highly conserved sugar transporter signature sequence, which, in combination with a PESP motif occurs in all members of the sugar transporter family from prokaryotes to eukaryotes. This motif was not present in permease S of *Z. bailii*, as well as in the previous published sequence of the *Z. bailii* Ffz1 transporter (Pina *et al.*, 2004).

The secondary structure of permease S can be projected as follows: Helix=47.0%, Strand=3.6%, Loop=49.4%, and one non-regular secondary structure (Guharoy and Chakrabarti, 2007). An intriguing feature of permease S is the predicted extremely long cytoplasmic N-terminal tail, which is composed of a sequence of 198 amino acids. A predicted protein ribbon was created, using the swissmodel protein structure homology-modeling server (<http://swissmodel.expasy.org/>; Appendix 4.4, page 184).

4.3.1.2 Permease B description

Permease B was the second putative transporter, isolated from the insert of plasmid ZT9. The ORF contains 2361 base pairs and codes for a putative peptide of 787 amino acids (see Appendix 4.3, page 182). When expressed in *S. cerevisiae*, this permease is able to mediate the functional complementation of an hxt null mutant on fructose as well as on glucose. Permease B is predicted to have 11 transmembrane domains (www.PredictProtein.org; Rost, 1996). For a predicted protein structure, see Appendix 4.4, page 185.

In turn, the closest homolog of ZbpermeaseB is an uncharacterized protein of *Zygosaccharomyces rouxii* (ZYRO0D17974p) with a 78% identity. In addition, several lower similarities with uncharacterized, hypothetical proteins of other organisms were found. Putative permease B shares a low homology to other transporters (below 67%) and to members of the *Candida albicans* oligopeptide transporter system (Appendix 4.3.1.1, transport protein alignments, page 165). However, members of the OPT transporter superfamily are thought to have 12-14 transmembrane domains and contain the following highly conserved motif, SPYxEVRxxVxxxDDP (Lubkowitz *et al.*, 1998), which is not present in the permease B sequence. Similarities to sugar transport systems of yeast or other organisms were not found.

Permease B has a predicted molecular weight of 89501 Da and a theoretical pI of 7.57. Several putative protein kinase C and casein kinase II phosphorylation sites are present, but these motifs are found in the majority of all known protein sequences. As was found in permease S, no conserved sugar transporter signature sequence (Bairoch, 1993; Kruckeberg, 1996) is present in permease B.

The secondary structure of permease B can be projected as follows: Helix=48.9%, Strand=7.0%, Loop=44.1% and also one non-regular secondary structure (Guharoy and Chakrabarti, 2007).

Many secreted and integral membrane proteins of yeast are glycosylated on asparagine residues. This modification occurs posttranslationally in the endoplasmic reticulum. The domains of integral membrane proteins that are the substrates for asparaginyl (N-) glycosylation are situated on the extracellular face of the membrane. The consensus site for N-glycosylation in yeast seems to be Asn-Xaa-Thr (Bisson *et al.*, 1993). Permease B contains two putative Asn-glycosylation sites between the predicted transmembrane domains 5 and 6 on the extracellular face of the plasma membrane. The position of the asparaginyl glycosylation motif in this topological region is highly conserved in yeast hexose transporters (Bisson *et al.*, 1993).

4.3.1.3 Functional complementation of JT5330 hxt null mutant via heterologous expression of *Zygosaccharomyces bailii* permease S and permease B

Cultures of JT5330, transformed with the *Z. bailii* permease S or permease B expressed from the pCEL13 vector and under transcriptional the control of the *S. cerevisiae* *PGK1* promoter and terminator (for constructs see page 174), are able to complement the growth defect of the Hxt null mutant on fructose and glucose (Figure 4.3.1). Expression of permease S establishes better growth on fructose compared to permease B, which allows a better growth on glucose.

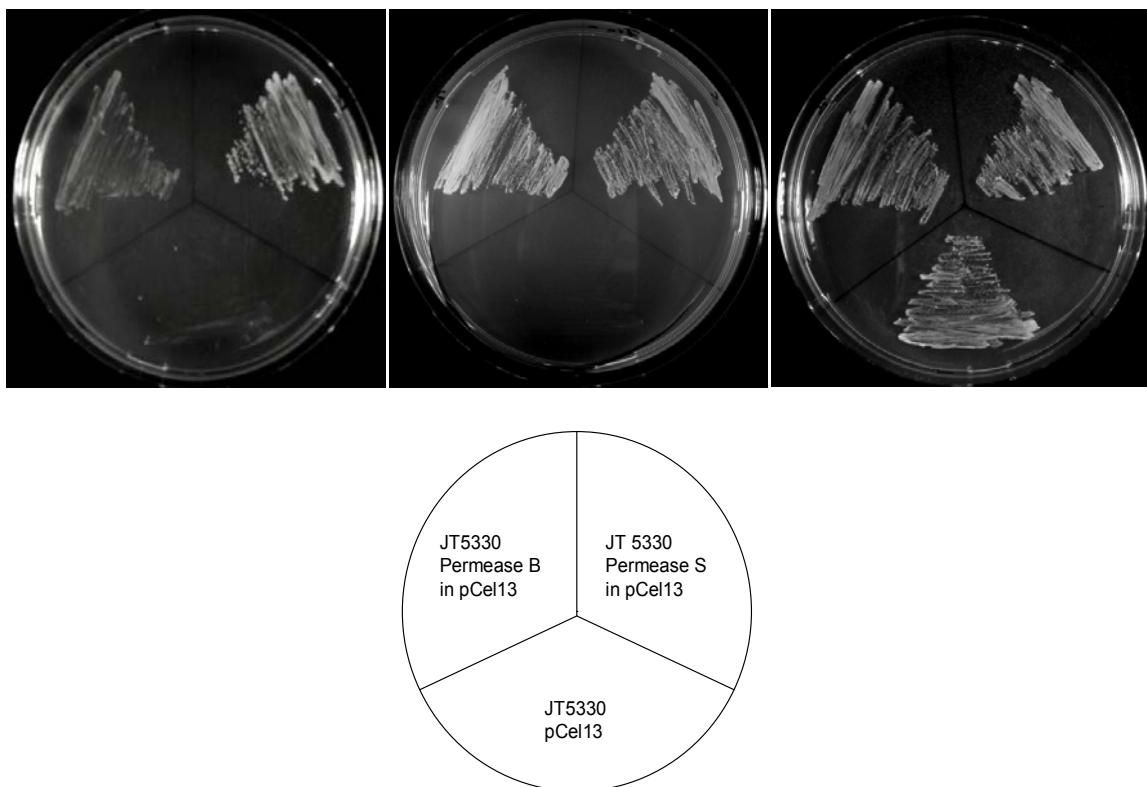
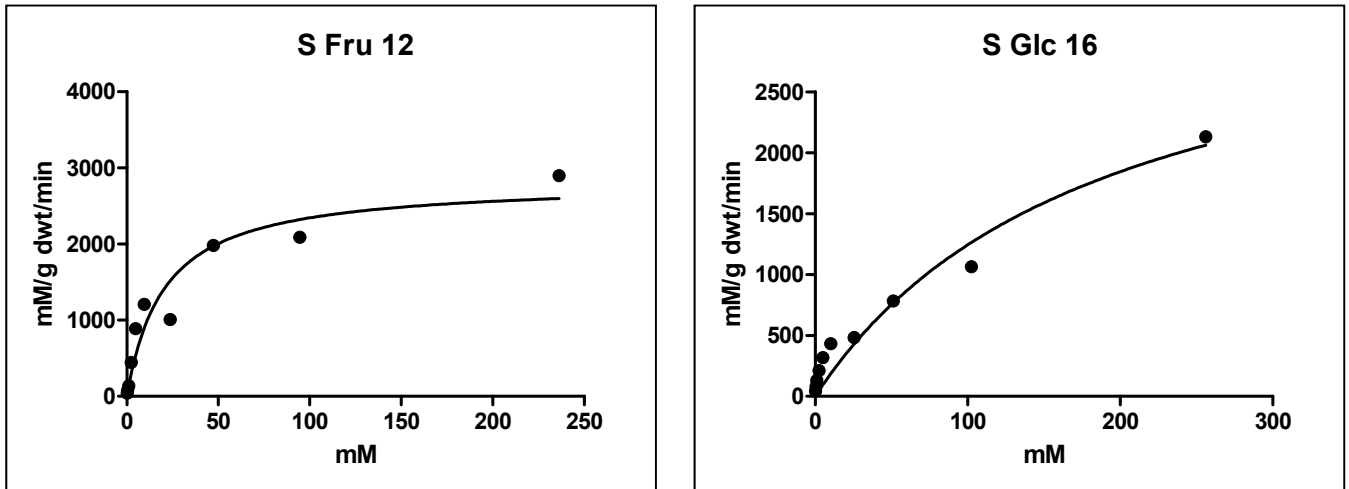


Figure 4.3.1: *S. cerevisiae* hxt null mutant JT5330, transformed with *Z. bailii* plasmids encoding permease B and permease S under the control of PGK1 promoter and terminator, and the empty vector pCel13. Growth on fructose, glucose and maltose media is shown from left to right

4.3.1.4 Permease S kinetic assays

The data obtained from fructose and glucose uptake experiments are shown in Figure 4.3.2. Strains transformed with pCel13 carrying the gene encoding permease S (TPS) were grown in liquid YNB medium containing 1% fructose as well as 1% glucose as sole carbon and energy source, respectively. TPS was able to take up both D-[U-¹⁴C] fructose and D-[U-¹⁴C] glucose. The kinetic parameters calculated for fructose uptake were $V_{\max}=2.822 \pm 0.15$

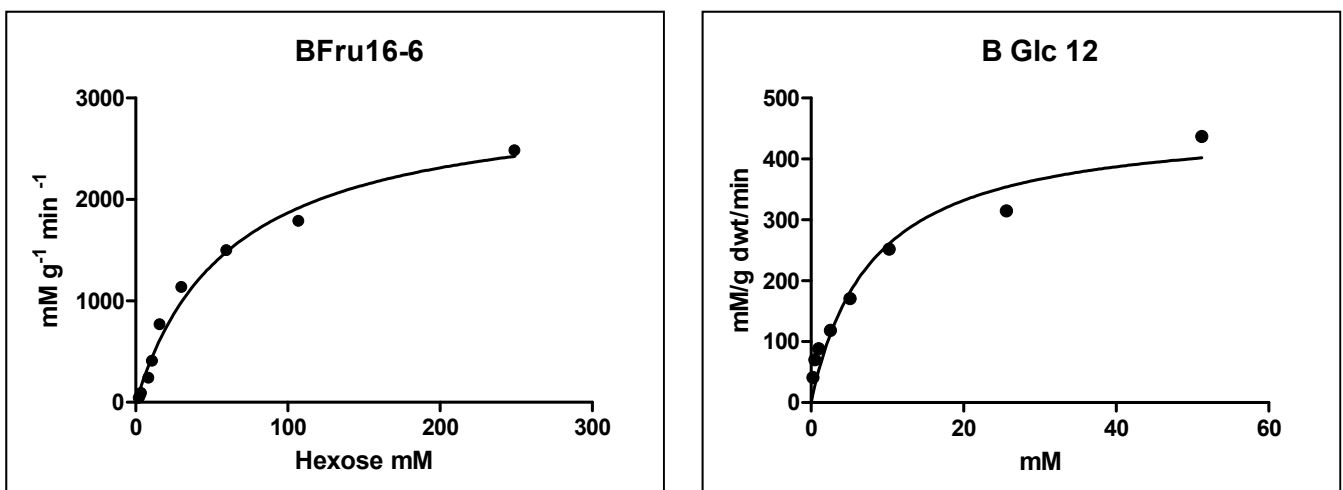
mmol/mg dwt/min and $K_m=20.4 \pm 3.5$ mM at 30°C. Glucose uptake was measured with a $V_{max}=3.567 \pm 0.4$ mmol/mg dwt/min and $K_m = 186 \pm 15$ mM at 30°C.



Figures 4.3.2: Direct representation and of glucose and fructose initial uptake rates in permease S transformant cells of the *S. cerevisiae hxt* null mutant.

4.3.1.5 Permease B kinetic assays

Yeast strains transformed with pCel13 carrying the gene encoding permease B (TPB) were grown in liquid YNB medium containing 1% fructose as well as 1% glucose as sole carbon and energy source, respectively. TPB was able to take up D-[U-¹⁴C] fructose and also D-[U-¹⁴C] glucose uptake could be measured. The kinetic parameters calculated for fructose uptake were $V_{max}=2.628 \pm 0.07$ mmol/mg dwt/min and $K_m= 31.4 \pm 2.5$ mM at 30°C. Glucose uptake was measured with a $V_{max}= 0.464 \pm 0.022$ mmol/mg dwt/min and $K_m = 7.9 \pm 1.1$ mM. The data are shown in Figure 4.3.3.



4.3.3: Direct representation and of glucose and fructose initial uptake rates in permease B transformant cells of the *S. cerevisiae hxt* null mutant.

No uptake could be measured with the negative control experiments, represented by the hxt null mutant JT5330, transformed with the empty vector pCEL13.

4.3.2 Screening for hexokinase encoding genes

The mutant strain YSH327 lacks both hexokinase encoding genes, *HXK1* and *HXK2*, but still contains a functional copy of the glucokinase *GLK1*. Therefore, this mutant yeast is able to grow on glucose, but not on fructose media. After transformation with the genomic library of *Z. bailii* strain 210, approximately 2.1×10^6 Ura⁺ transformants were selected on glucose-containing medium and were subsequently replica-plated onto YNB fructose medium. Four transformants, containing the same plasmid with an insert of 7193 bases, as assessed by the restriction pattern of the purified plasmids, were able to establish growth on fructose. The insert of plasmid ZK1 includes two complete ORF's, one of them could be identified as a putative hexokinase in a BLAST screen (see Appendix 4.3.2, page 179).

4.3.2.1 *Zygosaccharomyces bailii* hexokinase description

The putative hexokinase gene has an ORF of 1458 bases coding for a predicted peptide of 486 amino acids (Appendix 4.3, page 183), which is able to complement the growth on fructose media of the mutant strain YSH327. A BLAST search of the protein sequence indicated 76% identity to *S. cerevisiae* hexokinase isoenzyme 1 and 75% identity to hexokinase isoenzyme 2. In general, yeast hexokinases contain two structurally similar domains. Interestingly, the search for conserved domains reveals some significant similarity to the hexokinase 1 superfamily domain in the sequence up to amino acid 225 (domain 1), whereas in the rest of the sequence (domain 2) significant similarity to hexokinase 2 superfamily domain can be observed (Figure 4.3.2.1).

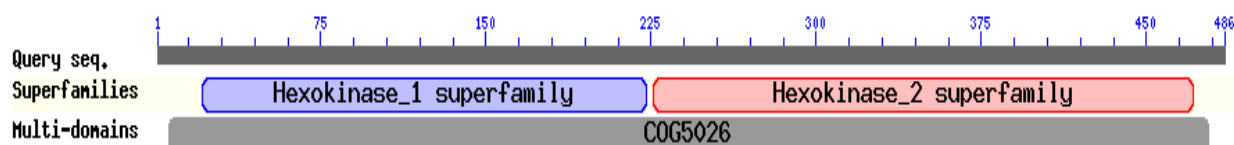


Figure 4.3.2.1: Graphical summary of conserved domains identified on the query sequence (HXK *Z. bailii*). Hexokinase contains two structurally similar domains represented by this family.

The similarity in the putative ZbHxk domain1 with Hxk1 can be demonstrated with the block of highly conserved amino acids in the hexokinase gene family, which has been proposed to function in sugar binding (Schirch and Wilson, 1987) and possibly is involved in the distinction

between glucose and fructose (Hohmann *et al.*, 1999). The *Z. bailii* hexokinase sequence is identical to the *S. cerevisiae* Hxk1p (152-plgfffsyp-160) sequence block. On the other hand, the sequence of ScHxk2p is given as 152-plgfffsfp-160. There is one more difference in the 20 most conserved amino acid residues when compared to ScHxk2p, namely that tryptophan 128 is changed to phenylalanine. Additionally, compared to ScHxk1p the highly conserved phenylalanine 240 is changed to tyrosine in ZbHxkp (Kuser *et al.*, 2000 and 2008). The complete alignment is displayed at Appendix 4.3.2.1, page 171.

The closest amino acid sequence identity (83%) was found with the uncharacterized putative protein ZYRO0E09878p from *Z. rouxii*.

The theoretical isoelectrical point pI was predicted to be at 5.85, and the average molecular weight is predicted to be 53819.72 Da. The motive search at Prosite database clearly identified the hexokinase signature (153 LGFTFSYPASQSKINEGILQRWTKGF). Furthermore, several putative phosphorylation sites exist in the sequence, including sites for protein kinase C (PKC), casein kinase II and cAMP or cGMP phosphorylation. A predicted protein ribbon, created by using the swissmodel protein structure homology-modeling server (<http://swissmodel.expasy.org/>) is displayed at Appendix 4.4, page 186.

4.3.2.2 Functional complementation of hexokinase deleted mutant strain YSH327 via heterologous expression of *Zygosaccharomyces bailii* hexokinase

Transformation of YSH327 with the pCEL13 plasmid containing the *Z. bailii* derived hexokinase allows this strain to grow on fructose (Figure 4.3.2). It also seems to accelerate the use of glucose as carbon source, since the only existing sugar kinase, the glucokinase of YSH327, only mediates a slow growth of this mutant.

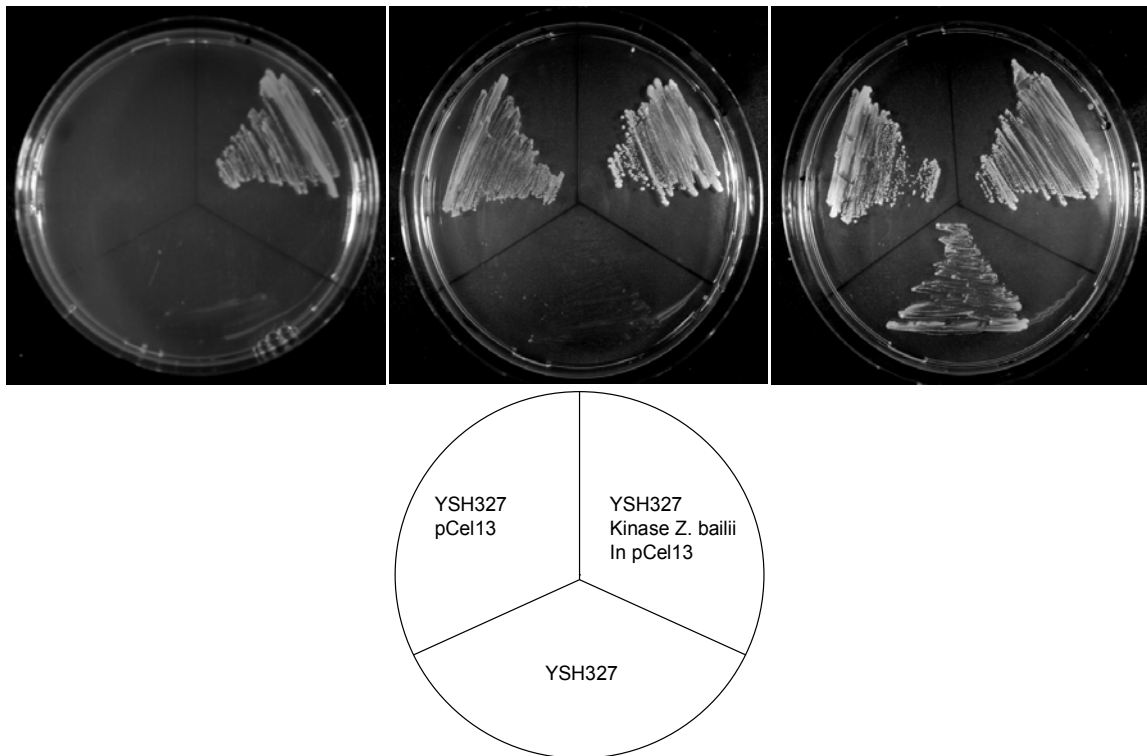
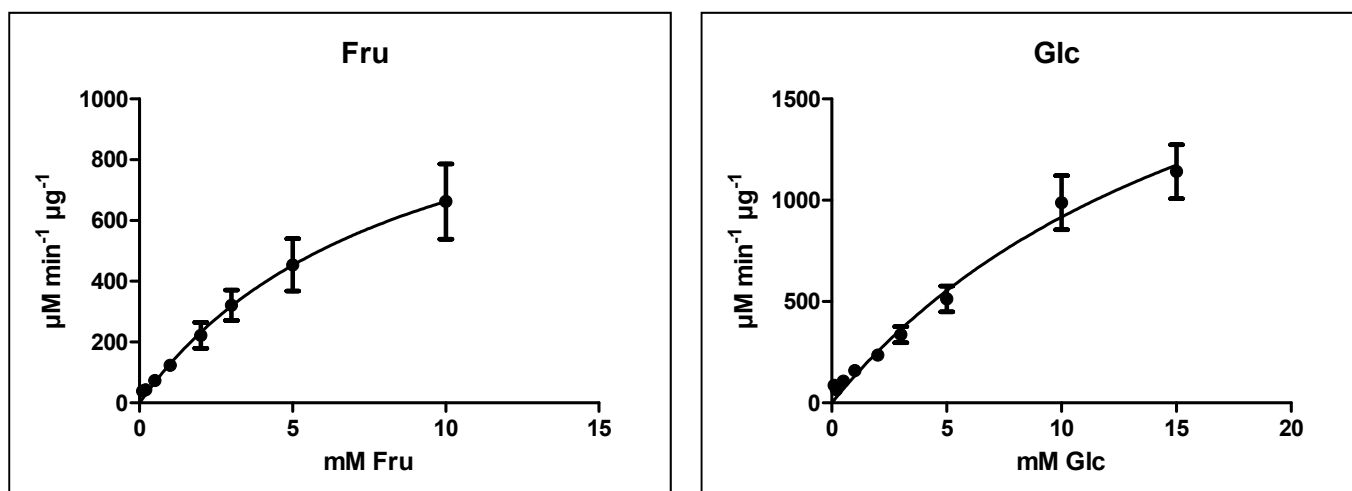


Figure 4.3.2: *S. cerevisiae* *hvk1* and *hvk2Δ* mutant YSH327, transformed with the cloned *Z. bailii* hexokinase gene under the control of the *PGK1* promoter and terminator and the empty vector pCel13, grown, from left to right, on fructose (+ura), glucose (–ura) and glucose (+ura) media).

4.3.2.3 Hexokinase assays

This experiment determined the kinetic parameters (K_m and V_{max}) of hexose phosphorylation using cell extracts of the yeast strain YSH327, transformed with the putative ZbHXK fragment and grown in an YNB medium with either 2% glucose or fructose as carbon source. Negative control experiments were carried out using empty vector transformed cells and also untransformed cells, which were subtracted from the results due to the elimination of Glucokinase activity from the measurements of glucose. On fructose, a V_{max} of 1.237 ± 0.15 mmol/mg dwt/min and a K_m of 8.6 ± 1.9 mM could be determined. For glucose, a V_{max} of 2.667 ± 0.34 mmol/mg dwt/min and a K_m of 19.1 ± 3.5 mM was calculated, with the use of the software Graph Pad Prism. The data are shown in Figure 4.3.2.2.



Figures 4.3.2.2 Kinetic assays of heterologously expressed *Z. bailii* hexokinase activity

Table 4.3.2.3 : Kinetic parameters of transporters and hexokinase, derived from *Z. bailii* and overexpressed in *S. cerevisiae* mutants. K_m values are given in mM, and V_{max} in $\text{mmol mg}^{-1} \text{min}^{-1}$

	Glucose		Fructose	
	K_m	V_{max}	K_m	V_{max}
Permease S	186 ± 15	3.567 ± 0.4	20.4 ± 3.5	2.822 ± 0.15
Permease B	7.9 ± 1.1	0.464 ± 0.022	31.4 ± 2.5	2.628 ± 0.07
Hexokinase	19.1 ± 3.5	2.667 ± 0.34	$8,6 \pm 1.9$	1.237 ± 0.15

4.3.3 Promoter region and binding sites for regulatory elements

Even though no information is published on the regulation or regulatory elements in *Zygosaccharomyces sp.*, the isolated and characterized genomic sequences were screened for some common regulator binding sites which are known to function on regulatory sequences of transporters, hexokinases and other important glycolytic proteins, based on published results of *S. cerevisiae*. This search for highly conserved binding sites in the putative promoter regions of the isolated ORF's was done with the *Saccharomyces cerevisiae* Promoter Database (<http://rulai.cshl.edu/SCPD/>; Zhu and Zhang, 1999).

In *S. cerevisiae*, the mediator component Med8 is a subunit of the mediator CTD complex of RNA polymerase II and appears to bind directly to both positively and negatively acting glucose regulatory elements (Chaves *et al.*, 1999). Med8p was found to bind to the *HXK2* and *SUC2* regulatory region and also to the promoters of *HXK1*, *GLK1* and *HXT1* through a heptameric motif. Among these genes, *HXT1* and *HXK2* are induced, while *HXK1*, *GLK1* and *SUC2* are repressed by high glucose levels (de la Cera *et al.*, 2002). The regulatory mediator was identified in *S. cerevisiae*, with mediator-homolog complexes having subsequently been purified from many eukaryotes. In yeasts, genes encoding for mediator subunits were previously identified in *S. pombe* (Miklos *et al.*, 2005), *K. lactis* (Dujon *et al.*, 2004) and *Z. rouxii* (Comparative genomics of protoploid *Saccharomycetaceae*, unpublished). These biochemical

data revealed a core set of Mediator subunits that are conserved in most, if not all eukaryotes. Since these elements were identified by genome wide sequencing experiments and characterized by homology or comparison, the information on differences between homologue binding sites of non-*Saccharomyces* and *Saccharomyces* are limited. Nevertheless, in *Z. bailii* several putative Med8 sites ((C/A)(G/A)GAAAT) could be identified, that are located up- and downstream off all three isolated ORF's. The fragment of ZT9 contains 16 putative Med8 sites in its entire length, which is statistically highly above the probability of a random sequence for a heptameric basepair motif. One of these binding sites is in the neighborhood of the start codons, namely 25 bases upstream of the probable permease S start codon (Appendix 4.3.1, page 177).

Rgt1 is a glucose-responsive transcription factor that binds to the promoters of several *HXT* genes in *S. cerevisiae* and regulates their expression in response to glucose. Rgt1 contains a Zn₂Cys₆ binuclear cluster responsible for DNA binding. Furthermore, the transcription factor Rgt1 is essential for repression of the *S. cerevisiae* *HXK2* gene in the absence of glucose. Rgt1 represses *HXK2* expression by binding specifically to the motif (CGGAAAA) located at -395 bp relative to the ATG translation start codon in the *HXK2* promoter (Palomino *et al.*, 2005). In the putative ZbHxk promoter region, a similar motif is present -439 bp upstream of the start codon (Appendix 4.3.2, page 180). Rgt1 binding sites are also located upstream of both ORF's encoding putative permeases in the ZT9 fragment of *Z. bailii*. One site is in close proximity to the permease S ORF (Appendix 4.3.1, page 177).

Of additional importance are the binding sites for Gcr1, 16 bp upstream of the putative ZbHXK START codon and 22 bases upstream the predicted permease S ORF. Gcr1p and Gcr2p are transcriptional activators of glycolytic genes in *S. cerevisiae*. Gcr1p is a DNA-binding protein, interacting with the consensus sequence CTTCC, whereas Gcr2p interacts with Gcr1p. Both factors are required for normal transcriptional activation. Null and point mutants have decreased expression levels of most of the glycolytic enzymes (Uemura and Jigami, 1992; Uemura and Fraenkel, 1999).

However, further investigations are required to establish if these regulatory elements play the same role in *Zygosaccharomyces* as in *Saccharomyces*.

4.3.4 Presence of the isolated ORF's in *Z. bailii* strains 210 and 3a

Since both isolated strains of *Z. bailii* differ in the highly conserved ITS1-4 sequence, further differences concerning the genetic code cannot be ruled out. The genomic library was constructed with *Z. bailii* strain 210. It was therefore necessary to establish if the isolated ORF's are indeed present in both strains. The affiliation of both strains to the species *Z. bailii* was verified by the Centraalbureau voor Schimmelcultures, Delft, Netherlands. To verify the

presence of the isolated genes in both strains, PCR was performed with primers flanking the ORF's of the isolated genes. The presence of the *Z. bailii* hexokinase gene as well as of the permease B gene could be confirmed in both strains (Figure 4.3.2.3), , whereas the fragment of the permease S could not be amplified when using the genomic DNA of *Z. bailii* strain 3a as template.

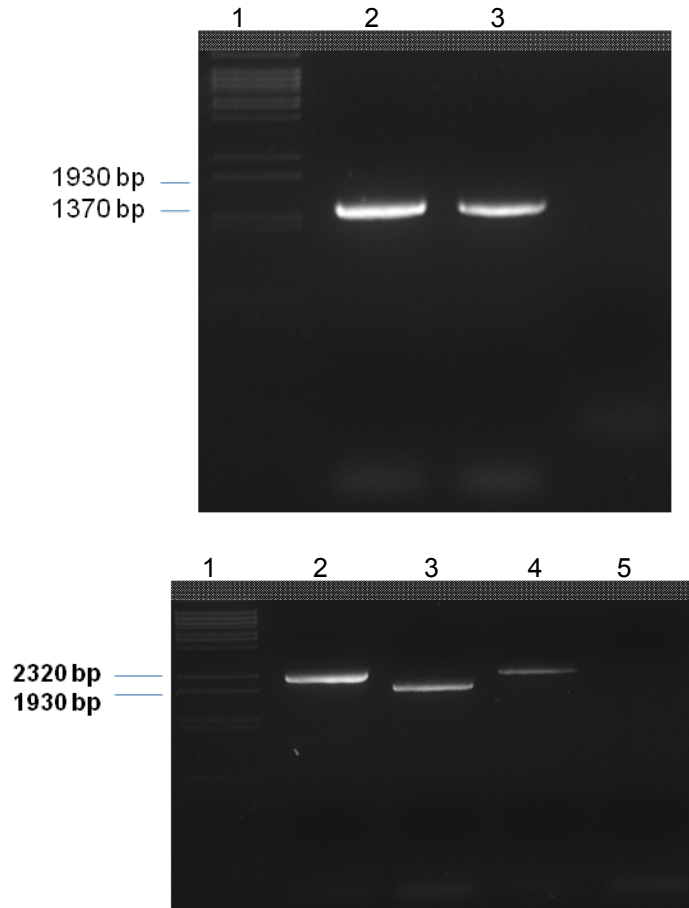
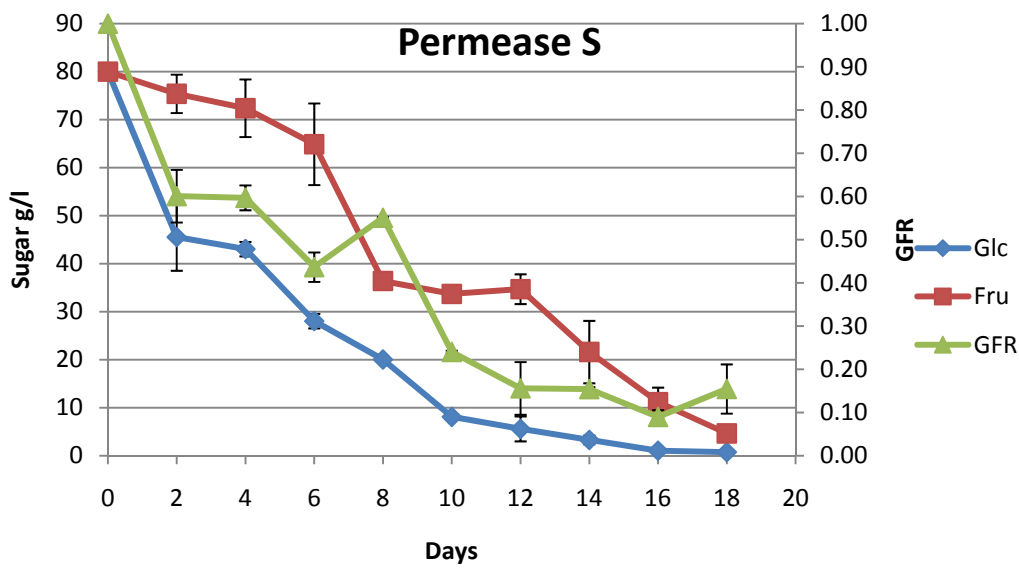
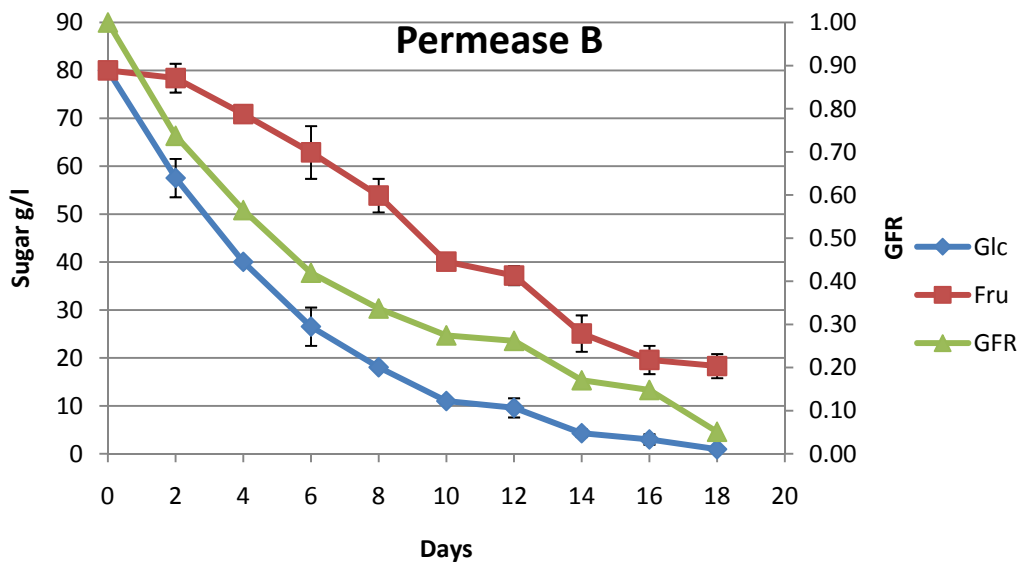
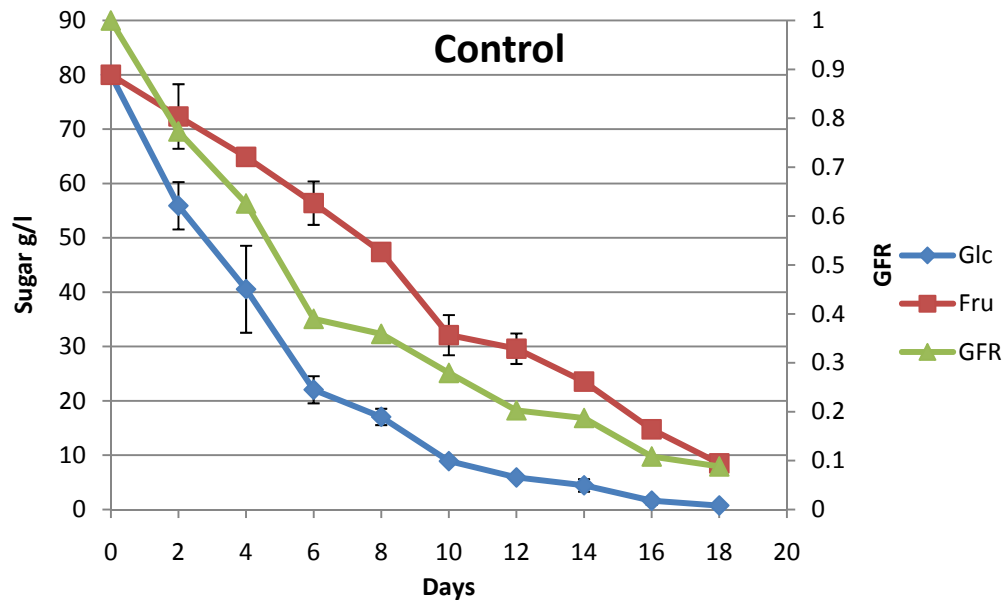
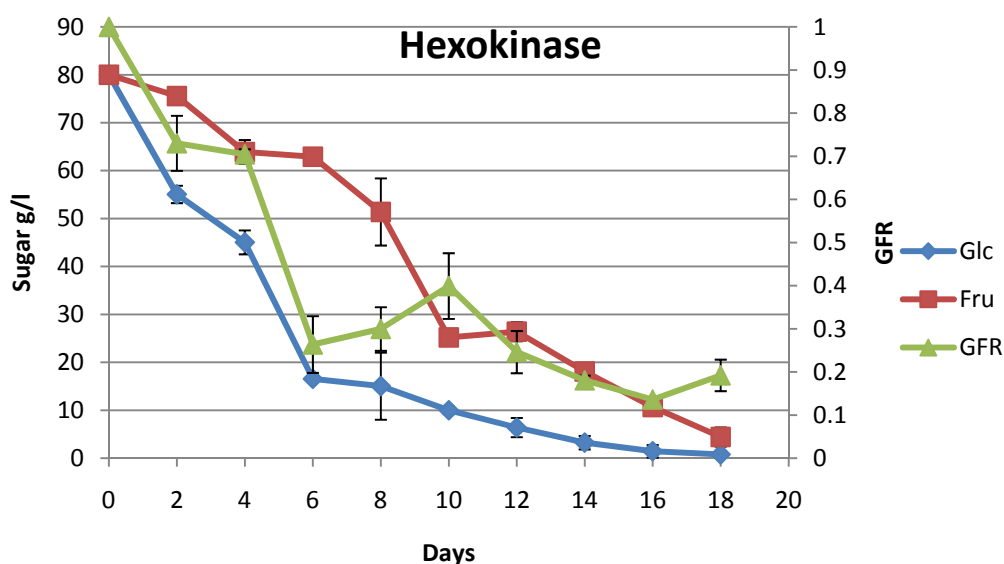


Figure 4.3.2.3: Appearance of the isolated ORF's in the genomic DNA of *Z. bailii* 210 and *Z. bailii* 3a. Upper gel: 1. Lane: Marker; 2. Lane: Hexokinase *Z. bailii* 210; 3. Lane Hexokinase *Z. bailii* 3a. Lower gel: 1. Lane: Marker; 2. Lane: permease B *Z. bailii* 210; 3. Lane permease S *Z. bailii* 210; 4. Lane: permease B *Z. bailii* 3a; 5. Lane permease S *Z. bailii* 3a (no signal).

4.3.5 Fermentation experiments

The *S. cerevisiae* lab strain FY2 (*Mata ura3Δ*), isogenic to *S. cerevisiae* strain S288c, was transformed with the two putative permeases and the hexokinase from *Z. bailii* and inoculated into MS300 synthetic must. Figure 4.3.5 shows the content in g/l of glucose and fructose during the fermentation process, and also indicates the resulting glucose-fructose ratio GFR. The fermentation experiments were carried out in two biological and two technical repeats. All fermentations were followed for 18 days.





Figures 4.3.5: Fermentation characteristics in synthetic must MS300 of Lab strain FY2. From top to bottom: control fermentation, FY2 transformed with pCel13 encoding either *Z. bailii* permease S, permease B or hexokinase.

4.4 Discussion

This study employed a functional complementation strategy using *S. cerevisiae* mutants defective in fructose utilisation, transformed with a genomic library of *Z. bailii*, which were screened for restoration of growth on media containing fructose as carbon source. This strategy enabled the isolation of genes which are thought to be responsible for the sugar preference phenotype of fructophilic yeasts (For review see: Santangelo, 2006). All transport proteins as well as all sugar kinases of the glucophilic yeast *S. cerevisiae* display a preference towards glucose, and in particular a higher affinity to glucose than to fructose (Entian and Mecke, 1982; Reifenberger *et al.*, 1997). A higher affinity towards fructose of the corresponding enzymes in fructophilic yeasts may therefore be expected.

Transformation of the *hxt* null mutant JT5330 with the genomic library reveals a putative transporter cassette of *Z. bailii* that contains three permeases. The open reading frames of two of these genes could be isolated and expressed. Both permeases are to varying degrees able to complement the *hxt* null mutant growth deficit on fructose and glucose. Based on BLAST searches of the corresponding sequences, these proteins are predicted to be putative transmembrane transport proteins. These presumptions were confirmed by the characterization with kinetic uptake experiments. Permease S is characterized as a permease with a medium affinity with fructose (apparent $K_m = 20\text{mM}$). If transformed with this permease, the *hxt* null mutant is also able to grow on glucose, however, the affinity of the permease towards glucose is shown to be low (apparent $K_m = 186\text{mM}$). This permease seems to be a nearly fructose-specific transporter with a medium affinity and high capacity, which also allows poor growth on glucose, when heterologous expressed in the *S. cerevisiae* *hxt* null mutant.

The second isolated transporter, permease B displays a higher affinity for glucose. The apparent K_m value for this sugar was determined as 7 mM, and this glucose preferring carrier also accepts fructose as substrate with a low affinity, indicated by an apparent K_m for this sugar of 31.4 mM. These results correlates with the study of Sousa-Dias *et al.* (1996), which analyzed the uptake kinetics of *Z. bailii*, grown on glucose and on fructose, respectively and reported the K_m for glucose uptake of *Z. bailii* to be 7 mM. In the same study it was shown that glucose uptake is inhibited by the presence of fructose.

Taken together with the previous data of Sousa-Dias, permease B might be the only glucose uptake system of *Z. bailii*, which is downregulated in the presence of fructose, but additionally allows low affinity fructose uptake, when overexpressed in *S. cerevisiae*.

Fructose uptake of *Z. bailii* may be the result of different fructose specific transport systems. This presumption is substantiated by the previous isolation of a fructose specific transporter Ffz1 of *Z. bailii* by Pina *et al.* (2004), which is not able to transport glucose. This transporter was characterized with an apparent K_m for fructose at 80 mM. In the study of Sousa-Dias, fructose uptake of *Z. bailii* was determined to have a K_m of 65 mM. Our kinetic data reveals a $K_{m\text{ App}}$ of 20 mM for the isolated fructose permease S. These data lead to the conclusion that fructose uptake of *Z. bailii* is mediated by at least three different carrier systems, namely, the two permeases, isolated in this study and the previously isolated fructose specific facilitator Ffz1. It certainly cannot be ruled out that further transporters are involved in the uptake of fructose.

As illustrated by sequence alignments (Appendix 4.3.1.1, page 165) and by phylogenetic relationship (Figure 4.4.1), both isolated permeases share a poor homology and therefore relationship with main hexose transporter families, members of the Major Facilitator Superfamily (MFS) or the ATP-Binding Cassette (ABC) superfamily. The closest relatives are two previously uncharacterized proteins, derived from the sequencing project of *Z. rouxii*.

A protein structure prediction, which has to be interpreted with precaution, indicates the presence of 11 transmembrane domains (TMD) for permease B and 9 TMDs for permease S, which seems to be unusual, but not unknown for hexose transporters. For example, 11 TMDs are predicted for the *Torulaspota delbrueckii* low-affinity glucose transporter (Alves-Araújo *et al.*, 2005) and the sugar phosphate permease of *Cryptosporidium parvum* (Abrahamsen *et al.*, 2004), whereas the glycerol/H⁺ symporter Stl1 of *S. cerevisiae* is predicted to have 9 TMD (Ferreira *et al.*, 2005). Some contradictory results are published about the number of transmembrane domains of the *S. cerevisiae* Hxt carriers. While most publications distinguish between 12 transmembrane domains, however, several internet databases are in conflict about the precise number (SGD; TMHMM; PredictProteins). For example, using TMHMM server, the number of Hxt2 TMD's are predicted at 12, whereas the PredictProtein server assumes the protein to have 11 TMD's. On the other hand, hydropathy analysis of protein sequences and topological studies with gene-fusion constructs predicted that almost all MFS proteins possess a

uniform topology of 12 transmembrane α -helices (TMs) connected by hydrophilic loops, with both their N- and C-termini located in the cytoplasm (Law *et al.*, 2008). However, as mentioned above, exceptions to the 12 TM rule do exist. Many transporters have arisen by intragenic duplication, triplication and quadruplication events, in which the numbers of transmembrane α -helical hydrophobic domains have increased. Gene fusion, splicing, deletion and insertion events have also contributed to protein topological diversity (Heylighen *et al.*, 1999). Therefore, several numbers of TMD's can and do form helical bundles that function efficiently. The reason why 12 TMD carriers predominate, is maybe in part statistical rather than functional, due to the fact that there are more routes to six than to five or seven (Saier, 2003).

Information about substrate binding cannot be predicted by the amino acid primary structure. Data concerning fructose binding of sugar transporters are scarce in general and, in addition, both of the isolated *Z. bailii* transport systems have low homology to characterized sugar transporters. A highly conserved sugar transporter signature sequence, which occurs in all members of the sugar transporter family from prokaryotes to eukaryotes (Bairoch, 1993; Kruckeberg, 1996), is not present in any of the newly characterized *Z. bailii* permeases. However, this sequence is also absent in the Ffz1 fructose specific transporter, isolated by Pina *et al.* (2004), so all newly isolated *Z. bailii* transporter may function differently with regards to sugar recognition. Like permease S and B, Ffz1 has a low homology to other yeast sugar uptake systems, and the relationship between Ffz1, permeases S and permease B is very low as well. A similarity search reveals 17% identities between Ffz1 and permease B; 18% identity between the sequence of Ffz1 and permease S; whereas the similarity between the two permeases is 14%. One small sequence of 12 amino acids in the outside region between putative transmembrane domains 5 and 6 of permease B (predicted asn-glycosylation site) reveals a 63% similarity to Ffz1. When taking these results in consideration, functional and regulatory motifs of the newly isolated permeases of *Z. bailii* have to be determined via amino acid substitutions or mutated alleles. The identification and characterization of especially the site of substrate recognition and/or binding would be of great interest. The characterization of the uncharacterized proteins of *Z. rouxii* which have a high similarity to the *Z. bailii* permeases would also be helpful for the further characterization of these comparatively unknown transport systems. Gene deletions in the host strain could furthermore help to update the kinetic data, since three sugar uptake systems have now been characterized to be involved in sugar uptake of this fructophilic yeast. In addition, such experiments could establish if there are more genes which encode fructose and glucose transport systems in this yeast.

Heterologous expression of the *Z. bailii* genomic library in the *hvk* deleted mutant YSH327 allowed the identification of one ORF that allows the growth of this mutant on fructose. The isolated open reading frame contains a predicted coding sequence of 1458 bp that corresponds to a predicted peptide of 486 amino acids, encoding for a hexokinase of *Z. bailii* (ZbHxk), with a homology of 75-76% to both hexokinases of *S. cerevisiae*. This hexokinase

phosphorylates both sugars, but displays a preference to fructose, which was revealed by the kinetic data as shown with a higher affinity of 8.6 mM for fructose, compared to 19.1 mM for glucose, when overexpressed under the control of the PGK promoter. Compared to the affinities of *S. cerevisiae* hexokinases, ZbHxk shows a lower affinity for both sugars. The affinity of Hxk1 for glucose ($K_m=0.12$ mM) is higher than for fructose ($K_m=1.5$ mM), in spite of its higher V_{max} with fructose. Hxk2 also displayed a higher affinity for glucose ($K_m=0.25$ mM) than for fructose ($K_m=1.5$ mM). Furthermore, the affinity for glucose is slightly higher for Hxk1 than it is for Hxk2, while the affinity for fructose is similar (Bernard, 1975; Entian and Mecke, 1982).

The isolated sugar kinase may not be classified as a fructokinase or ketohexokinase. This special group of fructose specific sugar kinases, mainly occurring in plants and bacteria, contains conserved domains for substrate recognition and ATP binding, which are absent in the ZbHxk sequence (Kanayama *et al.*, 1997). A database homology comparison with typical fructokinases reveals identities below 25%. ZbHxkp possesses the typical sequence and structural characteristics of a hexokinase, with the additional interesting feature that the structure of domain 1 is similar to *S. cerevisiae* Hxk1 and domain 2 has a higher homology to ScHxk2. A block of highly conserved amino acids in the hexokinase gene family, which seems to function in sugar binding (Schirch and Wilson, 1987) and provides a possible distinction between glucose and fructose (Hohmann *et al.*, 1999) is identical with the ScHxk1p sequence block (Appendix 4.3.2.1, page 171). The relationship between ZbHxk and the closest relatives is shown in the phylogenetic tree presented in Figure 4.4.2.

The particular substrate specificity of the *Z. bailii* hexokinase is reflected in a higher affinity for fructose than for glucose. A previously published sugar kinase, Hxk1 from the fission yeast *Schizosaccharomyces pombe* was observed to have similar characteristics, however, the SpHxk1 was shown to contain a lower K_m for both sugars, fructose as well as glucose (Petit *et al.*, 1996). However, although both of the sugar kinases of *S. pombe* and *Z. bailii* attest similar sugar specificities, the sequence similarity is below 50%. Substrate binding or sugar specificity of hexokinases seems not to be highly conserved, since a higher homology to hexokinases from closely related yeasts can be observed than to hexokinases with similar sugar affinities.

In the case of the permeases, a clearer understanding of the procedure of ZbHxk sugar binding could reveal the reason for a hexose preference of the catalytic activity. However, even with the substrate glucose there are still unsolved questions concerning the ScHxk substrate recognition and binding affinities (Blume *et al.*, 2009).

An open question is whether there are other isoenzymes of the isolated hexose phosphorylation enzyme of *Z. bailii*. With the selected strategy we did not succeed in isolating more than this single hexokinase-encoding gene. This could be due to several reasons: firstly the isolated hexokinase may be unique in *Z. bailii*; secondly, possible isoenzymes are not present or not entirely present in the constructed genomic library; or, thirdly, the method of functional complementation in the *S. cerevisiae* mutant does not allow the isolation of additional

sugar kinases, because of non-expression of the *Z. baillii* genes in *S. cerevisiae*. Furthermore, the glucose transport system of *Z. baillii* seems to be repressed in the presence of fructose, so it can not to be ruled out that a putative conventional hexokinase or glucokinase may be also repressed by growth on fructose in *S. cerevisiae*, also the existence of such a repression pathway in *S. cerevisiae* is rather unlikely. Therefore, further studies are required to answer these additional questions. The question about further hexokinases or glucokinases could be solved by gene disruption after haploidization, which was observed to be difficult due to the peculiar life cycle of the fructophilic yeast, in which mitotic but no meiotic spores appear to be formed (Rodrigues *et al.*, 2003).

Heterologous overexpression of a single isolated *Z. baillii* gene in the *S. cerevisiae* lab strain FY2 did not lead to a clear alteration of the hexose preferring phenotype of this strain. All fermentations, performed with MS300 synthetic must, followed a similar pattern of glucose preference, comparable to the control fermentation, which was transformed with the empty vector. In all samples, incomplete fructose consumption at the late phase of fermentation could be observed. Glucose was always consumed faster, except between day 6 and 8 of fermentation, where the permease S transformants showed accelerated fructose consumption resulting in a slight increase of the GFR between these time points. The same effect, but to a lesser extent, was observed with the hexokinase transformants between days 6 and 10. Between these time points, permease S and ZbHxk transformants consumed more fructose than glucose. It is not clear, if the heterologous expression of the *Z. baillii* genes causes these slight changes in the fermentation pattern, since accelerated rates of fructose consumption could also occur in standard fermentations. In total, all fermentations exhibited a form of glucophilic fermentation, since glucose was utilized faster.

The fructophilic phenotype of yeast is likely to involve a complex network of sugar sensing, transport, phosphorylation, induction and repression similar to the glucophilic sugar pathway of *S. cerevisiae*. It will be challenging to dissect this network, and our understanding of how the proteins involved in this network interact and achieve substrate binding and recognition requires significant research. It is rather sobering to consider that our knowledge has progressed in only a limited way from the models proposed more than 50 years ago when since Otto Fritz Meyerhof purified hexokinase from yeast in 1927, or when the first step of the glycolytic pathway was clarified in the 1940 by Sidney Colowick and Herman Kalckar (1940), and the first mention of yeast permeases in the 1950 by Monod and his colleagues (Barnett, 2003).

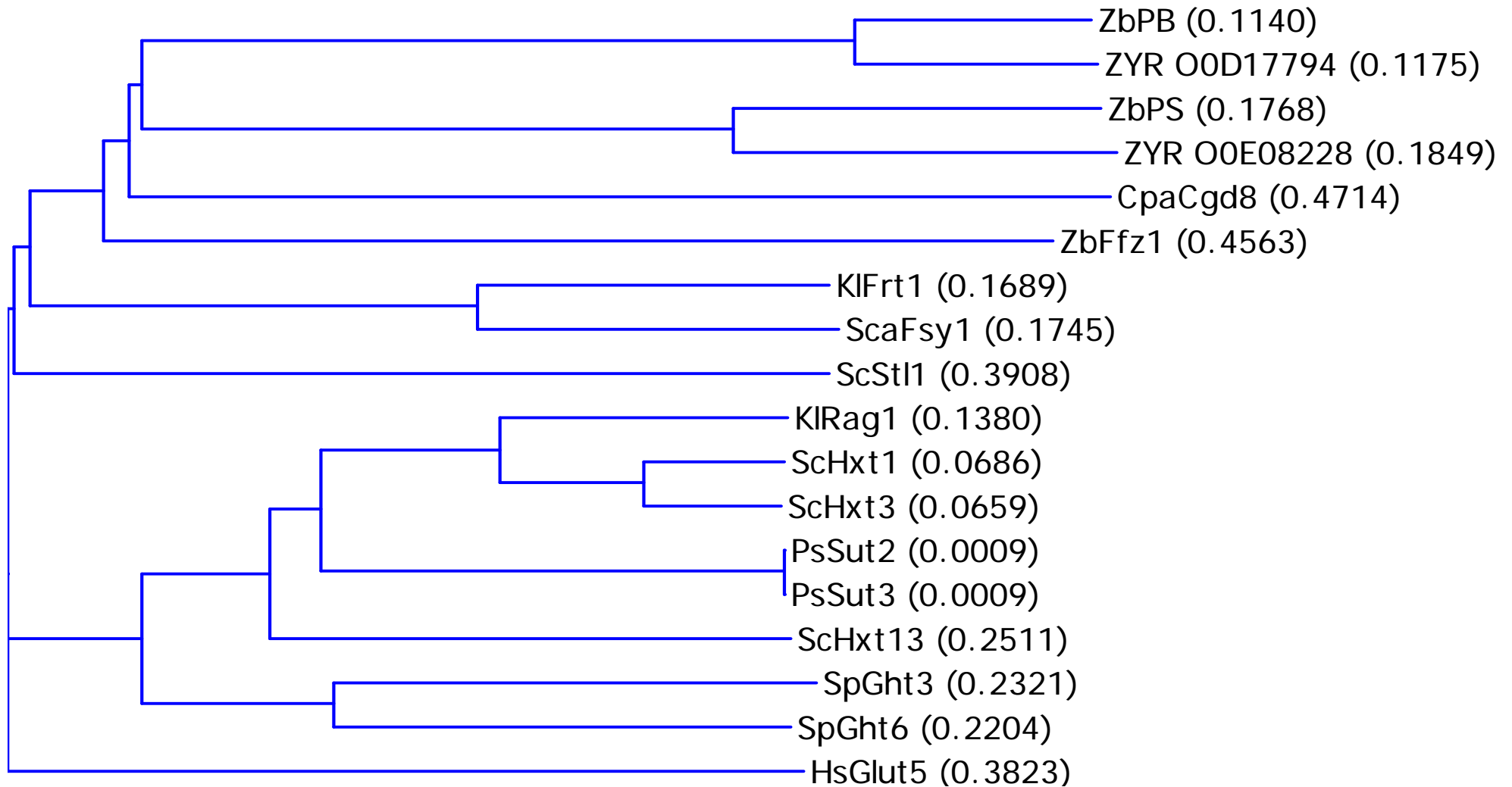


Figure 4.4.1: Phylogenetic tree based on primary sequence similarity depicting predicted evolutionary relationship of *Z. bailii* (Zb) permeases (ZbPS and ZbPB) to *S. cerevisiae* (Sc), *P. stipitis* (Ps), *S. pombe* (Sp), *K. lactis* (Kl), *S. carlsbergiensis* (Sca), *Z. rouxii* (Zyr) yeast and fungal hexose transporters as well as related membrane proteins from *Cryptosporidium parvum* (Cpa) and *H. sapiens* (Hs). The tree was built using the Vector NTI algorithm for multiple alignments

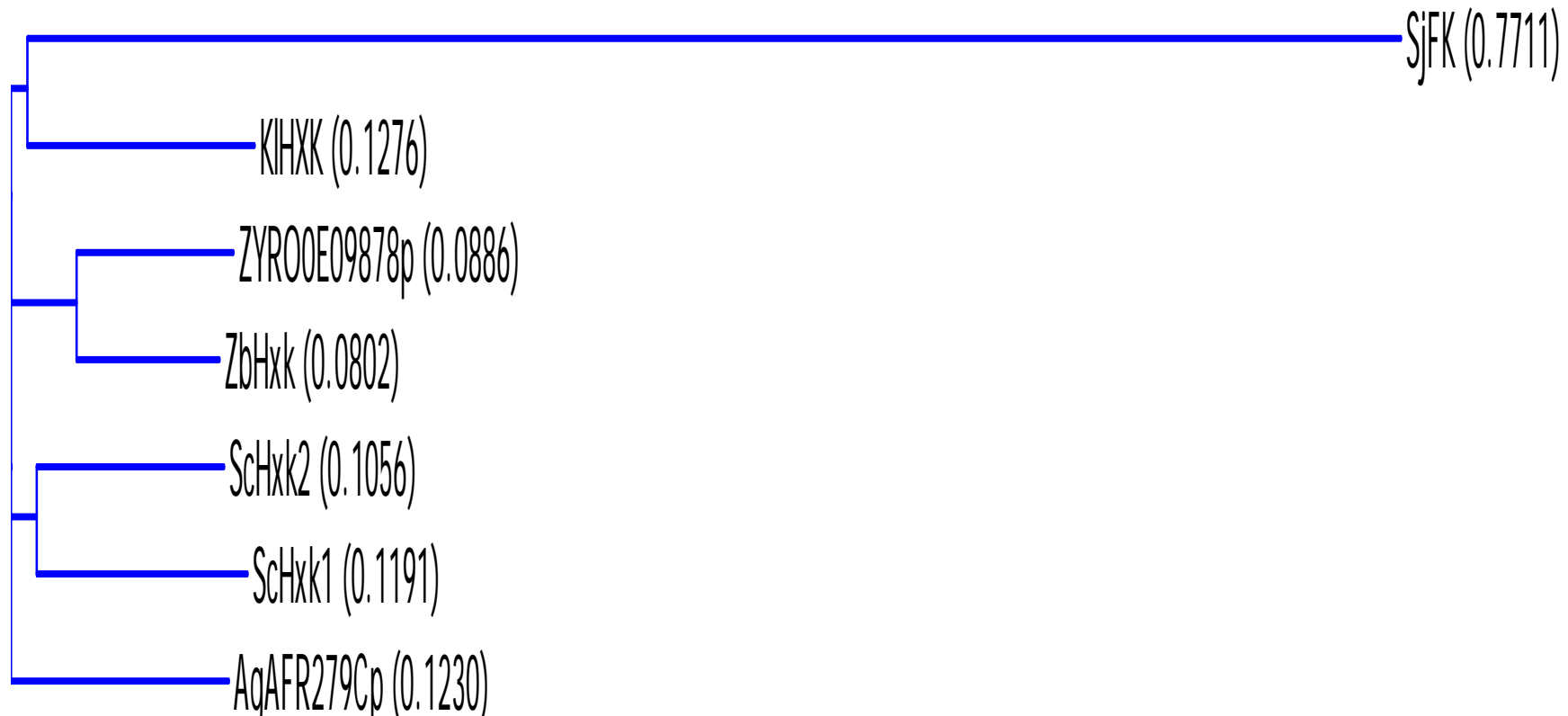


Figure 4.4.2: Phylogenetic tree based on primary sequence similarity depicting predicted evolutionary relationship of *Z. bailii* (Zb) Hexokinase to *S. cerevisiae* (Sc), *K. lactis* (Kl), *Z. rouxii* (ZYR), *Ashbia gosypii* (Ag) yeast and fungal hexokinases as well as an uncharacterized fructokinase from *Schizosaccharomyces japonicus* (Sj). The tree was built using the Vector NTI algorithm for multiple alignments.

4.5 References part 4:

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Chapter 5

General discussion and conclusions

5. General Discussion and Conclusions

5.1 Introduction

This study has significantly contributed to our understanding of the role of yeast in the onset of stuck fermentations, which causes significant economical losses every year in international wine industries. In particular, it could be confirmed that incomplete wine fermentations are usually characterized by undesired residual fructose content. The conventional wine yeast, *Saccharomyces cerevisiae*, is a glucophilic yeast and our data suggest that a low glucose/fructose ratio (GFR) presents an inhibiting factor for fermentations. We show that stuck fermentations can be cured in small, medium as well as large scale fermentations, probably due to an increase of the GFR in the last stage of fermentation. This increase can be achieved by glucose addition, which is internationally forbidden in industrial winemaking, or by the inoculation with fructophilic yeasts. This study furthermore describes the isolation of two candidate strains of the fructophilic yeast *Zygosaccharomyces bailii*, and the first trials in small scale test fermentations of stuck wines. The study also experiments up to the point of industrial scale winemaking, and shows the applicability of this strategy to cure stuck fermentation in industrial settings. It is important to highlight that such an endeavour is unusual, since few research projects in the wine industry can be carried out in such a large scale. A good relation with industry partners enabled such a study, and the combined data are clearly providing statistically solid evidence for our claims.

The molecular part of this thesis contributes to the understanding of the fructophilic phenotype of *Z. bailii* by characterization of some of the proteins responsible for the first two steps in sugar metabolism, the transport of sugar into the cell by transmembrane proteins and the sugar phosphorylation by hexokinase, which are thought to be responsible for sugar preference.

5.2 Methodology for curing stuck fermentations- critical discussion

Although non-*Saccharomycetes* are members of the natural microflora of grapevine (Schütz and Gafner, 1993; Mills *et al.*, 2002) and they furthermore are contributing to the complex aroma profile in natural fermented wines (Jolly *et al.*, 2006), it is very unusual to inoculate them as a treatment in winemaking. Members of this diverse group are often seen as a source of microbial spoilage and especially yeasts from the genus *Zygosaccharomyces* are described as major spoilage organisms in industrial food workflows (Sousa *et al.* 1996, Prudencio *et al.*, 1998). However, this research shows evidence pointing to a positive contribution in treatments of defective wine fermentations, since our data illustrates that a

treatment with fructophilic yeast species seems to cause a restart of the fermentation activity. Moreover, spoilage with undesired metabolic by-products and in particular with acetic acid could not be detected.

Methods for restarting stuck fermentations are described in the literature and in manuals for sound enological practices. For example, Ribéreau-Gayon *et al.* (2006a and 2006b) advises the re-inoculation with an active yeast starter with a tolerance to ethanol in combination with a temperature shift to 20-25°C. In various cases, as shown in this study, these conventional winemaking methods are adequate to restart the fermentation until the wine becomes dry. However, in several cases there is no effect after this first aid-method, or the fermentation completely stops after this treatments. Hence, we amended the definition of stuck fermentations in the course of this work to better discriminate between stuck fermentations and struggling fermentations.

This newly described methodology is adapted for curing stuck fermentations exclusively when conventional winemaking treatments fail. Therefore, it is not possible to compare this method with other, previously described options, such as physical treatments. Frequently, incompletely fermented wines are blended with other wines. Alternatively, stuck wines can also undergo a re-fermentation in a large quantity of grape must in the course of the following vintage. However, both options are linked with specific conditions in a given winery and depend on the qualities of the desired wines. Nevertheless, all of this options are associated with an economic loss and sometimes the loss of a special wine vintage. The costs for the described treatment can be calculated with about 0.1 €/1 Rand per Liter of treated wine for the yeast product (approx. 100 €/1000 Rand for 500g package). Additionally there are costs for the facilities and the energy for warming and the manpower, which are not as easy to analyse for costs due to the variable circumstances in wineries.

Success rate of this newly described treatment with *Z. bailii* is approximately 90%, if using active yeasts as in this study. First trials with a cryopreserved dry yeast product indicate, however, a drop in the success rate. In the course of this study it was not possible to substantiate this decrease, as the treatments were no longer under our control and the methodology was the individual responsibility of the concerned wine producers. Secondly, research about the production of cryopreserved dry yeast products of non-*Saccharomyces* is at the beginning and will be the subject of further investigations.

The exact trigger of this new treatment, which enables *Z. bailii* and the co-inoculated *S. cerevisiae* to restart the fermentation is not fully understood. Additionally, the uncertainty of the reason(s) for stuck fermentations in industrial winemaking, which seems to be a result of several synergistic acting factors, make it difficult to substantiate the reasons for several failed treatments. In some cases it was not possible for the responsible producers to increase the temperature, as recommended by the standard methodology, due to special conditions in the winery.

One big disadvantage of this method is time consumption, as treatment can take up to 12 weeks. Due to the increased temperature during the treatment, an enhanced risk for microbial spoilage exists. Therefore, microscopical monitoring is an obligation during the treatment, which adds to the effort required and creates a need for additional equipment or consultation.

The best alternative to this method would be the use of a *S. cerevisiae* strain with fructophilic properties. Firstly, a fructophilic *S. cerevisiae* strain, inoculated for a standard wine fermentation could avoid a low GFR. Therefore, a fructophilic *S. cerevisiae* could prevent the occurrence of stuck fermentations in probably many cases, because the prevention of stuck fermentations is essential to winemaking and it should take into account all of the recommendations previously stated. And secondly, such a potential fructophilic *S. cerevisiae* strain could also be used for the cure of stuck fermentations in the same manner as *Z. bailii*.

5.3 Fructophilic phenotype

The development of a fructophilic or glucophilic phenotype seems to be regulated at the initial steps of sugar metabolism, since after the formation of fructose-6-phosphate the pathways of both sugars are unified. All Hxt hexose transporters (Reifenberger *et al.*, 1997) and also the sugar kinases (Entian and Mecke, 1982) of the glucophilic yeast *S. cerevisiae* display a higher affinity to glucose, whereas the newly isolated and characterized proteins of *Z. bailii* display a higher affinity to fructose, with the exception of permease B. Permease B seems to be downregulated in the presence of fructose, since glucose uptake in *Z. bailii* only proceeds in the absence of fructose (Sousa-Dias *et al.*, 1996).

All previously identified fructophilic yeasts are yeasts with a relatively weakly fermentative metabolism, when compared to *S. cerevisiae* (Torija *et al.*, 2001; Mills *et al.*, 2002; Yu *et al.*, 2006; Zott *et al.*, 2008). In summary, the fructophilic phenotype is characterized by a weak metabolic rate when compared to the glucophilic phenotype. The specification of fructophilic yeasts as slow fermenters could result from the low substrate affinity of the enzyme- and the protein-equipment of such microorganisms. For example, all identified, characterized and published proteins of *Z. bailii* indicate medium or low affinities to their respective substrates (Sousa-Dias *et al.*, 1996; Merico *et al.*, 2001; Branduardi, 2002; Pina *et al.*, 2004; Rodrigues *et al.*, 2004; This study) and in every sense a lower enzyme activity compared to *S. cerevisiae*. Another mentionable aspect is that the hitherto characterized high affinity fructose transporter of yeasts are energy dependent (Frt1, Fsy1), whereas the high affinity glucose transporter not (Hxt2). This is definitively a disadvantage for the growth rate on fructose, since glucose transport in environments with low substrate concentrations is energy-independent. Up to now,

no high affinity fructose transporter was characterized which acts by an energy-independent facilitated diffusion system.

The newly isolated hexokinase of *Z. bailii* seems to be the second hexokinase of yeasts, apart from Hxk1 of *S. pombe* (Petit *et al.*, 1996), which has a higher affinity to fructose than to glucose. Both hexokinases are characterized as having medium affinities to both sugars. It cannot be excluded that the preference for fructose is bought at the expense of the affinity for different substrates. It has not yet been possible to create an exact model for substrate binding and recognition, for either the transport proteins or the hexokinases. It would be essential for the elucidation of the fructophilic phenotype, if exact substrate binding sites within the whole available selection of fructose preferring proteins could be detected.

With the triose phosphate isomerase, one more glycolytic enzyme of *Z. bailii* has been characterized (Merico *et al.*, 2001), beneath the hexokinase and the different transport proteins, which allocate sugars for glycolysis. The clear consideration of fructose mediated glucose repression and the generally low activities of the glycolytic enzymes leads to the presumption that *Z. bailii* seems to be assigned to a biological niche. Fructophilic behaviour and several other characteristics could act as growth advantages in specific natural environments, without evolving a metabolism turnover as fast as *S. cerevisiae*. *Z. bailii* is described as osmophilic (Corry, 1978) and extraordinarily resistant to common preservatives like Sulfur Dioxide (>3 mg/l at pH 3.4), Sorbic Acid (600-800 mg/l), Benzoic Acid (600-1'000 mg/l), Ethanol (>18% (v/v)) and Acetic Acid (20-25 g/l) (Thomas and Davenport, 1985; Fugelsang, 1998). The combination of the mentioned properties would certainly contribute to securing a growth advantage in a specific biological niche. Under this assumption it would be difficult to identify and transfuse a hexose preferring phenotype, since the advantage of a fructose preference would not be critical to the assertiveness of the yeast. With the aim to define a fructophilic principle, a comparison with other fructose preferring yeast species or organisms seems to be essential.

It is not possible to superimpose a fructophilic phenotype into a glucophilic yeast strain by a heterologous expression of a single gene, encoding for a protein with preference for fructose. Possible reasons therefore includes that the total affinity for fructose is higher with the existing proteins from *S. cerevisiae* than with the heterologous expressed from *Z. bailii*, although the proteins from the latter yeast have been characterized to exhibit a preference for fructose, while the *S. cerevisiae* proteins prefer glucose. Consequently, it would not be possible to force the turnover of fructose with the expression of a protein with lower affinity. Furthermore, the whole hexose metabolism is a strongly and complexly regulated network of sensing, recruiting, expression and repression systems (for review see: Santangelo, 2006). Several binding sites of regulatory elements are located in promoter regions, which are absent in the heterologous expressed ORF's. It can, however, not be ruled out that binding sites for regulatory elements

inside the coding region are slightly different between the two species and therefore not efficient, which would account for the absence of regulatory elements. The probability of expressing a fructophilic phenotype would increase with the number of simultaneously expressed “fructophilic” genes. The simultaneous expression of several genes would be potentially a helpful tool for the characterization of the fructophilic phenotype.

It is also possible, that the fructophilic phenotype only occur because of an inhibition or at least partial absence of glucose metabolism. An absence of the glucose metabolism can be excluded in *Z. bailii*, since this yeast is able to grow on glucose as well, with a similar growth rate than on fructose (Merico *et al.*, 2003). The inhibition of glucose transport was confirmed by the study of Sousa-Dias *et al.* (1996), however, further regulatory processes of fructophilic yeasts have not been identified to date. It would indeed be peculiar if the genes of the transport systems are the only systems which are affected by a sugar mediated repression.

We are still far away from the clarification of the fructose preference. It has been possible to isolate and characterize transport proteins and a hexokinase, with catalytically active binding sites displaying a preference to fructose, from the fructophilic yeast *Z. bailii*. The role of fructose in sugar mediated gene regulation has to be clarified and would be an interesting topic for future studies. It might be possible to draw parallels between the hexose signal transduction pathways of *S. cerevisiae* and the putative pathways of *Z. bailii*, but even in the case of *S. cerevisiae* there are still significant gaps in our understanding of hexose signal transduction.

5.4 Innovation medal award

The isolation of *Zygosaccharomyces bailii* and the development of a successful methodology for the second most important enological problem results in the product Fructoferm W3©, which was awarded in 2007 with the Innovation Medal for Enological Products on Intervitis/Interfructa in Stuttgart, Germany.

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Appendices

Transporter Alignments 1

		Section 1						
		1	10	20	30	40	53	
ZbPB	(1)	MSDENHSI	YBGMNHR	EEVGPTFK	KD PVS	INVVKSEDD	STGDGADETR	SI ESL
ZbPS	(1)	-----	-----	-----	-----	-----	-----	MTYT
ZYR O0E08228	(1)	-----	-----	-----	-----	-----	-----	MNTP
ZYR O0D17794	(1)	-----	-----	-----	-----	-----	-----	MMAVDET
ZbFfz1	(1)	-----	-----	-----	-----	-----	-----	KPSYV
CpaCgd8	(1)	---	MEELKLN	NAINSNY	CEEBEID	L CANDEL	R LFRDR	SNYLEK
KIFrt1	(1)	-----	-----	-----	-----	-----	-----	MSSNLSE
ScaFsy1	(1)	-----	-----	-----	-----	-----	-----	MSHVNAS
KIRag1	(1)	-----	-----	-----	-----	-----	-----	MS
ScHxt1	(1)	-----	-----	-----	-----	-----	-----	MNSTPDL
ScHxt3	(1)	-----	-----	-----	-----	-----	-----	MNSTPDL
ScHxt13	(1)	-----	-----	-----	-----	-----	-----	MSS
SpGht3	(1)	-----	-----	-----	-----	-----	-----	-----
ScStl1	(1)	-----	-----	-----	-----	-----	-----	-----
HsGlut5	(1)	-----	-----	-----	-----	-----	-----	-----
SpGht6	(1)	-----	-----	-----	-----	-----	-----	-----
PsSut2	(1)	-----	-----	-----	-----	-----	-----	-----
PsSut3	(1)	-----	-----	-----	-----	-----	-----	-----
		Section 2						
		54	60	70	80	90	106	
ZbPB	(54)	TTNHEBEL	LPEDLKS	IPKMVRQ	VVTLLEDD	PHPCFTFR	VVVL S	IIFILPG
ZbPS	(5)	LKNFFREI	PIDPWGR	LKYGILP	VRRIVDE	CRDDSLGS	NDBFDQ	NCNTTEA
ZYR O0E08228	(5)	TTSLMKEI	FVDPWRL	KFGIIPV	KRVLEBD	NLVKTG	STE	-----
ZYR O0D17794	(42)	DEDTTEK	ELPDDLK	DI PSIV	KGVVTL	LEDDPQ	EPCTFR	MVILS
ZbFfz1	(1)	-MVKIDAS	ADLDW	TNLPPQ	RLGRIG	GKYIND	SVDEAG	PI S
CpaCgd8	(50)	TSTGFPS	CDGSSK	SSRNYS	IDNLCML	MGNNGS	NGVIK	PGI V
KIFrt1	(16)	FGLLKST	SSVDSV	HEVEAG	VKVGED	DDNY	-----	FFSEDQ
ScaFsy1	(19)	SRVEMEK	PATKEV	FEDPTP	KKFEL	DDDBAG	--	KTSSV
KIRag1	(3)	NQMTDST	SAGSGT	EHSDT	NTALKA	GS	-----	PNDLK
ScHxt1	(8)	ISPQKSN	SSNSY	LESGR	SKAMNT	PEG	-----	KNE
ScHxt3	(8)	ISPQKSS	SENSN	ADLPS	SSSQVM	NMPBE	-----	KG
ScHxt13	(4)	AQSSIDS	DGDV	RDA	I HVAP	PEKE	WS	-----
SpGht3	(1)	-----	-----	-----	-----	-----	-----	-----
ScStl1	(1)	-----	-----	-----	-----	-----	-----	-----
HsGlut5	(1)	-----	-----	-----	-----	-----	-----	-----
SpGht6	(1)	-----	-----	-----	-----	-----	-----	-----
PsSut2	(1)	-MSSQDL	PSGAQ	TPIDG	SSILE	DKV	BQ	-----
PsSut3	(1)	-MSSQDL	PSGAQ	TPIDG	SSILE	DKV	BQ	-----
		Section 3						
		107	120	130	140	159		
ZbPB	(107)	DTLNSYR	TTSAAYS	VFPVQI	CSYWF	GKWLAK	VL PD	IKIGI
ZbPS	(58)	NELVKVD	TDVIVQ	KEKNLT	NSENISE	DAGDV	INDQEA	L T
ZYR O0E08228	(47)	-----	-----	-----	-----	-----	-----	-----
ZYR O0D17794	(95)	DTMNSYR	TTSAAYS	IPFVQI	CSYWF	GKWLAK	IL P	SKQ
ZbFfz1	(53)	LPLTYTE	SVBGD	GREVV	LTFAP	ADPQD	PYNW	LSRK
CpaCgd8	(103)	LENVNIE	NILSSK	KRMIN	LRYB	WFISK	LMDL	TM
KIFrt1	(62)	IANTPVT	KTLRQ	RMRME	FCEVN	FSNKTY	MVIM	LG
ScaFsy1	(70)	LGDSFK	RDRS	FWGKM	KALB	FEVEF	ENKKN	MVLM
KIRag1	(37)	H---	EEDLND	LEKT	ABETL	QKPA	KEYI	FVSL
ScHxt1	(38)	S---	FHDNLS	ESQVQ	PAVAPP	NTGK	GVYV	TVSI
ScHxt3	(37)	---	VQDDF	QAEAD	QVLT	NPNTG	KGAY	VT
ScHxt13	(31)	-DG---	FDDNE	VINGD	NVEPP	KRGLI	GYL	LV
SpGht3	(1)	-----	-----	-----	-----	-----	-----	-----
ScStl1	(1)	---	MKDLK	LSNFK	GKPI	SRTSH	WG	L
HsGlut5	(1)	---	MEQQD	QSMKE	GR	LT	VL	AL
SpGht6	(1)	---	MAKIL	TIV	MLV	FV	S	M
PsSut2	(27)	---	SSSNS	QSDL	ASIP	TG	I	K
PsSut3	(27)	---	SSSNS	QSDL	ASIP	TG	I	K

Section 4

	(160)	160	170	180	190	200	212
ZbPB (160)	IKENV	LITLAAAS	GATGNQGTTPIS	LAEL	YYKTKV	NPAVAIFFMWS	INFSGYA
ZbPS (111)	WK	FDEEYRI	RRK----	GARKNH	WY	SWFNGSPSQEKKL	ILKLDVLLAFYSC
ZYR O0E08228 (90)	WK	FDEQEYRIT	TKK----	KGKSNH	WY	SWFNGSPSTQEKKL	ILKLDILLALYAC
ZYR O0D17794 (148)	IKENV	LITLAAAS	GATGNQGTTPVS	LAEL	YYKTKV	NPAVAIFFMWC	INFSGYA
ZbFfz1 (106)	ATT	VYSSTISR	MVLS-----	FDRVAE	IGQLGL	LFTFNMTC	AIAPLFLA
CpaCgd8 (156)	LS	YLVLYSTR	KPF	SVVKLQIQEDLS	LS	TSVLGWIDT	TFLGSAFQQLIIP
KlFrt1 (115)	G-	ASYGMKASL	KLT-----	S--	DEDSL	LISSIMPL	LGAVGSSILLT
ScaFsy1 (123)	G-	ASIGMNKML	HLS-----	T--	HEASL	VSSIMPL	LGAVAGSILLT
KlRag1 (86)	G-	FVNQTD	FLRRFG--	QEKADGSH	YLSNV	RTGLIVS	IFNIGCAVGGIV
ScHxt1 (88)	G-	FVAQTD	FLRRFG--	MKHHDGSH	YLSKV	RTGLIVS	IFNIGCAIGGIV
ScHxt3 (85)	G-	FVAQTD	FLRRFG--	MKHKDGSY	YLSKV	RTGLIVS	IFNIGCAIGGII
ScHxt13 (80)	G-	FINMDNF	KMNF	GS-YKHSTGEY	YLSNV	RMGLLVAMP	SIGCAIGGLIFAR
SpGht3 (29)	G-	ILGMRD	FQSRFA	DRYNPISNSYS	SAWRQ	ALLTGTIN	AGLFGAMLSSPFT
ScStl1 (51)	S-	LITGKQ	FNYEF	PATKEN-GDHR	HATV	VQGATT	SCVELGCFAGS
HsGlut5 (41)	S	PALLMQQ	FYNETYYGRT	GEFMED	FPLTL	LWSVTVSM	FPFGGFIGSLLV
SpGht6 (29)	G-	ITNMRD	FQSRYA	DRYDPVTD	TYSYS	SARQGLLV	GMVNTGTTVGC
PsSut2 (73)	G-	FLNMSD	FLSRFG--	QDGSEGK	YLSDI	RVGLIVS	IFNIGCAIGGIF
PsSut3 (73)	G-	FLNMSD	FLSRFG--	QDGSEGK	YLSDI	RVGLIVS	IFNIGCAIGGIF

Section 5

	(213)	213	220	230	240	250	265
ZbPB (213)	LS	VIARNFLI	YEPQFIW	PQALMQ	TNLFNSMKSAD	-----	ANDSKEASR
ZbPS (160)	VAV	WVKYLD	TNVNNA	YVSGMKEDL	GMKGNDLVD	-----	LQNMWNI
ZYR O0E08228 (139)	MS	YWKYLD	SVNLNNA	YVSGMKESL	GMHGNDLVH	-----	VQNMFSI
ZYR O0D17794 (201)	MS	VIARNFLI	YDPQFVW	PRALMQ	TNLFNSM	KKADLP	-----
ZbFfz1 (151)	ELV	GRYVIY	VGAYL	CFSLCF	IGLALGRN	-----	IATILVLR
CpaCgd8 (209)	ENF	KVNEYI	CTISFI	CSAITSLI	FGISYS	STSLLLS	WFPNGLFHAGV
KlFrt1 (154)	EYF	GRKVAL	VISCIFY	TIGGIL	CAAAQD	-----	VHTMYAGR
ScaFsy1 (162)	EYF	GRKKS	LAISCV	FYTIGAI	VCAASN	-----	HEMYAGR
KlRag1 (136)	DRW	GRRIGL	ITVII	IYVIGI	IIQIAS	VDK	-----
ScHxt1 (138)	DMY	GRRIGL	IIVVV	IYTI	GIIIQI	ASINK	-----
ScHxt3 (135)	DMY	GRKMGL	IIVVV	IYTI	GIIIQI	ASINK	-----
ScHxt13 (131)	DTL	GRRLA	IIVV	LVYV	CAIIC	ISNHK	-----
SpGht3 (81)	ERI	GKKYSI	CFFSGV	IIAELL	LVTAV	PS	-----
ScStl1 (102)	ERI	GRKPLI	LMGSV	ITII	GAVIST	CAFRGYW	-----
HsGlut5 (94)	NKF	GRKGAL	LFN	IFSV	PAIL	MGCSR	VATS
SpGht6 (81)	DRF	GKRKC	IMGW	TLVY	ITGV	IVQL	TTIPS
PsSut2 (122)	DVY	GRRIGI	ISAM	VVYV	GIII	QIS	SQDK
PsSut3 (122)	DVY	GRRIGI	ISAM	VVYV	GIII	QIS	SQDK

Section 6

	(266)	266	280	290	300	318	
ZbPB (256)	KL	KYFFIA	VIGMTV	WEFL	PEYV	FPLSSLS	SFICWVAPENY
ZbPS (203)	II	FQLPFI	FVLNKV	PLNYLL	PCLDIG	WSLITV	GQYVK
ZYR O0E08228 (182)	IV	SQVPFL	FVLNKV	PLNYLL	PCLD	CWSLMTL	GQFAH
ZYR O0D17794 (245)	KM	KVFFML	IIGMTV	WEFL	PEYV	FPLSSLS	SFICWVAPN
ZbFfz1 (187)	TL	LGLFG	CVGTIL	VGGT	FD	DMFKPED	RAIPMSL
CpaCgd8 (262)	LIT	CFGV	SERGRIL	LGWTT	SQV	GAMASTAF	SASICLKL
KlFrt1 (190)	FLI	GVGV	IEGGV	GVYIA	ESV	STVRG	SLVSLYQFNI
ScaFsy1 (198)	FLI	GVGV	LEGGG	GVYIA	ESV	SSVRG	SLVSLYQFNI
KlRag1 (173)	II	SGLGV	GITVLS	PMLI	SETAP	KHLRGT	LVSCYQLMI
ScHxt1 (175)	II	SGLGV	GITVLS	PMLI	SEVAP	SEM	RGTLVSCYQVMI
ScHxt3 (172)	II	SGLGV	GIAVLS	PMLI	SEVAP	KEM	RGTLVSCYQLMI
ScHxt13 (168)	II	YGLG	AGCSVL	CPML	LSEI	APTDR	GLVSLYQLNM
SpGht3 (118)	II	AGVG	IGALS	SVLSP	GYQ	SEVAPQ	IRGAVVATYQIFS
ScStl1 (142)	VVT	GVGTG	LNTST	IPVW	QSEMS	KAENR	GLLVNLEGSTI
HsGlut5 (133)	LL	VGIC	AGVSN	VVPM	YL	GELAF	KNLRGAL
SpGht6 (118)	IWT	GLG	IGALS	VIA	PGY	QSESS	PHIRGAV
PsSut2 (159)	GVT	GLAVG	TVSVLS	PMFIS	ESAP	KHLRGT	LVYCYQLCI
PsSut3 (159)	GVT	GLAVG	TVSVLS	PMFIS	ESAP	KHLRGT	LVYCYQLCI

Section 7

	(319)	319	330	340	350	360	371
ZbPB (309)	FLNQSL	DWSNITS	--SV	LLSPYWT	ICIQFAAF	VFSCWLI	IPAAKWGNLSSYKH
ZbPS (247)	ALRFFV	GAFEAPS	--YL	AYQYLF	CFYKHDEM	VRRSAFY	YFGQYIGLSSGGI
ZYR O0E08228 (226)	ALRFFV	GFFEGPS	--YL	AQYLF	CFYKHDEV	VRRSAFY	YFGQYIGLSSGGI
ZYR O0D17794 (228)	FLNHS	LDWANITS	--SV	MLSPYWTT	CVQFAAFF	FFSCWVLI	IPAAKWGNMSSYKH
ZbFfz1 (298)	TVGAPI	IYAGFVDE	--NV	GWRWTEGI	IQGLANTP	LLVVIV	IFFRETRGGVTLRKR
CpaCgd8 (313)	VFLFGI	IIVLKYLK	--K	PKNLYIYD	NVGNKEL	NENNMVLV	MMEDSNNNKEVNNE
KIFrt1 (234)	GYVIGV	IIFFDVK	---	GGWRYMLG	SSLVFS	TILFVGLF	FLPESPRWLIHKGYD
ScaFsy1 (242)	GYIVGV	IIFFDVK	---	GGWRYMVG	SSLVFS	TTLFI	GLLFLPESPRWLMHKGRV
KIRag1 (217)	GYCTNY	GTKNYSN	--SV	QWRVPLGL	CFAWAIF	FMVLGMMF	VPESARFLVETDOI
Schxt1 (219)	GYCTNF	GTKNYSN	--SV	QWRVPLGL	CFAWALF	MIGGMMF	VPESPRYLVEAGRI
Schxt3 (216)	GYCTNF	GTKNYSN	--SV	QWRVPLGL	CFAWALF	MIGGMTF	VPESPRYLVEAGQI
Schxt13 (212)	GYCSVY	GTRKYDN	--T	AQWRVPLGL	CFLWALI	IIIGML	LVPEsprYLIECERH
SpGht3 (162)	AACINM	GTHKLRK	--T	ASWRTSFG	INMLWGI	LLMVGVL	FLPESPRYLIYKGRD
ScStl1 (186)	AYWIDF	GLSYTNS	--SV	QWRFPVSM	QIVFALF	LLAFMIK	LPESPRWLISQSRT
HsGlut5 (177)	AQIFGL	LRNLLAN	--V	DGWPILGL	TGVPAALQ	LLLLP	FFPESPRYLLIQKRD
SpGht6 (172)	AACINM	GTHKYTHP	EAQ	WRVPIG	INLLWGI	LMFFGML	FLPESPRYLAVKGRN
PsSut2 (203)	GYCVTY	GTKDLND	--S	RQWRVPLGL	CFLWAI	FLVVGML	LAMPESPRFLIEKKRI
PsSut3 (203)	GYCVTY	GTKDLND	--S	RQWRVPLGL	CFLWAI	FLVVGML	LAMPESPRFLIEKKRI

Section 8

	(372)	372	380	390	400	410	424
ZbPB (360)	GLMSNHLLT	SNGTLYPVNDL	ITYD	-----	-----	-----	HSSTANGEVSNHT
ZbPS (298)	QAGVYGTLS	-----	-----	-----	-----	-----	GKNGLSGWRWNF
ZYR O0E08228 (277)	QSAVYKSLN	-----	-----	-----	-----	-----	GRNGLAGWRWNF
ZYR O0D17794 (349)	GLMSNKLLTR	NGTVYPSS	-----	-----	-----	-----	LIYDEGASKNGKVSFNEK
ZbFfz1 (279)	-----	-----	-----	-----	-----	-----	AQLLRKDTGDDRW
CpaCgd8 (364)	IKPNREEELEA	IIVKPARIDRVE	KEEDYEELG	SIAEGTHQ	EEIENG	MTKYSS	-----
KIFrt1 (283)	-----	-----	-----	-----	-----	-----	VEAYKVWRRLRD
ScaFsy1 (291)	-----	-----	-----	-----	-----	-----	GESWNVWKRRLRD
KIRag1 (268)	-----	-----	-----	-----	-----	-----	EEARKSLAKTNK
Schxt1 (270)	-----	-----	-----	-----	-----	-----	DEARASLAKVVK
Schxt3 (267)	-----	-----	-----	-----	-----	-----	DEARASLSKVVK
Schxt13 (263)	-----	-----	-----	-----	-----	-----	EEARASIAKINK
SpGht3 (213)	-----	-----	-----	-----	-----	-----	EEALRIMCNMAE
ScStl1 (237)	-----	-----	-----	-----	-----	-----	EEARYLVGTLDD
HsGlut5 (227)	-----	-----	-----	-----	-----	-----	EEAAKKALQTLR
SpGht6 (215)	-----	-----	-----	-----	-----	-----	EECMKILTRNAG
PsSut2 (254)	-----	-----	-----	-----	-----	-----	EEAKKSLARSNK
PsSut3 (254)	-----	-----	-----	-----	-----	-----	EEAKKSLARSNK

Section 9

	(425)	425	430	440	450	460	477	
ZbPB (398)	AYEKHG	EVYIGA	QQSWNI	FFDYAA	FSAITW	IVLFG	GRKEIVSSFKKLERFLN	
ZbPS (319)	VIDAIVSA	VGVIGF	YSLPGD	PRNCYS	IFLTDD	DEIRLARKRL	QBEHTQEN---	
ZYR O0E08228 (298)	IFDFIISI	IVALIGF	YSLPGD	PQNCYS	IFLTDD	DEIRLARTRL	KKENKTEGN---	
ZYR O0D17794 (387)	AYQLHG	EYIGA	QKAWNM	FFDYAA	FSAISW	MLLFG	GRKDIAKNFKKLQRLFH	
ZbFfz1 (292)	VSR	EL	EAPGLKDAL	YASSV	KAIQMLCTE	PVVFF	EGLWIAFAWFITFLFLS--	
CpaCgd8 (417)	NLLDS	RSNPL	LASSSH	LINGE	EBEEEE	GEAYLN	EPDNLGFENHQIQDMSANSSS	
KIFrt1 (295)	TSD	-LGNK	REFLEM	KHAAEQ	DROLKE	QESRFKSM	FDL-----ILIPRNRRA-	
ScaFsy1 (303)	VKE	-EGNK	IEFLE	LRDVASH	DRELHANE	SREFQSL	FDL-----VRVPRNRRA-	
KIRag1 (280)	VSI	-DDPV	VKYEL	LKIQSS	IELEKA	AGNASW	GELITG-----KPSMFR---	
Schxt1 (282)	CPP	-DHPY	IQYELE	TI	EASVE	EMRAAGTASW	GELFTG-----KPAMFQR---	
Schxt3 (279)	VAP	-DHPFI	QQELEV	IEASVE	EARAAGS	ASW	GELFTG-----KPAMFKR---	
Schxt13 (275)	VSP	-EDPW	VLKQADE	INAGV	LAQREL	GEASW	KELFSV-----KTKVLQR---	
SpGht3 (225)	LSP	-ESEI	IQTNFNT	IKSDIE	IEIEMAGG	KARWIE	EIFG-----KDIRYR---	
ScStl1 (249)	ADP	-NDEE	VITEV	AMLDH	AVNRTK	HEKHS	LSSLFSRG-----RSQNLQR---	
HsGlut5 (239)	GWD	---	SV	DREVAE	IRQED	EAEKA	AGFISVLKLF	R-----MRSLRWQ-
SpGht6 (227)	LPA	-DHP	IMQKEY	NAIQAD	VEAELAGG	PCSWPQ	IFSN-----EIRYR---	
PsSut2 (266)	LSP	-EDPG	VYTEV	QLIQAG	IDREAA	AGSASW	MELITG-----KPAIFRR---	
PsSut3 (266)	LSP	-EDPG	VYTEL	QLIQAG	IDREAA	AGSASW	MELITG-----KPAIFRR---	

Section 10

(478) 478 490 500 510 520 530
 ZbPB (451) KRNINKLYNDR LNDIQSQYEEV PVWFFAVLW LITVVIFMTIFATNRMFIPWWT
 ZbPS (369) -----DFHKKIFDLTALKGILLDKI WVL SVWAVFCWDBSNAGSGSYILWLD
 ZYR O0E08228 (348) -----DFHKKVFDIGVWKKIIFDFKI WIVSIW AIFCWDN S NVSGGCY I LWLK
 ZYR O0D17794 (440) NKNINL LYNDR LNKIQSNYEEV PVWFFALLFLITLVVVFVTFATNTMFIPWWT
 ZbFz1 (343) -----VISITFSEGGKHWAEGLIAGLPYIGLVLGCTLAFGANFLQIRKYDSLRA
 CpaCgd8 (470) NSGSSSQLLMQKSLSEKENDSM TDLHMEDGDKFINDGFGTSLSIEKSEFSDHL
 KIFrt1 (340) -----LLYSIMMVSLGQLTGINAIMYYMSTLMGQIGFSPKQA-VAMSMVGGGA
 ScaFsy1 (348) -----LVYSCMMIGLGQLTGINAIMYYMSTLMKNIGFDDKQS-VAMSMVGGGA
 KlRag1 (323) -----TLMGIMI QSLQQLTGDNYFFYYGTTIFQSVGMDDS---FETSIVLGI
 ScHxt1 (325) -----TMMGIMI QSLQQLTGDNYFFYYGTTIVFQAVGLSDS---FETSIVFGV
 ScHxt3 (322) -----TMMGIMI QSLQQLTGDNYFFYYGTTVFNAGVMSDS---FETSIVFGV
 ScHxt13 (318) -----LITGILVQTFQLTGENYFFYYGTTIFKSVGLTDG---FETSIVLGT
 SpGht3 (296) -----TCLGF LVMLFRELI GNYYFYATQVFKGTGMTDI---FLPAVILGA
 ScSt1 (262) -----ALIAASTQFFQCF TGCNAIYYSTVLFNKTIKLDYRLSMIGGFAT
 HsGlut5 (278) -----LLSITVLMGGQQLSGVNAIYYADQIYLSAGVPEEH-VQYVTAGTGA
 SpGht6 (268) -----TLLGMGVMAFQQLTGNNYFFYYGTQVFRGTGLNSP---FLAALILDA
 PsSut2 (309) -----VIMGIILQSLQQLTGVNYFFYYGTTIFQAVGLQDS---FQTSIILGT
 PsSut3 (309) -----VIMGIILQSLQQLTGVNYFFYYGTTIFQAVGLQDS---FQTSIILGT

Section 11

(531) 531 540 550 560 570 583
 ZbPB (504) YFVALGISCIIIIPLAYLYAISN--FQLAIGAFDELLYGVMVQNLKSHKHPAG
 ZbPS (416) SLKNSSEGEKRYSEKVNQLSMITPGLGLVYLAL AALVADKLSHRWLAICLTQI
 ZYR O0E08228 (395) SLTNSNGEQRYSIPKVN TLSMITPALGLVYLTV TALIADKLSHRWSAILFTQI
 ZYR O0D17794 (493) YFVAVGVSFIIIIPLAYLYAISN--FQLAIGTFDELLYGVMVQNLSTHKHPAS
 ZbFz1 (390) KSNGLP LPPENRLYGAMCGALFLP--IGLFIYSFSQYPRLHWMGPMVGLTICIF
 CpaCgd8 (523) SYISELSPTKKNRQQLNNDTTN--GSDGFTQNPNLKENNVENNIYSSFDIQ
 KIFrt1 (386) ALLIGTIPAILYMDKFGRR-----PWSMTIIG-FSVGLVLVGVGYQID
 ScaFsy1 (394) ALLIGTVPAI LWMDFGRR-----TWAMTIIIFIVGLVLVGVGYQIN
 KlRag1 (367) VNFASTFFALYTVDFGRRNCLL--YGCVMVACYVVYASVGVTRLWPDGPDH
 ScHxt1 (369) VNFSTCCSLYTVDRFGRNCLM--WGAVMVCCYVVYASVGVTRLWPNQDQ
 ScHxt3 (366) VNFSTCCSLYTVDRFGRNCLL--YGAIGMVCCYVVYASVGVTRLWPNGEEN
 ScHxt13 (362) VNFSTIIAVMVVDKI GRRKCLL--FGAAGMMACMVIFASIGVKCLYPIIGQDG
 SpGht3 (310) INFGTTFGALYTIDNLGRRNPLI--FGAAFQSTCFFIYAAV GDRKLIYKNGTS
 ScSt1 (339) IYALSTIGSFFLIEKLGRRKFL--LGATGOAVSFTITFA CLVKENKEN----
 HsGlut5 (324) VNVVMTFCAVFVVELLGRLLLL--LGFSICLIACCVLTAALALQD TVSWMP--
 SpGht6 (312) VNFGCTFGAIFVLEYFGRRGPLI--VGGVWQSI CFFIYASVGDRAITRPNGTS
 PsSut2 (353) VNFSTFVGIWAIERFGRRQCLL--VGSAGMFVCFI IYSVIGTTHLFIDGVVD
 PsSut3 (353) VNFSTFVGIWAIERFGRRQCLL--VGSAGMFVCFI IYSVIGTTHLFIDGVVD

Section 12

(584) 584 590 600 610 620 636
 ZbPB (555) ASTYGAMSGDLWYRAQYMLQDQKIGHYMHIPP KAVFMSQILGQLIGVPMNYGT
 ZbPS (469) FNIIGNVILSVWYVAEGA KWF AFMLQYMGYAMAFVLYG VVNDICRRDSEARAV
 ZYR O0E08228 (448) FNIIGNVILAVWNVQEGA KWF AFMLQCMGWAMAFVLYG VVNDICRRDSESRGI
 ZYR O0D17794 (544) ASTYGALSGDLWYRAQYMLQDQKIGHYMHIPP KAVFLSQVILGQLIGVPMNYGT
 ZbFz1 (441) GIFFIFESCYSYTSDCY GQSASSA IAGQG FMRNTLGAVSPLFAHQFFRQVHSQ
 CpaCgd8 (574) NMDDVG FVEKLIILLKIPN I LNIAISY YFIKLIRYSMLFWLPYYLIRELNYGP
 KIFrt1 (428) LN-----TNLAAAEGVYLTGQ ILYNI FFGTYATLTWVVPSESF-SLATRS
 ScaFsy1 (436) LK-----THMAAAAEGVYLTGQ I IYNMAFGSYAALTWVLPSESF-SLGTRS
 KlRag1 (418) PD-----ISSK GAGNCMIVFACFYIFCFATTWAPIAYVVISESY-PLRVKG
 ScHxt1 (420) P-----SSK GAGNCMIVFACFYIFCFATTWAPIAYVVISECF-PLRVKS
 ScHxt3 (417) G-----SSK GAGNCMIVFACFYIFCFATTWAPIAYVVISETF-PLRVKS
 ScHxt13 (413) P-----SSK GAGNAMIVFTCFYIFCFATTWAPVAVIVVAESF-PSKVKS
 SpGht3 (361) D-----HRAGSVMIVFSCFLFESYCCSWGPMGVI VGETE-PIRYRS
 ScSt1 (386) -----ARGAAVGLFLFITFFGLSLLSLPWIYPP EIA-SMKVRA
 HsGlut5 (374) -----YISTVCVISYVIGHALGFSPIPAL LITBIFLQSSRPS
 SpGht6 (363) N-----HRAGAVMIVFSCFLFIFSAQTWAPAAYVIVGESY-PIRYRS
 PsSut2 (404) NDN-----TRQSSGNAMIFITCLFIFFFACTWAGGVFTI ISESY-PLRIRS
 PsSut3 (404) ND-----NTRQSSGNAMIFITCLFIFFFACTWAGGVFTI ISESY-PLRIRS

Section 13

	(637)	637	650	660	670	689
ZbPB (608)	VRWV	LDTKMP	YLRGI	IKDPLHQ	WTGQGL	TSYN-----TEAVLYV
ZbPS (522)	II	VTLNIA	AGTVFNT	WSSVVF	FFPT	VEAPRYLKGYAHTAANAFALV
ZYR O0E08228 (501)	IMT	AMNIA	ASVFSV	WTSVVF	FFPT	TTEAPRYLKGYSEFAANALALIL
ZYR O0D17794 (597)	VRWV	LDTKMD	YLRGI	TAKDPLHQ	WTGQSL	TSYN-----TNVLYV
ZbFfz1 (494)	Y	AGLIC	SVVATGLT	FLPYILFK	EGPTIRAKS	-----KRAIVYPADNGD
CpaCgd8 (627)	SVAGY	SSMLFD	IGGI	IAGAISAGA	IADTY	FGGKRILVACYM
KIFrt1 (472)	IGMT	ICSA	FLYLWAF	VTYTNFNK	MKDAFT	YTGLTLG
ScaFsy1 (480)	AGMT	VSSALL	YLFAPT	VTYTNFEK	MKEAMT	YTGLTLG
KIRag1 (463)	KAMAI	ASASN	WIWGF	LIGF	FTPFIT	SAIHFY---
Schxt1 (463)	KCMSI	ASASN	WIWGF	LIGF	FTPFIT	SAIHFY---
Schxt3 (460)	KAMSI	ATAAN	NLWGF	LIGF	FTPFIT	SAIHFY---
ScHxt13 (456)	RAMSI	STACN	NLWQF	LIGF	FTPFIT	SAIHFY---
SpGht3 (402)	KCASV	ATS	GNWLG	NFMIS	FTPFIN	NAIGFK---
ScStl1 (423)	STNA	AFST	CTNWL	CNFAVVM	FTPI	FIGQSGWG---
HsGlut5 (411)	-	AFMV	VGGSVH	WLSNFT	VGLIF	PFPIQ
SpGht6 (404)	KCA	AVATA	SNWF	NFMIS	FTPFIS	NSIGFK---
PsSut2 (449)	KAMSI	ATAAN	NMWGF	LISF	CTPFI	VNAINFK---
PsSut3 (449)	KAMSI	ATAAN	NMWGF	LISF	CTPFI	VNAINFK---

Section 14

	(690)	690	700	710	720	730	742
ZbPB (656)	SSK	TKV	IPFGL	LVGF	EAPFI	IYVLY	KLVPKMRFDLW
ZbPS (575)	AKR	DDRKHS	ESGIT	LYNSK	KGGLPE	EKPD	TNSFTSMKV
ZYR O0E08228 (554)	YKNE	ERKYAK	ENGI	ILYNS	NVGVPP	KE--ER	QQLGNIYHDD
ZYR O0D17794 (645)	SGHN	KIIPF	GLLVGF	EAPFI	IYALY	KLFP	PRKFNLW
ZbFfz1 (541)	AEQPS	PDDSE	EQISS	PVEKERS	QEEGHI	ENANI	PETTTT
CpaCgd8 (680)	ITK	TGLNLL	ILFGIA	FMGFCV	SGPDS	SILGST	AQAQD
KIFrt1 (525)	MP	ETKDKT	LEEDI	DDIF	EMPTR	QLVK---	QNLK
ScaFsy1 (533)	MP	ETKNRT	LEEDI	DDIF	EKPTS	SQIIK---	ENIAYL
KIRag1 (513)	VP	ETKGLT	LEEVN	EMYSE	GVLP	PKS---	SSWVPS
Schxt1 (513)	VP	ETKGLS	LEEVN	DMYA	EGVLP	PKS---	ASWVPS
Schxt3 (510)	VP	ETKGLT	LEEVN	DMYA	EGVLP	PKS---	ASWVPT
ScHxt13 (506)	LP	ETIGLS	LEEI	QLLY	EEGI	KPWKS---	ASWVPP
SpGht3 (452)	AK	ETKGLT	LEEVN	DLMSN	IKPWE	SYKYV	REIESHR
ScStl1 (473)	YP	ETAGRS	LEEDI	DIIF	AKAYE	DGTQP	PWRVAN
HsGlut5 (460)	VP	ETKAKT	FIENQ	LFTK	MNKV	SEVYP--	EKEELKE
SpGht6 (454)	AK	ETKGLT	LEEI	NQLY	LSN	IKPWNT	GAYQR
PsSut2 (499)	VS	ETKGLS	LEEV	DELY	AEG	IAPWKS	-----
PsSut3 (499)	VS	ETKGLS	LEEV	DELY	AEG	IAPWKS	-----

Section 15

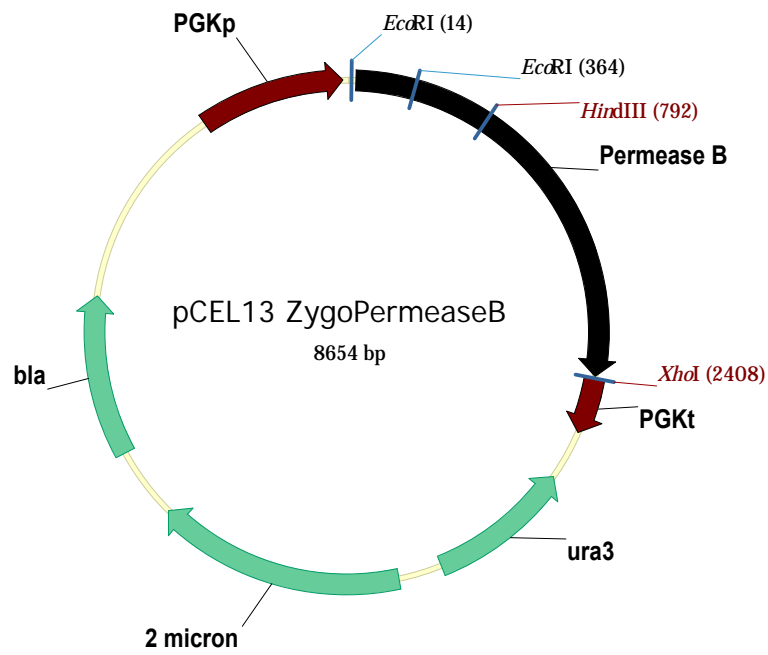
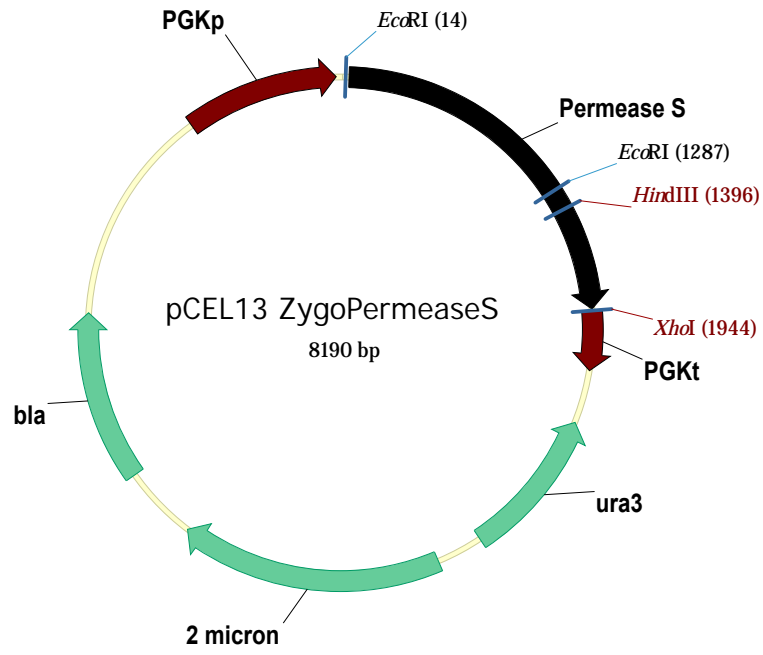
	(743)	743	750	760	770	780	795
ZbPB (709)	TGYST	QFIV	GTFS	MYFL	FNYK	PQIWR	KYNYL
ZbPS (621)	-----	-----	-----	PGKS	-EELD	AG	NEQ
ZYR O0E08228 (598)	-----	-----	-----	SPAAE	AGEHV	HIV	GDKGDKI
ZYR O0D17794 (698)	TGYAT	QFV	GTFS	MYLY	NHKK	PKFWR	NYNYL
ZbFfz1 (588)	-----	-----	-----	GAE	LHSS	DESS	NSTED
CpaCgd8 (733)	IVNGL	GAF	GA	VTQ	GLTAY	ISE	YYGWS
KIFrt1 (563)	-----	-----	-----	RKSI	-----	-----	-----
ScaFsy1 (571)	-----	-----	-----	-----	-----	-----	-----
KIRag1 (549)	-----	-----	-----	EYD	VDAL	QHDD	KPWYK
Schxt1 (549)	-----	-----	-----	DYN	ADLM	HDDQ	PFYKSI
Schxt3 (546)	-----	-----	-----	NYD	ADAL	MHDD	QPFYK
ScHxt13 (542)	-----	-----	-----	SSE	SKTE	KKDW	KKFLK
SpGht3 (505)	IRG	QEEEF	IEN	AED	NND	SSSS	SGSV
ScStl1 (526)	EME	KEDF	GED	RVED	TYNQ	ING	DNS
HsGlut5 (502)	-----	-----	-----	-----	-----	-----	-----
SpGht6 (500)	-----	-----	-----	TS	KLHE	YVEH	APNS
PsSut2 (535)	-----	-----	-----	MQN	STY	GAB	AKEQE
PsSut3 (535)	-----	-----	-----	MQN	STY	GAB	AKEQE

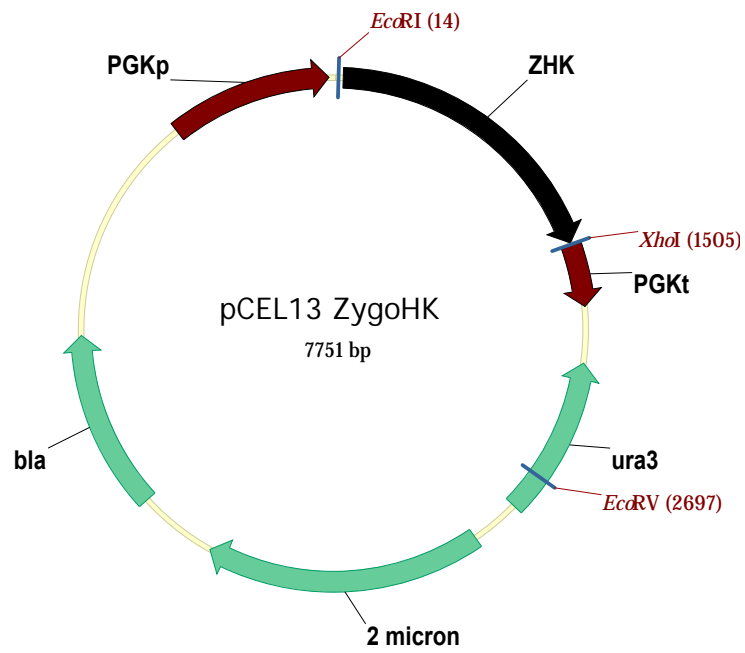
	(796)	<u>796</u>	<u>810</u>	<u>821</u>
ZbPB (762)	G	K	T	I
ZbPS (635)	-	-	-	-
ZYR O0E08228 (621)	-	-	-	-
ZYR O0D17794 (751)	H	K	P	I
ZbFfz1 (617)	-	-	-	-
CpaCgd8 (786)	K	S	L	V
KlFrt1 (567)	-	-	-	-
ScaFsy1 (571)	-	-	-	-
KlRag1 (568)	-	-	-	-
ScHxt1 (571)	-	-	-	-
ScHxt3 (568)	-	-	-	-
ScHxt13 (565)	-	-	-	-
SpGht3 (552)	P	T	Y	I
ScStl1 (570)	-	-	-	-
HsGlut5 (502)	-	-	-	-
SpGht6 (536)	-	-	-	-
PsSut2 (551)	-	-	-	-
PsSut3 (551)	-	-	-	-

Appendix 4.3.1.1: Transport protein sequence alignments, using Vector NTI software. Based on primary sequence similarity depicting homology of *Z. bailii* (Zb) permease B (ZbPB) and permease S (ZbPS) to *S. cerevisiae* (Sc), *P. stipitis* (Ps), *S. pombe* (Sp), *K. lactis* (Kl), *S. carlsbergensis* (Sca), *Z. rouxii* (ZYR) yeast and fungal hexose transporters as well as related membrane proteins from *Cryptosporidium parvum* (Cpa) and *H. sapiens* (Hs) (Sequences from NCBI database, <http://blast.ncbi.nlm.nih.gov/>).

Hxk alignments

		Section 1					
	(1)	1	10	20	30	40	52
ZbHxk	(1)	MVHLGPKKPP	PARKGSMAD	IPEDLM	BQIRGLE	VLFVSP	EKLRAVTKHFIS
ZYRO0E09878p	(1)	MVHLGPKKPP	PARKGSMAD	MPQDLL	LDVIRGLE	VIFSVST	EKLRAVTKHFIDEL
AgAFR279Cp	(1)	MVHLGPKKPP	TRKGSMA	DVPKTL	VBQIAS	FERIFT	VSAEKLQEI
KIHXX	(1)	MVRLGPKKPP	PARKGSMAD	VANLMB	BQIHGLE	TLFTVSS	EKMRSIVKHFIS
ScHxk1	(1)	MVHLGPKKPP	QARKGSMAD	VPKELM	DEIHQ	LEDMFT	VDSSETLRKVVKHFIDEL
ScHxk2	(1)	MVHLGPKKPP	QARKGSMAD	VPKELM	QQIEN	FEKIFT	VPTETLQAVTKHFIS
SjFK	(1)	-----	-----	-----	-----	-----	-----
		Section 2					
	(53)	53	60	70	80	90	104
ZbHxk	(53)	GKGLSKKGGNIP	MIPGWV	LEYPTG	KBRCH	HYLAID	LGGTNLRVVL
ZYRO0E09878p	(53)	QKGLSKKGGNIP	MIPGWV	LEYPTG	KETGN	NYLAID	LGGTNLRVVL
AgAFR279Cp	(53)	DKGLSKKGGNIP	MIPGWV	MDYPTG	NETGD	YLAID	LGGTNLRVVL
KIHXX	(53)	DKGLSKKGGNIP	MIPGWV	VEYPTG	KETGD	FLALD	LGGTNLRVVL
ScHxk1	(53)	NKGLTKKGGNIP	MIPGWV	MEFPTG	KESGN	YLAID	LGGTNLRVVL
ScHxk2	(53)	EKGLSKKGGNIP	MIPGWV	MDFP	TGKESGD	FLAID	LGGTNLRVVL
SjFK	(1)	-----	MVP	SKTDK	TVEFSS	SGMFI	IDDVEHEDGS
		Section 3					
	(105)	105	110	120	130	140	156
ZbHxk	(105)	FDSSQSKYK	LPHHMR	TTRDSK	ELFFI	ADSLKS	FLHEE
ZYRO0E09878p	(105)	FDSSQSKYK	LPHHMR	TTRNPK	DLDFI	ASLKN	FIEE
AgAFR279Cp	(105)	FDTTQSKYR	LPNRMR	TQNAS	ELWDFI	ESLKD	FLHEE
KIHXX	(105)	FDTTQSKYR	LPHHMR	TGTG	-EQLW	SFIAK	CLKEFVD
ScHxk1	(105)	FDTTQSKYK	LPHDMR	TTKHQE	ELWSFI	ADSLKD	FMV
ScHxk2	(105)	FDTTQSKYR	LPDAMR	TQNP	DELW	EFIADSL	KAFIDE
SjFK	(38)	---GG	TWALL	GART	WTPG	AEARL	GLVID
		Section 4					
	(157)	157	170	180	190	208	
ZbHxk	(157)	FSYPASQS	KINEGIL	QRWTK	GFDIP	GVEGHD	VVPM
ZYRO0E09878p	(157)	FSYPASQG	KINEGV	LQRWTK	GFDIP	GVEGHD	VVPM
AgAFR279Cp	(157)	FSYPASQD	KINMGIL	QRWTK	GFDIP	GVEGHD	VVPM
KIHXX	(156)	FSYPASQK	KINSGV	LQRWTK	GFDIE	GVEGHD	VVPM
ScHxk1	(157)	FSYPASQN	KINEGIL	QRWTK	GFDIP	NVEGHD	VVPL
ScHxk2	(157)	FSFPASQN	KINEGIL	QRWTK	GFDIP	NHDV	VVPM
SjFK	(85)	FRANPDR	LTHGY	NYVHN	NGFR	HFEYK	SPKRAI
		Section 5					
	(209)	209	220	230	240	250	260
ZbHxk	(209)	INDTTGTL	VASLYT	DPA	TRMG	VIFGT	GVNGAY
ZYRO0E09878p	(209)	INDTTGTL	VASHY	TDPE	TQMG	VIFGT	GVNGAY
AgAFR279Cp	(209)	INDTTGTL	VASLYT	DAET	KMGV	I	FGTVNG
KIHXX	(208)	INDTTGTL	VASLYT	DPQ	TMG	I	I
ScHxk1	(209)	INDTVGTL	I	ASYY	TDPE	T	KMGV
ScHxk2	(209)	INDTTGTL	VASYY	TDPE	T	KMGV	I
SjFK	(137)	TNP	TKFRR	MHAR	LM	HR	TAA





Appendix 4.2: Yeast expression vectors containing the putative permease S, permease B and hexokinase of *Zygosaccharomyces bailii* strain 210 (from up to down). The genes are under regulation of the PGK1 promoter and terminator sequence. The vector contains an ampicillin resistance gene for bacterial selection (*bla*; β -lactamase), and a uracil selection gene (*URA3*) for selection of yeast transformants.

Z9 complete

1 ATCCAACGACTGCAAATCCAGCCCCAGCTACCGAGACTAACGTCGAATCCAGATTGTTTATCCCTCCCAT
71 ACCTTCATAAAGTAGGTTTTGTTGGTAAGGAAGATTGTCGCTATCTGTTGCAACACGAAAAAAGGAAA
141 AAAAGCCAAGTAATGCAGTCCCTAACAGCCTCTCTGAATTGGTGTTTTTTGAACACCTTTTGCTCGATTG
211 ATGAACCCGTTAGATTTCTGTACTCTTCGGATGATCCACACTTTTTCTCCACAGAAAAAGAACTTTGCCTC
281 TGCAGGATTATTGGGAAAAATAGAAAACTATTATTGTCATTAGAAAAGTAAGCCCTCCAATAATGATA
351 TGGAATATCTTCCATTGCGCAATAGGGCTATCTTTGATATAAAGCACGCCGTAAGCAATGAAAGCCTGTGG
421 GTATTGGGGCTCCCATGCATGTAGCAAAGAAAAAGGGCTGAGCTGCGGCA CGCTCAGTAGCATTGAAGAA
491 CATTCCCATGACAAACCATCAGTATCGGCATTACAATACTTTCAAAAAATCTAAAAAAAATCGCAGAGCT
561 GCAACCCCGAAAAATTGAAGGCAGCACAAATGCAAAAA CACAATAACTGTCCAATGAACAATAACATCG
631 TTAAAAACAGCCTGAGAGGCAGCTTTTGTGCGAGATATTGACCTGAAACTGTCCAAGTGATAGCCAAC
701 GTAAAATAGTGTATTCACAGTGTATAAGTGTCTGAGTAAGACCAGTGTCTCCAAAAAATCCATGATA
771 GAATCATAGGACAAAGGTAGCTTTATCATGTAAAGAAGCAGATTAATAGCAAAAACAAAGCTGTAGACGC
841 CAAAGAAAAGTTTTAGTTTCAGTTTTTTCTTGTGTGAGTAATTGGAGAAA CTGCTCTGAATATTC
911 TTCATAAAAGTTAGGGTGCATCTGCATACAGAGGGGATAAATATTTTCATCATGCGAAGAAATCACTT
981 CTGTTTTACTCTTTGGTCTCTGCTGATTCTTCTAATCTTTCAGAAACTGACTATTAACTTCAGAGTC
1051 AGAAAGGGTGGTATCCGTGATATCCAACACCTTTTGAGCAGATTGACTTTTGGTCACCATCATATATTAT
1121 CTGCGGTCCCGGAAATCAGTCTGTGGAAAAACAAGTGTGAGGCTGCGTGTGCTTCATCTCTTGTGTG
1191 CTTTCTGGTTTTAAATACTATCACAGAGAACATTGATCA CAGTTACAATCTATAGATTTCAATAATGTACG
1261 AGCATATGGCAGTAGTTAATGTGGAATAATCAAGATAATTTCAAGTATCATACGATGTTTCAGTCTCG
1331 AGTCATTGGAGGCGATTCTTGAAATGCTTTTTGAGAAAGCGTTAACTCTCCA TTTTTTCTTCCTGAAC
1401 TTTGGTCAGTGACACATGTTTCATTATCGCACAGATCGTTGCTGCGTTGTAGCCGCACAAGTGTGGCTG
1471 ACCAAAAAATATAGTACGTTTCGATGTTTTACTTGGTT CATGTGACAATATAAAGCTTTATTATCCA
1541 TAGTGAATTTTGGAAAGTCTCCAAGGGCGTTGTACATACGTCTTGGTCA TTTAACGGCTTTTTTTCTTT
1611 TCAACAGAGTTCGAAAAATCCAAGAAATAGTCTCTATG TTTTTCTCCTCTGCGCTGCGAGCAATATGAC
1681 ACATCTGTTTCATGAACGCAAAGCGCGCTGTTGCAACCTAAGTGTGGCTGAGGAAAAACAGCATTGTGTTT
1751 TCTTGGTTAGACTTCTACGGCATTTTTTTTCAAAGAAAGGACCTGAACGGCTAAGGTTAA CAGCAGGG
1821 AAAAAAGTTAAAGATTTGCCCTGACATGGGTATTTCA TTTTCAGACTTGTGGGCCAGTCCGGGTTACCCA
1891 AACATTTCCGTTGAATGTGATGAGAGAACAGAATTTCT CTTAATTCGGCATCTATTTGTTGGTAATAATC
1961 GACTTGGAAGTCTATGACATTAACGAAGGGATTTTAA CAGGGCTCAATCCCAATTTTGGGAAAAAGATTT
2031 TTTCTAATGTACGAACTATAGAGAGTTTGCTCTGTGTG TCTCGCCTAGCTATACTAACTTCTAACTTGA
2101 AAACGAAGCAATATTTTGGCAGTCCGAAAAAATCTAAG ATCCGCATCAAAGATTTTTTTCTT CATGAGTT
2171 CTAGCATGTCCGAA CAACCGATTAATGTTCTGCATAAGT CACAAAGTCTCGATCAGTTGACCAATCATC
2241 TT CAGGCAAACAAA CAAAAAAGCTGTCCGAGGCCGAAA ACATCCGTGTGGTTACTATTTCAAATTTGTATG
2311 GGTTAGCTTCTTAGTAAAGCGCGCAAGTTTTCACTTCTA GTGGTATCTCTGGAACGATTTTTCGACAC
2381 TTTTTCGCCTCGTAGCGCCTCAGGTT CGTGAGTGACGC CATGTGGGTGATTGGAGTTTCGTATTTCTCTT
2451 TTTTGACACATTTTAACCAGACTTGTCTGACGTTTATCTG AGAGCCGCCATGCGGATGCATTGTGATTTG
2521 TGTCTTTGTTTTGATACTTTCCCCCTTAACTGGGTGCAG CCATGCTGTGTACCATCGCCAGTTGTCTGCA
2591 TGCAACCATGTAATATGTTACTTGGAGAAGAATCTTTGA AATGTTGTATTCAGAAATCTGTCAACGGAT
2661 ATATAAGGAGCGGGAAAAACATAACTTGCAGAAGTTTCT CAGAACATTTTTAATTTACATAAAATCAGTAA
2731 TATTTCTCACAGTCTTGAAAAATTAATTGCTGACAGAGGAG GACCTGTATATCATGAGTGATGAAAACCA
2801 TTCAATTTATGAAGGCGAGATGAACCATCGTGAAGAAGTTGGCCCAACTTTAAAAAAGACCAGTTAGT
2871 ATAAACGTGGTGAAATCCGAAGATGATTCACCGGAGATGGTGTGATGAAACCCGGTCCATTGAATCTT

+3
 2941 TGACAACAAACCATGAAGAAGAGTTATTACCAGAAGACCTCAAGTCGATTCCCAAATGGTCAGACAGGT
 +3
 3011 GTGCACATAGAAAGATGACCCACATGAACCTTGTTTACTTTTCAGGGTGGTGTCTTATCAATTATTTTT
 +3
 3081 ATTTTACCAGGGGCCTTTTAGATACTTTGAATTCATACCGAACCCTTACAGCCGCTTACTCAGTTTTTT
 +3
 3151 TTGTACAAATTTGTTTCGTATTGGTTTGGAAAATGGTTGGCA AAGGTTTTACCTGACATAAAAAATTGGCAT
 +3
 3221 TGGAAACTTCAAGTTTAGTTAAACCCTGGTCCCTGGTCAATAAAGGAGAATGTTTTGATCACTTTGGCA
 +3
 3291 GCTGCTTC CGGTGCAACTGGCAATCAAGGTACTACACCAATTTCTCTGGCAGAAATCTATTACAAAACAA
 +3
 3361 AAGTAAACCTGCAGTTGCAATTTTTTTCATGTGGTCTATTAACCTTCTCCGGTTACGCACTCAGTTATAT
 +3
 3431 TGCACGGAATTTCTTGATATATGAGCCACAATTTATCTGGC CGCAGGCATTAATGCAAACCTAACCTCTTT
 +3
 3501 AACTCCATGAAAAGTGCAGATGCAAATGACTCTAAAGAAGCTTCCAGGAAGTTAAAATATTTCTTTATCG
 +3
 3571 CTGTAATTGGAATGACTGTTGGGAATTTCTCCCGAGTATGTTTTTCTATGCTTTCTTCACTTTCCCT
 +3
 3641 CATTTGCTGGGTAGCGCCTGAAAATTACGTGCAAACTTTGTTGGCGGAGGAATGGGTGGTATGGGGTTT
 +3
 3711 CTGAACCAATCTTTGGACTGGTGAACATTACGTCTTCAGTCTTGCTGT CACCATACTGGACAATTTGTA
 +3
 3781 TTCAATTTGCTGCCTTCGTCTTTTCGTGTTGGATATTAATTCCAGCAGCGAAATGGGGAAATCTGTGCTC
 +3
 3851 TTATAAGCATGTTTGATGTCAAATCATCTGCTGACATCGAATGGAACCTTTTACC CGGTGAATGATTTG
 +3
 3921 ATCACATATGACCACAGCTCTACCGCAAATGGTGAAGTCTCGTTTAAACCATACAGCATATGAAAAACATG
 +3
 3991 GCCCTGTTTATATCGGTGCGCAACAATCTTGAACATTTTTTTTCGATTATGCTGCATTCACTTCGGCAAT
 +3
 4061 TACTTGATAGTACTTTTTTGAAGGAAGGAGATAGTTAGCAGTTTCAAAAAGTTGAAGGAAAGATTTCTG
 +3
 4131 AACAAAAGAAATATTAACAAACCTTTACAATGACAGACTTAA CGATATTCAATCACAATATGAGGAGGTAC
 +3
 4201 CAGTATGGTGGTTTGCAGTATTGTGGCTAATAACTGTAGTCATTTTTTATGACAATTTTTGCTACAAATAG
 +3
 4271 AATGTTCA TTCGTGGTGGACTTACTTTGTGCTTTGGGAATTAGTTGCATTATTATTATCCACTGGCT
 +3
 4341 TATCTTTATGCAATTTCTAATTTCCAATTAGCCATAGGTGCCTTTGATGAATTGCTATACGGTGTATGG
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 4411 TACAGAATCTGAAATCCATAAACATCCTGCCGGTGGCTTCGACGTATGGAGCAATGTCCGGAGATTTGTG
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 4481 GTACAGAGCTCAATACATGTTACAAGATCAAAAAATTGGTTCATTACATGCACATTCCTCCGAAAGCTGTG
 +3
 4551 TTCATGTCACAAATCTGGGGCAATTAATTGGTGTCCCAATGAATTATGGTACTGTCCGTTGGGTTTTGG
 +3
 4621 ATACTAAGATGCCATACTTGCGTGAATAATTAAGATCCGTTACATCAATGGACCGGTCAAGGATTAAC

+3
 4691 ATCTTACAATACAGAAG CAGTCCTCTATGTACTAATTTGGTCTACGAAATTTTTTCTTCCAGCAAAACA
 +3
 4761 AAGGTTATAACCATTTGGACTGTTGGTTGGATTTTTTGCACCTTTCATAATCTATGTGCTTTACAACTGG
 +3
 4831 TCCCCAAAATGCGTTTTGATCTCTGGAATGTTACTATTTTCTGTTCAACTATGTGCAATTTTTTACGGGAA
 +3
 4901 TCTTTCTACTGGATACT CAACTCAGTTTATTGTTGGAACGTTTTCCATGTACTTTCTGTTCAACTACAAA
 +3
 4971 CCGCAAATCTGGAGAAAATATAATTATCTGACTGCTGCGGCTTTTGACACAGGATACAATTTGGCAGTGC
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 5111 TAGCGTTGAACGCTGTTTTGCGTTGGAGAAAGCCTGATTTTGCATCTCTATTATTTTAAATTTGGGCTGA
 5181 AATGTTTGAAGATATGCAGGTCCATCAAAAAGTAAATCATGTAAAAAAGAAAACGTTGTGACT
 5251 ATTTGAGAAAAGCTTACAGTTGTTGAATCCAGGAATGTAAGTTAGCAGATCTATAATAGTAAATGTAAA
 5321 CATTGAATGCGCTTATTTTTGGGGTTCTGGTGTAGATGAAAGAAATAATTCAAACGGAAAATGCAGATC
 5391 TAAAAATTGTAACGAAAGGTGTAGGTGTTCCATCTGAAGAGTACTTTGCGAAAAATGAAACTCAAAGACA
 5461 AATAGATGATTACAGACGTAAGCAGTGGATCAGGGTTATTTAGTTTCGATAATCCACAATAAATCTAGCTC
 5531 TACTTTTGTCTAACGGATGTAATGTAATGTATACGATTTTGTAAAGCAAAC TAGAAAATTTGAGCGGCT
 5601 TTATCGTTGAGCTGAAATAGAAAACCATAATGTTGGATCTACAACAGTTGTTTTAAAATTTGTGTTATCGT
 5671 CTTAATCAGAAAGTAATTTTACCAGTCTTGACTTATAAACATT CAGTATTTGACAAATTTGAAGCATCATC
 5741 GTGTTTATAATTTTTTCTGGCTCCAGATCCTTTATACAACCCCGAAGCGAGTATTACACAGCGCATTTC
 5811 AAGCACTGTAAACGTTTTCTAAGTAATGCTATCTTCTGGAAGCGGGCTGAGAAACAAATTTCTTATTG
 5881 ATTGTCGATTTTGGCTGAGTTTCTTGACTTGCCACGGCACGACGGCATT TTTCTTGGCATGATTTTATCG
 5951 TTTCTTTTTCGAATCTATGCAGAGTTTTTTTTCTCGGTTGACACGACAATGCCAAGGCATTTAAGACACT
 6021 AACCGGCATAATTTTAGCTCAGAAGCCATGAAAAAGGGTGTCAAAC TGTGCGCTGGGGAGCGCAGTATGT
 6091 GTCAGGGTAGTTAAATCGAGGGCAATGCATAAGTGGGGTCGAAATGGCTAAGGCATTTGAGACATTGACG
 6161 GGCATAATTTTAGCACAGAAGCCATGAAAAAGGTGTCAAAC TGTGCGCTGGGGAACACAGTATGTGTCAG
 6231 GGTAGTCAAATCGAGGG CAATACATAAGTAGGGTCGAAATGGAAGTCATTTCCCTTAATTATTCTAATAT
 +3
 6301 AATGGAAAACAAAGATTTTACTCAGACTGTTAAAATAGCGGGTTCAAACATGACGTATACTCTGAAGAA
 +3
 6371 TTTCTTTCGAGAAATTTTATCGATCCATGGGGAAGGCTTAAGTACGGCATT TTTACCCGTCAGAA GAATT
 +3
 6441 GTTGATGAATGCAGAGATGATAGCCTCGGAAGTAATGATGAATTTGATCAGAATTGTAATACTACAGAAG
 +3
 6511 CCGATGCTTCGAATGAACTGGTAAAAGTCGATACCGATGTAATTGTACAGAAAGAAAAGAACCTTACCAA
 +3
 6581 CTCTGAAAATATCTCTGAGGATGCAGGGGATGTTATTAATGACCAGGAAGCTCTTACTTTGCGTAATGTC
 +3
 6651 GAATATAGAGATGAAGCAAATAAGCCATGGTGGAAAGTTTTTTGATGAAGAGGAGTATCGCATCCGACGGA
 +3
 6721 AAGGTGCCAGAAAAGAAATCATTGGTATTCATGGTTAATGGAAGTCCTTCAATGCAAGAGAAAAGTTGAT
 +3
 6791 TTTGAAGCTGGATGTTCTTTTGGCTTTTTATTTCATGTGTTGCATATTGGGTAAAATATCTTGATA CCGTA
 +3
 6861 AACGTCAACAATGCATATGTTTCTGGAATGAAGGAAGATTTGGGCATGAAAGGCAATGATTTAGTCGATT

+3
 6931 TACAGAACATGTGGAACATAGGGAATATAATATTCCAGCTACCGTTCATATTTGTTTTGAACAAGGTACC
 +3
 7001 TCTAAATTACCTTTTACCCTGTTTAGATATAGGATGGTCACTCCTCA CAGTAGGT CAGGGTTATGTTAAA
 +3
 7071 ACCTACGGGGTATGAAAGCACTGCGTTTCTTTGTGGGCGCGTTTGAAGCCCCATCTTACTTAGCATAACC
 +3
 7141 AGTATCTTTTGGTTGTTTTTACAAACATGATGAAATGGTCCGCCGTTCTGCTTTCTACTACTTTGGACA
 +3
 7211 GTACATAGGCTCTCTATCCTCCGGAGGTATTCAGGCAGGAGTTTATGGAACATTGAGTGGTAAGAATGGA
 +3
 7281 CTATCGGGCTGGAGATGGAATTTTGTCAATTGATGCTATTGTTTCCGCAGTGGTGGGAGTGATAGGTTTTT
 +3
 7351 ACTCTTTGCCAGGTGATCCCAGGAACTGTTATTCCATTTTTCTAACTGATGACGAAATTAGACTGGCTCG
 +3
 7421 GAAGAGGTGCGAGGAGGACACTCAGGAAAATGATTTTCATCACAAAATTTTTGATTTAACTGCACTT
 +3
 7491 AAAGGGATTTTATTGGATTGGAAGATATGGGTATTGTCGGTCTGGGCAGTGTTTTGGCTGGGATGACAGCA
 +3
 7561 ATGCAGGTAGTGGCTCTTATATCTTGTGGCTCGATTGCTCAAGAATTCTGAAGGAGAAAAACGGTATTC
 +3
 7631 TGTGGAGAAGGTTAACCAGTTGAGCATGATTACGCCCGGTTTGGGTTGGTCTACCTTGCTCTAGCAGCA
 +3
 7701 CTGGTGGCGGACAAGCTTCATTCAGGTGGCTGGCAATCTGTCTCACGCAGATTTTCAATATCATTGGTA
 +3
 7771 ATGTAATTTTGT CAGTGTGGTATGTTGCTGAAGGTGCAAAGTGGTTTGCTTTTATGCTT CAGTATATGGG
 +3
 7841 TTATGCGATGGCCCCTGTTCTTTATGGATGGGTCAATGACATATGTCGTGCGGATTCGGAGGCAAGGGCT
 +3
 7911 GTTATAATCGTGACCCTGAATATTGCAGGTACAGTATTTAATACTTGGAGTAGTGTGGTATCTTTCCCTA
 +3
 7981 CGGTGGAAGCTCCTCGTTACTTGAAGGGGTATGCATTCACCGCTGCTAATGCTTTTGCATTAGTCGTTTG
 +3
 8051 GACTTTCGTGGTGTGTGGCTTGCAAAGCGGGATGATCGCAAACACTCGGCAGAAAGCGGAATTATACTC
 +3
 8121 TATAATCCAAAAAAGGAGGCTTGCCCGAAGAGAAGCCTGACACTAACAGCTTCACAAGCATGAAGGTAT
 +3
 8191 CGGGATCTATTACTATAGAACCAGGGAAGTCTGAAGAACTAGATGCTGGTTTTAAACGAA CAGGGTTTTAAA
 +3
 8261 CGAACAGTAGTGTAGTATCAAGCTATGTAATTGCAGTTGGTACTCGAACTATAGTGCCAATTTTGCATA
 8331 ACTGCTGCAAGATCATATACGCAGCTCATGATTTGTTTCAAGCGTAATTTCTTTCTTGCTCTAGTTATTG
 8401 AATAAATATGGTCTCTTTGAGGTGGGCGGATGGTGGTGAACGAAAGATGGGTATCATTGAAATATTCTA
 8471 TCAAGTCATTATTGTTTCGTTCCAAGCAAAGATCACAGTCAAAAACTATAGCCCTTATTATCAGACGTGA
 8541 AAATTC AATTGAAACGTGGGAGAGAGGGACATGACC AAAATCAACGAAATGGATCCGGT GACTAAATTTT
 8611 TTTGACTTGACACGTAACGTGCCAAAAACCTGTATTAGGCTAACACCAGCAAGAATACAGAAA CTAC
 8681 TTACCAAGTATATATTAGTAAGTCAAGTCGACAGGTAGCTGCTACGTGTAGAGCTCAAA CATTGATTTA
 8751 CTTGTTTAAATGGTACTACGCATTTTTTGGTCATGTTTTGAAAGAGCATTTTTAGTTGACCTTTTTAATTT
 8821 TGCTCCTTTTTTACTAACCTTTTTTCTTTTCACTCAGGTATTTATGTAGAAGAAGAAAAGATGTACTAACA
 8891 TACGTGCCTTTGATGAATATCTACTTCAAAAGATAATGGGTGATTCAGAGAGATTTTTAAATTTGAAAA
 8961 TGTTGCTAAAAAGAAATGAAATTGGACAAGAAGAGACAATCCCAACTGTAAATTTGAAATCATTCTTATA
 9031 GTAATGTTCCAAACAAGCGATCC

Appendix 4.3.1: Complete DNA sequence of fragment ZT9, containing two transporter of *Z. bailii*. ORF Permease B: Base 2784- 5144; ORF Permease S: 6351- 8267.

1 ATTTCCACAGGTATCAGTCTATGACCTGATTACGCCAAGCTTGCAATGCCTGCAGGTGCGACTCTAGAGGAT
 71 CTCGAAAAAGACCATCGTTTTCTTCGTGCTAGACCAATTGTGAGGGTGTCTCAGGGTCTCAGGCCAGAG
 141 AAAGTCACCAGGAAAGGGTCCGGCTCTGCCTGTTTTCTCACCGTCAATCACCTGCTGTGTTCCGCCGAATC
 211 CACTAGCACTGCTCGCTGCCTCGGCCAACGACCTCGGTGCTCACCTTCTCAAGCGCCTCCAGGTGATATT
 281 CGATCCCTCCCTCAGGCTCGAGTCCGAACCTCCGCTCTGAGGCCAACTCATAGTCGATTTTACTAAATTA
 351 AAATATTCCAGTATGTCAACTGCAAACGTATTTCTGTATGTATCAACATACATTGCGAGCCGGCGTTGT
 421 CTTGTGTACTTGACTACGGCACAGATCGTCTGGCTAATAACACCAAACCGGGCTCAACTTATACCTGG
 491 TGAATCTCACACCCACCATCAGCCACTCTTTGACCACGTGATGAGCAATTCGTTGAACTCCGTCAAATG
 561 CCCTTCCCTGTTAATAAGCTCATGCTCACGATCCATGGCTGAATCACTGGCTGTGGTGGACGGGTTGTGG
 631 CCTTCCATCGCTTTTCCCTTTCATCTCCGTGAGCTAGAACGGGAACGGCGCTGTGCAACTCCGGGGAAGA
 701 CGCTTCGTCCGTTGTAGGGGCCCTGCTCGGCCACGGAGCGAGGACGGGACAAAATCTTGGTGACACACAC
 771 GGAGTGACGCAGGCAAATCTGAAGCTTTTTCAAGCCGTTTGCTCTAATCCTTGCTGAATAATATGTAAG
 841 CAGACCGGATTTCTGCCCTCAGATTTGGCAATGTTTCATAAGATAATGGCCGGAAACCGTGGCAACTGAGATA
 911 CGGCTTGACGGGGACGTAGTTCACCCACTCTTGTGCGTTAAGCGCGCGCTCCAGTAGCGGAAGTAG
 981 CACGCAGTTTCTACCCTGTAAACGACGACTTGCTGCGCAGGTAGGGTCCGTACTTGTACAGAACAAATGG
 1051 AATCAGGGCAAGACCCA CAGAAAAGAAGCCAGGATAGACGACCCCAACCGACTGGATAGCCCTTGATA
 1121 GCCAGATTGTTGTACATCGCCTGGCCAAA CAGCGGAATGCGCAGGCAAACCTGCACGACAGACGCCAT
 1191 TCCCTGCAAAGACAGAGGCCATGTACTTGGGGTATGACATCGCCAGGTAGGAGAAGGTCCGCTGAAAGAG
 1261 GTTGAACACCGCATTACGAAGAATATTTCTGCAAGCATCGGCAGCGTCCAGTGACAGAGGCGGCCAAC
 1331 CAAACAGGAACAACGAAAGCGGCAGGCAC CAGCATACCCCATGGCCAGGATCAGAAACGTCTCTGGGGT
 1401 GAACGAGTTGTCCGCGAACTTCTTCGCGACCACTTTGGCCAGGAACA CAGAGGCAGATAGCATAGGCAAAG
 1471 CAGCACCCGACGCAAAA CCCCATATATGCGAGCCCGAGCTCTATCAGAGAGAAATTGTACACGCCGATGA
 1541 ACACGATGGGAAACGCCTCAAAGAACAGGTAGAACGTGCCGTAGCACAGCGCGATGTACACGTGAGCGC
 1611 CAGCAGATGGGCTCTTTATAATGATCTCGAATGGCCGGTACAGCGTGATGACCGCATGTCTCTGGCG
 1681 GTCAGTTTCGCTCCGCTCGTTCCTTCACTGTGTAGTACCTGTTGTGCGCGGTCTGCTTTCTCAAACGCT
 1751 GGCAGCGCGGTACAGGATGTTGTCCACGCTGGTCTCTGGGAAAAAGAAA ACTAGCAGCACCCAGCGTCCG
 1821 CGCGCTGGCCACAGCAGCAGCCAGAATATCCACCTCCAGTCCCTGGCCACCACCATTGACGCCCCGATG
 1891 AGCGGCCCCACGCGAGGGTGGCGGACAGCCCCGATGGACCA CAGCCCAGTGAAGATCGGCACCACCTCCG
 1961 GCCTGACAAATGTGCGCGACGCTCGCCCCGCTGTGCGCCAGCGAGGGCGAGCAGAGGAACCGGTGAGAAA
 2031 CCGCAGGATCACCGACCCCTGCAATGTTGCGCACTGTGCGGCACCCGATCTGCAGCATGGCGTACAGGAC
 2101 AGCGTCACGATGTACAGCTGCTGACGCCCCGAAGATCGCGAACTCCGACAGCGGCGAGAAGATCAAACGCC
 2171 CTATACCATACCCAAAGCACGTACAGCGATAGGTTACAGCGTCCCGGTCACGTGACCCACCCGAACTCCTG
 2241 CTGGATCTCCTCCTGGCCCGCGGTGATATCGAAGAGCCCATGTATGTACGCACGTGAGCAGCATCACC
 2311 TGTGCGATTACCACTGCTGTCTTGCAATTGGACCAAGTTGAGCGGGTTTTCCGGCGTCTCTGCGCCCCGCA
 2381 ACTCCACCAAGAACGGGTCTGCCGGCGCCAGCACGCTGCCCTCGCTGCTCCGCACTTCTGGGACACCTT
 2451 CAGGCCCTCCAGGTCCAGGTCCAGCACGCTGCGCCGCGACTCGCCCGTGTGCTGCTACTGCTTCTGCTA
 2521 CTACTGCTGCTGCGAGTGCCGCTGTGCCCCGCTCCGCGCGCCCTCCCGACTGCGTGGATCTCCACCACCC
 2591 CGAACTGTTCTAGCATGTCCACAAAGAACGTGTTTTTGAAGTGTCTGTATACCCCATTTTCTCTTGAT
 2661 CGCTAACACACGGGTCTTGGCCGTT CAGAGATCCTGCTGCCAGAGCCAACTTTCTCTTATACCGCTGC
 2731 AGCGCTAACCGGAGAGCTAGCGCAGTAACCCCAACTGCTGTCCCCTGTTTAGCCCTGTGCATCTTGTCTT
 2801 GACTCGACAGTTGCTAGGGTCCGCACCCGCCAAGTGCCTCTAGTTCTTTGTCCAAA ACTCCGTGGCAATT
 2871 CCACTCTGCGCGTT CAGCCGTTCCGCCATCTGGGATCCAGCTTCCC CGCTCATCCGGTACGTGACTA
 2941 TCACGTGGTGGAGTAACTGGGTGCGCTGTTTCATTATCCGCGT CAGTGAACACCAACCAAGTAGCGGCC
 3011 GGTATCCGCTGGCCCCACATCTCCCCACACATGCCCTA TAGTGTCCCTTATACAAATGTGTAACGC
 3081 TCGCCCCCACATCTCCCCATATGCCGACAGCCAAAGGGCGCACTGCACCCCTGCAACCCGACGTCAGGCT

3151 ACTCGCGACGCTCGGCGTC CAGCGGAGGGCGGTGCCCTCTCCCCCTGAAGAGACTAAGGGGTGCTCTCG
 3221 CAGGGGAGGAGATAGGGAATCAGCTTTGCGGCAGAGGCGTCCAGACACACAGCAGCGCATATTACGTGT
 3291 GGTTACACGGTGCAACCCAGTGCCTGGACTTCACACACAGGCGTGTCCAACACAGCAGTGTAAAGCGTGTTC
 3361 ATTACAGCGGCGGGTGGATTGTGGCAGACAGAGGGAGCGTGGGTGAGAAGCGGAGGGCGGGATCGGGGTC
 3431 GGGGTGCGGATGCGCGGTTTGGCACAGTACACGATCCCATTGTCGCAGCGCAGTGGGTCTCCCCCTCGG
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 4761 CCACCTACCTCTCCAATGGCTATCAACTGTGAGTACGGTTCGTTGACAAACGAGCACTTGGTGTGCCAA
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 4831 GAACCAAGTACGATGTCATGGTTCGACGAGCAGTCCCCAAGACCCGGCCAGCAGGCCTTCGAGAAGATGTC
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+3
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+3
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+3
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 6441 GACACACTAAGTCCCTAAATCATTATACACGGTCATGTGACCAACGCTCTTATGGAGCTCTAGATACAGT
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 6861 GCATATGACGTACATGATTGTTCCAATGTACTTAGTCCAGAATATCACCCCTTTTCTCTAGCTTGTCA
 6931 CCATCACGCAGCACTACATTCTCAAGAGATCCATGTATTTTTCTCACTTGTGGAATATCACCCACCC
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 7351 GCCTTGACGACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCA
 7421 ACAGTTGCGCAGCCTGAATGGCGAATGGCGCCTGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTATT
 7491 TCACACCG

Appendix 4.3.2: Complete DNA sequence of fragment ZK1, containing an ORF of hexokinase from *Z. bailii* from base 3987- 5444.

Translation of B

1	MSDENHSIYE	GEMNHREEVG	PTFKKDPVSI	NVVKSEDDST	GDGADETRSI
51	ESLTTNHEEE	LLPEDLKSIP	KMVRQVVTLE	DDPHEPCFTF	RVVVLSTIFI
101	LPGAFDLTLN	SYRTTSAAYS	VFFVQICSYW	FGKWLAKVLP	DIKIGIGNFK
151	FSLNPGPWSI	KENVLITLAA	ASGATGNQGT	TPISLAEIYY	KTKVNPAAVI
201	FFMWSINFSG	YALSYIARNF	LIYEPQFIWP	QALMQTNLFN	SMKSADANDS
251	KEASRKLKYF	FAVIGMTVW	EFLPEYVFPM	LSSLSFICWV	APENYVANFV
301	GGGMGGMGFL	NQSLDWSNIT	SSVLLSPYWT	ICIQFAAFVF	SCWILIPAAK
351	WGNLSSYKHG	LMSNHLLTSN	GTLYPVNDLI	TYDHSSTANG	EVSFNHTAYE
401	KHGPNYIGAQ	QSWNIFFDYA	AFTSAITWIV	LFGRKEIVSS	FKKLERFLN
451	KRNINKLYND	RLNDIQSQYE	EVPVWVFAVL	WLITVVI FMT	IFATNRMFIP
501	WWTYFVALGI	SCIIIIPLAY	LYAISNFQLA	IGAFDELLYG	VMVQNLKSHK
551	HPAGASTYGA	MSGDLWYRAQ	YMLQDQKIGH	YMHIPPKAVF	MSQILGQLIG
601	VPMNYGTVRW	VLDTKMPYLR	GIKDPHQQW	TGQGLTSYNT	EAVLYVLIGP
651	TKFFSSSKTK	VIPFGLLVGF	FAPFIIYVLY	KLVPKMRFDL	WNVTIFCSTM
701	SNFYGNLSTG	YSTQFIVGTF	SMYFLFNYKP	QIWRKYNLYT	AAAFDTGYNL
751	AVLLIFIIFS	SGKTISMPNW	WGNNAVSVER	CFALEKA	

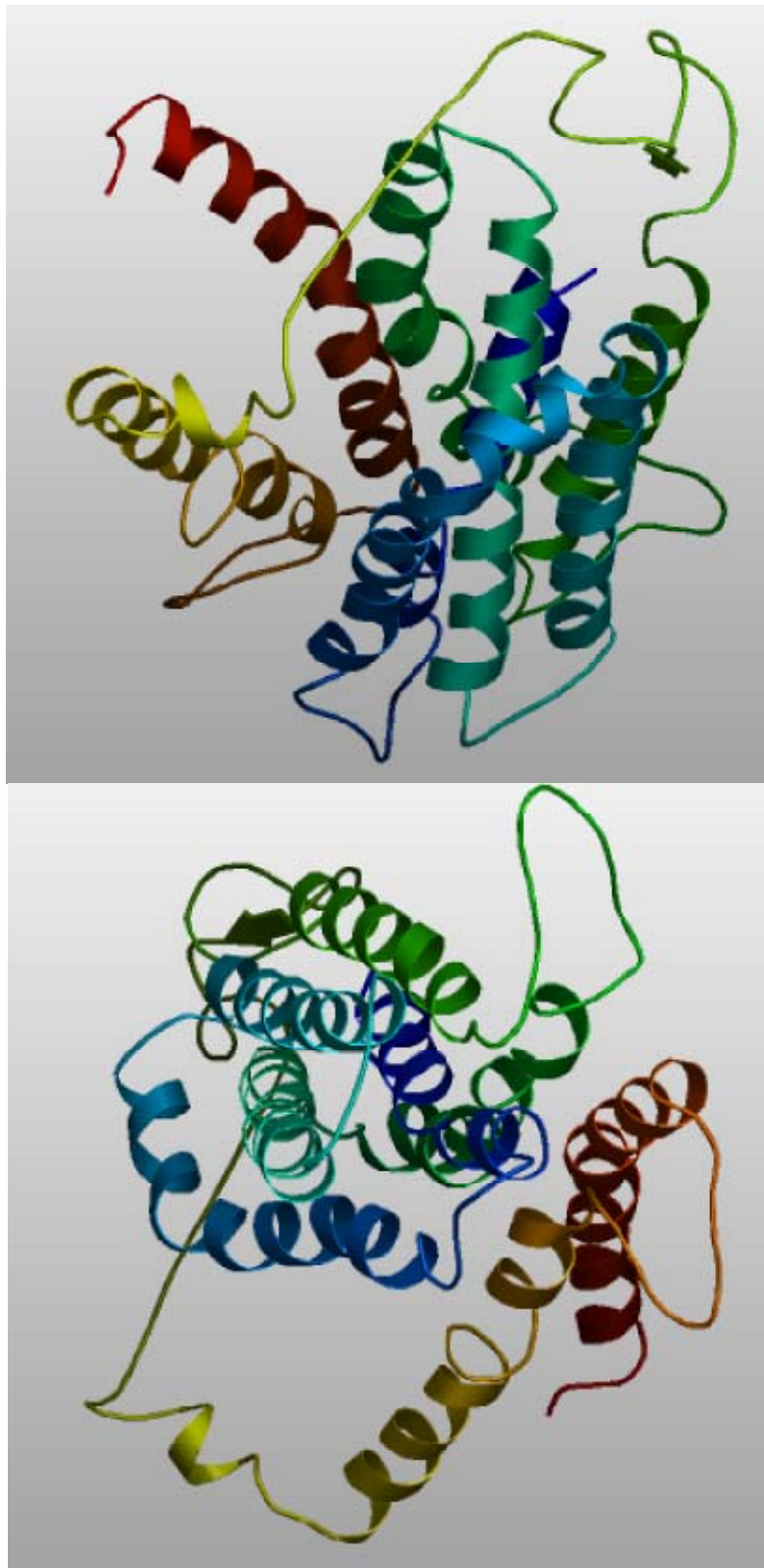
Translation of S

1	MTYTLKNFFR	EIFIDPWGRL	KYGILPVRR I	VDECRDDSLG	SNDEFDQNCN
51	TTEADASNEL	VKVDTDVIVQ	KEKNLTNSEN	ISEDAGDVIN	DQEALTFGNV
101	EYRDEANKPW	WKFFDEEEYR	IRKKGARKNH	WYSWFNGSPS	MQEKKLILKL
151	DVLLAFYSCV	AYWVKYLDTV	NVNNAYVSGM	KEDLGMKGND	LVDLQNMWNI
201	GNIIFQLPFI	FVLNKVPLNY	LLPCLDIGWS	LLTVGQGYVK	TYGGMKALRF
251	FVGAPEAPSY	LAYQYLFDCF	YKHDEMVRRS	AFYYFGQYIG	SLSSGGIQAG
301	VYGTLSGKNG	LSGWRWNFVI	DAIVSAVGVV	IGFYSLPGDP	RNCYSIFLTD
351	DEIRLARKRL	QEEHTQENDE	HKIFDLTAL	KGILLDWKIW	VLSVWAVFCW
401	DDSNAGSGSY	ILWLDSLKNS	EGEKRYSV EK	VNQLSMITPG	LGLVYLALAA
451	LVADKLHSRW	LAICLTQIFN	IIGNVILSVW	YVAEGAKWFA	FMLQYMGYAM
501	APVLYGWVND	ICRRDSEARA	VIIVTLNIAG	TVFNTWSSVV	FFPTVEAPRY
551	LKGYAFTAA N	AFALVVWTFV	VLWLAKRDDR	KHSAESGIIL	YNSKKGGLPE
601	EKPDTNSFTS	MKVS GSITIE	PGKSEELDAG	LNEQ	

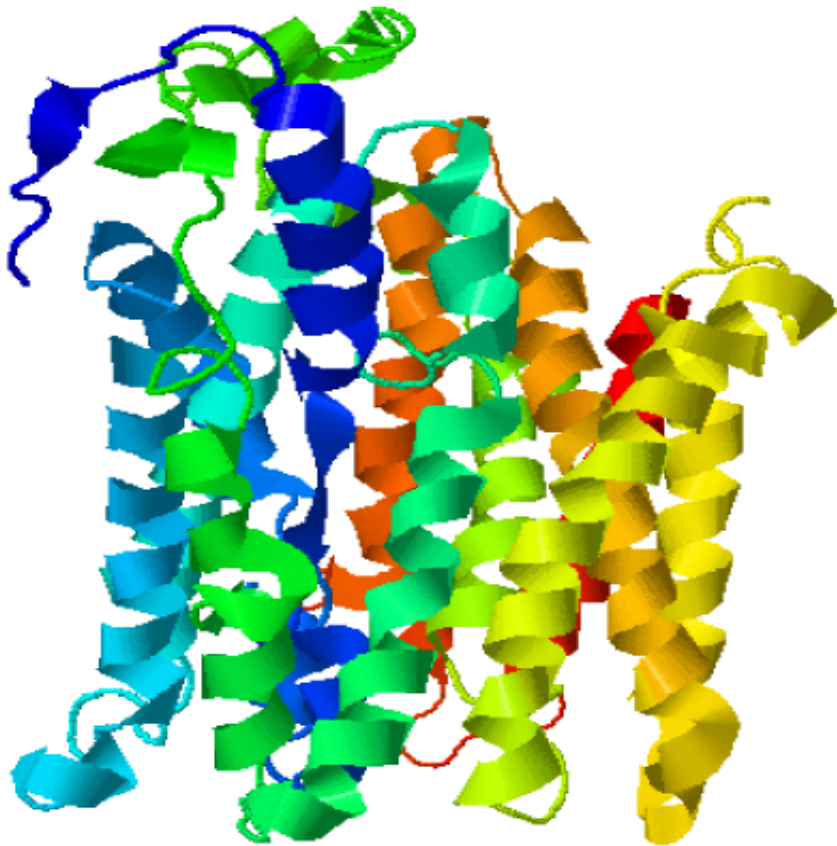
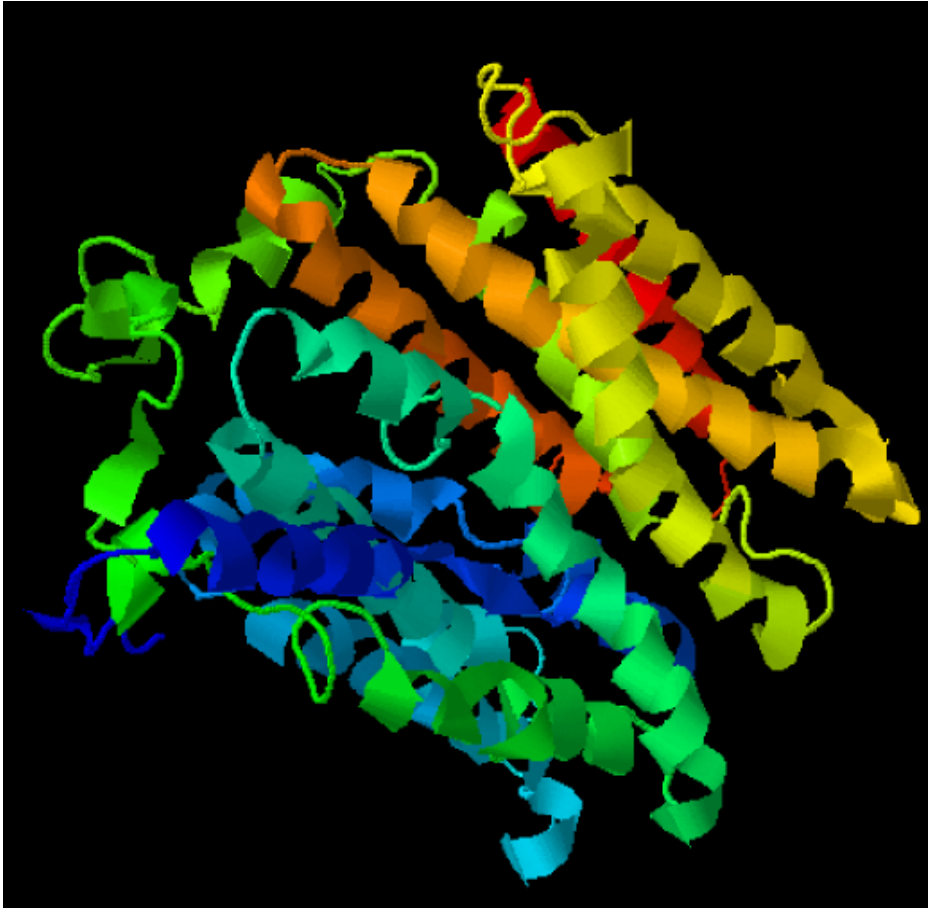
Translation of KORF

1	MVHLGPKKPP	ARKGSMADIP	EDLMEQIRGL	EVLFTVSPEK	LRAVTKHFIS
51	ELGKGLSKKG	GNIPMIPGWV	LEYPTGKERG	HYLAIDLGGT	NLRVVLIKLN
101	GDRTFDSSQS	KYKLPHEMRT	TRDSKELFEF	IADSLKSFLE	EEFPKGTKEV
151	LPLGFTFSYP	ASQSKINEGI	LQRWTKGFDI	PGVEGHDVVP	MLQREIAARN
201	VPIEVVALIN	DTTGTLVASL	YDTPATRMGV	IFGTGVNGAY	YDVCSDIEKL
251	EGKLPPDIPP	TSPMAINCEY	GSFDNEHLVL	PRTKYDVMVD	EQSPRPGQQA
301	FEKMSGYL	GEIVRLTLLD	LHDQGFIFKD	QDITKLKEPY	IMDTTYPISKV
351	EDDPFENLED	TFDLFQKDLG	ISTTVPERKL	IRRLCELIGT	RSARLSVCGI
401	AAICQKRGYE	TAHIAADGSV	FNKYPGFKER	AAHALNDIYG	WGIDDPKHP
451	IIIVPAEDGS	GAGAAVIAAL	TMNRLREGKS	VGVLTK	

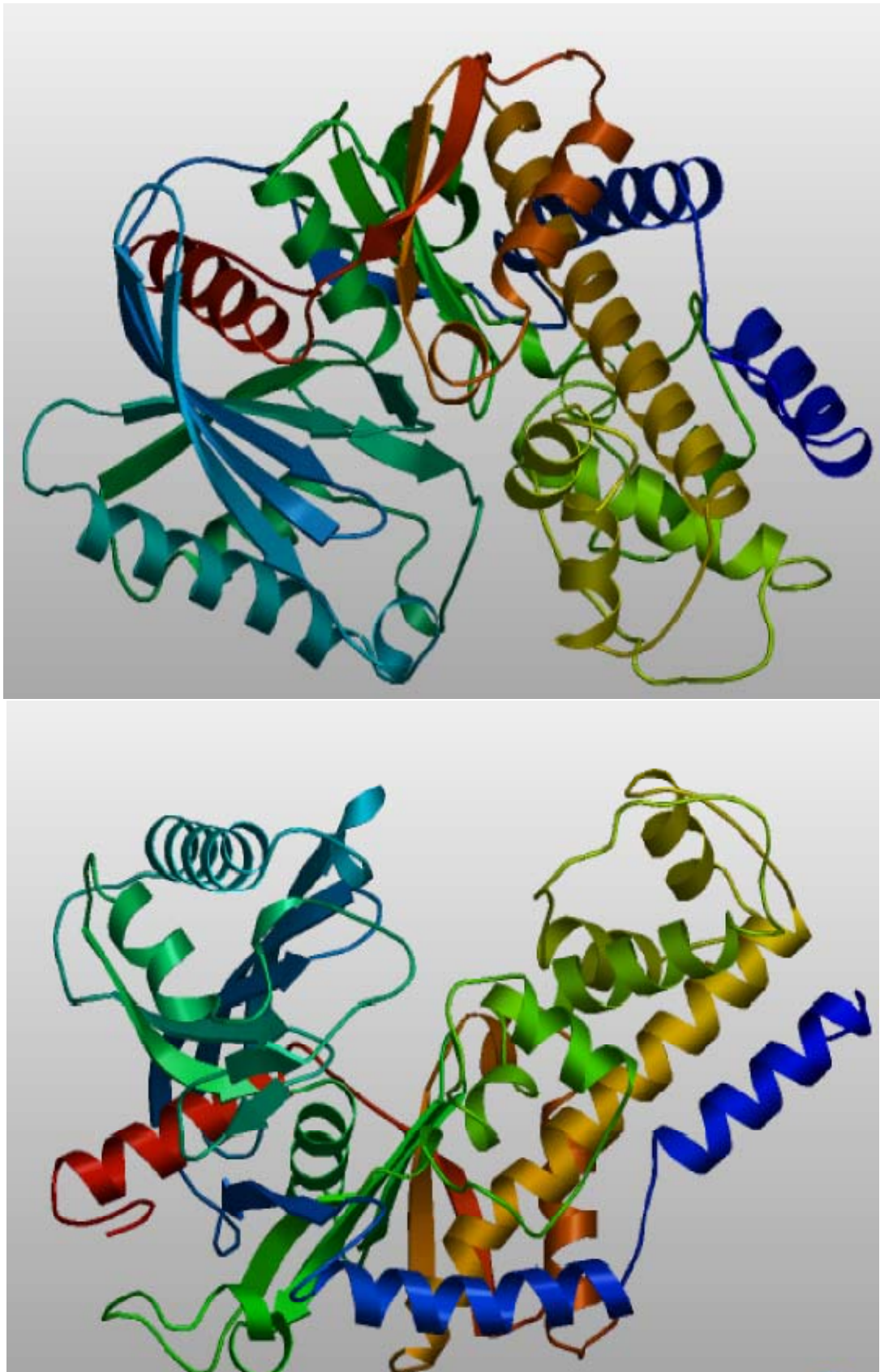
Appendix 4.3: Protein Translations of the isolated ORF's from *Z. bailii*.

Appendix 4.4: Predicted protein models of isolated *Z. bailii* proteins

Swissmodel protein ribbons from permease S, based on template of the crystal structure of the glycerol-3-phosphate transporter from *E.coli*, illustrated from different perspectives.



Permease B on template of the Glycerol-3-phosphate transporter *E. coli* (www.predictprotein.org)



Swissmodell protein ribbons from Zb hexokinase, based on template crystal structure of yeast hexokinase pi in complex with glucose