Evaluation of evolutionary engineering strategies for the generation of novel wine yeast strains with improved metabolic characteristics

by

Heidi K Horsch

Dissertation presented for the degree
of
Doctor of Philosophy at Stellenbosch University.

Supervisor:
Prof. FF Bauer
Co-supervisor:
Prof. J Gafner

December 2008

DECLARATION

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the owner of the copyright thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: 8 December 2008

SUMMARY

The occurrence of sluggish and stuck fermentations continues to be a serious problem in the global wine industry, leading to loss of product, low quality wines, cellar management problems and consequently to significant financial losses. Comprehensive research has shown that many different factors can act either in isolation, or more commonly synergistically, to negatively affect fermentative activity of wine yeast strains of the species *Saccharomyces cerevisiae*. The individual factors most commonly referred to in the literature are various nutrient and oxygen limitations. However, other factors have been shown to contribute to the problem. Because of the mostly synergistic nature of the impacts, no single factor can usually be identified as the primary cause of stuck fermentation.

In this study, several strategies to evolutionarily engineer wine yeast strains that are expected to reduce the occurrence of stuck and sluggish fermentations are investigated. In particular, the investigations focus on improving the ability of wine yeast to better respond to two of the factors that commonly contribute to the occurrence of such fermentations, nitrogen limitation and the development of an unfavorable ratio of glucose and fructose during fermentation.

The evolutionary engineering strategies relied on mass-mating or mutagenesis of successful commercial wine yeast strains to generate yeast populations of diverse genetic backgrounds. These culture populations were then exposed to enrichment procedures either in continuous or sequential batch cultivation conditions while applying specific evolutionary selection pressures.

In one of the stragegies, yeast populations were subjected to continuous cultivation under hexose, and especially fructose, limitation. The data show that the strains selected after this procedure were usually able to out-compete the parental strains in these selective conditions. However, the improved phenotype was not detectable when strains were evaluated in laboratory scale wine fermentations.

In contrast, the selection procedure in continuous cultivation in nitrogen limiting conditions proved to be highly efficient for the generation of yeast strains with higher total fermentative capacity in low nitrogen musts.

Furthermore, yeast strains selected after mutagenesis and sequential batch cultivation in synthetic musts with a very low glucose on fructose ratio showed a fructose specific improvement in fermentative capacity. This phenotype, which corresponds to the desired outcome, was also present in laboratory scale wine fermentations, where the discrepancy between glucose and fructose utilization of the selected strains was significantly reduced when compared to the parents.

Finally, a novel strategy for the rectification of stuck fermentations was adjusted to industrial conditions. The strategy is based on the use of a natural isolate of the yeast species *Zygosaccharomyces bailii*, which is known for its preference of fructose. This process was successfully established and implemented in the wine industry.

OPSOMMING

Die voorkoms van sloerende en gestaakte gistings bly ‘n ernstige probleem in die globale wynbedryf, waar dit lei tot ‘n verlies van produk, wyn van ‘n lae kwaliteit, probleme met kelderbestuur en gevolglik tot betekenisvolle finansiële verliese. Omvattende navorsing het getoon dat verskeie faktore op hulle eie of meer algemeen sinergisties kan werk, om ‘n negatiewe invloed op die gistingsaktiwiteit van wyngisrasse van die spesie *Saccharomyces cerevisiae* uit te oefen. Die individuele faktore waarna daar die algemeenste in die literatuur verwys word, is verskeie voedingstof- en suurstofbeperkings. Daar is egter baie ander faktore wat ook ‘n bydrae tot die probleem maak. Vanweë die hoofsaaklik sinergistiese aard van die invloede kan geen enkele faktor gewoonlik as die vernaamste oorsaak van gestaakte gisting uitgewys word nie.

In hierdie studie is verskeie strategieë ondersoek om wyngisrasse wat verwag word om die voorkoms van gestaakte en sloerende gistings te verlaag, evolusionêr te manipuleer. Die ondersoeke het veral gefokus op die verbetering van die vermoë van wyngis om beter te reageer op twee van die faktore wat algemeen bydra tot die voorkoms van sulke gistings, naamlik stikstofbeperking en die ontwikkeling van ‘n ongunstige glukose-tot-fruktose verhouding tydens gisting.

Die evolusionêre manipulasiestrategieë het staatgemaak op die massaparing of mutagenese van suksesvolle kommersiële wyngisrasse om gispopulasies met diverse genetiese agtergronde te genereer. Hierdie populasies is daarna aan verrykingsprosedures blootgestel in kontinue of in opeenvolgende lotkultuurtoestande terwyl spesifieke evolusionêre seleksiedruk uitgeoefen is.

In een van die strategieë is gispopulasies opgegroei onder kondisies van heksose- en veral fruktosebeperking. Die data toon dat die rasse wat ná hierdie prosedure geselekteer is, gewoonlik die stamrasse onder hierdie selektiewe toestande kon uitkompeteer. Die verbeterde fenotipe kon egter nie opspoorbaar word toe die rasse in laboratoriumskaal fermentasies geëvalueer is nie.

In teenstelling het die seleksieprosedure onder stikstofbeperkende toestande getoon dat dit hoogs doeltreffend is vir die generering van gisrasse met hoër totale gistingskapasiteit in mos met lae stikstofinhoud.

Verder het gisrasse wat ná mutagenese en opeenvolgende lotkultuur in sintetiese mos met ‘n baie lae glukose tot fruktose verhouding geselekteer is ‘n fruktose-spesifieke verbetering in gistingskapasiteit getoon. Hierdie fenotipe, wat met die gewenste uitkoms ooreenstem, was ook in laboratoriumskaal fermentasies teenwoordig. Híér is die verskil tussen die verbruik van glukose- en fruktose deur die geselekteerde rasse betekenisvol verminder in vergelyking met dié van die stamrasse.

Laastens is ‘n nuwe strategie vir die regstelling van gestaakte gistings by industriële toestande aangepas. Hierdie strategie is gebaseer op die gebruik van ‘n natuurlike isolaat van die gisspesie *Zygosaccharomyces bailii*, wat bekend is om sy voorkeur vir fruktose. Hierdie proses is suksesvol in die wynbedryf gevestig en geïmplementeer.

This dissertation is dedicated to my grandma

Klara Schätzle

Her way of dealing with life – always full of energy and joy for work – is an ideal to me. She never lost faith in her doing and living, despite a lot of strokes of fate on her way. Her vitality, although already at a remarkable age, is admirable and desirable to me.

Diese Doktorarbeit widme ich meiner Grossmutter

Klara Schätzle

Ihre Art das Leben anzupacken - immer mit Tatkraft und Freude an der Arbeit – ist ein Ideal für mich. Trotz schwerer Schicksalsschläge hat sie nie die Freude am Schaffen und am Leben verloren. Ihre Vitalität bis in ihr inzwischen beachtliches Alter ist für mich bewunderns- und erstrebenswert.

„Wende dein Gesicht der Sonne zu, dann fallen die Schatten hinter dich“

Sprichwort aus Südafrika

BIOGRAPHICAL SKETCH

Heidi Horsch (maiden name: Gut) was born in Freiburg im Breisgau, Germany, on 16 September 1971. She received her university-entrance diploma (Abitur) in 1991 from the “Technisches Gymnasium Freiburg” in Germany. She then entered the „Mannheim University of Applied Sciences” and graduated as „Diplom-Ingenieur FH“ in Biotechnology in 1997. She was then appointed as „Diplom-Ingenieur Biotechnology“ by „Pharmacia Diagnostics GmbH“ in Freiburg im Breisgau, Germany in the Cell biology department for the development of production processes of recombinant proteins in the Baculovirus expression system. In 1999 she changed to the department of Assay Development and was appointed as Manager Assay Development in 2001. In 2002 she joined the Institute for Wine Biotechnology, University Stellenbosch, South Africa as a Research Student. She worked on the breeding of commercial wine yeast strains. She matriculated in 2004 as PhD Student in Wine Biotechnology at the Institute for Wine Biotechnology, University of Stellenbosch, South Africa. She joined Agroscope Changins-Wädenswil ACW, Microbiology Research, Switzerland in 2004 in order to conduct the experimental work for the PhD thesis jointly with Stellenbosch University.

ACKNOWLEDGEMENTS

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

Professor Florian Bauer, Institute for Wine Biotechnology, University Stellenbosch, for giving me the opportunity to study as a PhD student in his research group. Additionally, I want to thank him for his continuing support as my supervisor.

Professor Jürg Gafner, Agroscope Changins-Wädenswil ACW, Research Station Wädenswil, Switzerland for giving me the opportunity to conduct the practical work for this thesis at his laboratory as well as for his continuous support as my co-supervisor.

My colleagues at the Research Station Wädenswil, Switzerland for creating an interesting and lively work environment as well as numerous discussions about my work and helping hands if necessary.

Special thanks to Dr. M Kidd and Dr. D. Baumgartner for their support in statistics.

My fellow students and personnel at the Institute for Wine Biotechnology in Stellenbosch, South Africa, for always giving support from far and always giving a warm welcome, if I visited the Institute.

Ich möchte meinen Familien danken, die immer an mich geglaubt und mich in allen Phasen dieser Dissertation unterstützt haben. Ganz besonderen Dank gilt meinen Eltern Barbara und Hans, da ich ohne ihre Unterstützung meine beruflichen Ambitionen nicht verwirklichen hätte können. Sie haben immer hinter mir gestanden und mir dadurch eine Sicherheit vermittelt, die von unschätzbarem Wert für mich war.

My husband Kay, for never loosing faith in me and my work. He was always standing by my side and supporting me through all phases of this thesis. This was of utmost importance to me.

PREFACE

This thesis is presented as a compilation of seven chapters. Each chapter is introduced separately and is written according to the style of the journal Applied and Environmental Microbiology.

Chapter 1 **Introduction and Project Aims**

Chapter 2 **Literature Review**
Hexose metabolism in *Saccharomyces cerevisiae* and the evolutionary engineering of improved microorganisms

Chapter 3 **Research Results I**
Mass-mating, enrichment and selection: a novel strategy to generate new wine yeast strains

Chapter 4 **Research Results II**
Evaluation of mutagenesis and mass-mating in combination with selection in continuous cultivation for the generation of wine yeast strains exhibiting reduced discrepancy in glucose and fructose utilization.

Chapter 5 **Research Results III**
Selection of wine yeast strains exhibiting reduced discrepancy in glucose and fructose utilization in sequential batch cultivations

Chapter 6 **Concluding remarks and future prospects**

Chapter 7 **Appendix**
The fructophilic yeast *Zygosaccharomyces bailii* in its application in curing industrial stuck fermentations

CONTENTS

Chapter 1

INTRODUCTION AND PROJECT AIMS

1 Introduction and project aims

Evaluation of evolutionary engineering strategies for the generation of novel wine yeast strains with improved metabolic characteristics

1.1 Introduction and project aims

In most wineries today, the alcoholic fermentation of grape must is conducted by specifically selected commercial strains of yeast, mostly of the species *Saccharomyces cerevisiae* and generally referred to as “wine yeast strains”. This fermentation process can be considered as a collection of metabolic pathways or processes. The most central pathway in this system is glycolysis, the metabolization of the grape sugars, mainly glucose and fructose, to alcohol and carbon dioxide. Many other metabolic pathways are however active during wine fermentations and several have important impacts on the organoleptoc qualities of the final product. Such metabolic activities relate for example to the conversion of aroma precursors found in the grape, as well as to the *de novo* synthesis of many metabolites that contribute significantly to the aroma, flavor and mouth feel of the finished wine (2, 9).

The conversion of the sugars of grape must by *S. cerevisiae* wine yeast strains is however not always proceeding smoothly or reaching completion. Wine fermentations that are characterized by significantly decreased fermentative activity are commonly known as sluggish fermentations, while cases of a total arrest are considered as “stuck” fermentations (6). Wineries globally continue to experience significant economic losses related to stuck and sluggish fermentations, since wines obtained from such problem fermentations are frequently of low quality and characterized by high residual sugar. Problem fermentations are also frequently characterized by off-flavor production, either by the stressed wine yeast itself or by spoilage organisms that benefit from the reduced fermentative activity. Additional costs are incurred because of the inefficient utilization of fermenter space and the necessity of extra measures in order to re-start fermentative activity or to ensure stabilization of the wine (12). Despite the continuous development and release of new commercial wine yeast strains for the inoculation of wine fermentations, the

occurrence of stuck and sluggish fermentations continues to be a challenge to the global wine industry.

Investigations by many research groups have revealed several factors that negatively influence the fermentative activity of *S. cerevisiae* in winemaking conditions. It is generally thought that in most cases synergistic effects of several of such factors are responsible for the occurrence of problematic fermentation. Indeed, the identification of a specific cause for any given industrial problem fermentation *a posteriori* is in most cases not possible (6). Nutrient limitations (in particular of nitrogen sources), oxygen limitations, toxicity of ethanol and fatty acids, low pH, elevated concentrations of acetic acid and sulfites, residual concentration of fungicides and enological practices such as too intense clarification and extremes of temperature were all described for their contribution to stuck and sluggish fermentations (1, 5, 17). At the same time, many of these factors have been shown in model fermentations to have the potential to act as single causative factors.

The development of the ratio of glucose and fructose concentrations (GFR) in wine fermentations was also shown to be one such causative factor affecting fermentative activity (10, 18). The difference in the concentration of glucose and fructose in ongoing fermentations are mainly caused by the fact that *S. cerevisiae* displays a preference for glucose metabolization. Therefore, the initially equimolar or close to equimolar amounts of glucose and fructose that are present in most grape juices are utilized at different rates. While the discrepancy in glucose and fructose utilization was found to be yeast strain dependent, the molecular mechanisms responsible for the preferred utilization of glucose by *S. cerevisiae* have not been identified (4). It is however common opinion that the first two steps in glycolysis, namely hexose transport and hexose phosphorylation, are responsible for the difference. Yet, this has not been unambiguously proven to date (3, 11).

Data by Schütz and Gafner (13) and Wucherpfennig et al. (15) suggest that if the GFR drops below a certain value, at a given stage of fermentation, an arrest in the fermentative activity of *S. cerevisiae* is observed. This finding was supported by the fact that on-going fermentation could be inhibited by addition of fructose and artificially decreasing the GFR to below a value of approximately 0.1. Furthermore, fermentative activity in such cases could be re-stimulated by glucose addition (16). Due to the legal restrictions that prohibit glucose supplementation in wine

fermentations, it is however not possible to use glucose addition as a strategy for the rectification of stuck fermentations. Therefore, other strategies for either preventing or treating low GFRs have to be found.

In this dissertation, several such strategies are presented. One strategy, which is described in the Annexe (Chapter 7) of this thesis, since it was an entirely industry-based project, is based on the use of natural isolates of the fructophilic yeast *Zygosaccharomyces bailii* (*Z. bailii*). This yeast was found to specifically metabolize fructose in the harsh conditions of stuck fermentations without influencing adversely the aroma profile of the treated wine (Sütterlin et al., in preparation). The convincing results of the laboratory scale evaluation of the potential of these *Z. bailii* yeast strains for rectifying stuck fermentations led to the implementation of this process in the commercial wine industry. It is the adaptation of this strategy to industrial conditions that is described in Chapter 7 of this thesis. Due to the success of this strategy, a *Z. bailii* strain was commercialized as a dried yeast product in Europe in the harvest season of 2005.

However, the experiences gained through the use of *Z. bailii* for the rectification of stuck fermentations also revealed significant draw-backs of this strategy. Due to its physiological characteristics, *Z. bailii* can only be inoculated by the time that the problem has already emerged. The strategy therefore involves several additional measures that need to be taken by the winemaker and additional expenses linked to the yeast inoculation. In addition, the fermentation time of the problematic fermentation will exceed generally accepted durations.

These drawbacks in the use of *Z. bailii* for the rectification of stuck fermentations indicate the desirability of other strategies. One strategy is to prevent the occurrence of the problem in the first place. This could be achieved by generating new wine yeast strain that would show a very reduced or even no discrepancy in glucose and fructose utilization. A second aim of this thesis was therefore the evaluation of different strategies of evolutionary engineering for their potential to generate such a trait.

To achieve such an aim, a strategy of metabolic engineering using recombinant DNA technology could have been considered. However, the general skepticism of the wine industry and of many consumers towards the use of genetically modified organisms (GMO) led to the decision not to pursue such an approach. Furthermore, metabolic

engineering through genetic modification, although more directed and frequently of high efficiency, would not have been easily applicable in this case, since the mechanisms responsible for the glucophilic behaviour of wine yeast strains are not well understood, and no obvious target for genetic modification is available (15). Instead, in this study genetic variability was generated through mutagenesis by ethylmethane sulfonate (EMS) or hybridization of two successful commercial wine yeast strains. Both methods generate genetically highly diverse yeast populations of non-GM status (14).

These newly generated populations were subsequently grown in either continuous or sequential batch cultivation conditions while applying specific selection pressures in order to enrich the population for strains best adapted to the selection criteria. The strategy therefore uses highly diverse populations at the start of the selection, with the assumption that some of the mutated or hybridized strains should already be carrying beneficial adaptations. This process can therefore be referred to as “enrichment”. However, data have shown that by extending the selective cultivation over more than 20 generations, evolutionary adaptation processes can become an important contributing factor to the selection of improved phenotypes of the strains under selection pressure (8, 15) and our selection strategy therefore combines enrichment with “directed” or “adaptive” evolution.

In the first section of the results, the suitability of evolutionary engineering to generate new yeast strains is assessed by generating novel yeast strains of improved fermentative capacity in nitrogen limiting conditions. Since this project was entirely funded by a single company and led to marketable new yeast strains, the chapter does not present all data, but only those of relevance to judge the success of the strategy. Several new yeast strains of the desired characteristics were obtained by an approach that combined mass-mating, evolutionary enrichment and the final selection of individual strains. Although nitrogen metabolism in *S. cerevisiae* is complex, it is well established which nitrogen sources are utilized preferably under winemaking conditions (7). The strategy was specifically based on continuously cultivating a mass mated yeast population under fermentative conditions mimicking wine fermentation in a synthetic grape juice containing only limited amounts of nitrogen. The selected strains showed a significant improvement in total fermentative capacity under nitrogen limiting conditions (defined as the total amount of sugar consumed for a given amount of available nitrogen).

Since the strategy proved highly successful, a similar approach was adopted to evolutionary engineer novel wine yeast strains that would exhibit reduced discrepancy in glucose and fructose utilization. The second chapter of the results section describes the evaluation of a strategy that combines either mass-mating or mutagenesis with a selection in continuous cultivation in fructose limiting conditions.

The conditions applied in the continuous cultivation were dominated by hexose limitation, with the feeding medium containing a high, and continuously increased proportion of fructose. The total sugar concentration was kept at a level representative of the amounts of sugars encountered during many stuck fermentations. All other nutrients were supplied in excess, as would frequently be the case in a natural grape juice. Since most of the fermentation process is characterized by a metabolically active stationary phase, the growth rate during the continuous selection process was maintained at a low level. Therefore, the conditions in this selection procedure were attempting to be more representative for the late stages of grape must fermentation, when the discrepancy in glucose and fructose concentrations is usually already significant.

However, since it is not known which part of the fermentation process has the highest impact on the glucose and fructose utilization pattern, a second selection strategy was evaluated, which is described in the third results section of this thesis. The strategy is based on sequential batch cultivation in synthetic grape juice containing a high sugar concentration, with an excess of fructose, and a sufficient concentration of all other nutrients. Batch cultivation represents best the winemaking process since the yeast population is exposed to the continuously changing conditions that characterize such fermentations, including the increasing amount of ethanol. By repeatedly exposing the highly diverse mutagenised population to this cycle, it can be expected to select for strains that exhibit phenotypes that are best adapted for the entire process.

The results of these studies allow to draw some conclusions regarding the suitability of these strategies to address fermentation related problems in *S. cerevisiae* strains.

The initial evaluation of the evolutionary engineering approach, which generated yeast strains of improved nitrogen utilization, was focused on developing a strain for use in the wine industry. Since the commercial potential of these strains was of

importance, experimental scale fermentations of Pinotage and Colombard juice were conducted, determining the potential of those strains for industrial scale winemaking.

The various approaches for the generation of yeast strains exhibiting decreased discrepancy in glucose and fructose utilization under fermentative conditions were focused on yielding yeast strains that could serve as tools for further investigations regarding the physiological and molecular mechanisms that define the hexose preference in *S. cerevisiae*. Suitability for industrial use was a secondary consideration. These studies are currently on-going.

1.2 Literature cited

1. **Alexandre, H., and C. Charpentier.** 1998. Biochemical aspects of stuck and sluggish fermentation in grape must. J. Ind. Microbiol. Biotechnol. **20:**20-27.
2. **Amerine, M. A., H. W. Berg, and W. V. Cruess.** 1972. The technology of wine making, 2nd ed. Avi Pub. Co., Westport, Conn.
3. **Berthels, N. J., R. R. Cordero Otero, F. F. Bauer, I. S. Pretorius, and J. M. Thevelein.** 2008. Correlation between glucose/fructose discrepancy and hexokinase kinetic properties in different *Saccharomyces cerevisiae* wine yeast. Appl. Environ. Microbiol. **77:**1083-1091.
4. **Berthels, N. J., R. R. Cordero Otero, F. F. Bauer, J. M. Thevelein, and I. S. Pretorius.** 2004. Discrepancy in glucose and fructose utilisation during fermentation by *Saccharomyces cerevisiae* wine yeast strains. FEMS Yeast Res. **4:**683-689.
5. **Bisson, L. F.** 1999. Stuck and sluggish fermentations. Am. J. Enol. Vitic. **50:**107-119.
6. **Bisson, L. F., and C. E. Butzke.** 2000. Diagnosis and rectification of stuck and sluggish fermentations. American Journal of Enology and Viticulture **51:**168-175.
7. **Cooper, T. B.** 1982. Nitrogen metabolism in *Saccharomyces cerevisiae*, p. 39-99. *In* J. N. Strathern, E. W. Jones, and J. R. Broach (ed.), The molecular biology of the yeast *Saccharomyces*: Metabolism and gene expression. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
8. **Dykhuizen, D. E., and D. L. Hartl.** 1983. Selection in chemostats. Microbiol. Rev. **47:**150-68.
9. **Fleet, G. H.** 2003. Yeast interactions and wine flavour. International Journal of Food Microbiology **86:**11-22.
10. **Gafner, J., and M. Schütz.** 1996. Impact of glucose-fructose-ratio on stuck fermentations. Vitic. Enol. Sci. **51:**214-218.
11. **Guillaume, C., P. Delobel, J.-M. Sablayrolles, and B. Blondin.** 2007. Molecular basis of fructose utilization by the wine yeast *Saccharomyces*

cerevisiae: a mutated HXT3 allele enhances fructose fermentation. Appl. Environ. Microbiol. **73:**2432-2439.

12. **Henschke, P. A.** 1997. Wine yeast, p. 527-560. *In* F. K. Zimmermann and K.-D. Entian (ed.), Yeast sugar metabolism: biochemistry, genetics, biotechnology, and applications. Technomic Publishing Co, Lancaster, PA.

13. **Parekh, S., V. A. Vinci, and R. J. Strobel.** 2000. Improvement of microbial strains and fermentation processes. Appl. Environ. Microbiol. **54:**287 - 301.

14. **Pretorius, I. S., and F. F. Bauer.** 2002. Meeting the consumer challenge through genetically customized wine-yeast strains. Trends Biotechnol. **20:**426-432.

15. **Sauer, U.** 2001. Evolutionary engineering of industrially important microbial phenotypes, p. 129-169. *In* T. Scheper (ed.), Advances in Biochemical Engineering/Biotechnology, vol. 73. Springer Verlag, Berlin Heidelberg.

16. **Schütz, M., and J. Gafner.** 1993. Sluggish alcoholic fermentation in relation to alterations of the glucose-fructose ratio. Chemie, Microbiologie, Technologie der Lebensmittel **15:**73-78.

17. **Viviani-Nauer, A., P. Hoffmann-Boller, and J. Gafner.** 1997. In vivo detection of folpet and its metabolite phthalimide in grape must and wine. Am. J. Enol. Vitic. **48:**67-70.

18. **Wucherpfennig, K., K. Otto, and Y.-C. Huang.** 1986. Aussagekraft des Glucose-Fructose-Verhältnisses. Die Weinwirtschaft-Technik **122:**254-260.

Chapter 2

LITERATURE REVIEW

Hexose metabolism in *Saccharomyces cerevisiae* and the evolutionary engineering of improved microorganisms

2 Literature Review

Hexose metabolism in *Saccharomyces cerevisiae* and the evolutionary engineering of improved microorganisms

2.1 Introduction

Despite our comprehensive knowledge of the fermentation process, stuck and sluggish fermentations continue to be a severe problem in winemaking. Such problem fermentations cause significant economic losses to the global wine industry. In particular, stuck and / or sluggish fermentations result in a prolonged duration of the winemaking process, and will frequently lead to a shortage of fermentation capacity during the harvest season. The reduced fermentative activity also results in lower CO_2 production, causing reduced protection and leading to a higher risk of spoilage of the wine by undesired microorganisms. Such microorganisms may than cause the production of off-flavors. In addition, and even if these problems can be avoided, the resulting wines will usually be of an undesired style and can not be commercialized as intended. They often need to be blended and are of lower quality, resulting in a reduced price on the market (63).

Various factors were found to negatively influence the fermentative activity of wine yeast strains, which are mainly of the species *Saccharomyces cerevisiae* (*S. cerevisiae*) (3, 16). Besides nutrient limitation and lack of oxygen, factors that are most frequently referred to in the relevant literature, the evolution of the glucose to fructose ratio (GFR) during fermentation was also identified as a contributing factor (162). The initially close to equimolar concentrations of glucose and fructose in the grape juice are fermented at different rates due to the glucophilic character of *S. cerevisiae*. If the yeast strain dependent discrepancy in glucose and fructose utilization is leading to an excessive imbalance, the likelihood of an arrest in fermentative activity is increased (12, 45, 204). Therefore, the availability of a yeast strain with reduced or even no discrepancy in glucose and fructose utilization under fermentative conditions would be a significant advantage to the winemaking industry. Since the main focus of the studies in this dissertation is on the generation of a yeast strain of such traits, the first part of this literature review gives an in depth summary of the hexose metabolism in *S. cerevisiae.*

In this study, an evolutionary engineering approach was employed. The strategy is based on the generation of genetic variation followed by enrichment and adaptive evolution of the yeast population to select for desired phenotype through specific selection pressures. A more direct strategy would have been metabolic engineering. However, due to the current lack of knowledge about the biochemical factors responsible for the preferential utilization of glucose and therefore the lack of suitable targets for the genetic engineering, this methodology could not be considered. The second section of this literature review therefore focuses on the evolutionary engineering of improved microorganisms.

2.2 Sluggish and stuck fermentation – definition and contributing factors

The metabolic basis of stuck and sluggish fermentation was defined as a decrease in sugar uptake capacity correlated with a decrease in sugar consumption rate, while the rest of the glycolytic pathway remains intact and fully active (21, 91, 151). Salmon (152) employed an isogenic set of industrial strains of *Saccharomyces* differing only in ploidy to show that the plasma membrane transport capacity is controlling the CO_2 production rate and not the level of intracellular enzymes. Schaaff et al. (158) showed already in 1989 that the overproduction of key enzymes of the glycolytic pathway and their increased enzyme activity did not facilitate elevated ethanol production. The concentrations of key glycolytic metabolites were also found to be the same as in the reference strain. Therefore, the rate of alcohol production by wine yeasts is considered to be primarily limited by the glucose and fructose uptake rate (136).

A significant amount of research has already been undertaken, revealing factors exhibiting a negative impact on the fermentation rate of sugars present in grape must. The best characterized condition leading to stuck or sluggish fermentations is nutrient limitation. Other factors were found to be ethanol toxicity, presence of organic and fatty acids in unfavorable concentrations, toxins as by-products of microbial activity, cation imbalance, residues of pesticides and fungicides, extremes in temperature, competition of the microbial culture population and certain enological practices (189, 190). Some of the factors are interacting synergistically, thereby increasing the inhibitory potential of each other. The presence of various factors within a problematic fermentation makes it also difficult to identify the original source

of the fermentation problem. The factors that lead to stuck and sluggish fermentation were reviewed extensively by Bisson (16) and Alexandre and Charpentier (3).

2.3 Glucose and fructose utilization under enological conditions

Relatively little attention has thus far been paid to the importance of the relative concentrations of glucose and fructose under fermentative conditions (16). Wucherpfennig (204) summarized the first observations made with different grape musts, yeast strains and yeast species regarding the difference in glucose and fructose utilization rate during fermentation. A general co-evolution of fermentation rate and the percentages of fructose and glucose in the residual sugar concentration was observed. This allowed the definition of ranges for the ratio of glucose to fructose (GFR) at various stages of the fermentation progress. These ranges were suggested to indicate the “natural” or “normal” range of the difference in glucose and fructose utilization rates under enological conditions. Based on these findings it was possible to verify whether the residual sugar found in commercialized wines was due to natural fermentation progression or due to the addition of clarified must (“Suessreserve”) or sucrose. The latter practice is in many winegrowing regions not permitted and the source of clarified must has to be documented (5, 204).

Berthels et al. (12) further verified the finding of a yeast strain dependent difference in glucose and fructose utilization. In addition, these authors showed that environmental changes have a hexose specific impact on the sugar utilization rates. Addition of 40 g/l ethanol to the must revealed a higher inhibition of glucose utilization, while fructose utilization was significantly more inhibited after a second addition of ethanol after 5 days of fermentation. Supplementation of the must with the nutrient di-ammonium phosphate resulted in a stronger stimulation of fructose utilization. These effects were generally found to be true for all tested strains, and variations only concerned the degree of impact (12).

Besides establishing yeast strain and fermentation condition dependent glucose and fructose utilization rates, the resulting increase in the glucose to fructose ratio (GFR) during fermentation was proposed to have a negative impact on the fermentative activity (162). The examination of samples of stuck fermentations from wineries revealed that stuck or sluggish fermentations occurred mostly when more than 80 % of the initial sugars have been fermented. These fermentations all exhibited a GFR of

0.1 and lower, while in regularly progressing fermentations values of about 0.1 are only found when at least 90 % of the initial sugars have been metabolized (45, 204). By decreasing the GFR within a normally ongoing fermentation to values below the range by addition of glucose oxidase, it was shown that the fermentative activity dropped significantly. This result was further confirmed by the finding that co-inoculation of lactic acid bacteria, which consume additional glucose, led to a decline in the fermentative activity of yeast. However, inhibition of yeast fermentative activity in the presence of bacteria was also reported to be caused by the competition for other nutrients and the production of toxic compounds (16). These findings led the authors Schütz and Gafner (162) to propose a low GFR as a cause of stuck and sluggish fermentations. However, other authors suggested that a high residual fructose concentration may be a symptom rather than the cause of stuck and sluggish fermentations (16).

This argument could be in part resolved by showing that it was possible to practically rectify stuck fermentations by addition of sucrose, and, if applicable, by re-inoculation of carefully, freshly prepared yeast and temperature elevation to at least 22 °C (45). If these measures were not sufficient, the inoculation of a selected yeast strain of the species *Z. bailii* facilitated the degradation of excess fructose to a favorable GFR, enabling the re-inoculated wine yeast strains to increase fermentative activity, ensuring fermentation to dryness within a time span that would be acceptable to the majority of winemakers (173). Due to the experience gained by the application of this strategy in practice, two weeks can be considered to be an optimum time-frame, and up to four weeks was still considered acceptable by most winemakers (practical implementation of the treatment strategy by the working group of J. Gafner).

As mentioned above, the exact reasons for the difference in glucose and fructose utilization rates in yeast under fermentative conditions are not well characterized. The general assumption however is, that one of the first two steps in hexose catabolism, the hexose transport system and the phosphorylation of hexoses (12, 52), has to be involved. The following section presents a more detailed analysis of these two initial steps of the hexose metabolism.

2.4 Hexose metabolism in *S. cerevisiae*

The yeast *S. cerevisiae* is able to ferment various sugars almost completely to ethanol and CO_2 under anaerobic as well as aerobic conditions (208). Under high hexose concentrations of above 1 %, catabolism is solely facilitated by glycolysis, while the tricarboxylic acid cycle is not involved (42, 208). Figure 2.1 shows the general pathway of hexose fermentation and the enzymes involved (80). Only when sugar levels drop below this threshold and oxygen is available, does the yeast use a respiro-fermentative system, where pyruvate generated from hexose catabolism is decarboxylated to acetyl-CoA which enters the TCA cycle.

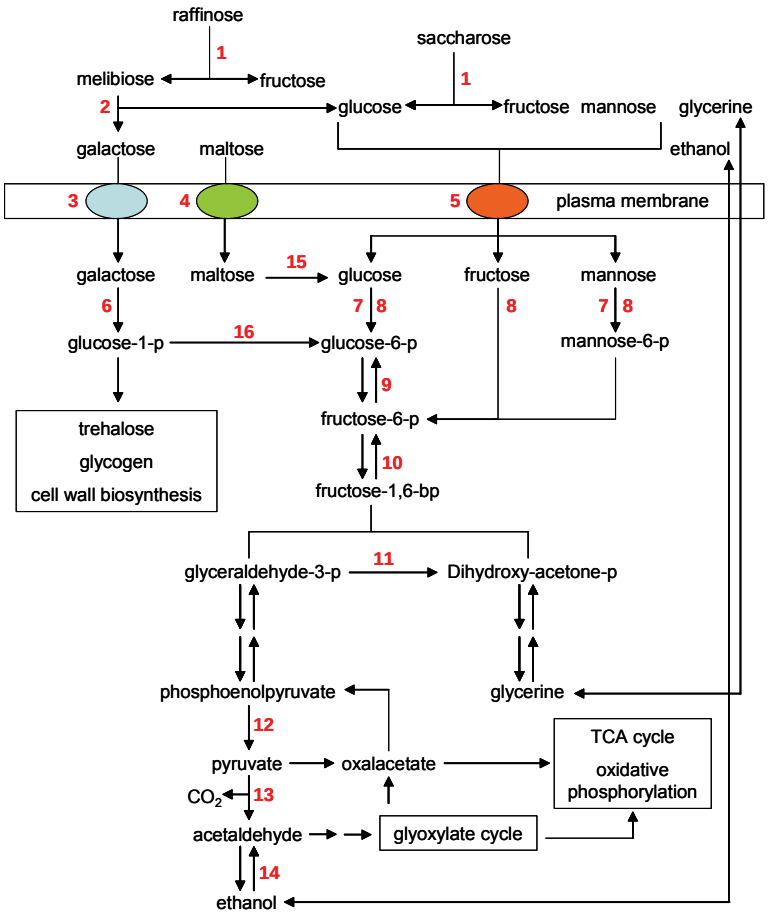


Figure 2.1: General mechanisms and pathways involved in hexose uptake and utilization in *S. cerevisiae* (80). Numbers indicate enzymes important for hexose catabolism. Invertase (1, *SUC1-5*/*7*), α-galactosidase (2, *MEL1-10*), galactopermease (3, *GAL2*), maltose permease (4, *MALx1*), hexose transporters (5, *HXT1-17*), Leloire pathway (6), glucokinase (7, *GLK1*), hexokinases (8, *HXK1*, *HXK2*), phosphoglucoisomerase (9, *PGI1*), phosphofructokinase (10, *PFK1*, *PFK2*), triose phosphate isomerase (11, *TPI1*), pyruvate kinase (12, *PYK1*, *PYK2*), pyruvate decarboxylase (13, *PDC1*, *PDC5*, *PDC6*), alcohol dehydrogenase (14, *ADH1*), α-glucosidase (15, *MALx2*), phosphoglucomutase (16, *PGM1*, *PGM2*).

The rate of sugar fermentation in yeast was suggested to be mainly controlled by the hexose transport. This was proposed based on the finding that sugar concentrations in the cytoplasm are extremely low (9). Under adverse conditions, the fermentation rate is decreased by specific degradation of *HXT* transporters. If the proteolysis of

the transporters is blocked, the consequence will be apoptosis due to the toxic conditions caused by continued hexose catabolism (16). The yeast sugar permease family consist of 34 proteins, twenty of which belong to the hexose transporter subfamily (82). Glucose, fructose and mannose are transported across the plasma membrane by the hexose transporter family. This protein family also includes a galactose permease facilitating the transport of galactose. Maltose permease enables the yeast cell to take up maltose. The disaccharides sucrose and melobiose as well as the trisaccharides raffinose and maltotriose undergo extracellular hydrolysis before transport into the yeast cell (8, 31, 35).

2.4.1 Hexose transporter family in *S. cerevisiae*

The hexose transport system of *S. cerevisiae* comprises 20 genes encoding proteins similar to hexose transporters: *HXT1* to *HXT17*, *GAL2*, *SNF3* and *RGT2* (17, 20, 27, 82, 116). These proteins are part of the major facilitator superfamily (MFS) of transporters whose members transport their substrates through passive, energy-independent facilitated diffusion. Of any organism described so far, *S. cerevisiae* has the largest number of MFS transporters (122). Table 2.1 gives an overview of the function and regulation of the different proteins belonging to the hexose transporter family.

The Hxt proteins facilitate the transport of hexoses in general. In the case of glucose, the transcription of the *HXT* genes is dependent on the intracellular signal generated by Rgt2p and Snf3p, two proteins functioning as sensors for extracellular glucose (17, 20, 27). *GAL2* encodes a galactose permease and is more than 60 % identical to the Hxt proteins. *GAL2* is only expressed in the presence of galactose and a strain lacking this gene does grow poorly on medium containing galactose as the sole carbon source (181).

Seven members of the *HXT* gene family have thus far been shown to encode for functional proteins. Indeed, for the generation of a laboratory *S. cerevisiae* strain (MC996A background) that is unable to grow on glucose, fructose or mannose, the deletion of *HXT1* to *HXT7* (*hxt1-7* null mutant) was shown to be sufficient. In addition, this strain did not show any glycolytic flux (20, 96, 140, 141). Expression of any of the genes *HXT1*, *2*, *3*, *4*, *6* or *7* in the *hxt1-7* null mutant is sufficient to restore glucose utilization, but to various degrees (140). In further experiments with the yeast strain

CEN.PK it was shown that additional deletions of other transport proteins are needed to ensure abolition of glucose consumption or transport completely. It was concluded that the higher respiration rate of CEN.PK compared to MC996A enables glucose catabolism even at very low uptake rates (96, 199). Deletion of *HXT1-4* and *HXT6/7* in a third laboratory strain did not completely eliminate glucose transport either (96). In summary, it was shown that 20 transport genes need to be knocked-out in order to block all hexose uptake in *S. cerevisiae*. Besides *HXT1* to *HXT17*, *GAL2*, *AGT1* (maltose permease) and two genes encoding alpha-glucoside permeases (*YDL247w* and *YJR160c*) were deleted generating a strain unable to show glucose consumption or transport activity. If *SNF3*, one of the glucose sensor genes, was also deleted, a partial restoration of growth on hexoses was observed, indicating the presence of even more proteins exhibiting hexose transport ability (199).

Two different systems for hexose transport were described in *S. cerevisiae*. One is a low-affinity system that is constitutively expressed and exhibits high K_m values of 15 to 20 mM. The second system shows high-affinity characteristics accompanied by low K_m values of 1 to 2 mM and is known to be glucose-repressed (15, 17-19, 138). These values are valid for glucose. The affinities of the transport systems are different for fructose and were found to be 2.5 to 5-fold higher than for glucose resulting in a decreased affinity. The low-affinity system exhibits K_m values for fructose of 20 to 50 mM and for the high-affinity system values of 5 to 10 mM. In contrast, the V_{max} values of the transport of fructose are always higher than for glucose (16). At any given moment, the transport system will consist of different combinations of the various hexose transport proteins. Low- and high-affinity glucose transport is each facilitated by several transport proteins. Furthermore, since none of the individual transporters is essential for growth on glucose, they can be considered to be at least in part functionally redundant. However, the expression of the appropriate transporters is dictated by the available glucose concentration regulating the *HXT* gene expression (116). Their transcription is regulated in response to glucose and is consistent with their function as low- and high-affinity transporters. Derepression of the expression of high-affinity hexose transporters is mediated by the Snf3p glucose sensor in the presence of low hexose concentrations, while the expression of the low-affinity hexose transporters is mediated by Rgt2p in the presence of high glucose concentrations.

Table 2.1: Overview of the hexose transporter protein family

Protein	Properties in standard laboratory conditions	Regulation in standard laboratory conditions	Properties under enological conditions
Hxt1p	low-affinity glucose transport, K_m of 100 mM	induced by high glucose via Rgt2p-Rgt1p and an independent pathway; induced by hyperosomotic stress	abundant in the beginning; declining significantly after 17 h of fermentation; clearly associated with initial and lag phase of fermentation
Hxt2p	high-affinity glucose transport, exhibiting two affinity systems: K_m of 1.5 /60 mM and 10 mM	induced by low glucose via Snf3p-Grr1p; repressed by high glucose via Mig1p	activation just after inoculation of cells to must, peak at 8 h after inoculation; very low level through growth phase, no induction towards end of fermentation
Hxt3p	low-affinity glucose transport, K_m of 30 to 60 mM	induced by glucose independent of concentration	present throughout fermentation process; maximum at end of growth phase and abundant throughout stationary phase
Hxt4p	low-affinity glucose transport, K_m of 6 to 9 mM	induced by low glucose via Snf3p-Grr1p; restores growth in *hxt1-7* null mutant only on high glucose concentrations	*HXT4* promoter induced by high glucose concentrations; protein activity not detectable in the strain used in the study
Hxt5p	glucose transporter; restores growth on 2 % glucose, fructose and mannose; expression growth rate dependent; expressed due to osmotic stress; expressed in presence of non-fermentable carbon sources	low levels of expression	no expression detected throughout the fermentation process
Hxt6p	high-affinity glucose transport, K_m 1.1 mM to 2.1 mM	induced by low glucose; repressed by high glucose via Snf3p	induction at entry of stationary phase, present in high abundance throughout stationary phase and after end of fermentation
Hxt7p	high-affinity glucose transport, K_m 1.1 mM to 2.1 mM	induced by low glucose; repressed by high glucose	induction at entry of stationary phase, present in high abundance throughout stationary phase and after end of fermentation

Hxt8p	restores growth on 2 % glucose, fructose and mannose;	low expression level	so far, no importance under enological conditions revealed
Hxt9p	restores growth on 2 % glucose, fructose, mannose and galactose; involved in pleiotropic drug resistance	induced by drugs via Pdr1p and Pdr3p	so far, no importance under enological conditions revealed
Hxt10p	restores growth on 2 % glucose, fructose, mannose and galactose	repressed by glucose	so far, no importance under enological conditions revealed
Hxt11p	restores growth on 2 % glucose, fructose, mannose and galactose; involved in pleiotropic drug resistance	induced by drugs via Pdr1p and Pdr3p; low expression level; not induced by glucose	so far, no importance under enological conditions revealed
Hxt12p	possibly pseudogene	low levels if expressed; not induced by glucose	so far, no importance under enological conditions revealed
Hxt13p	restores growth on 2 % glucose, fructose and mannose;	low expression level	so far, no importance under enological conditions revealed
Hxt14p	specific transport of galactose	not known to date	so far, no importance under enological conditions revealed
Hxt15p	restores growth on 2 % glucose, fructose and mannose;	repressed by high glucose concentrations	so far, no importance under enological conditions revealed
Hxt16p	restores growth on 2 % glucose, fructose and mannose;	repressed by glucose	so far, no importance under enological conditions revealed
Hxt17p	restores growth on 2 % glucose, fructose and mannose;	repressed by glucose	so far, no importance under enological conditions revealed
Gal2p	high-affinity galactose and glucose transporter	Induced by galactose via Gal1p-Gal3p-Gal4p	so far, no importance under enological conditions revealed
Rgt2p	high-glucose sensor	low expression level; constitutive	not specifically described to date
Snf3p	low-glucose sensor	low expression level; glucose-repressed	not specifically described to date

Summary of the characteristics known to date for the different proteins belonging to the hexose transporter family in *S. cerevisiae* as published in the review articles of Santangelo (153), Boles and Hollenberg (20) and Özcan and Johnston (116). Properties found in enological conditions were published by Luyten et al. (100) and Rossignol et al. (149).

When considering the different industrial applications of yeasts, it is not very surprising to find this organism being able to adapt efficiently and specifically to a very broad range of glucose concentrations. For example, fermenting a grape juice to dryness requires the ability of the yeast strain to metabolize very high sugar concentrations of more than 200 g/l to depletion.

2.4.1.1 Proteins of the low-affinity transport system

Of the twenty known hexose transport proteins, Hxt1p, Hxt3p and Hxt4p were characterized as low-affinity transport proteins. Introduction of *HXT1*, *HXT3* or *HXT4* in the *hxt1-7* null mutant restores growth on glucose concentrations of more than 1 % (140). *HXT1* was shown to be induced only by high glucose concentrations, and the corresponding transporter has a K_m for glucose of 100 mM. Hxt3p was shown to have a K_m of 30 to 60 mM, and the *HXT3* gene is induced by high and by lower concentrations of glucose. Therefore, the two transporters, Hxt1p and Hxt3p, were proposed to be responsible for glucose transport in cells growing in high glucose concentrations (20, 102, 140).

HXT4 encodes for a protein that exhibits a K_m for glucose of 6 to 9 mM (102, 140). However, it was also shown that *HXT4* does not restore growth of the *hxt1-7* null mutant on 0.09 % glucose, but is able to do so at high glucose concentration. It therefore appears to belong to the low-affinity transporters, although its K_m value is low compared to those of *HXT1* or *HXT3*. This inconsistency was explained by altered transcriptional regulation of *HXT* genes in the *hxt1-7* null mutant (140). Comparing the kinetic parameters for the glucose and fructose uptake, a difference can be observed between cells grown in 5 mM glucose or in 100 mM glucose. The K_m values increase for the cells grown in the higher glucose concentration. When the kinetic parameters for the fructose uptake were established, a 2 to 5-fold increase in K_m values was observed in cells that were grown in medium containing 100 mM glucose. This indicates a generally lower affinity of the tested transporters for fructose compared to glucose in these conditions (140).

The expression of the Hxt transporters Hxt1p and Hxt3p was also examined under enological conditions in laboratory scale fermentations in a synthetic grape must as described by Bely et al. (10).

Hxt1p was abundant at the beginning of fermentation and the beginning of the exponential growth phase, followed by a rapid decrease already after 17 h of fermentation. No Hxt1p was found during stationary phase although promoter activity was found to be significant. *HXT1* seems to be specifically associated with the lag phase and the beginning of growth phase (131). These results correlate well with the known characteristic of *HXT1* as it is induced by high glucose concentrations. The strong induction of Hxt1p expression in the presence of high sugar concentrations could also be supported by the induction of the HOG pathway, which is activated by the osmotic stress that is caused by high glucose concentrations. It was shown that full induction of *HXT1* indeed requires, the glucose signaling pathway and the HOG pathway (177). The early degradation of Hxt1p under enological conditions was discussed to be possibly triggered by nitrogen starvation in the presence of sugar. Indeed, it was suggested that the Tor pathway may also be able to contribute to Hxt1p degradation under nitrogen deficiency (131).

Hxt3p was found in the membrane fraction throughout the whole fermentation process, exhibiting a maximum level at the end of the exponential growth phase and significant abundance during stationary phase. The promoter activity of *HXT3* was found to be in good agreement with the protein levels, although activity levels varied more significantly than the protein levels. Considering the transport-kinetic data, Hxt3p can be suggested to be the main low-affinity transporter during the metabolically active stationary phase of wine fermentation. This shift of low-affinity transporters between the growth and stationary phase enables the expression of a more stable protein under nitrogen deficient conditions (131).

Indeed, the expression of only Hxt3p in a the *hxt1-7 null* mutant is characterized by the capacity to maintain a high fermentation rate during starvation, supporting the findings obtained under enological conditions (100).

The *HXT4* promoter activity was found to be induced by high sugar concentrations under enological conditions. This is in contrast to the induction observed in low sugar concentrations reported for laboratory conditions in rich growth medium. Although *HXT4* promoter activity was induced, the transporter protein Hxt4p of the yeast strain V5, used in this study, appeared not to be functional. It was found not to be able to restore growth in the *hxt1-7* null mutant. This was explained by a nonsense mutation at codon 123 in the V5 *HXT4* gene. Expression of this gene results in the production

of truncated, inactive protein, about 20 % in size of the wild-type protein. So far it is unclear whether this mutation is specific to V5 or whether this feature is commonly found among wine yeast strains (100, 131). In two genome-wide studies by Rossignol et al. (149) and Varela et al. (185) transcription of *HXT4* was also not found to be elevated in winemaking conditions. Further studies on the regulation of *HXT4* in winemaking conditions will have to be done to be able to define its function.

2.4.1.2 Proteins of the high-affinity transport system

The transport proteins Hxt2p, Hxt6p and Hxt7p were suggested to represent the most relevant high-affinity transporters among the twenty hexose transport proteins. *HXT2*, *HXT6* and *HXT7* were found to be induced only by low concentrations of glucose. Expression of any of the three *HXT* genes enables the *hxt1-7* null mutant to grow on 0.1 % of glucose (140).

The amino acid sequences of *HXT6* and *HXT7* are almost identical and it was therefore not surprising to find very similar values for the kinetic parameters regarding glucose and fructose uptake. The K_m value for glucose uptake was 1.1 mM and 2.1 mM in cells grown in 5 mM or 100 mM glucose. The values for fructose were also determined in cells grown in 5 mM and 100 mM glucose, and the corresponding K_m values were found to be 2.6 mM and 4.6 mM. The lower affinity for fructose is therefore also found for the high-affinity Hxt transporters (140).

Hxt2p was found to exhibit biphasic behavior. Evaluation of uptake kinetic parameters revealed a high- and a low-affinity activity in Eadie-Hofstee plots. For cells grown on 5 mM glucose a high-affinity component with a K_m value of 1.5 mM and low-affinity component with a K_m value of 60 mM was detected. For cells grown up in 100 mM glucose a K_m value of 10 mM was determined. The K_m values for the fructose uptake were 2 to 3-fold higher than those observed for glucose (140). This distinct characteristic of Hxt2p might be caused by modulation of its affinity in response to the sugar concentration. Another explanation could be a regulatory function of Hxt2p in the *hxt1-7* null mutant activating other transporters with various affinities for glucose.

In conclusion Hxt2p, Hxt6p and Hxt7p are considered to be responsible for the high-affinity transport of glucose. The reason why all of them would exhibit almost identical affinity for glucose has not been explained to date (116).

Analysis of the expression of *HXT2*, *HXT6* and *HXT7* under enological conditions revealed a different induction profile than seen under standard laboratory conditions (131). The *HXT2* promoter was found to be active within the first hours after inoculation of the cells to the grape must. After a peak in activity after 8h in the lag phase of growth, the activity declined rapidly and was only present at a very low level during the growth phase. Contrary to the prediction based on the results gained under laboratory conditions, the *HXT2* promoter is not activated at the end of fermentation in the stationary phase when glucose concentrations become derepressive. The expression levels of Hxt2p are in line with the promoter activity (131). The mechanism enabling *HXT2* to bypass glucose repression during lag phase is not yet understood. Deletion of *HXT2* led to a prolonged lag phase but no further alterations in fermentation kinetics (100).

HXT6 and *HXT7* are also induced at a stage of fermentation that is still high in sugar concentration. The induction indeed occurs at the entry of stationary phase of the culture population, when sugar levels may be above 100 g/l. The expression of Hxt6p and Hxt7p is characterized by a strong induction just after the cell culture enter stationary phase. Both proteins are found in abundant concentrations throughout stationary phase and are still present after sugar depletion. When considering the results that indicated degradation of Hxt7p in nitrogen limiting conditions in the presence of high sugar concentration, the expression of Hxt6p and Hxt7p during stationary phase of fermentation seems to be contradictory (81). The explanation for the expression of the two transporters in these conditions might be due to a higher rate of synthesis than degradation. Furthermore, cultivation conditions under enological conditions differ significantly with respect to the availability of nitrogen sources. Assimilable nitrogen is depleted in stationary phase of grape must fermentation but proline is present in very high concentrations and although it can not be utilized in the absence of oxygen it might be sufficient to trigger a specific signal (131). In addition, the autolysis of yeast cells during stationary phase of grape must fermentation may lead to the release of amino acids that can be utilized by active yeast cells (29).

2.4.1.3 Other *HXT* transporter genes

The other *HXT* genes (*HXT5* and *HXT8* to *HXT17*) are not as well characterized as the major *HXT* genes (*HXT1* to *HXT4*, *HXT6* and *HXT7*).

Regarding *HXT5*, two distinct sets of data can be found with respect to its glucose transport abilities. Expression of *HXT5* derived from strain V5 did not restore growth in the *hxt1-7* null mutant on glucose at various concentrations (100). If *HXT5* is expressed in CEN.PK2-1C exhibiting a deletion for *hxt1-17 gal2*, growth was however detected on 2 % of glucose, fructose and mannose, while growth on 2 % galactose was not detectable (199). The ability of *HXT5* to restore growth on hexoses seems to be dependent on the type of deletion strain it is expressed in. Due to the more comprehensive deletions in the CEN.PK2-1C, inhibitory mechanisms to the induction of *HXT5* might be by-passed. Another possibility would be sequence differences of the two *HXT5* genes derived from V5 and CEN.PK2-1C, resulting in distinct enzyme conformations and therefore distinct substrate affinities or activities. Finally, the conditions tested may not have favored the induction of *HXT5* activity. Recently, *HXT5* expression was found to be growth rate dependent. Expression of Hxt5p was detected in conditions of low glucose consumption rates accompanied by low growth rates. *HXT5* is expressed during stationary phase of the cell cycle (187). The mechanism of Hxt5p degradation is distinct from the mechanism known for the major Hxt proteins. Degradation of Hxt5p occurs in ubiquitine-independent manner via the endocytic pathway (183). Recent results show *HXT5* to be expressed due to osmotic stress induced by high sugar concentrations (50). The same study revealed *HXT5* expression under growth on non-fermentable carbon sources. Under enological conditions, *HXT5* gene expression was not observed at any time during fermentation when the V5 yeast strain was used (131).

For the transporter genes *HXT8* to *HXT17* and *GAL2*, Luyten et al. (100) did not find any evidence for a role under enological conditions. It was pointed out, that these transporters appear not to be able to facilitate sufficient hexose uptake to start or terminate fermentation.

Wieczorke et al. (199) expressed *HXT8* to *HXT17* in the CEN.PK2-1C strain deleted for *hxt1-17* and *gal2*. Their results show Hxt8p, Hxt13p, Hxt15p, Hxt16p and Hxt17p to be able to restore growth on 2 % glucose, fructose and mannose but not on galactose. Hxt9p, Hxt10p and Hxt11p in addition showed galactose transport. Hxt14p

specifically transported galactose, but none of the other hexoses. In the same conditions, it was not possible to functionally express Hxt12p. This is supporting the opinion that *HXT12* may be a pseudogene (4, 82).

Özcan and Johnston (116) evaluated the expression of the remaining *HXT* genes in an experiment using promoter-*lacZ* fusion proteins in multicopy plasmids. They found that *HXT5* and *HXT13* were up to 300-fold less expressed than *HXT1*, while the others showed expression levels comparable to *HXT1* to *HXT4*. They found *HXT11* and *HXT12* to be expressed at a very low level and apparently not regulated by glucose. *HXT10*, *HXT16* and *HXT17* appeared to be repressed by glucose at various degrees. *HXT5*, *HXT8*, *HXT13*, *HXT14* and *HXT15* are induced by low levels of glucose, while being repressed by high glucose levels at various degrees. Their results led to the conclusion that none of these *HXT* genes exhibit the same regulation as *HXT1* to *HXT4*. It was further discussed by Özcan and Johnston (116) whether these transporters indeed encode for glucose transporters. Some of these genes could be involved in the transport of other sugars or exhibit a regulatory function. These hypothesis can be partly answered by the results of Wieczorke et al. (199).

Further evidence for the function of *HXT9* and *HXT11* were gained by the finding that both genes appeared to be regulated by Pdr1p and Pdr3p, two zinc-finger-containing transcription factors of the ABC transporter genes. Therefore, it was suggested that *HXT9* and *HXT11* might be involved in pleiotropic drug resistance. This suggestion was supported by the fact that deletion of *HXT9* and *HXT11* caused resistance to several drugs like cycloheximide and sulfometuron methyl. Overexpression of *HXT11* increased sensitivity of the yeast cells to these drugs (111).

HXT13 was reported to be co-regulated by Hap2p and therefore with respiration. Hap2p is a transcriptional regulator of genes involved in respiration and *HXT13* promoter was found by screening for targets of Hap2p (27). In a recent study, evaluating the expression of *HXT* genes by osmotic stress due to high sugar concentrations, *HXT13* was found to be induced by low concentrations of glucose but also by non-fermentable carbon sources. The authors concluded a regulation of *HXT13* expression independent of glucose concentration (50).

Further investigations are needed in order to get more information about the activity and regulation of these less intensively studied *HXT* genes in order to elucidate their

possible specific function for hexose transport or regulation in general, but also to determine any eventual roles in winemaking conditions.

2.4.1.4 The two glucose sensors *SNF3* and *RGT2*

Snf3p and Rgt2p were found to be glucose sensors rather than hexose transporters. They are about 70 % identical to each other but show only 30 % homology to the other proteins belonging to the Hxt transporter family (20, 82, 115).

Evidence for the absence of transporter activity in Snf3p and Rgt2p is based on the fact that the expression of either of them in the *hxt1-7* null mutant strain did not restore growth on glucose (96, 114). On the other hand, the presence of Snf3p is essential for the expression of *HXT2* and *HXT4* but not for *HXT1*. The expression of Snf3p reaches its maximum if glucose concentrations are low, suggesting that it is functional as a sensor for low glucose concentrations. High glucose concentrations were found to repress Snf3p expression (115). Rgt2p on the other hand proved to be necessary for the induction of *HXT1* expression in high glucose concentrations and it was shown to be expressed in cells growing on high glucose concentrations. Rgt2p expression is not controlled by glucose concentration and the gene is therefore considered to be expressed constitutively (115). Strong evidence that the two proteins act as glucose sensors is provided by the observation that dominant mutations in *RGT2* (*RGT2-1*) and *SNF3* (*SNF3-1*) cause constitutive expression of *HXT1* to *HXT4* in the absence of the inducer glucose. These mutations are proposed to lead to a permanently glucose bound form of the proteins and thereby facilitating constitutive signaling for *HXT* activation (115).

The activity of Rgt2p and Snf3p results in derepression of the Rgt1p repression of *HXT* expression which is present in the absence of glucose. At low glucose concentrations, the signaling via Snf3p enables expression of *HXT1* to *HXT4*. Rgt2p facilitates in high glucose concentrations the modification of the *RGT1* suppressor to a *HXT1* activator resulting in a strong expression of Hxt1p (118). The detailed regulatory mechanisms are described later within this literature review.

2.4.1.5 Fructose specific transport systems in yeast

Since yeast species, other than *S. cerevisiae*, were found to exhibit a more fructophilic character or even a clear preference for fructose utilization, it is

worthwhile to take a closer look at the specific fructose transport systems in these yeast species.

S. cerevisiae facilitates fructose transport by the Hxt transporter system and therefore by facilitated diffusion. Fructose transport occurs in competition to glucose transport accompanied by a lower affinity of the Hxt transporters for fructose (140). In *S. bayanus* and *Saccharomyces pastorianus* (*carlsbergensis*) a specific fructose/H^+ symporter is produced in addition to the Hxt transporter system (142). The transporter is encoded by the gene *FSY1*, which is not found in *S. cerevisiae* (75). The transport through proton symport is energy-dependent, and the activity of this system is observed in low fructose concentrations. It has been shown that the expression of Fsy1p fructose/H^+ symporter is only induced if fructose transport via the facilitated diffusion system is proceeding at a very low rate. Expression of Fsy1p can be induced by low levels of glucose and fructose. However, only its induction by fructose is physiologically important, since glucose is not a substrate of Fsy1p.

Diezemann and Boles (37) described such a specific fructose/H^+ symporter in *Kluyveromyces lactis* (*K. lactis*), the predominant yeast in milk. The transporter is encoded by a gene identified as *FRT1*. Glucose transport in *K. lactis* is facilitated usually by the two transporters Rgt1p and Hgt1p. Some natural isolates of *K. lactis* appear not to harbor the *HGT1* gene and exhibit instead two tandemly arranged genes, *KHT1* and *KHT2* (13, 198). Rag1p, Hgt1p and Ftr1p were found to be involved in fructose transport. No other fructose transporting proteins were found in *K. lactis* (37).

Specific fructose transport was also reported in another species of the genera *Kluyveromyces*. Evaluation of the regulation of glucose and fructose transport in *Kluyveromyces marxianus* (*K. marxianus*) revealed a constitutively expressed low-affinity system specific for both glucose and fructose. In addition, it was shown that high-affinity transport is facilitated by two proton symport mechanisms specific for either glucose or fructose (33, 135).

Z. bailii is known to consume fructose faster than glucose. Due to this property which is contrary to most yeast species, *Z. bailii* deserves the attribute fructophilic yeast. The fructophilic character of *Z. bailii* was revealed by Sousa-Dias et al. (169). Two different and independent transporters were described in this study. One of those was found to be specific for fructose transport while the other transported glucose

and fructose. Three mechanisms were identified in order to enable *Z. bailii* to favor fructose transport over glucose transport. The fructose specific transport system shows higher capacity and at high fructose concentrations the other transporter is inhibited significantly and therefore glucose uptake is impeded. In addition, it was shown that fructose can also be transported by the glucose transporter, generating competition with glucose and therefore also diminishing the glucose uptake in the presence of fructose (169). Recently, a new transporter was identified in *Z. bailii* encoded by the *FFZ1* gene. Ffz1p does not transport glucose, but fructose specifically at a V_{max} of 3.3 mmol h^{-1} g^{-1} and a K_m of 80.4 mM and can therefore be classified as low-affinity transporter (133).

In the fission yeast *Schizosaccharomyces pombe* (*S. pombe*), glucose uptake was shown to occur in a energy-dependent H^+-symport way (65). Other reports suggested however that glucose uptake occurs by facilitated diffusion (184). Investigation of the kinetics and affinities of the hexose transporters Ght1p to Ght6p revealed a preference of Ght6p for fructose compared to glucose. Ght6p was shown to be the only transporter in *S. pombe* exhibiting a preference for fructose (61).

Strains of *Candida stellata* (*C. Stellata*) were shown to utilize preferably fructose if inoculated to stuck wine fermentations. However, no information is available about the molecular characteristics of the fructose preference of this yeast (134, 173).

2.4.2 Hexose phosphorylation in *S. cerevisiae*

Phosphorylation of glucose and fructose is the enzymatic step following the transport of the sugars through the plasma membrane of the yeast cell (Figure 2.1). The phosphorylation of glucose to glucose-6-phosphate is followed by conversion to fructose-6-phosphate by phosphoglucoisomerase (*PGI1*). From this intermediate glycolysis is proceeding identically for glucose and fructose.

Three different kinases were shown to facilitate phosphorylation of glucose and fructose. Hexokinases 1 (A or PI, Hxk1p) and 2 (B or PII, Hxk2p) show phosphorylation of glucose and fructose, while Glucokinase 1 (Glk1p) is only phosphorylating glucose specifically (42). Kinetic parameters were reported to be the same for Hxk1p and Hxk2p for phosphorylation of fructose and were published with K_m values of 1,5 mM (7, 41) or 5 mM (19). Both hexokinases were found to exhibit a higher affinity for glucose with a slightly higher affinity of Hxk1p than Hxk2p. The

reported K_m values differ between 1 to 2 mM (19) for both kinases while other authors report K_m values of 0.12 mM for Hxk1p and 0.25 mM for Hxk2p (7, 41). Determination of the V_{max} values for both sugars revealed a 3-fold higher velocity of Hxk1p for fructose, while Hxk2p exhibits a slightly higher V_{max} for glucose (7, 41). If yeast is grown on high concentrations of glucose, fructose or mannose, Hxk2p is expressed, while expression of Hxk1p and Glk1p are repressed (19). Derepression of *HXK1* and *GLK1* occurs under cultivation in non-fermentable carbon sources. Under these conditions, *HXK2* is repressed (143). Hxk1p and Glk1p are found in the cytosol (2), while Hxk2p shows two locations, in the cytosol and in the nucleus (139).

Hxk2p exhibits specific properties in the yeast sugar metabolism. Present in the cytosol it can be considered to be the key enzyme for glycolysis. Present in the nucleus it contributes to the glucose-induced repression of several genes including *HXK1* and *GLK1*. Additionally, it was postulated that Hxk2p induces the expression of its own gene *HXK2* (139, 143). The role of Hxk2p in the regulation of the sugar metabolism in yeast is discussed more comprehensively later on in the following chapter. Recently, a contribution of Hxk2p in replicative cell aging was shown by Kaeberlein et al. (71). Deletion of *HXK2* in yeast increases longevity of the cells.

The investigation of the expression and activity of Hxk1p, Hxk2p and Glk1p under enological conditions is also of significant interest, since phosphorylation of glucose and fructose could be responsible for the difference in glucose and fructose utilization rates by *S. cerevisiae* (12, 52). Investigations by Berthels et al. (11) revealed a higher fructose/glucose phosphorylation ratio in cell extracts and a lower K_m for both sugars to correlate with a lower glucose preference of the yeast strain. Overexpression of *HXK1*, the hexokinases exhibiting a 3-fold higher V_{max} for fructose than for glucose, in a laboratory strain of *S. cerevisiae* caused a more significant acceleration of fructose consumption than of glucose consumption. The same overexpression in a commercial wine yeast strain reduced fructose consumption less than glucose consumption.

Schütz and Gafner (161) postulated a correlation between hexose uptake and hexokinase expression. Different wine yeast strains of the species *S. cerevisiae* and *S. bayanus* (as defined by physiological characterization available at the time of investigation) were characterized for their glucose and fructose uptake capacity, after growth to stationary phase in different conditions. The *S. cerevisiae* strains were

found to be generally less glucophilic than the strains considered being of the *S. bayanus* species. Evaluation of the *HXK1* by Southern blot revealed similar bands for three *S. bayanus* strains exhibiting the most glucophilic character. The two tested *S. cerevisiae* strains and the *S. bayanus* showing the least pronounced glucophility, exhibited double bands in the Southern blot analysis as a distinct difference to the more glucophilic strains (161). Both results support the hypothesis of hexose phosphorylation to contribute significantly to the hexose utilization character of yeasts.

A quantitative analysis of wine yeast gene expression profiles under enological conditions by Varela et al. (185), revealed a dominance of *HXK2* expression in the first phase of fermentation, when cells are still actively growing. The expression of *HXK1* and *GLK1* is increasing towards the end of fermentation, when conditions are characterized by lower sugar and higher ethanol concentrations and often nitrogen limitations. *HXK2* expression is significantly decreasing during this latter stage of fermentation. The shift of Hxk2p to Hxk1p towards the end of fermentation was also observed by Rossignol et al. (149). These authors discussed this shift in expression to be advantageous for fructose utilization, since Hxk1p has a higher affinity for fructose than Hxk2p.

The following description of the regulation and signaling mechanisms in hexose metabolism in *S. cerevisiae* highlights the involvement of the hexose transporters and hexose kinases in these crucial pathways.

2.4.3 Regulation and signaling of glucose metabolism in *S. cerevisiae*

The application of yeast genomics and proteomics allows the characterization of the yeast cell glucose response in a comprehensive way. A recent study, employing DNA microarray transcriptome analysis, revealed a fast change in expression pattern after addition of glucose. A higher than 3-fold change was recognized for approximately 20 % of the 6.200 genes in *S. cerevisiae* only 20 min after glucose addition. Another 40 % showed a change in expression rate of at least 2-fold (196). Generally, glucose repression largely affects genes involved in alternative carbon source metabolism, respiration, gluconeogenesis and the glyoxylate cycle. Glycolytic genes and genes encoding ribosomal proteins (RP) are subject to glucose activation (146, 153). Global

molecular analysis of *S. cerevisiae* recently revealed two surprises regarding the signaling and regulation of gene expression in response to glucose.

First, the glucose repression and induction mechanisms seem to exhibit a much more pronounced overlap than commonly accepted. The overlap in glucose induction and repression was revealed by the analysis of regulators found to be active rather “down-stream” in the signaling cascade and mostly function within the yeast nucleus (153). Rgt1p is known to act as repressor of *HXT1-4* in the absence of glucose. It was also found to exhibit activating properties for *HXT1* transcription in the presence of high glucose concentrations (56, 77, 110). Induction of *HXT1* was also shown to be facilitated by Hxk2p, a hexokinase. But this enzyme was also known for a long time to be involved in glucose repression (117). Snf1p, a serine-threonine kinase, was also recently found to exhibit derepressing as well as repressing activities of glucose regulated genes (178, 206). Finally, Grr1p was also shown to exhibit regulatory function as repressor or derepressor of transcription in response to the presence of glucose (153, 155, 182). Eight proteins influenced in their function by glucose signaling, were shown to interact with RNA polymerase II in both ways of regulation, positive and negative (24, 84). Over all, these results indicate the overlapping circuit to extend from the plasma membrane through to the induction or shutoff of transcription initiation by RNA polymerase II (153).

The second novelty was the finding that yeast cells can generate the full amplitude of the glucose adaptation, both induction and repression, in the complete absence of glucose. The underlying experiment revealing this novelty was based on the analysis of the yeast transcriptome, after induction through activated alleles of Ras2p and Gpa2p. Ras2p and Gpa2p are considered to be signal transmitters exhibiting activity in parallel in the early stages of the Ras/cyclic AMP (cAMP)/protein kinase A (PKA) pathway (196). 92 % of the genes exhibiting an at least 3-fold change in expression due to glucose addition were found to change at least 2-fold after activation of Ras2p. The activation of Gap2p caused the same pattern of regulation, although the intensity of the transcriptional response was lower than seen for activated Ras2p. Additionally, the authors were able to show the change in transcript levels due to Ras2p and Gap2p activation to be mediated by PKA (196). Due to this enlarged knowledge, it was suggested to place the G-protein signaling and therefore the Ras/cAMP/PKA pathway in the centre among the cellular components participating in the mechanisms of glucose response (153). So far it was commonly accepted that

the transport of glucose by the Hxt transporters and glucose phosphorylation by the three known hexokinases Hxk1, Hxk2 and Glk1 is potentially essential for glucose response (46, 145, 147). In the context of the novel results, transport and phosphorylation appear at most to participate in redundant signaling operating through or in parallel with the cAMP-PKA pathway (153).

Although research revealed many different pathways to be involved in the glucose signal transduction, the Ras/cAMP/PKA pathway and the pathway via Snf3p/Rgt2p/Yckp are considered to play the central roles (153).

2.4.3.1 The Ras/cAMP/PKA pathway

The Ras/cAMP/PKA pathway (Figure 2.2) is the major pathway involved in glucose-signaling, via posttranslational regulation by phosphorylation. Central to the activity of this pathway is the cAMP concentration in the cell, which depends on the carbon source provided for growth. Growth on rapidly fermentable sugars, especially glucose, causes an increase in cAMP concentration. This leads to an activation of PKA by binding of cAMP to its regulatory subunit Bcy1p, followed by the release and activation of the catalytic protein kinase subunits Tpk1p, Tpk2p and Tpk3p. PKA is a heterotetramer containing two of the protein kinase subunits and two molecules of the regulatory subunit Bcy1p. Finally, the activated Tpk subunits are conducting the phosphorylation of the PKA target proteins (146). The targets of activated PKA were found to be proteins involved in transcription, energy metabolism and cell cycle progression (51). The downstream gene expression pattern depends on the catalytic subunit of PKA via which signaling is facilitated. However, further research is needed for the identification of the distinct target proteins eventually leading to transmission of the regulatory signal to the yeast cell nucleus (153).

Adenylate cyclase (Cyr1p) is responsible for the synthesis of cAMP from ATP and its activity is controlled by activated G-proteins (GTPases) binding to Cyr1p. The two *RAS* genes (*RAS1* and *RAS2*) in *S. cerevisiae* encode for monomeric GTPases that are functioning as switches. GTP-binding activates the Ras proteins (guanosine triphosphate), while inactive G proteins show a bound GDP (guanosine diphosphate). Switching form the active to the inactive form requires GTPase activating proteins (GAPs) stimulating hydrolysis of GTP by intrinsic GTPase. Inactivation of the Ras proteins was found to be facilitated by Ira1p and Ira2p. To revert the inactive G-

proteins to their active form, guanine nucleotide exchange factors (GEFs) are required (153). Cdc25p and Sdc25p were identified as GEFs for the Ras1p and Ras2p. Although, Ras proteins are found in the cytosol, anchoring to the cytoplasmic face of the yeast plasma membrane by a palmitoyl moiety is required for correct function (69, 87). A second G-protein in *S. cerevisiae* is known to be able to activate adenylate cyclase (Cyr1p). It is a heterotrimeric G-protein consisting of α, β and γ subunit. The α subunit Gap2p was shown to interact with Gpb1p and Gpb2p, the putative β subunits. A corresponding γ subunit has not yet been identified. Gpa2p, together with its negatively acting regulator Rgs2p (GAP), is participating in glucose signaling in *S. cerevisiae*. A protein that would exhibit GEF activity of Gpa2p also remains to be identified (54, 79, 186). In a two-hybrid screen, interaction of Gpa2p with a G protein-coupled receptor (GPCR), encoded by *GPR1*, was detected. Gpr1p exhibits seven transmembrane domains and is therefore found in the plasma membrane of the yeast cell (205). Since Gpr1p is considered to be the only receptor coupled to Gpa2p and the resulting complex binds to Cyr1p, it was suggested to directly contribute to the activation of the cAMP pathway in response to glucose (79). These results were confirmed by studies of *gpr1*, *ras2* and *gpa2*, *ras2* double mutant strains. All of them showed severe growth defects indicating Gpr1p to facilitate glucose sensing via Gpa2p (205). The activation of PKA via the Gpr1p/Gpa2p protein complex is independent of Ras proteins but facilitated via increasing cAMP concentrations. The two arms of the Ras/cAMP/PKA pathway were shown to interact via the two putative β subunits of the heterotrimeric G-Protein, Gpb1p and Gpb2p. The β subunits bind to a C-terminal sequence of Ira1p and Ira2p, the known Ras-GAP proteins (53).

The activation of PKA in a cAMP independent way is not well studied. The pathway requires both a fermentable carbon source as well as complete growth medium, and was therefore called the “Fermentable carbon source growth medium pathway” (FGM pathway). The FGM pathway was shown to be mediated by Sch9 kinase. So far, only the Gpr1p/Gpa2p complex was shown to participate upstream of this pathway (175, 176).

The Ras/cAMP/PKA pathway was also found to be involved in regulation of morphogenetic changes, such as pseudohyphal differentiation, in diploids of *S. cerevisiae*. This phenomenon occurs under high glucose concentrations accompanied of limiting nitrogen supply. Haploids of *S. cerevisiae* show invasive

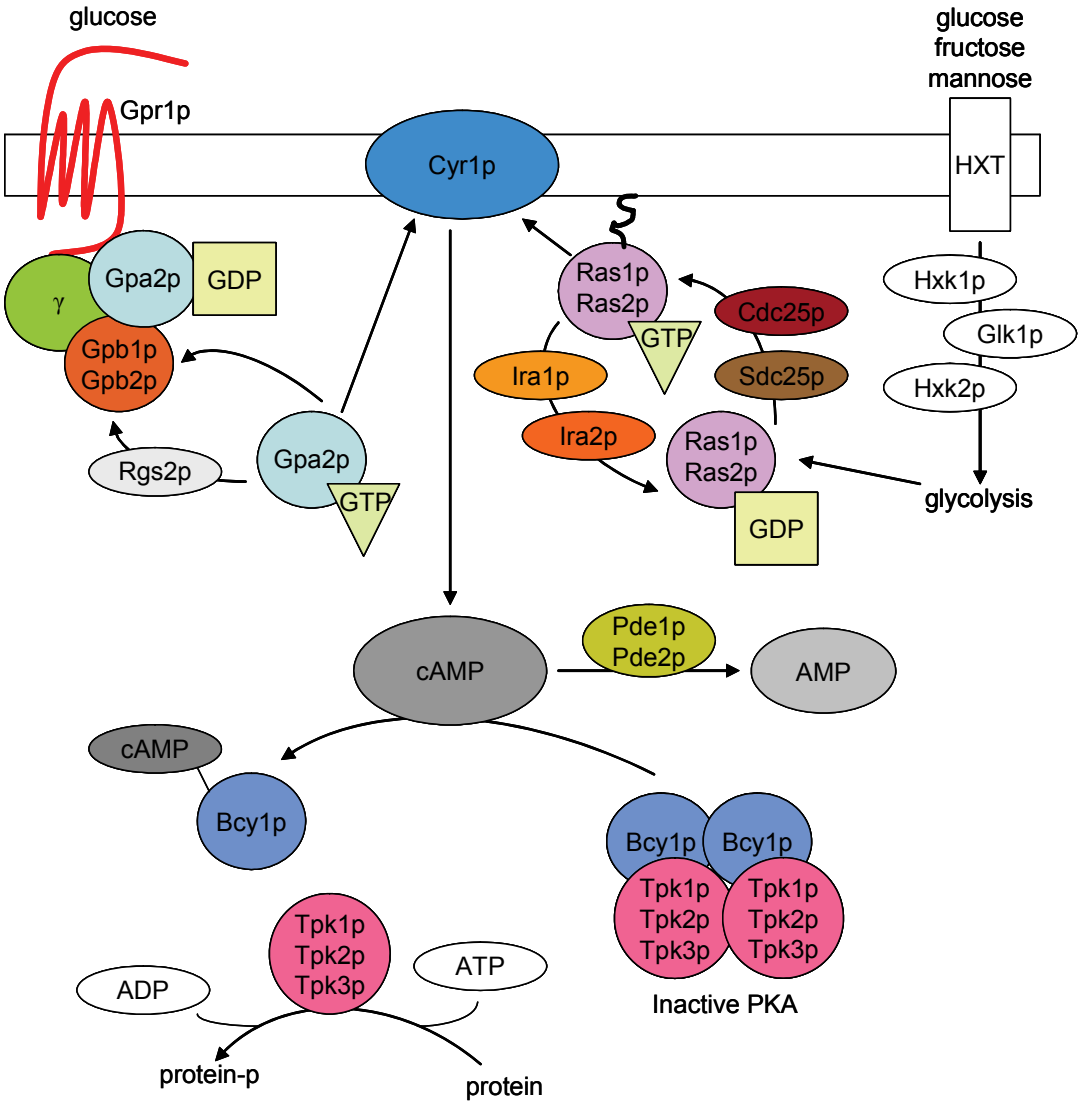


Figure 2.2: Modified illustration of the Ras/cAMP/PKA pathway according to Santangelo (153) and Rolland et al. (146). Upon addition of glucose to cells growing on non-fermentable carbon sources or to stationary phase cells, an increase in cAMP concentration is followed by activation of PKA, in order to facilitate phosphorylation of target proteins, exhibiting functional properties for transport of the glucose signal to the nucleus. Elevation of the cytoplasmic cAMP concentration is facilitated by Cyr1p, which can be activated by G-proteins. Gpa2p, α subunit of a heterotrimeric G-protein interacts with the seven-transmembrane protein Gpr1p. Gpr1p was shown to sense extracellular glucose and transmitting the glucose signal via Gpa2p to the cytoplasm. A second G-protein is monomeric and encoded by *RAS1* or *RAS2*. A solely intracellular glucose signaling, dependent on hexose phosphorylation, was proposed for the Ras proteins. Correct function of Ras proteins is dependent on membrane anchoring by a palmitoyl residue. The exact mechanisms of glucose signal transmission between the G-proteins and Cyr1p remains to be elucidated. Activation of PKA requires binding of cAMP to the Bcy1p regulatory subunits of PKA and thereby releasing the catalytic subunit comprised of the Tpk proteins. Three Tpk proteins are known in *S. cerevisiae* (Tpk1p, Tpk2p and Tpk3p) and the catalytic subunit of PKA contains two of them. Pde1p and Pde2p antagonize glucose signaling by conveying cAMP to AMP.

growth on agar plates under nutrient rich conditions. The involvement of the Ras/cAMP/PKA pathway in pseudohyphal and invasive growth was suggested due to the defects in both morphogenic changes if elements of the pathway were mutated (47).

2.4.3.2 Activation of Ras and Gpa2p in presence of glucose

Extracellular sensing of glucose in yeast could be facilitated by the Hxt transporters or the seven-transmembrane receptor Gpr1p. In the *hxt1-7* null mutant, glucose induced cAMP synthesis can be restored by constitutively expressed galactose permease (*GAL2*). This has led to the suggestion, that the Hxt transporters are not involved in signaling but only required for the transport of glucose (144). Clear evidence for glucose sensing by Gpr1p requires prove of interaction of Gpr1p with glucose. A recent study suggested this interaction due to results gained by mutagenic analysis. It is expected that interaction of Gpr1p and glucose activates Gpa2p. Therefore activation of Gpa2p is suggested to be facilitated by extracellular glucose sensing (92). Since activation of Ras2p in the presence of glucose is not diminished in the absence of Gpr1p or Gpa2p, its activation is considered to be independent of glucose signaling by Gpr1p. Paidrini et al. (119) recently found an involvement of Cdc25p, a Ras-GEF, in glucose induced cAMP-signaling, apparently mediated by Gpr1p/Gap2p. On the other hand, hydrolysis of Ras-GTP to the inactive Ras-GDP was found to probably be mediated by binding of Gpb1p and Gpb2p to Ira1p and Ira2p and therefore stabilizing the GPAs and their activity (53). Since activation of Ras2p is missing in a strain deficient in glucose phosphorylation (*hxk1 hxk2 glk1*) it is considered to be dependent on phosphorylated glucose rather than on extracellular glucose (28).

This implies a solely intracellular signaling mechanism responsible for Ras2p activation (153). Rose et al. (148) showed that the metabolic steps downstream of glucose phosphorylation are not essential for the activation of Ras2p. Considering the recent finding of a full glucose-like gene expression response by activated Ras2p and Gap2p only, it was suggested that glucose phosphorylation needs to generate the activating signal through G-proteins and/or through a yet unknown pathway (153). Colombo et al. (28) discussed an inhibition of Ira proteins, facilitating the

inactivation of Ras2p, by glucose phosphorylation as a possible mechanism for increasing Ras2-GTP concentrations.

2.4.3.3 The Snf3p/Rgt2p signaling pathway

Signaling via Snf3p/Rgt2p is the second important pathway in hexose metabolism in *S. cerevisiae*, besides the already described Ras/cAMP/PKA pathway. The central proteins in the Snf3p/Rgt2p signaling pathway are Mig1p and Rgt1p. In the absence of glucose, Rgt1p represses expression of the HXT genes, while Mig1p represses its target genes in the presence of high glucose concentrations (Figure 2.3, Figure 2.4). These two mechanisms allow a cross-regulation of the signaling pathway facilitated by Snf3p and Rgt2p (153).

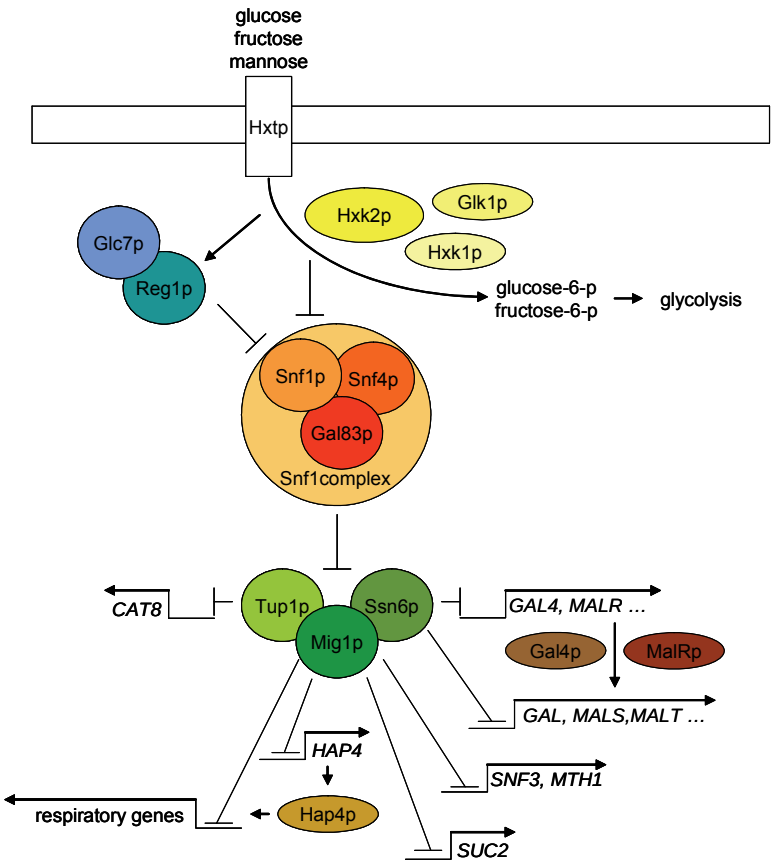


Figure 2.3: Glucose-repression pathway in a modified, simplified schematic illustration according to Rolland et al (146). In the presence of a fermentable carbon source, the Snf1 complex is inactivated through dephosphorylation mediated by Glc7p/Reg1p activity. Inhibition of phosphorylation of Mig1p by Snf1 complex is leading to glucose repression of several genes mediated by Mig1p/Ssn6p/Tup1p repressor complex.
Repression of *CAT8* inhibits the expression of the gluconegenic genes. Repression of transcription of *GAL4* and *MALR* genes inhibits expression of the proteins needed in order to metabolize galactose and maltose. Mig1p was also shown to repress transcription of *SNF3* and *MTH1* indicating its involvement in high-affinity hexose transport.

Genes repressed by Mig1p (Figure 2.3) are involved in high-affinity hexose transport or activities that are generally not required for growth on glucose (e.g. *SUC2* or *GAL1*). Mig1p is a DNA-binding protein and interacts with Ssn6p and Tup1p for repression of its target genes (179). Under high glucose conditions, Mig1p was shown to move to the nucleus and subsequently binds to the promoters of the glucose-repressible genes. Phosphorylation of Mig1p by the Snf1-protein kinase complex, in the absence or presence of low concentrations of glucose, initiates translocation of Mig1p to the cytoplasm and therefore derepression of the glucose-repressed genes (34, 113, 180). Recently, it was shown that the nuclear portion of Hxk2p builds a repressor complex with Mig1p in the presence of glucose. Additionally, Hxk2p was shown to be essential for nuclear translocation of Mig1p in addition to Snf1p (Figure 2.4). Snf1p appears to interact constitutively with Hxk2p and with Mig1p under low glucose concentrations. The interpretation of the results led to the suggestion that Hxk2p could be involved in the repression of Mig1p phosphorylation under high glucose conditions (1).

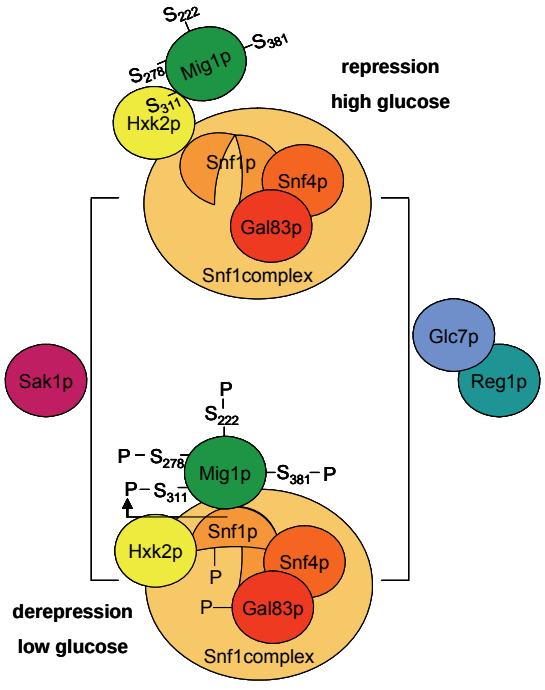


Figure 2.4: Model of the mechanism of Hxk2p regulation of Mig1p by phosphorylation of serine 311 by Snf1p complex. This model was proposed by Ahuatzi et al (1).
Hxk2p was found to be located in the nucleus in high glucose concentrations interacting Snf1 kinase subunit of the Snf1p complex as well as with Mig1p. Binding of Hxk2p to serine 311 of Mig1p causes dephosphorylation resulting in maintenance of repression of several glucose-repressed genes. Under low glucose conditions the interaction of Hxk2p with Mig1p is inhibited and serine residue 311 is phosphorylated by Snf1 kinase. Following phosphorylation Mig1p is exported out of the nucleus and thereby derepression of glucose-repressed genes is mediated. P, phosphate groups

The Snf1-protein kinase complex was shown to be in an autoinhibited state in the presence of glucose. This is brought about by a dephosphorylation of the active

Snf1-protein complex by the Reg1p/Glc7p complex, the protein phosphatase PP1. The active Snf1-protein complex is comprised of the α subunit Snf1p consisting of a phosphorylated catalytic domain and a regulatory domain, a scaffolding β subunit encoded under glucose regulation conditions by *GAL83* and a γ subunit, Snf4p (55). Dephosphorylation of the catalytic domain by PP1 in the presence of glucose causes cleavage of Snf4p of the active Snf1p-protein complex, binding of the regulatory domain to the catalytic domain of Snf1p and therefore conveys it to the inactive state accompanied by translocation to the cytoplasm. Once glucose is depleted, phosphorylation of the Snf1p catalytic domain by Sak1p occurs and the activated complex enters the nucleus. In this state Snf4p binds, accompanied by a release of the catalytic domain enabling the phosphorylation of the protein targets mediated by Std1p. In addition, Reg1p is phosphorylated by the Snf1p-protein complex and thereby stabilizes the interaction between PP1 and Snf1p complex. This step enables a rapid response in the case of sudden availability of glucose, which will be followed by the described cycle. Nuclear localized Hxk2p was shown to interact with Reg1p. Due to reduced or even missing phosphorylation of Hxk2p by PKA in response to increasing glucose concentrations, Hxk2p dimerizes and mediates Glc7p for dephosphorylation of the Snf1p complex and therefore inactivation. This is consequently causing Mig1p mediated repression of glucose-repressed genes (57, 139, 153, 154, 188, 191).

Derepression of the Rgt1p mediated repression of the *HXT* genes depends on signal transduction via Snf3p and Rgt2p (Figure 2.5). Snf3p mediates signal transduction in conditions of low glucose, while Rgt2p is required as well for signaling under high glucose concentrations and especially for the full expression level of *HXT1* (17, 104, 117). The cytoplasmic C-terminal residues of Snf3p and Rgt2p were found to exhibit central significance for glucose signal transduction. Originally these domains were thought to play an important role for reception of the glucose signal. Recent results although suggest the dynamic interaction of the residues with Mth1p and Std1p as their key function (90, 109, 160). Mth1p and Std1p are basically anchored to the membrane by the cytoplasmic C-terminal tails of Snf3p and Rgt2p. This interaction is considered to mediate phosphorylation of Mth1p and Std1p by the kinases Yck1p and Yck2p (109). These kinases are tethered to the plasma membrane at their C-terminal sequence, thereby facilitating an interaction with Snf3p and Rgt2p eventually leading to activation after conformational modification of Snf3p and Rgt2p due to

interaction with glucose (70, 109). The target of the kinase activity of Yck1p and Yck2p are Mth1p and Std1p the corepressors interacting with Rgt1p (109). Phosphorylated Mth1p and Std1p are ubiquitinated and degraded by the SCF^{Grr1} complex and the S26 proteasome. Degradation of Mth1p and Std1p eventually facilitates a conformational change of Rgt1p, followed by its inability to bind to the consensus sequences of the *HXT* genes and therefore derepression of their transcription (44, 95, 170).

The two glucose signaling pathways via Mig1p/Snf1p and via Snf3p/Rgt2p/Rgt1p are interconnected. In the absence of glucose the Snf1 complex is active and therefore phosphorylating Mig1p causing translocation of it to the cytoplasm. Since *SNF3* and *MTH1* are target genes of glucose repression mediated by Mig1p, those genes are expressed if glucose is absent (44, 72, 160). This ensures availability of Mth1p, binding to Rgt1p and therefore facilitating its repressor function by binding to the promoter sequences of the *HXT* genes. In contrast, in the presence of glucose Mig1p is present in the nucleus repressing the expression of *MTH1* and *SNF3*. Repression of *MTH1* transcription in the presence of glucose reinforces the inhibitory effect of glucose on Mth1p in addition to its degradation mediated by the SCF^{Grr1} complex and therefore ensures full activity of Rgt1p derepression (72).

The repression of *SNF3* transcription by Mig1p in high glucose concentrations supports the common opinion that Snf3p acts as a high-affinity glucose sensor (117).

Std1p, known as corepressors of Rgt1p, was shown to stimulate Snf1p activity by interaction with its catalytic domain and therefore antagonizing autoinhibition by PP1 (85). Since active Snf1p facilitates derepression of *MTH1* by translocation of Mig1p to the cytoplasm, the interaction of Std1p supports the expression of Mth1p, the second corepressors of Rgt1p. In a further study, Std1p degradation was also shown to be inhibited in the presence of Snf1p (126). How these two mechanisms can be connected remains to be elucidated. *STD1* expression itself was shown to be feedback regulated by Rgt1p. In the presence of glucose, degradation of Std1p is stimulated. On the other hand *STD1* expression is stimulated by the Snf3p/Rgt2p/Rgt1p pathway in the presence of glucose (76). The feedback regulation was discussed to have a dampening effect to the protein degradation due to glucose induction and additionally facilitating a fast response to glucose depletion (72).

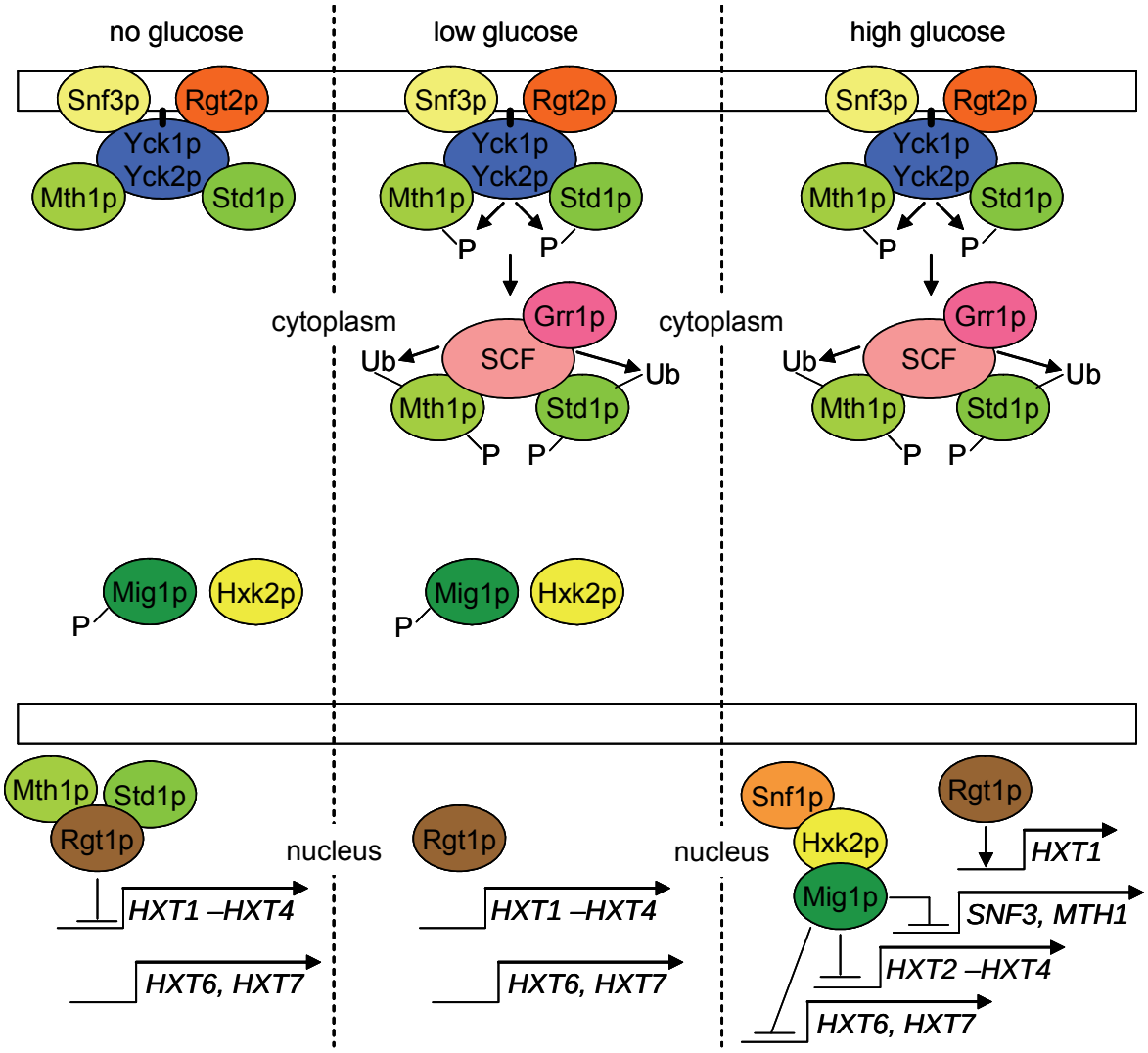


Figure 2.5: Schematic illustration of the derepression of HXT expression in the presence of glucose according to Santangelo et al. (153) and Rolland et al. (146). Modifications were included according to the latest results by Ahuatzi et al. (1). In the absence of glucose the transcription of *HXT1* - *HXT4* is repressed by Rgt1p and its corepressors Mth1p and Std1p. Under low glucose concentrations, Snf3p and Rgt2p mediate glucose signaling stimulating phosphorylation of Mth1p and Std1p by Yck1p and Yck2p, two casein kinases. Phosphorylation of Mth1p and Std1p is followed by their degradation via SCF^{Grr} complex mediated ubiquitination and the 26S proteasome. Rgt1p is therefore released from its binding sites and results in expression of *HXT1 – HXT4*. Under high glucose conditions, Mig1p was shown to be present in the nucleus. Dephosphorylation of serine 311 of Mig1p by Hxk2p allows the repression of its target genes including *SNF3* and *MTH1*. The Snf1 complex was shown to be inactive in the presence of glucose and therefore inactivation of Mig1p is prevented. Removal of Mth1p and Std1p, accompanied by interaction of Grr1p with Rgt1p was found to mediate conversion of Rgt1p to a transcriptional activator of *HXT1*. This process is probably further supported by an activator that awaits elucidation.

Mig2p was found to mediate glucose repression of the same genes targeted by Mig1p. The proteins exhibit redundant function but their induction is regulated

differently. While Mig1p is inactivated by phosphorylation through Snf1p, Mig2p is regulated by the Snf3p/Rgt2p/Rgt1p pathway (99). These two pathways of repression and their interaction may serve to integrate the response of the yeast cell with various other glucose signaling events (72). Similarly, the activity of Mig3p was also detected but shown to be of less importance (72).

An additional network connection of the glucose signal transduction via Snf3p/Rgt2p/Rgt1p and Snf1p/Mig1p was proposed by Palomino et al. (120). They were able to show *HXK2* expression to be regulated by Rgt1p. Repression of *HXK2* expression was found to be facilitated by Rgt1p and Med8p in a Snf1p dependent manner in the absence or under low concentrations of glucose. Contrarily, in high glucose conditions, Rgt1p is phosphorylated in a Tpk3p dependent manner, a catalytic subunit of the active PKA. Phosphorylation of Rgt1p leads to release of DNA and therefore expression of the Rgt1p target genes including *HXK2* (121). These results allow the establishment of a further mechanism of interaction between the two pathways of glucose signaling via glucose repression. Additionally, a connection to the Ras/cAMP/PKA pathway is given by the involvement of Tpk3p (153).

2.5 Engineering of microorganisms for the improvement of industrial processes

After recognizing the role of microorganisms as biological catalyzers for the conversion of biological substrates to industrially interesting products of high value, research has been directed towards the improvement of these strains in order to make conversion processes more efficient and economical. Prokaryotes as well as eukaryotes are sources for the development of successful biocatalysts. Before the establishment of molecular biology, improvement of strains could only be conducted through classical breeding and screening methods. In the case of yeast, such methods were already described by Lindegren and Lindegren (97) in 1943. Already then it was apparent that naturally occurring microorganisms that exhibit interesting biocatalytic properties would generally benefit from experimental selection and manipulation in order to improve commercial processes and applications (25).

Today, many efforts are directed towards the improvement of production rates in terms of yields on substrate, rate of reaction, tolerance towards inhibitors and products and the adaptability of the microorganism to process conditions that may

differ significantly from natural habitats. Improved microorganisms are used in processes of the chemical, environmental, pharmaceutical, health care, food and enological industry (127). The efficient production of biofuel can be considered one of the most recognized fields of development in this regard. Looking at the number of industries employing biocatalysts in their processes, one can estimate the large number of strains that have been developed. In the following sections, a closer look will be taken at achievements that are related to strains of the species *S. cerevisiae* that were improved for their application in the food and beverage industry.

S. cerevisiae arguably enabled the world's first biotechnological processes: brewing beer, wine fermentation, producing sparkling wine and leavening bread dough (136). After the advent of molecular genetics, *S. cerevisiae* has been intensively used for the application of genetic engineering approaches in order to improve its properties for industrial use. This development was supported by the tremendous progress in research on the genetics in *S. cerevisiae*. Strains of this yeast species were established as model systems for genetic studies, followed by the establishment of laboratory strains allowing comprehensive investigation of its genetic background. The laboratory strain S288c was the first eukaryotic genome to be completely sequenced in 1996 (49).

In the food industry, the first genetically modified organism (GMO) that was cleared for utilization in food production, was a transgenic strain of *S. cerevisiae* (194): A transgenic *S. cerevisiae* strain, containing constitutively expressed maltose permease and maltase genes, allowing a much faster CO_2 production and leavening of the dough, was approved in the baking industry (168). The same author also described the development of a brewer's yeast transformed with a glycoamylase encoding gene allowing partial hydrolysis of maltodextrins and therefore yielding a beer of low carbohydrate content (168). Volschenk et al. (192, 193) generated a transgenic wine yeast strain, which has the ability to not only conduct alcoholic but also malolactic fermentation. This yeast (ML01) was approved by the Food and Drug Administration (FDA) with the status of a substance generally recognized as safe (GRAS; http://www.cfsan.fda.gov/~rdb/opa-g120.html) in 2003 and therefore cleared for use in the American wine industry. The same yeast was approved by the Canadian regulatory bodies, Health Canada and Environment Canada, for use in this country.

Many other properties of wine yeast strains were targeted for the development of improved production strains. Pretorius (136) reviewed comprehensively the properties of wine yeast strains, potentially important for improved wine production and already subject to scientific investigation: quality control and strain handling, processing efficiency, wine sensory qualities, health aspects of wine and control of wine spoilage microorganisms.

However, despite the successful generation of yeast strains by genetic engineering, the public acceptance of their application in the production processes has not followed. This can especially be seen by the controversial discussions in various media platforms related to the commercial application of the genetically modified wine yeast strain ML01. Due to such consumer concerns, classical methods that yield non-GM yeast strains are still of high importance for the development of new yeast strains, including for the wine industry.

2.5.1 Evolutionary engineering of complex phenotypes

Evolutionary engineering is an approach for the generation of complex improved phenotypes in microorganisms. The term evolutionary engineering was coined only recently in the 1990s to describe a process that is employed for the selection of improved phenotypes by introduction of genetic variation and selection in defined, selective culture conditions (23, 132). This methodology has the advantage that no a priori knowledge of the molecular and regulatory nature of a given phenotype is required. This is of particular relevance when considering the complexity of interactions of metabolic and regulatory pathways that are responsible for the expression of most industrially relevant phenotypes, such as the regulation underlying sugar utilization described in this review. For this reason, evolutionary engineering is frequently the preferred strategy in comparison to metabolic engineering.

Metabolic engineering relies on the knowledge of molecular and functional properties of the target enzymes in the complete context of a metabolic active cell in order to generate the desired phenotype (127). The knowledge required to successfully apply metabolic engineering is often not available. Although there is substantial progress in using holistic approaches based on genomics, proteomics and systems biology approaches, there is still limited knowledge about regulatory and biochemical

processes, localization of enzymatic reactions, fluxes and transport of precursor, intermediates and products. The missing information impairs the rational application of recombinant DNA techniques for the improvement of cellular functions (132).

Evolutionary engineering is based on variation and selection and therefore is using natural processes (157). It is regarded as a process that applies a combination of different evolutionary techniques to microbial properties in a biotechnological context (23, 157). Among the methods established for evolutionary engineering, traditional ones, leading to a non-GMO strain, are used as well as methods based on genetic engineering, dependent on the process to be improved (25, 127, 132, 157, 165). Combining metabolic engineering with evolutionary engineering was also shown to be a potentially successful strategy yielding desired phenotypes of industrially interesting strains. Both routes, starting with metabolic engineering followed by improving the generated strain further by evolutionary engineering or vice versa, were reported to lead to improved strains with the desired traits (88, 89, 157). It is commonly accepted that the strategy chosen for the generation of genetic variability and the type of selection or screening methods are decisive for the success of whole-cell evolutionary engineering (132, 157).

2.5.2 Methods in evolutionary engineering for the generation of genetic variability

Since methods generating microorganisms referred to as GMO are not applicable to the project of this study, the content of this chapter is limited to the description of classical approaches for the generation of genetic variability in microorganisms. These methods are less directed than genetic engineering approaches, but this property also exhibits advantages, by introducing random genetic modifications that may reveal unknown genetic traits. In addition, classical approaches are the strategy of choice if the genetic targets to be modified are unknown for the desired phenotype (25, 125, 127, 137, 157). Recent projects aiming for the improvement of *S. cerevisiae* yeast strains for the bread and wine industry employed non-recombinant techniques successfully (64, 106, 108).

2.5.2.1 Mutagenesis

Spontaneously occurring mutations are the source of genetic variation on which evolutionary processes depend. The large majority of mutations are either harmful or have no apparent effect on the cellular functions. Advantageous mutations are a rather rare event.

Spontaneous mutations are found to occur at a low rate of $10^{-5} – 10^{-10}$ changes per replicated nucleotide per generation. But it has been suggested that their frequency is increased considerably under exposure to adverse environmental conditions such as stationary phase or metabolic stress. The ability of a microorganism to mutate is an important property since it ensures the generation of variability in the gene pool and therefore increases the ability to adapt to new environmental conditions. Like any other property of a microorganism, mutability is genetically determined and can therefore be affected by environmental influences and genetic manipulations.

Mutations refer to various changes in the DNA. They may affect only a single nucleotide as a point mutation by substitution or deletion, or refer to rearrangements of one or more nucleotide base pairs, translocation of chromosome parts and others. Evolutionary engineering does not only rely on naturally occurring mutagenesis. Mutations are also induced by exposure of strains to chemical or physical sources known to exhibit mutagenic properties (125, 157).

2.5.2.1.1 Spontaneous mutations

Three categories of spontaneous mutations in proliferating cells were defined: small local changes, DNA rearrangements and horizontal DNA transfer (157). In addition, adaptive mutation is considered to be the process of microorganisms to acquire mutations in the non-proliferating or very slow proliferating status under prolonged exposure to non-lethal stress conditions. These mutations were described to be non-random, therefore specific for the selective conditions and lead to a growth advantage of the exposed cells. It is generally accepted that also in the non-proliferating status some sort of DNA processing is required in order to introduce the observed mutations to the genome. These mechanisms were shown to be in part the same as those observed to be responsible for the introduction of mutations in proliferating cells (25, 58, 166).

Certain environmental conditions cause an induction of enzyme systems and in particular DNA polymerases. In contrast to the replicative DNA polymerases, known to precisely copy the DNA sequences, the induced DNA polymerases are introducing replication errors at a high rate. By introducing the copy mistakes, the genetic variety and therefore the potential for adaptation of the exposed cell population is increased, eventually allowing the survival of cells which acquired the mutations exhibiting the highest advantage in order to survive the specific environmental conditions (58, 157). In the case of adaptive mutations, antagonistic mechanisms were defined that inhibit the appearance of adaptive mutations as long as all components of the involved pathways are fully and correctly functional. It was however reported that under certain conditions DNA polymerases δ and ε and members of the mismatch repair (MMR) pathway could be malfunctional and therefore contribute to the fixation of adaptive mutations (58).

The heterogeneous Rad6 epistasis group functions in postreplicative repair and was shown to be error-prone since it is based on bypassing DNA lesions rather than correcting them. Nucleotide excision repair and homology dependent double-strand repair were reported to have the potential to directly contribute to the incorporation of adaptive mutations. *RAS2* is considered to contribute only in an indirect way by influencing other mechanisms involved (58). Heidenreich (58) described two mechanisms found to directly produce mutations. The mechanism of non-homologous end joining (NHEJ) is one of the major repair mechanisms for double strand breakages and is considered to be responsible for the introduction of frame shifts. The property of inducing mutations directly was also found for the translesion synthesis enzymes (TLS) facilitated by error-prone bypasses of DNA lesions.

2.5.2.1.2 Chemical or physical induced mutations

Due to the technical simplicity and applicability to almost any organism, chemical or radiation mutagenesis is frequently used for the generation of genetic variability of a microorganism. Commonly used mutagens are listed in Table 2.2 according to Parekh et al. (125) and Sauer (157). The damages to the DNA, caused by these agents range from deletion, addition, transversion and substitution of bases or breakage of DNA strands (125). Ultraviolet radiation (UV), ethylmethane sulfonate (EMS) and nitroso-methyl guanidine (NTG) are the agents used most commonly for

Table 2.2: Overview of commonly used mutagenic agents.

Mutagen	Mutation induced	Impact on DNA	Effect
Radiation			
X rays, gamma rays	Single or double-strand breakage of DNA	Deletions, structural changes	High
Ultraviolet rays	Diamerization of pyrimidines; DNA cross links	Transversion, deletion, frame shift, GC→AT transitions	Medium
Chemicals			
Base analogs			
5-chlorouracil	Results in faulty pairing	GC→AT, AT→GC transitions	Low
3-bromouracil	Results in faulty pairing	GC→AT, AT→GC transitions	Low
2-aminopurine deaminating agents	Errors in DNA replication		Low
Hydroxylamine (NH_2OH)	Deamination of Cytosine	AT→GC transitions	Low
Nitrous acid (HNO_2)	Deamination of A, C and G	Bi-directional translation, deletion, GC→AT, AT→GC transitions	Medium
Alkylating agents			
N-methyl-N'-nitro	Methylation, high pH	GC→AT transitions	High
N-nitrosoguanidine	Alkylation of C and A	GC→AT transitions	High
Ethyl methanesulfonate	Alkylation of bases C and A	GC→AT transitions, deletions	High
Intercalating agents			
Ethidium bromide, acrinidinedyes	Intercalation between two base pairs	Frame shift, loss of plasmids and microdeletions	Low
Biological			
Phage, plasmid, DNA transposing	Base substitution, breakage	Deletion, duplication, insertion	High

Mutagens commonly used for development of new strains for evolutionary engineering according to Parekh et al (125) and Sauer (157).

many applications due to their property of inducing a great variety of molecular alterations, without specificity for genomic subregions (150, 164).

The critical step in the induction of mutations by chemical or physical agents is the determination of the dose and time of incubation to be applied. The optimum dose will yield a culture population comprised of the highest number of desirable mutants. Subopitmal doses of a mutagen will lead to a culture population of lower genetic diversity, while a too high dose will kill the culture population. Another drawback of a slightly too high dose is the creation of strains carrying too many mutations, many of which may be unfavorable and will be masking potentially advantageous mutations (73, 157). The estimation of an optimal dose of a chosen mutagenic agent is sometimes complicated due to the complexity or the difficulty to detect phenotypes. Therefore, commonly used protocols advise to rely on the evaluation of the lethality of different combinations of dose and incubation time (157).

Experimental exposure of starved cell populations to UV or IR showed both a significant increase in adaptive mutations. Exposure to UV is commonly known to cause pyrimidines dimers and therefore for blocking replication in proliferating cells. The mechanism how UV induced mutations arise in non-proliferating cells is not clearly understood to date (59, 60, 171). Ionizing radiation is known to induce strand breaks in DNA, including double-strand breaks. Heidenreich and Eisler (59) revealed the requirement of non-homologous end joining (NHEJ), not only for the processing of spontaneously occurring DNA strand breaks, but also for the mutagenic processing of artificially imposed DNA strand breaks.

Naturally evolving mutations and induced mutations by chemical and physical agents of proliferating culture populations, as well as adaptive mutations of non-proliferating or slowly proliferating culture populations, can be considered to be most suitable for the introduction of genetic variability into a wild-type yeast culture population.

2.5.2.1.3 Other strategies for mutagenesis

Further methodologies for mutagenesis are available. So-called mutator strains can be used for the generation of strain diversity. These strains have the advantage of exhibiting a much higher frequency of spontaneous mutations. While useful for experimental purposes, those strains are however usually unsuitable for most

specific applications, in particular in industrial environments. If a known mutator strain is not suitable for the specific application, suitable mutator strains could be generated by genetic manipulation, but would therefore be considered as GMOs. In addition, mutator strains obviously exhibit inherent phenotypic instability not desired for industrial applications (157).

Tagged mutagenesis is an approach that enables the transfer of an evolved phenotype by metabolic engineering to other strains or organisms. This process also involves genetic manipulation and therefore the improved strain has to be considered a GMO (98, 157).

2.5.2.2 In vivo recombination

In vivo recombination is an important strategy for the generation of genetic variety. If classical, non-directed approaches such as hybridization or inbreeding are involved, the culture population that is generated is non-GM. More directed approaches involve specific genetic manipulation that can be facilitated by various methods. These approaches are considered to yield GMO strains and are therefore of no importance to this work. The main focus of the following description will therefore be on classical strategies applicable to this project.

2.5.2.2.1 Hybridization

Eukaryotic microorganisms, such as *S. cerevisiae*, offer a high potential for breeding independently improved variants. The strains originating from the hybridization of two parental strains may combine advantageous traits of the parental strains (157). Table 2.3 lists the possible strategies for in vivo hybridization which were employed in various projects for the improvement of yeast strains and in particular for wine yeast strains (137).

Breeding strategies for industrial yeast strains have a long tradition. Already in 1943 Lindegren and Lindegren (97) published successful breeding strategies for industrial yeast strains used for the baking industry. The authors outlined three steps required to yield an improved yeast strain exhibiting the desired properties. The selection of strains with various useful characteristics was highlighted as the first essential step.

Inbreeding of the selected strains was described as enhancing the advantageous properties even further. In a third step the different desirable qualities of the yeast strains were combined by hybridization. Due to the asexual propagation of the newly generated hybrids, they were considered to be highly stable in their genetic background and phenotypic characteristic (97).

Table 2.3: Strategies for in vivo hybridization

Description of method	Application in wine industry
Sexual reproduction:	
Intra-species hybridization	
sporulating diploids, recovering individual haploid ascospores, mating of haploids of opposite mating type, production of a new heterozygous diploid	hybridization is the most effective method for improving and combining traits, particularly if the molecular basis of the mechanism was not revealed; inclusion or elimination of a specific property can be facilitated by hybridization; several hybrid wine yeast strains already commercially used
Asexual reproduction:	
Spore-cell mating	
homothallic yeast strains require a direct spore-cell mating procedure	heterothallic haploid strains are crossed with homothallic ascospores of wine yeast strains
Rare mating	
strains not expressing a specific mating type can be forced for mating	combination of properties exhibited by two non-mating diploid or polyploidy strains to a hybrid
Cytoduction	
cytoplasmic genetic elements are introduced without transfer of nuclear genes	killer/zymocin dsRNA or mitochondrial DNA located in the cytoplasm or plasmids are introduced
Spheroplast fusion	
asexual direct technique; production of hybrids or used in cytoduction	fusion of cells with different level of ploidy; diploid and haploid wine yeast strains can be fused to generate a triploid strain

Commonly used hybridization methods used in the wine industry for the generation of new commercially applied yeast strains according to Pretorius and Bauer (137). These methods were widely used due to their trait of yielding yeast strains not referred as GMO.

Sexual reproduction by hybridization of haploid ascospores was shown to be not only applicable to intraspecific crossings, but also to a limited number of interspecific matings of closely related species. Evaluation of naturally occurring strains of the *Saccharomyces* sensu stricto complex revealed the existence of chimeric genomes and suggests introgression to take place within this complex (32). Hybrids of *S. cerevisiae* and *S. bayanus* were shown to have been generated successfully for different applications in the beverage industry (78, 156, 163). In a recent study conducted by Marullo et al. (106) the potential of hybridization in a breeding program was evaluated by following up 11 relevant enological traits. The results of this study showed that successive hybridization is a powerful methodology in order to obtain wine yeast strains exhibiting a range of improved characteristics.

The question of whether haploid or diploid strains should be used for evolutionary experiments is controversially discussed. Paquin and Adams (124) found the fixation of mutations to be increased by 1.6-fold in diploid strains of *S. cerevisiae*, compared to haploid strains of the same species. It appears as if diploidy is advantageous in asexually propagated populations, when the number of favorable mutations per generation is very small, as it is highly likely to be the case in evolutionary engineering (157).

In summary, classical breeding by hybridization of desired phenotypes enables us to generate new improved yeast strains for the application in various industrial fields.

2.5.2.2.2 Whole genome shuffling

Whole genome shuffling is one of the newest contributions to the field of non-recombinant methods for industrial strain improvements and was first described by Patnaik et al. (128, 207) in 2002. This procedure relies on a novel protoplast fusion process, optimized for iterative multiparental fusion followed by a screening process, in this case facilitated by high-throughput-screening. New and improved industrial strains of *Streptomyces fradiae* and *Lactobacillus* were generated by whole genome shuffling within a remarkably short time (128, 207).

The technique of protoplast fusion itself was already applied in the late 1970s for the modification of phenotypic properties of prokaryotic and eukaryotic cells (66, 159). By fusing complete protoplast genomes, a high frequency of gene transfer and

recombination can be achieved. It is possible to then target phenotypes at the genetic level for which limited information is available (25, 132). Whole genome shuffling can considerably contribute to the engineering of polygenic phenotypes (127).

So far, whole genome shuffling was applied to mixed populations of one bacterial species (30, 128, 207). The application to the shuffling of genomes originating from different species as well as the application of the method to yeast species still has to be evaluated. Nevertheless, the fact that whole genome shuffling is regarded as a natural genome-altering mechanism, organisms yielded by this methodology are defined as non-GMO. This broadens the range of applications to many industrial fields including the food and beverage industry (25, 127, 132).

The application of this methodology for the improvement of wine yeast strains was discussed by Giudici et al. (48) in a recent review about this topic. It can therefore be expected that studies employing this method in the field of wine yeast strain improvement will be published soon.

2.5.2.2.3 Mapping of quantitative trait loci and introgression

An additional recent development in the industrial yeast strain generation is the application of the strategy of mapping quantitative trait loci (QTL-mapping). Individual diploid strains of *S. cerevisiae* are frequently highly heterozygous and therefore exhibit a huge genetic and genomic variability, showing the genetic potential for metabolic diversity and specialization of specific strains for various industrial purposes (14, 38, 200). Most industrial yeast traits are distributed within a yeast population in a complex and continuous way. The quantitative distribution of the traits is determined by their polygenic nature as well as by the various allelic forms existing within a yeast population. These loci, usually unidentified, are named quantitative trait loci (QTL) (101).

The approach of QTL-mapping has two main advantages. First, there is no necessity for a priory knowledge about gene function and sequence variation. Second, it is often possible to detect multiple genes determining the value of a single quantitative trait (105).

Winzeler et al. implemented this technique in 1998 with *S. cerevisiae* by using the powerful genomic tools of DNA-micro arrays (201). Hybridization of fragmented and labeled genomic DNA, of yeast strains with diverging phenotypes for a specific trait, to oligonucleotide arrays enabled the identification of the underlying genetic differences. By this technique and thanks to the compactness and comprehensive knowledge of the yeast genome, genes and even single nucleotide polymorphisms (SNP) were identified which control quantitative traits (22, 36, 105, 167, 172). The identified, suitable alleles may then be introduced into a yeast strain of interest by DNA technology. Thereby the desired trait is improved, while all other qualities of the strain are preserved (129).

If DNA technology can not be applied, an alternative approach for the introduction of a desired allele would be introgression. This technique is based on backcrossing of suitable alleles in a recipient variety, called “elite”, which is considered to be one of the best on the market. Specific markers are used for the detection of the successful integration of the desired alleles. This strategy is already routinely used in agronomy (67).

The approach of QTL-mapping in combination with molecular marker assisted introgression was recently applied for the improvement of wine yeast strains by Marullo et al. (105, 107). The authors were able to define significant loci for the production of acetic acid, hydrogen sulphide and phenolic off-flavor as well as for the kinetic trait determining the duration of the lag phase. In their study, five successive backcrosses between an “elite” strain and appropriate segregants were sufficient for the improvement of three different trait values. However, a weakness of these results was that the identified traits in fact behaved as monogenic, and not polygenic traits, indicating that the techniques of combining various multigenic traits in a single strains requires additional significant efforts. The results nevertheless illustrate the power and the potential of this strategy for a more directed improvement of wine yeast strains.

2.5.3 Methods in evolutionary engineering for the selection of improved microorganisms

The selection process, as the second step in the evolutionary engineering approach, facilitates the enrichment and identification of strains adapted best to the selection conditions.

The screening of individual strains for their phenotypic characterization is the most direct way to select for a specific application, and is to some degree a part of every selection program, whatever approach has been chosen. When screening individual strains directly, any cellular function for which an appropriate assay exists can be targeted. (97). Due to the latest developments in the field of high-throughput screening and the employment of multi-well plates, screening of single strains for certain purposes has in addition become much more efficient and less time demanding. If direct screening of individual strains is chosen as the selection strategy, evolutionary progression can be achieved in a step-wise manner. Disadvantages include that direct screening can usually not address more complex phenotypes, or traits that depend on complex environmental conditions to which the strains may have to be adapted. It is also difficult to predict whether strains selected under high-throughput conditions will also exhibit the desired phenotype under production conditions.

In contrast, continuous evolution can be conducted under process relevant conditions and therefore offers the possibility to meet the most important process parameters throughout the evolution of the adapted and improved microorganism. Continuous evolution is based on the competition among the various strains present in the treated culture population and it therefore selects for the competitive fitness of strains, under selective conditions. The appearance of advantageous mutations is not controllable by the experimenter and therefore also not predictable. However, it has to be considered that the competitive fitness might not be identical to a truly improved desired property at the single cell level (39, 132, 157).

The selection process can be accomplished through various cultivation modes, including cultivation on solid media, in batch cultivation and in continuous cultivation. Independent of the cultivation method, natural evolution will occur due to the

appearance of spontaneous mutations and therefore strains of higher fitness that will be able to take over an increasing portion of the culture population (39, 40).

2.5.3.1 Selection on solid media

Selection on solid media is useful for experiments expected to provoke large differences in fitness occurring with low frequency by evolutionary adaptation. It also displays a very direct detection of the adaptation processes (195). However, this method is likely to be inefficient for the selection of complex phenotypes, requiring multiple mutations. Additionally, most industrially used microorganisms will be employed in some sort of fermentation process in a bioreactor. Therefore one of the most important aspects of the selection process, to be as close as possible to the conditions of the process the improved strain is supposed to be used in, is not met. This suggests a rather low rate of success for many selection strategies on solid media (157).

Therefore, selection procedures conducted in liquid media are commonly considered to exhibit a higher success rate for the selection of complex phenotypes.

2.5.3.2 Selection in batch cultivation

Liquid media selection procedures, in general, should yield fitter variants for any particular condition over time, due to adaptation by selection. In order to study these adaptation processes, batch culture is often employed. Cultivation in batch fermentation differs dramatically from continuous cultivation in chemostats. The difference is brought about by the fact that the conditions in batch cultivation are constantly varying. Nutrients are available in excess at the beginning and are depleted continuously over fermentation time. The culture population goes through various periods of growth beginning with a lag phase, followed by exponential phase and finally stationary phase. In most batch fermentation, the stationary phase will be a result of nutrient limitation, and will be characterized by low metabolic activity. In much industrial fermentations, such as wine fermentation, the stationary phase is however characterized by high metabolic activity. In this case, entry into stationary phase is in most cases due to high ethanol levels, while sugar fermentation continues (103). Repeated exposure of the culture population to these changing

conditions will eventually yield strains best adapted to the overall process and therefore to all phases of the progressing fermentation. Evolutionary improvements may therefore be expected for every phase of cultivation (157).

In a study evaluating a single ancestor of *Escherichia coli* (*E. coli*) in glucose containing minimum medium over 10,000 generations, 12 independent improved *E. coli* populations were found. The average fitness of these clonal variants was about 50 % increased in comparison to the parental strain when determined in direct competition assays in the same fermentative conditions. The genetic diversity between the 12 progenies was significant, suggesting that the phenotype was facilitated by highly different genetic changes. Most of the advantageous adaptation events occurred within the first 2,000 generations and the gain of fitness in the following selection phases was significantly decreased (93, 94, 123).

In a recent study by McBryde et al. (108) sequential batch fermentation was employed, in order to asses the potential of the adaptive evolution events occurring under these cultivation conditions for the generation of novel wine yeast strains. Mutants arising from the cultivation of a wild-type commercially available wine yeast strain after 350 generations exhibited altered production of metabolites like ethanol, glycerol, succinic acid and acetic acid. The results of this study led McBryde et al. (108) to suggest adaptive evolution under sequential batch cultivation as a potential procedure for non-recombinant modification and optimization of industrial yeast strains.

2.5.3.3 Selection in continuous cultivation

Culture conditions in a continuous cultivation are stable over the entire fermentation time, in contrast to the constantly varying conditions in batch cultivation. Most frequently used in continuous cultivation is the chemostat. Chemostat cultivation of microorganisms was invented in the 1950’s and has since been widely used for various applications. The conditions in a chemostat are physiologically stable and a constant cell culture density is maintained by the constant influx of growth limiting nutrients. The same flux rate is chosen for the volume taken out of the fermenter in order to maintain constant culture volume. The dilution rate resulting from culture

volume and flux of in- and out-flow of fresh medium and excess culture volume defines the growth rate of the culture population (39).

The well defined culture conditions allow independent variation of growth parameters and therefore the maintenance of specific and tightly regulated environmental conditions over a long period of time (157). This form of chemostat cultivation, in a mode of steady-state, was already reported by Novick and Szilard in 1950 to apply a strong selective pressure to the culture population, eventually triggering evolutionary events leading to an enrichment of evolved genotypes better adapted to the conditions applied (112).

If chemostat cultivation is employed for physiological studies, adaptive events are impairing the results of such a study. Similarly, in continuously operating production processes, the occurrence of adaptive evolutionary events is rather a threat, since most production strains are specifically genetically engineered and adaptive mutations might reduce the product formation rate (157). Kubitschek (83) and Ferea et al. (43) proposed a cultivation period of 20 generations to be suitable for physiological studies. Experiments conducted for more than 20 generations can be considered as study of evolution in action.

Chemostat cultivation is employed for the enrichment of strains exhibiting an improved phenotype. For example, when run under carbon limiting conditions, evolutionary adaptation will trigger an increased efficiency of the culture population with regard to a higher conversion of carbon to biomass. If nutrients other than the carbon source are limiting, the carbon flux in the exposed culture population will be less tightly regulated (157). Nutrient limitation will lead to a decrease in culture density. Addition of the limiting nutrient will increase culture population proportional to the added amount of nutrient (39).

Continuous cultivation under prolonged carbon limitation was shown to trigger two general types of evolutionary events. The maximum specific growth rate was found to increase while the K_m value for the limiting nutrient decreases (40, 86). A recent study of Jansen et al. (68) confirmed the findings for the elevation of specificity for the limiting nutrient in an experiment of prolonged cultivation of *S. cerevisiae* under aerobic, glucose limiting conditions. But in contrast to already reported results, the specific growth rate of the strains was found to be lower, and accompanied by a

decrease in fermentative activity. This led to the conclusion that enzymes downstream in the glycolysis pathway were affected by this treatment. This also suggests a much more complex response of the cultured population to the applied conditions than discussed before.

However, the complexity of the cellular response to the continuously applied chemostat conditions displays one of the major advantages of this system: Its application for the enrichment of populations or for the selection of improved strains for phenotypes of undefined genetic traits. Since missing information about essential genetic properties of a desired phenotype excludes genetically directed approaches, the less directed strategy of evolutionary engineering by continuous chemostat cultivation is the preferred method due to the potential of triggering complex evolutionary events (40, 157).

Two phenomena were reported that can be disadvantageous for the selection in continuous chemostat cultivations. One of them is co-metabolism in polymorphic populations that may evolve during continuous cultivation. Selection of a specific phenotype is complicated by arising co-metabolism, since it was observed that whole culture populations display the desired phenotype facilitated through symbiotic properties of the subculture populations. But none of the single strains evaluated exhibited the phenotype selected for (62, 197). The second drawback is caused by the strictly sequential appearance and fixation of adaptive mutations. Since in continuous cultivation competition only takes place between the immediate present strains, older variants already counter selected are not available for competition. This circumstance was reported to possibly lead to the selection of strains exhibiting a lower fitness than earlier predecessors not present any longer in the culture population. Paquin and Adams (124) observed this phenomenon for glucose limited chemostats of haploid and diploid strains of *S. cerevisiae*. Therefore it has to be taken in account, that a combination of adaptive mutations might lead to maladapted clones if compared to progenitors of earlier generations. In contrast, sequential batch cultivation exhibits a steady, although hyperbolic, improvement in fitness. However, fitness in chemostats evolves due to the specific conditions, accompanied by the competition with other variants present at the given time. But depending on these two parameters, the increase in fitness might not be identical with an improved phenotype for the biotechnological application (94).

In the process of a project in evolutionary engineering, complex phenotypes might be generated slowly due to the property of natural evolution, to always direct the culture population towards a fitness optimum. Reaching such a fitness optimum might not represent the global phenotypic optimum the experimenter is looking for. Wright (202, 203) invented the commonly accepted, but nevertheless controversially discussed, theory of fitness landscapes. It is defined by the advantageous adaptation of subpopulations within a treated culture population to the applied conditions in co-evolution with other subpopulations. That means that one organism depends on another organism present in the system, while each organism changes and adapts to the applied conditions individually. Therefore each subpopulation will exhibit distinct genetic adaptations enabling mutual survival and improvement. Changes in the environment will additionally support the restart of the adaptation process until the peaks of fitness for each subpopulation is reached again (74). This theory leads to the assumption that progress of an evolutionary engineering process can be influenced in an advantageous way by distinct modifications of the selection scheme if the adaptation process becomes very slow or even stops before the desired phenotype is reached, on the single strain level. Additionally, slight modifications in the selection scheme over the experimental time were also reported to avoid evolution of strains adapted too specifically, thereby exhibiting the acquired phenotype only in the exact conditions of selection (157).

The success of an evolutionary engineering project might also be impaired by the requirement of too many adaptive changes of high impact, like the generation of several novel enzyme activities, all essentially simultaneously needed in order to express the desired phenotype. The appearance of several independent deregulatory mutations in one strain is considered to be highly unlikely. For projects of such nature, recombination of already “pre adapted” phenotypes would be a strategy with a likely higher success rate. Table 2.4 shows advantageous conditions for successful evolutionary engineering.

Independent of the cultivation mode chosen, the selection conditions, applied to the culture population to be improved, are the most critical issue in order to direct the adaptive evolution processes towards the desired phenotype (40, 157).

In order to ensure successful evolutionary adaptation, brought about by either selection in batch or in continuous cultivation in a chemostat, evaluation on the

single strain level has to be done under the process conditions the strain is supposed to be applied in (157).

Table 2.4: Conditions for evolutionary engineering

Conditions supportive for successful evolutionary engineering
▪ the desired phenotype can be detected at least in rudimentary form ▪ a close relative of the organism to be treated exhibits the desired phenotype ▪ a related phenotype can be detected in the organism to be treated ▪ important aspects of the desired phenotype are susceptible to recombinant approaches

Supportive conditions for the successful application of an evolutionary engineering approach according to Sauer (157).

2.5.4 Further aspects of evolutionary engineering strategies

Screening on the single strain level is a crucial part of an evolutionary engineering strategy, since it is required to assess the success of the applied procedure (39, 157). The assessment of the desired phenotype on single strain level can be very laborious dependent on the biotechnological process to be improved. But nowadays, for many industrial applications small scale systems have been developed, allowing to increase the number of single strains to be tested simultaneously. High-throughput technology contributes significantly to screening procedures as outlined previously (130). Despite this tremendous progress it has to be kept in mind that high-throughput approaches are not applicable to all biotechnological processes. The highly reduced volume might not be suitable to mimic all important process parameters to a degree that the experimenter can expect to get a representable result for the performance under production process conditions. Furthermore, for many industrial processes, even small improvements may be of economic interest. However, such small improvements, such as increases in yield of les than one percent, can frequently not be monitored in small scale, high-throughput systems. Therefore, the screening method has to be chosen carefully and evaluated in the sense of being representative of the biotechnological process that is to be improved.

In 1996, the concept of “inverse metabolic engineering” was introduced by Bailey et al. (6). In this strategy, a desired phenotype is first identified and generated by evolutionary engineering, followed by the identification of the pivotal genetic and

environmental basis and subsequent transfer of these pivotal properties to another strain or organism in order to generate the desired strain in the host organism of choice. Proteomics, transcriptomics and since recently QTL-mapping allow the identification of the genetic and molecular background of complex phenotypes, and have benefited this approach. Further progress in these fields will also support the more frequent employment of “inverse metabolic engineering” for industrial strain improvement (107, 157).

The combination of metabolic engineering followed by evolutionary engineering is also accepted as a strategy for the introduction of a desired phenotype in order to improve industrial production strains. This strategy is considered to be useful if two diverging properties would have to be adapted in order to yield the optimal production strain. For such applications, evolutionary engineering alone has a very limited rate of success or might even be impossible to realize. Therefore the combination of a directed genetic adaptation approach for the improvement of properties well understood with the evolutionary engineering approach for properties less well understood is discussed to be a strategy of higher rate for success (157).

Both strategies, combining metabolic engineering with evolutionary engineering, can be applied in industrial sectors where the application of GMOs for production purposes are accepted by the industry itself as well as by the consumers. As already mentioned, the acceptance for GMOs in the wine industry is limited, (136, 137). However, for laboratory use and basic research in wine biotechnology, the two described strategies can be considered to be of high potential, and will generate a better knowledge about metabolic pathways or wine yeast specific characteristics that are currently not well understood.

2.6 The bridge from the literature to the project

The discrepancy in glucose and fructose utilization by wine yeast strains, the resulting low ratio of glucose on fructose concentration and its impact on the successful progression of wine fermentations requires the elucidation of the origin of the glucose preference of yeast strains commonly employed for winemaking (12, 45). Despite the generally presented hypothesis of an involvement of the first steps in glycolysis, hexose transport and hexose phosphorylation, in the disproportionate

utilization of glucose and fructose by *S. cerevisiae*, and significant research efforts so far, the underlying mechanisms are not well understood to date (11, 19, 149, 161, 162).

In addition, it was shown that under conditions of a sluggish or stuck fermentation, a glucose to fructose ratio (GFR) of lower than 0.1, will result in a stop of fermentative activity in most commonly used wine yeast strains of the species *S. cerevisiae.* Laboratory scale experiments revealed the possibility to restart fermentation by *S. cerevisiae* if the GFR is artificially elevated with the addition of glucose (45, 162). Since the addition of glucose is not permitted in many winegrowing regions and the addition of sucrose, if allowed, leads to undesired elevated ethanol concentrations, other strategies need to be designed. A natural isolate of *Z. bailii* was found to be suitable under the conditions of stuck and sluggish fermentations, to reduce excess fructose specifically and to lead to an increase of the GFR in problematic wine fermentations. This enables cells of *S. cerevisiae* to reengage in fermentative activity and finalize fermentation to reach sugar depletion (45, 134, 173, 174). This strategy was further developed and scaled up for practical application. The process of implementing this strategy in practice is described in the experimental section of this thesis.

The strategy of employing *Z. bailii* if wine fermentations appears to lack fermentative activity has the drawback of treating the symptoms, instead of avoiding the problems before they appear. It also means extra effort by the winemaker accompanied by the costs for the second inoculation. Finally, *Z. bailii* shows a very low fermentative rate making the whole process rather time consuming (173, 174).

In order to provide the winemaking industry with a yeast strain of desired fermentative ability, the aim of this study was the generation of yeast strains that exhibit a reduced glucophilic character or in an optimal scenario, a similar glucose and fructose utilization (136, 137). To achieve this aim, evolutionary engineering, a classical approach employing mutagenesis and hybridization followed by selection in different cultivation modes, was the strategy of choice. Several strategies, using mutagenesis and hybridization to generate genetic variety, and chemostat and sequential batch-based set-ups for enrichment and directed evolution, were implemented. Finally, the assessment of the fermentative performance of single

strains isolated after the selection procedure was done. This evaluation did show the suitability of the different strategies to yield the desired industrial strains.

A yeast strain obtained through this methodology could immediately be employed in commercial wine production. From a research point of few, it could also be used for an “inverse metabolic engineering” approach. Evaluation of the genetic and molecular background of improved strains in comparison to the original strains can be used to identify the relevant molecular mechanisms responsible for the selected phenotype. This information could than be used to modify other *S. cerevisiae* strains.

2.7 Literature cited

1. **Ahuatzi, D., A. Riera, R. Pelaez, P. Herrero, and F. Moreno.** 2007. Hxk2 regulates the phosphorylation state of Mig1 and therefore its nucleocytoplasmic distribution. J. Biol. Chem. **282:**4485-4493.
2. **Alberts, B., A. Johnson, J. Lewis, M. Raff, K. Roberts, and P. Walter.** 2002. Molecular biology of the cell, Fourth Edition ed. Garland Publishing, New York.
3. **Alexandre, H., and C. Charpentier.** 1998. Biochemical aspects of stuck and sluggish fermentation in grape must. J. Ind. Microbiol. Biotechnol. **20:**20-27.
4. **André, B.** 1995. An overview of membrane transport proteins in *Saccharomyces cerevisiae*. Yeast **11:**1575-1611.
5. **Bailer, J.** 1990. Theoretische Überlegungen zum Glucose-Fructose-Verhältnis in restsüssen Weinen. Mitteilungen Klosterneuburg **40:**76-79.
6. **Bailey, J. E., A. Sburlati, V. Hatzimanikatis, K. Lee, W. A. Renner, and P. S. Tsai.** 1996. Inverse metabolic engineering: A strategy for directed genetic engineering of useful phenotypes. Biotechnol. Bioeng. **52:**109-121.
7. **Barnard, E. A.** 1975. Hexokinases from yeast. Methods Enzymol. **42:**6-20.
8. **Barnett, J. A.** 1981. The utilization of disaccharides and some other sugars by yeasts. Adv. Carbohydr. Chem. **39:**347-404.
9. **Becker, J.-U., and A. Betz.** 1972. Membrane transport as controlling pacemaker of glycolysis in *Saccharomyces carlsbergensis*. Biochim. Biophys. Acta **274:**584-597.
10. **Bely, M., J.-M. Sablayrolles, and P. Barre.** 1990. Automatic detection of assimilable nitrogen deficiencies during alcoholic fermentation in oenological conditions. J. Ferment. Bioeng. **70:**246-252.
11. **Berthels, N. J., R. R. Cordero Otero, F. F. Bauer, I. S. Pretorius, and J. M. Thevelein.** 2008. Correlation between glucose/fructose discrepancy and hexokinase kinetic properties in different *Saccharomyces cerevisiae* wine yeast. Appl. Environ. Microbiol. **77:**1083-1091.

12. **Berthels, N. J., R. R. Cordero Otero, F. F. Bauer, J. M. Thevelein, and I. S. Pretorius.** 2004. Discrepancy in glucose and fructose utilisation during fermentation by *Saccharomyces cerevisiae* wine yeast strains. FEMS Yeast Res. **4:**683-689.

13. **Betina, S., P. Goffrini, I. Ferrero, and M. Wesolowski-Louvel.** 2001. RAG4 gene encodes a glucose sensor in *Kluyveromyces lactis*. Genetics **158:**541-548.

14. **Bidenne, C., B. Blondin, S. Dequin, and F. Vezinhet.** 1992. Analysis of the chromosomal DNA polymorphism of wine strains of *Saccharomyces cerevisiae*. Curr. Genet. **22:**1-7.

15. **Bisson, L. F.** 1988. High-affinity glucose transport in *Saccharomyces cerevisiae* is under general glucose repression control. J. Bacteriol. **170:**4838-4845.

16. **Bisson, L. F.** 1999. Stuck and sluggish fermentations. Am. J. Enol. Vitic. **50:**107-119.

17. **Bisson, L. F., D. M. Coons, A. L. Kruckeberg, and D. A. Lewis.** 1993. Yeast sugar transport. Crit. Rev. Biochem. Mol. Biol. **28:**259-308.

18. **Bisson, L. F., and D. G. Fraenkel.** 1984. Expression of kinase-dependent glucose uptake in *Saccharomyces cerevisiae*. J. Bacteriol. **159:**1013-1017.

19. **Bisson, L. F., and D. G. Fraenkel.** 1983. Involvement of kinases in glucose and fructose uptake by *Saccharomyces cerevisiae*. Proc. Nat. Acad. Sci. U.S.A. **80:**1730-1734.

20. **Boles, E., and C. P. Hollenberger.** 1997. The molecular genetics of hexose transport in yeasts. FEMS Microbiol. Rev. **21:**85-111.

21. **Boulton, R. B., V. L. Singleton, L. F. Bisson, and R. E. Kunkee.** 1996. Principles and practices of winemaking. Chapman Hall, New York.

22. **Brem, R. B., G. Yvert, R. Clinton, and L. Kruglyak.** 2002. Genetic dissection of transcriptional regulation in budding yeast. Science **296:**752-755.

23. **Butler, P. R., M. Brown, and S. G. Oliver.** 1996. Improvement of antibiotic titers from *Streptomyces* bacteria by interactive continuous selection. Biotechnol. Bioeng. **49:**185-196.

24. **Carlson, M.** 1997. Genetics of transcriptional regulation in yeast: Connections to the RNA Polymerase II CTD. Annu. Rev. Cell Dev. Biol. **13:**1-23.

25. **Chatterjee, R., and L. Yuan.** 2006. Directed evolution of metabolic pathways. Trends Biotechnol. **24:**28 - 38.

26. **Ciani, M., L. Ferraro, and F. Fatichenti.** 2000. Influence of glycerol production on the aerobic and anaerobic growth of the wine yeast *Candida stellata*. Enzyme Microb. Technol. **27:**698-703.

27. **Ciriacy, M., and E. Reifenberger.** 1997. Hexose transport, p. 45-65. *In* F. K. Zimmermann and K.-D. Entian (ed.), Yeast sugar metabolism: biochemistry, genetics, biotechnology, and applications. Technomic Publishing Co, Lancaster, Pa.

28. **Colombo, S., D. Ronchetti, J. M. Thevelein, J. Winderickx, and E. Martegani.** 2004. Activation state of the Ras2 protein and glucose-induced signaling in *Saccharomyces cerevisiae*. J. Biol. Chem. **279:**46715-46722.

29. **Cooper, T. B.** 1982. Nitrogen metabolism in *Saccharomyces cerevisiae*, p. 39-99. *In* J. N. Strathern, E. W. Jones, and J. R. Broach (ed.), The molecular biology of the yeast *Saccharomyces*: Metabolism and gene expression. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

30. **Dai, M., and S. D. Copley.** 2004. Genome shuffling improves degradation of the anthropogenic pesticide pentachlorophenol by sphingobium chlorophenolicum ATCC 39723. Appl. Environ. Microbiol. **70:**2391-2397.

31. **Day, R. E., P. J. Rogers, I. W. Dawes, and V. J. Higgins.** 2002. Molecular analysis of maltotriose transport and utilization by *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. **68:**5326-5335.

32. **de Barros Lopes, M., J. R. Bellon, N. J. Shirley, and P. F. Ganter.** 2002. Evidence for multiple interspecific hybridization in *Saccharomyces* sensu stricto species. FEMS Yeast Res. **1:**323-331.

33. **de Bruijne, A. W., J. Schuddemat, P. J. A. van den Broek, and J. van Steveninck.** 1988. Regulation of sugar transport systems of *Kluyveromyces marxianus*: the role of carbohydrates and their catabolism. Biochim. Biophys. Acta **939:**569-576.

34. **De Vit, M., J. Waddle, and M. Johnston.** 1997. Regulated nuclear translocation of the Mig1 glucose repressor. Mol. Biol. Cell **8:**1603-1618.

35. **Dequin, S.** 2001. The potential of genetic engineering for improving brewing, wine-making and baking yeasts. Appl. Environ. Microbiol. **56:**577 - 588.

36. **Deutschbauer, A. M., and R. W. Davis.** 2005. Quantitative trait loci mapped to single-nucleotide resolution in yeast. Nat. Genet. **37:**1333-1340.

37. **Diezemann, A., and E. Boles.** 2003. Functional characterization of the Frt1 sugar transporter and of fructose uptake in *Kluyveromyces lactis*. Curr. Genet. **43:**281-288.

38. **Dunn, B., R. P. Levine, and G. Sherlock.** 2005. Microarray karyotyping of commercial wine yeast strains reveals shared, as well as unique, genomic signatures. BMC Genomics **6:**53.

39. **Dykhuizen, D. E.** 1993. Chemostats used for studying natural selection and adaptive evolution, p. 613-631, Methods Enzymol., vol. 224. Academic Press Inc.

40. **Dykhuizen, D. E., and D. L. Hartl.** 1983. Selection in chemostats. Microbiol. Rev. **47:**150-68.

41. **Entian, K., and D. Mecke.** 1982. Genetic evidence for a role of hexokinase isozyme PII in carbon catabolite repression in *Saccharomyces cerevisiae*. J. Biol. Chem. **257:**870-874.

42. **Entian, K. D., and J. A. Barnett.** 1992. Regulation of sugar utilization by *Saccharomyces cerevisiae*. Trends Biochem. Sci. **17:**506-10.

43. **Ferea, T. L., D. Botstein, P. O. Brown, and R. F. Rosenzweig.** 1999. Systematic changes in gene expression patterns following adaptive evolution in yeast. Proc. Nat. Acad. Sci. U.S.A. **96:**9721-9726.

44. **Flick, K. M., N. Spielewoy, T. I. Kalashnikova, M. Guaderrama, Q. Zhu, H.-C. Chang, and C. Wittenberg.** 2003. Grr1-dependent Inactivation of Mth1 Mediates Glucose-induced Dissociation of Rgt1 from HXT Gene Promoters. Mol. Biol. Cell **14:**3230-3241.

45. **Gafner, J., and M. Schütz.** 1996. Impact of glucose-fructose-ratio on stuck fermentations. Vitic. Enol. Sci. **51:**214-218.

46. **Gancedo, J. M.** 1998. Yeast carbon catabolite repression. Microbiol. Mol. Biol. Rev. **62:**334-361.

47. **Gimeno, C. J., P. O. Ljungdahl, C. A. Styles, and G. R. Fink.** 1992. Unipolar cell divisions in the yeast *S. cerevisiae* lead to filamentous growth: Regulation by starvation and RAS. Cell **68:**1077 - 1090.

48. **Giudici, P., L. Solieri, A. M. Pulvirenti, and S. Cassanelli.** 2005. Strategies and perspectives for genetic improvement of wine yeasts. Appl. Environ. Microbiol. **66:**622-628.

49. **Goffeau, A., B. G. Barrell, H. Bussey, R. W. Davis, B. Dujon, H. Feldmann, F. Galibert, J. D. Hoheisel, M. Johnston, E. J. Louis, H. W. Mewes, Y. Murakami, P. Philippsen, H. Tettelin, and S. G. Oliver.** 1996. Life with 6.000 genes. Science **274:**563 - 567.

50. **Greatrix, B. W., and H. J. J. van Vuuren.** 2006. Expression of the HXT13, HXT15 and HXT17 genes in *Saccharomyces cerevisiae* and stabilization of the HXT1 gene transcript by sugar-induced osmotic stress. Curr. Genet. **49:**205-217.

51. **Griffioen, G., and J. M. Thevelein.** 2002. Molecular mechanisms controlling the localisation of protein kinase A. Current Genetics **41:**199-207.

52. **Guillaume, C., P. Delobel, J.-M. Sablayrolles, and B. Blondin.** 2007. Molecular basis of fructose utilization by the wine yeast *Saccharomyces cerevisiae*: a mutated HXT3 allele enhances fructose fermentation. Appl. Environ. Microbiol. **73:**2432-2439.

53. **Harashima, T., S. Anderson, J. R. I. Yates, and J. Heitman.** 2006. The Kelch proteins Gpb1 and Gpb2 inhibit Ras activity via association with the yeast RasGAP neurofibromin homologs Ira1 and Ira2. Mol. Cell **22:**819-830.

54. **Harashima, T., and J. Heitman.** 2002. The Ga protein Gpa2 controls yeast differentiation by interacting with Kelch repeat proteins that mimic Gß subunits. Mol. Cell **10:**163 - 173.

55. **Hardie, D. G., D. Carling, and M. Carlson.** 1998. The AMP-activated/Snf1 protein kinase subfamily: Metabolic sensors of the eukaryotic cell? Annu. Rev. Biochem. **67:**821-855.

56. **Hazbun, T. R., and S. Fields.** 2002. A genome-wide screen for site-specific DNA-binding proteins. Mol. Cell. Proteomics **1:**538-543.

57. **Hedbacker, K., R. Townley, and M. Carlson.** 2004. Cyclic AMP-dependent protein kinase regulates the subcellular localization of Snf1-Sip1 protein kinase. Mol. Cell. Biol. **24:**1836-1843.

58. **Heidenreich, E.** 2007. Adaptive Mutation in *Saccharomyces cerevisiae*. Crit. Rev. Biochem. Mol. Biol. **42:**285 - 311.

59. **Heidenreich, E., and H. Eisler.** 2004. Non-homologous end joining dependency of gamma-irradiation-induced adaptive frameshift mutation formation in cell cycle-arrested yeast cells. Mutat. Res. **556:**201 - 208.

60. **Heidenreich, E., H. Eisler, and F. Steinboeck.** 2006. Epistatic participation of *REV1* and *REV3* in the formation of UV-induced frameshift mutations in cell cycle-arrested yeast cells. Mutat. Res. **593:**187 - 195.

61. **Heiland, S., N. Radovanovic, M. Hofer, J. Winderickx, and H. Lichtenberg.** 2000. Multiple hexose transporters of *Schizosaccharomyces pombe*. J. Bacteriol. **182:**2153-2162.

62. **Helling, R. B., C. N. Vargas, and J. Adams.** 1987. Evolution of *Escherichia coli* during growth in a constant environment. Genetics **116:**349-358.

63. **Henschke, P. A.** 1997. Wine yeast, p. 527-560. *In* F. K. Zimmermann and K.-D. Entian (ed.), Yeast sugar metabolism: biochemistry, genetics, biotechnology, and applications. Technomic Publishing Co, Lancaster, PA.

64. **Higgings, V. J., P. J. L. Bell, I. W. Dawes, and P. V. Attfield.** 2001. Generation of a novel *Saccharomyces cerevisiae* strain that exhibits strong maltose utilization and hypersomotic resistance using nonrecombinant techniques. Appl. Environ. Microbiol. **67:**4346-4348.

65. **Höfer, M., and F. R. Nassar.** 1987. Aerobic and anaerobic uptake of sugars in *Schizosaccharomyces pombe*. J. Gen. Microbiol. **133:**2163-2172.

66. **Hopwood, D. A., H. M. Wright, M. J. Bibb, and S. N. Cohen.** 1977. Genetic recombination through protoplast fusion in *Streptomyces*. Nature **268:**171 - 174.

67. **Hospital, F., and A. Charcosset.** 1997. Marker-assisted introgression of quantitative trait loci. Genetics **147:**1469-1485.

68. **Jansen, M. L. A., J. A. Diderich, M. Mashego, A. Hassane, J. H. de Winde, P. Daran-Lapujade, and J. T. Pronk.** 2005. Prolonged selection in aerobic, glucose-limited chemostat cultures of *Saccharomyces cerevisiae* causes a partial loss of glycolytic capacity. Microbiology **151:**1657-1669.

69. **Jiang, Y., C. Davis, and J. R. Broach.** 1998. Efficient transition to growth on fermentable carbon sources in *Saccharomyces cerevisiae* requires signaling through the Ras pathway. EMBO J. **17:**6942-6951.

70. **Johnston, M., and J.-H. Kim.** 2005. Glucose as a hormone: receptor-mediated glucose sensing in the yeast *Saccharomyces cerevisiae*. Biochem. Soc. Trans. **33:**247-252.

71. **Kaeberlein, M., K. T. Kirkland, S. Fields, and B. K. Kennedy.** 2005. Genes determining yeast replicative life span in a long-lived genetic background. Mech. Ageing Dev. **126:**491-504.

72. **Kaniak, A., Z. Xue, D. Macool, J.-H. Kim, and M. Johnston.** 2004. Regulatory network connecting two glucose signal transduction pathways in *Saccharomyces cerevisiae*. Eukaryot. Cell **3:**221-231.

73. **Kaplan, N. L., R. R. Hudson, and C. H. Langley.** 1989. The ``hitchhiking effect'' revisited. Genetics **123:**887-899.

74. **Kauffman, S. A.** 1993. The origins of order self-organization and selection in evolution. Oxford University Press, New York.

75. **Kellis, M., N. Patterson, M. Endrizzi, B. Birren, and E. S. Lander.** 2003. Sequencing and comparison of yeast species to identify genes and regulatory elements. Nature **423:**241-254.

76. **Kim, J.-H., and M. Johnston.** 2006. Two glucose-sensing pathways converge on Rgt1 to regulate expression of glucose transporter genes in *Saccharomyces cerevisiae*. J. Biol. Chem. **281:**26144-26149.

77. **Kim, J.-H., J. Polish, and M. Johnston.** 2003. Specificity and regulation of DNA binding by the yeast glucose transporter gene repressor Rgt1. Mol. Cell. Biol. **23:**5208-5216.

78. **Kishimoto, M.** 1994. Fermentation characteristics of hybrids between the cryophilic wine yeast *Saccharomyces bayanus* and the mesophilic wine yeast *Saccharomyces cerevisiae*. J. Ferment. Bioeng. **77:**432-435.

79. **Kraakman, L., K. Lemaire, P. Ma, A. W. R. H. Teunissen, M. C. V. Donaton, P. Van Dijck, J. Winderickx, J. H. de Winde, and J. M. Thevelein.** 1999. A *Saccharomyces cerevisiae* G-protein coupled receptor, Gpr1, is specifically required for glucose activation of the cAMP pathway during the transition to growth on glucose. Mol. Microbiol. **32:**1002-1012.

80. **Krampe, S.** 2001. Molekularbiologische und physiologische Untersuchungen zum Hexosetransport unter Hungerbedingungen der Hefe *Saccharomyces cerevisiae*. Heinrich-Heine-Universität, Düsseldorf.

81. **Krampe, S., and E. Boles.** 2002. Starvation-induced degradation of yeast hexose transporters Hxt7 is dependent on endocytosis, autophagy and terminal sequence of permease. FEBS Lett. **513:**193-196.

82. **Kruckeberg, A. L.** 1996. The hexose transporter family of *Saccharomyces cerevisiae*. Arch. Microbiol. **166:**283-292.

83. **Kubitschek, H. E.** 1970. Introduction to research with continuous cultures. Prentice-Hall, Englewood Cliffs, N.J.

84. **Kuchin, S., I. Treich, and M. Carlson.** 2000. A regulatory shortcut between the Snf1 protein kinase and RNA polymerase II holoenzyme. Proc. Nat. Acad. Sci. U.S.A. **97:**7916-7920.

85. **Kuchin, S., V. K. Vyas, E. Kanter, S.-P. Hong, and M. Carlson.** 2003. Std1p (Msn3p) positively regulates the Snf1 kinase in *Saccharomyces cerevisiae*. Genetics **163:**507-514.

86. **Kurland, C. G.** 1992. Translational accuracy and the fitness of bacteria. Annu. Rev. Genet. **26:**29-50.

87. **Kuroda, Y., N. Suzuki, and T. Kataoka.** 1993. The effect of posttranslational modifications on the interaction of Ras2 with adenylyl cyclase. Science **259:**683-686.

88. **Kuyper, M., M. J. Toirkens, J. A. Diderich, A. A. Winkler, J. P. Dijken, and J. T. Pronk.** 2005. Evolutionary engineering of mixed-sugar utilization by a xylose-fermenting *Saccharomyces cerevisiae* strain. FEMS Yeast Res. **5:**925-934.

89. **Kuyper, M., A. A. Winkler, J. P. Dijken, and J. T. Pronk.** 2004. Minimal metabolic engineering of *Saccharomyces cerevisiae* for efficient anaerobic xylose fermentation: a proof of principle. FEMS Yeast Res. **4:**655-664.

90. **Lafuente, M. J., C. Gancedo, J.-C. Jauniaux, and J. M. Gancedo.** 2000. Mth1 receives the signal given by the glucose sensors Snf3 and Rgt2 in *Saccharomyces cerevisiae*. Mol. Microbiol. **35:**161-172.

91. **Lagunas, R.** 1993. Sugar transport in *Saccharomyces cerevisiae*. FEMS Microbiol. Rev. **10:**229-42.

92. **Lemaire, D., S. Van den Velde, P. Van Dijck, and J. M. Thevelein.** 2004. Glucose and sucrose act as agonist and mannose as antagonist ligands of the G protein-coupled receptor Gpr1 in the yeast *Saccharomyces cerevisiae*. Mol.Cell **16:**293-299.

93. **Lenski, R., and M. Travisano.** 1994. Dynamics of adaptation and diversification: A 10.000-generation experiment with bacterial populations. Proc. Nat. Acad. Sci. U.S.A. **91:**6808-6814.

94. **Lenski, R. E., J. A. Mongold, P. D. Sniegowski, M. Travisano, F. Vasi, P. J. Gerrish, and T. M. Schmidt.** 1998. Evolution of competitive fitness in experimental populations of *E. coli*: What makes one genotype a better competitor than another? Antonie van Leeuwenhoek **73:**35 - 47.

95. **Li, F. N., and M. Johnston.** 1997. Grr1 of *Saccharomyces cerevisiae* is connected to the ubiquitin proteolysis machinery through Skp1: coupling glucose sensing to gene expression and the cell cycle. EMBO J. **16:**5629-5638.

96. **Liang, H., and R. F. Gaber.** 1996. A novel signal transduction pathway in *Saccharomyces cerevisiae* defined by Snf3-regulated expression of *HXT6*. Mol. Biol. Cell **7:**1953-1966.

97. **Lindegren, C. C., and G. Lindegren.** 1943. Selecting, inbreeding, recombining and hybridizing commercial yeasts. J. Bacteriol. **46:**405-419.

98. **Lorenzo, V. c., M. Herrero, J. M. Sanchez, and K. N. Timmis.** 1998. Mini-transposons in microbial ecology and environmental biotechnology. FEMS Microbiol. Ecol. **27:**211-224.

99. **Lutfiyya, L. L., V. R. Iyer, J. DeRisi, M. J. DeVit, P. O. Brown, and M. Johnston.** 1998. Characterization of three related glucose repressors and genes they regulate in *Saccharomyces cerevisiae*. Genetics **150:**1377-1391.

100. **Luyten, K., C. Riou, and B. Blondin.** 2002. The hexose transporters of *Saccharomyces cerevisiae* play different roles during enological fermentation. Yeast **19:**713-726.

101. **Lynch, M., and B. Walsh.** 1998. Genetics and analysis of quantitative traits. Sinauer, Sunderland, Mass.

102. **Maier, A., B. Völker, E. Boles, and G. F. Fuhrmann.** 2002. Characterisation of glucose transport in *Saccharomyces cerevisiae* with plasma membrane vesicles (countertransport) and intact cells (initial uptake) with single Hxt1, Hxt2, Hxt3, Hxt4, Hxt6, Hxt7 or Gal2 transporters. FEMS Yeast Res. **2:**539-550.

103. **Marks, V. D., S. J. Ho Sui, D. Erasmus, G. K. van der Merwe, J. Brumm, W. W. Wasserman, J. Bryan, and H. J. J. van Vuuren.** 2008. Dynamics of the yeast transcriptome during wine fermentation reveals a novel fermentation stress response. FEMS Yeast Res. **8:**35-52.

104. **Marshall-Carlson, L., J. L. Celenza, B. C. Laurent, and M. Carlson.** 1990. Mutational analysis of the Snf3 glucose transporter of *Saccharomyces cerevisiae*. Mol. Cell Biol. **10:**1105 - 1115.

105. **Marullo, P., M. Aigle, M. Bely, I. Masneuf-Pomarede, P. Durrens, D. Dubourdieu, and G. Yvert.** 2007. Single QTL mapping and nucleotide-level resolution of a physiologic trait in wine *Saccharomyces cerevisiae* strains. FEMS Yeast Res. **7:**941-952.

106. **Marullo, P., M. Bely, I. Masneuf-Pomarede, M. Pons, M. Aigle, and D. Dubourdieu.** 2006. Breeding strategies for combining fermentative qualities and reducing off-flavor production in a wine yeast model. FEMS Yeast Res. **6:**268-279.

107. **Marullo, P., G. Yvert, M. Bely, M. Aigle, and D. Dubourdieu.** 2007. Efficient use of DNA molecular markers to construct industrial yeast strains. FEMS Yeast Research **7:**1295-1306.

108. **Mc Bryde, C., J. M. Gardner, M. de Barros Lopes, and V. Jiranek.** 2006. Generation of novel wine yeast strains by adaptive evolution. Am. J. Enol. Vitic. **57:**423-430.

109. **Moriya, H., and M. Johnston.** 2004. Glucose sensing and signaling in *Saccharomyces cerevisiae* through the Rgt2 glucose sensor and casein kinase I. Proc. Nat. Acad. Sci. U.S.A. **101:**1572-1577.

110. **Mosley, A. L., J. Lakshmanan, B. K. Aryal, and S. Özcan.** 2003. Glucose-mediated phosphorylation converts the transcription factor Rgt1 from a repressor to an activator. J. Biol. Chem. **278:**10322-10327.

111. **Nourani, A., M. Wesolowski-Louvel, T. Delaveau, C. Jacq, and A. Delahodde.** 1997. Multiple-drug-resistance phenomenon in the yeast *Saccharomyces cerevisiae*: involvement of two hexose transporters. Mol. Cell. Biol. **17:**5453-5460.

112. **Novick, A., and L. Szilard.** 1950. Experiments with the chemostat on spontaneous mutations of bacteria. Proc. Nat. Acad. Sci. U.S.A. **36:**708 - 719.

113. **Ostling, J., and H. Ronne.** 1998. Negative control of the Mig1p repressor by Snf1p-dependent phosphorylation in the absence of glucose. Eur. J. Biochem. **252:**162-168.

114. **Özcan, S., J. Dover, and M. Johnston.** 1998. Glucose sensing and signaling by two glucose receptors in the yeast *Saccharomyces cerevisiae*. EMBO J. **17:**2566-2573.

115. **Özcan, S., J. Dover, A. G. Rosenwald, S. Wolfl, and M. Johnston.** 1996. Two glucose transporters in *Saccharomyces cerevisiae* are glucose sensors that generate a signal for induction of gene expression. Proc. Nat. Acad. Sci. U.S.A. **93:**12428-12432.

116. **Özcan, S., and M. Johnston.** 1999. Function and regulation of yeast hexose transporters. Microbiol. Mol. Biol. Rev. **63:**554-69.

117. **Özcan, S., and M. Johnston.** 1995. Three different regulatory mechanisms enable yeast hexose transporter (HXT) genes to be induced by different levels of glucose. Mol. Cell. Biol. **15:**1564-1572.

118. **Özcan, S., T. Leong, and M. Johnston.** 1996. Rgt1p of *Saccharomyces cerevisiae*, a key regulator of glucose-induced genes, is both an activator and a repressor of transcription. Mol. Cell. Biol. **16:**6419-6426.

119. **Paiardi, C., F. Belotti, S. Colombo, R. Tisi, and E. Martegani.** 2007. The large N-terminal domain of Cdc25 protein of the yeast *Saccharomyces cerevisiae* is required for glucose-induced Ras2 activation. FEMS Yeast Res. **7:**1270-1275.

120. **Palomino, A., P. Herrero, and F. Moreno.** 2005. Rgt1, a glucose sensing transcription factor, is required for transcriptional repression of the HXK2 gene in *Saccharomyces cerevisiae*. Biochem. J. **388:**697-703.

121. **Palomino, A., P. Herrero, and F. Moreno.** 2006. Tpk3 and Snf1 protein kinases regulate Rgt1 association with *Saccharomyces cerevisiae* HXK2 promoter. Nucl. Acids Res. **34:**1427-1438.

122. **Pao, S. S., I. T. Paulsen, and M. H. j. Saier.** 1998. Major facilitator superfamily. Microbiol. Mol. Biol. Rev. **62:**1-34.

123. **Papadopoulos, D., D. Schneider, J. Meier-Eiss, W. Arber, R. E. Lenski, and M. Blot.** 1999. Genomic evolution during a 10.000-generation experiment with bacteria. Proc. Nat. Acad. Sci. U.S.A. **96:**3807-3812.

124. **Paquin, D., and J. Adams.** 1983. Frequency of fixation of adaptive mutations is higher in evolving diploid than haploid yeast populations. Nature **302:**495-500.

125. **Parekh, S., V. A. Vinci, and R. J. Strobel.** 2000. Improvement of microbial strains and fermentation processes. Appl. Environ. Microbiol. **54:**287 - 301.

126. **Pasula, S., D. I. Jouandot, and J.-H. Kim.** 2007. Biochemical evidence for glucose-independent induction of HXT expression in *Saccharomyces cerevisiae*. FEBS Lett. **581:**3230-3234.

127. **Patnaik, R.** 2008. Engineering complex phenotypes in industrial strains. Biotechnol. Prog. **24:**38-47.

128. **Patnaik, R., S. Louie, V. Gavrilovic, K. Perry, W. P. C. Stemmer, C. M. Ryan, and S. del Cardayre.** 2002. Genome shuffling of *Lactobacillus* for improved acid tolerance. Nat. Biotechnol. **20:**707 - 712.

129. **Pellerin, P., B. Blondin, J. M. Sablayrolles, and C. Guillaume.** 2005. Yeast strains with improved fructose fermentation capacity. WO patent 2005058947.

130. **Pereira, D. A., and J. A. Williams.** 2007. Origin and evolution of high throughput screening. Br. J. Pharmacol. **152:**53 - 61.

131. **Perez, M., K. Luyten, R. Michel, C. Riou, and B. Blondin.** 2005. Analysis of *Saccharomyces cerevisiae* hexose carrier expression during wine fermentation: both low- and high-affinity Hxt transporters are expressed. FEMS Yeast Res. **5:**351-361.

132. **Petri, R., and C. Schmidt-Danner.** 2004. Dealing with complexity: evolutionary engineering and genome shuffling. Curr. Opin. Biotechnol. **15:**298 - 304.

133. **Pina, C., P. Goncalves, C. Prista, and M. C. Loureiro-Dias.** 2004. Ffz1, a new transporter specific for fructose from *Zygosaccharomyces bailii*. Microbiology **150:**2429-2433.

134. **Porret, N. A., P. Coreth, P. Hoffmann-Boller, D. Baumgartner, and J. Gafner.** 2003. Reperaturhefen zur Behebung von Gärstockungen. Schweizerische Zeitschrift für Obst- und Weinbau **22:**4-7.

135. **Postma, E., and P. J. A. van den Broek.** 1990. Continuous-culture study of the regulation of glucose and fructose transport in *Kluyveromyces marxianus* CBS 6556. J. Bacteriol. **172:**2871-2876.

136. **Pretorius, I. S.** 2000. Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. Yeast **16:**675-729.

137. **Pretorius, I. S., and F. F. Bauer.** 2002. Meeting the consumer challenge through genetically customized wine-yeast strains. Trends Biotechnol. **20:**426-432.

138. **Ramos, J., K. Szkutnicka, and V. P. Cirillo.** 1988. Relationship between low-and high-affinity glucose transport systems of *Saccharomyces cerevisiae*. J. Bacteriol. **170:**5375-5377.

139. **Randez-Gil, F., P. Herrero, P. Sanz, J. A. Prieto, and F. Moreno.** 1998. Hexokinase PII has a double cytosolic-nuclear localisation in *Saccharomyces cerevisiae*. FEBS Lett. **425:**475-478.

140. **Reifenberger, E., E. Boles, and M. Ciriacy.** 1997. Kinetic characterization of individual hexose transporters of *Saccharomyces cerevisiae* and their relation to the triggering mechanisms of glucose repression. Eur. J. Biochem. **245:**324-333.

141. **Reifenberger, E., K. Freidel, and M. Ciriacy.** 1995. Identification of novel *HXT* genes in *Saccharomyces cerevisiae* reveals the impact of individual hexose transporters on glycolytic flux. Mol. Microbiol. **16:**157-167.

142. **Rodrigues de Sousa, H., I. Spencer-Martins, and P. Gonçalves.** 2004. Differential regulation by glucose and fructose of a gene encoding a specific fructose/H^+ symporter in *Saccharomyces sensu stricto* yeasts. Yeast **21:**519-530.

143. **Rodriguez, A., T. De La Cera, P. Herrero, and F. Moreno.** 2001. The hexokinase 2 protein regulates the expression of the GLK1, HXK1 and HXK2 genes of *Saccharomyces cerevisiae*. Biochem. J. **355:**625-631.

144. **Rolland, F., J. H. de Winde, K. Lemaire, E. Boles, J. M. Thevelein, and J. Winderickx.** 2000. Glucose-induced cAMP signalling in yeast requires both a G-protein coupled receptor system for extracellular glucose detection and a separable hexose kinase-dependent sensing process. Mol. Microbiol. **38:**348-358.

145. **Rolland, F., V. Wanke, L. Cauwenberg, P. Ma, E. Boles, M. Vanoni, J. H. Winde, J. M. Thevelein, and J. Winderickx.** 2001. The role of hexose transport and phosphorylation in cAMP signalling in the yeast *Saccharomyces cerevisiae*. FEMS Yeast Res. **1:**33-45.

146. **Rolland, F., J. Winderickx, and J. M. Thevelein.** 2002. Glucose-sensing and -signalling mechanisms in yeast. FEMS Yeast Res. **2:**183-201.

147. **Rolland, F., J. Winderickx, and J. M. Thevelein.** 2001. Glucose-sensing mechanisms in eukaryotic cells. Trends Biochem. Sci. **26:**310-317.

148. **Rose, M., W. Albig, and K.-D. Entian.** 1991. Glucose repression in *Saccharomyces cerevisiae* is directly associated with hexose phosphorylation by hexokinases PI and PII. Eur. J. Biochem. **199:**511-518.

149. **Rossignol, T., L. Dulau, A. Julien, and B. Blondin.** 2003. Genome-wide monitoring of wine yeast gene expression during alcoholic fermentation. Yeast **20:**1369-1385.

150. **Rowlands, R. T.** 1983. Industrial fungal genetics and strain selection, p. 346. *In* J. E. Smith, Berry, D.R., Kistiansen, B. (ed.), The filamentous fungi, vol. 4. Edward Arnold, London.

151. **Salmon, J. M.** 1989. Effect of sugar transport inactivation in *Saccharomyces cerevisiae* on sluggish and stuck enological fermentations. Appl. Environ. Microbiol. **55:**953-958.

152. **Salmon, J.-M.** 1997. Enological fermentation kinetics of an isogenic ploidy series derived from an industrial *Saccharomyces cerevisiae* strain. J. Ferment. Bioeng. **83:**253-260.

153. **Santangelo, G. M.** 2006. Glucose signaling in *Saccharomyces cerevisiae*. Microbiol. Mol. Biol. Rev. **70:**253-282.

154. **Sanz, P., G. R. Alms, T. A. J. Haystead, and M. Carlson.** 2000. Regulatory Interactions between the Reg1-Glc7 Protein Phosphatase and the Snf1 Protein Kinase. Mol. Cell. Biol. **20:**1321-1328.

155. **Sasaki, H., and H. Uemura.** 2005. Influence of low glycolytic activities in gcr1 and gcr2 mutants on the expression of other metabolic pathway genes in *Saccharomyces cerevisiae*. Yeast **22:**111-127.

156. **Sato, M., M. Kishimoto, J. Watari, and M. Takashio.** 2002. Breeding of brewer's yeast by hybridization between a top-fermenting yeast *Saccharomyces cerevisiae* and a cryophilic yeast *Saccharomyces bayanus*. J. Biosci. Bioeng. **93:**509 - 511.

157. **Sauer, U.** 2001. Evolutionary engineering of industrially important microbial phenotypes, p. 129-169. *In* T. Scheper (ed.), Advances in Biochemical Engineering/Biotechnology, vol. 73. Springer Verlag, Berlin Heidelberg.

158. **Schaaff, I., J. Heinisch, and F. K. Zimmermann.** 1989. Overproduction of glycolytic enzymes in yeast. Yeast **5:**285-290.

159. **Scheinbach, S.** 1983. Protoplast fusion as a means of producing new industrial yeast strains. Biotechnol. Adv. **1:**289 - 300.

160. **Schmidt, M. C., R. R. McCartney, X. Zhang, T. S. Tillman, H. Solimeo, S. Wolfl, C. Almonte, and S. C. Watkins.** 1999. Std1 and Mth1 proteins interact with the glucose sensors to control glucose-regulated gene expression in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **19:**4561-4571.

161. **Schütz, M., and J. Gafner.** 1995. Lower fructose uptake capacity of genetically characterized strains of *Saccharomyces bayanus* compared to strains of *Saccharomyces cerevisiae*: A likely cause of reduced alcoholic fermentation activity. Am. J. Enol. Vitic. **46:**175-180.

162. **Schütz, M., and J. Gafner.** 1993. Sluggish alcoholic fermentation in relation to alterations of the glucose-fructose ratio. Chemie, Microbiologie, Technologie der Lebensmittel **15:**73-78.

163. **Sebastiani, F., C. Barberio, E. Casalone, D. Cavalieri, and M. Polsinelli.** 2002. Crosses between *Saccharomyces cerevisiae* and *Saccharomyces bayanus* generate fertile hybrids. Res. Microbiol. **153:**53-58.

164. **Sega, G. A.** 1984. A review of the genetic effects of ethyl methanesulfonate. Mutat. Res. **134:**113-142.

165. **Sen, S., V. Venkata Dasu, and B. Mandal.** 2007. Developments in directed evolution for improving enzyme functions. Appl. Biochem. Biotechnol. **143:**212 - 223.

166. **Shapiro, J. A.** 1997. Genome organization, natural genetic engineering and adaptive mutation. Trends Genet. **13:**98 - 104.

167. **Sinha, H., B. P. Nicholson, L. M. Steinmetz, and J. H. McCusker.** 2006. Complex genetic interactions in a quantitative trait locus. PLoS Genet. **2:**e13.

168. **Smith, J. E.** 1998. The impact of genetic engineering on food and beverage fermentation, p. 838-852. *In* B. J. B. Wood (ed.), Microbiology of fermented foods, vol. 2. Blackie Academic & Professional, Glasgow.

169. **Sousa-Dias, S., T. Gancalves, J. S. Leyva, J. M. Peinada, and M. C. Loureiro-Dias.** 1996. Kinetics and regulation of fructose and glucose transport systems are responsible for fructophily in *Zygosaccharomyces bailii*. Microbiology **142:**1733-1738.

170. **Spielewoy, N., K. Flick, T. I. Kalashnikova, J. R. Walker, and C. Wittenberg.** 2004. Regulation and recognition of SCFGrr1 targets in the glucose and amino acid signaling pathways. Mol. Cell. Biol. **24:**8994-9005.

171. **Steele, D. F., and S. Jinks-Robertson.** 1992. An examination of adaptive reversion in *Saccharomyces cerevisiae*. Genetics **132:**9-21.

172. **Steinmetz, L. M., H. Sinha, D. R. Richards, J. L. Spiegelmann, P. J. Oefner, J. H. McCusker, and R. W. Davis.** 2002. Dissecting the architecture of a quantitative trait locus in yeast. Nature **416:**326-330.

173. **Sütterlin, K. A., H. Gut, P. Hoffmann-Boller, and J. Gafner.** 2005. Presented at the 14. Internationales Oenologisches Symposium, Konferenzzentrum Porto Carras, Chalkidiki, Griechenland, 08.05.2005 - 12.05.2005.

174. **Sütterlin, K. A., H. K. Horsch, J. Gafner, and F. F. Bauer.** in preparation; personal communication.

175. **Thevelein, J. M.** 1991. Fermentable sugars and intracellular acidification as specific activators of the RAS-adenylate cyclase signalling pathway in yeast: the relationship to nutrient-induced cell cycle control. Mol. Microbiol. **5:**1301-1307.

176. **Thevelein, J. M.** 1994. Signal transduction in yeast. Yeast **10:**1753-1790.

177. **Tomas-Cobos, L., L. Casadome, G. Mas, P. Sanz, and F. Posas.** 2004. Expression of the Hxt1 low affinity glucose transporter requires the coordinated activities of the HOG and glucose signalling pathways. J. Biol. Chem. **279:**22010-22019.

178. **Tomas-Cobos, L., and P. Sanz.** 2002. Active Snf1 protein kinase inhibits expression of the *Saccharomyces cerevisiae* HXT1 glucose transporter gene. Biochem. J. **368:**657-663.

179. **Treitel, M. A., and M. Carlson.** 1995. Repression by Ssn6-Tup1 is directed by Mig1, a repressor/activator protein. Proc. Nat. Acad. Sci. U.S.A. **92:**3132-3136.

180. **Treitel, M. A., S. Kuchin, and M. Carlson.** 1998. Snf1 protein kinase regulates phosphorylation of the Mig1 repressor in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **18:**6273-6280.

181. **Tschopp, J. F., S. D. Emr, C. Field, and R. Schekman.** 1986. *GAL2* codes for a membrane-bound subunit of the galactose permease in *Saccharomyces cerevisiae*. J. Bacteriol. **166:**313-318.

182. **Türkel, S., T. Turgut, M. C. López, H. Uemura, and H. V. Baker.** 2003. Mutations in GCR1 affect SUC2 gene expression in *Saccharomyces*. Mol. Genet. Genomics **268:**825-831.

183. **van Suylekom, D., E. van Donselaar, C. Blanchetoto, L. N. Do Ngoc, B. M. Humbel, and J. Boonstra.** 2007. Degradation of the hexose transporter Hxt5p in *Saccharomyces cerevisiae*. Biol. Cell **99:**13-23.

184. **van Urk, H., E. Postma, W. A. Scheffers, and J. P. Van Dijken.** 1989. Glucose transport in crabtree-positive and crabtree-negative yeasts. J. Gen. Microbiol. **135:**2399-2406.

185. **Varela, C., J. Cárdenas, F. Melo, and E. Agosin.** 2005. Quantitative analysis of wine yeast gene expression profiles under winemaking conditions. Yeast **22:**369-383.

186. **Versele, M., J. H. de Winde, and J. M. Thevelein.** 1999. A novel regulator of G protein signalling in yeast, Rgs2, downregulates glucose-activation of the cAMP pathway through direct inhibition of Gpa2. EMBO J. **18:**5577 - 5591.

187. **Verwaal, R., J. W. Paalman, A. Hogenkamp, A. J. Verkleij, C. T. Verrips, and J. Boonstra.** 2002. HXT5 expression is determined by growth rates in *Saccharomyces cerevisiae*. Yeast **19:**1029-1038.

188. **Vincent, O., R. Townley, S. Kuchin, and M. Carlson.** 2001. Subcellular localization of the Snf1 kinase is regulated by specific ß subunits and a novel glucose signaling mechanism. Genes Dev. **15:**1104-1114.

189. **Viviani-Nauer, A., P. Hoffmann-Boller, and J. Gafner.** 1997. In vitro detection of folpet and its degradation to phthalimide in aqueous solutions and in yeast suspensions. Am. J. Enol. Vitic. **48:**63-66.

190. **Viviani-Nauer, A., P. Hoffmann-Boller, and J. Gafner.** 1997. In vivo detection of folpet and its metabolite phthalimide in grape must and wine. Am. J. Enol. Vitic. **48:**67-70.

191. **Vojtek, A. B., and D. G. Fraenkel.** 1990. Phosphorylation of yeast hexokinases. Eur. J. Biochem. **190:**371-375.

192. **Volschenk, H., M. Viljoen-Bloom, R. E. Subden, and H. J. J. van Vuuren.** 2001. Malo-ethanolic fermentation in grape must by recombinant strains of *Saccharomyces cerevisiae*. Yeast **18:**963-970.

193. **Volschenk, H., M. Viljoen-Bloom, J. van Staden, J. Husnik, and H. J. J. van Vuuren.** 2004. Genetic engineering of an industrial strain of *Saccharomyces cerevisiae* for L-malic acid degradation via an efficient malo-ethanolic pathway. S. Afr. J. Enol. Vitic. **25:**63-73.

194. **Walker, G. M.** 1998. Yeast physiology and biotechnology. J. Wiley & Sons, Chichester; New York.

195. **Walker, J. E., and B. Miroux.** 1999. Selection of *Escherichia coli* hosts that are optimized for the expression of proteins, p. 575. *In* A. L. Demain and J. E. Davis (ed.), Manual of industrial microbiology and biotechnology. ASM Press, Washington, D. C.

196. **Wang, Y., M. Pierce, L. Schneper, C. G. Güldal, X. Zhang, S. Tavazoie, and J. R. Broach.** 2004. Ras and Gpa2 mediate one branch of a redundant glucose signaling pathway in yeast. PLoS Biol. **2:**e128.

197. **Weikert, C., U. Sauer, and J. E. Bailey.** 1997. Use of a glycerol-limited, long-term chemostat for isolation of *Escherichia coli* mutants with improved physiological properties. Microbiology **143:**1567 - 1574.

198. **Weirich, J., P. Goffrini, P. Kuger, I. Ferrero, and K. D. Breunig.** 1997. Influence of mutations in hexose-transporter genes on glucose repression in *Kluyveromyces lactis*. Eur. J. Biochem. **249:**248-257.

199. **Wieczorke, R., S. Krampe, T. Weierstall, K. Freidel, C. P. Hollenberg, and E. Boles.** 1999. Concurrent knock-out of at least 20 transporter genes is required to block uptake of hexoses in *Saccharomyces cerevisiae*. FEBS Lett. **464:**123-128.

200. **Winzeler, E. A., C. I. Castillo-Davis, G. Oshiro, D. Liang, D. R. Richards, Y. Zhou, and D. L. Hartl.** 2003. Genetic diversity in yeast assessed with whole-genome oligonucleotide arrays. Genetics **163:**79-89.

201. **Winzeler, E. A., D. R. Richards, A. R. Conway, A. L. Goldstein, S. Kalman, M. J. McCullough, J. H. McCusker, D. A. Stevens, L. Wodicka, D. J. Lockhart, and R. W. Davis.** 1998. Direct allelic variation scanning of the yeast genome. Science **281:**1194-1197.

202. **Wright, S.** 1982. Character change, speciation, and the higher taxa. Evolution **36:**427 - 443.

203. **Wright, S.** 1988. Surfaces of selective value revisited. Am. Nat. **131:**115 - 123.

204. **Wucherpfennig, K., K. Otto, and Y.-C. Huang.** 1986. Aussagekraft des Glucose-Fructose-Verhältnisses. Die Weinwirtschaft-Technik **122:**254-260.

205. **Xue, Y., M. Batlle, and J. P. Hirsch.** 1998. GPR1 encodes a putative G protein-coupled receptor that associates with the Gpa2p G subunit and functions in a Ras-independent pathway. EMBO J. **17:**1996 - 2007.

206. **Young, E. T., K. M. Dombek, C. Tachibana, and T. Ideker.** 2003. Multiple pathways are co-regulated by the protein kinase Snf1 and the transcription factors Adr1 and Cat8. J. Biol. Chem. **278:**26146-26158.

207. **Zhang, Y.-X., K. Perry, V. A. Vinci, K. Powell, W. P. C. Stemmer, and S. B. del Cardayre.** 2002. Genome shuffling leads to rapid phenotypic improvement in bacteria. Nature **415:**644-646.

208. **Zimmermann, F. K., and K.-D. Entian.** 1997. Yeast sugar metabolism. Technomic Publishing, Lancaster, PA.

Chapter 3

RESEARCH RESULTS I

Mass-mating, enrichment and selection: a novel strategy to generate new wine yeast strains

3 Research results I

Mass-mating, enrichment and selection: a novel strategy to generate new wine yeast strains

3.1 Abstract

Here we present an innovative strategy to generate a large number of new yeast strains that fulfill all the basic requirements for wine fermentation, while in addition being improved for fermentative activity in conditions of limited availability of assimilable nitrogen. The strategy is based on a mass-mating approach of two commercial wine yeast strains with known and widely diverging characteristics. Hybrid yeast strains exhibiting the desired enological characteristics were selected through continuous cultivation in a chemostat in a series of enrichment and adaptive evolution steps. The data show that the culture consisted of at least 95% hybrids at the end of this selection process, while the culture population after mass-mating showed a content of only 6 % of such strains. The assessment of single strains revealed that 52 % of the selected hybrids show an improved fermentative activity in nitrogen limiting conditions in comparison to the performance of the parental strains. In addition, all selected hybrids exhibited a high fermentative capacity in conditions mimicking a high sugar grape juice. Our results showed that this novel strategy has the potential to rapidly produce a large number of new yeast strains with specifically improved and enologically important characteristics.

3.2 Introduction

Today, the use of selected yeasts for inoculated wine fermentations is frequently preferred to the traditional method of spontaneous fermentation as the fermentation process can be better controlled and the final product is generally of more consistent, if not better, quality (18, 28). New wine yeast strains have in the past been selected through time and labor-intensive procedures that involve the individual assessment of large numbers of strains, which may either be natural isolates, hybrids obtained through breeding or mutants of such strains (28). Yeast strain selection requires the assessment of individual strains for all important enological characteristics.

Despite improvements in fermentation control, sluggish and stuck fermentations are still a major enological problem (29). Many factors responsible for stuck or sluggish fermentations have been elucidated, and include nitrogen deficiency (4), thiamine depletion of the must (3), lack of oxygen (11, 29), excessive clarification of juice (1, 2), unfavorable ratio of the residual glucose and fructose concentration (12, 31, 32) inhibition of yeast cells by fermentation by-products, especially octanoic and decanoic acids (36), by killer toxins (35) and by pesticides (7, 37, 38). While most of the above mentioned causes for a sluggish or stuck fermentation can be avoided or diminished by good enological practices the deficiency of assimilable nitrogen is a parameter that is frequently inherent to the grape juice. The content in grape must of yeast assimilable nitrogen (YAN), consisting of free amino acids, ammonium ions and di and tripeptides, is highly variable and ranges from 60 to 2,400 mg N/l (25). It has also been shown that the nitrogen demand of different yeast strains is genetically determined and varies significantly between different strains (22). For this reason, predicting the occurrence of stuck or sluggish fermentation due to nitrogen deficiency of the grape juice remains difficult.

In response to this situation, winemakers tend to supplement the must with at least 250 mg/l of YAN, mainly in the form of di-ammonium phosphate (DAP), if the must contains limited nitrogen. Managing must nitrogen is however rather difficult in cellar conditions. It has been speculated, that if nitrogen levels are too high, the risk of off-flavor and ethyl-carbamate formation increases (5). This speculation was however shown to be wrong (14, 34)

The pattern of nitrogen compound utilization varies among different yeast strains. However, a general preference for certain amino acids exists. Glutamate, glutamine, arginine and aspartatic acid are the preferentially used amino acids of most *Saccharomyces* strains, while lysine can not be used as nitrogen source, and proline cannot be degraded in the absence of molecular oxygen even if present in abundance in the grape juice (6). Yeast nitrogen demand (17) and utilization efficiency are thus an important selection criterion for wine yeasts. In particular, yeast with reduced nitrogen demand should be less likely to be involved in stuck or sluggish fermentation.

The aim of this study was the implementation of a novel strategy to generate new yeast strains that would all fulfill the essential technological trait of a wine yeast

strain - being able to ferment a high sugar must to dryness - while showing specifically improved nitrogen utilization.

Evolutionary engineering is a way to create new microbial strains showing desired phenotypes especially for industrial applications. It works on the principles of variation and selection and is a complementary strategy to metabolic engineering of single strains. The major steps within this strategy are the generation of variability and diversity of the genetic background and continuous selection in large populations for many generations, allowing in addition for accumulation of spontaneous mutations under selective conditions (30).

Mass-mating was chosen as a strategy for this study, since it is a non-recombinant method enabling the generation of a highly diverse genetic pool within a yeast population by randomly hybridizing the haploid spores of two well known wine yeast strains (16, 21, 23). Hybridization is an accepted technology within the wine industry for the generation of novel yeast strains (27). During the process of hybridization genetic variation is not only introduced by the combination of two haploid spores originating from different parents to form a novel diploid cell. In the case of hybridization of spores from a single parent, the meiotic recombination will ensure that the new diploids will be different from this parent (15, 24, 26). This method enables therefore to generate a culture population composed of an infinite number of new yeast strains with a highly diverse genetic background.

The next step of the strategy is the application of a strong selection pressure to enrich for yeast strains showing the desired characteristics within the newly generated culture population (10, 16, 21, 30). For this purpose, a chemostat set-up was used. The growth conditions and therefore the selection pressure are precisely defined and kept stable. The population can be maintained for the desired amount of generations in these conditions, ensuring that the strains best adapted to this specific environment will start to dominate the population (10). Besides the initial genetic variability generated through the mass-mating, random spontaneous mutations will also occur during mitotic divisions in the chemostat. The cells finally selected will therefore be the result both of enrichment for cells from the initial highly diverse culture population and of directed evolution within the chemostat (13, 19, 20).

The success of this selection process is dependent on the conditions applied to the population. In general, it is considered that continuous cultivation for up to 20 generations can be used for studying the physiological characteristics of populations or single strains qualitatively, whereas longer cultivation duration will introduce a significant element of evolutionary adaptation by spontaneous mutation (9, 13, 19, 20, 30). Although, the overall rate of spontaneous mutagenesis is usually rather stable and low, it may rise considerably under certain circumstances and modulation of environmental conditions, for instance metabolic stress, nutrient limitation or stationary phase (8).

In a third stage, single strains isolated at the end of the selection procedure are assessed for their fermentative characteristics in the specific selective conditions and in general wine fermentation.

In this study, the main focus was on generating yeast with improved nitrogen efficiency by using nitrogen as the limiting nutrient in the chemostat. The data show that the method generated a high percentage of improved yeast strains when compared to the parental strains.

To our knowledge, this is the first study employing a mass-mating approach combined with selection in chemostat cultivation for the generation of new yeast strains showing improved fermentative activity in nitrogen limiting conditions.

3.3 Material and Methods

3.3.1 Strains

Two wine yeast strains were used as parental strains: BM45 (Lallemand Inc) is a commercially available wine yeast of the species *Saccharomyces cerevisiae* and USM21 (University Stellenbosch, Institute for Wine Biotechnology, South Africa) also of the species *Saccharomyces cerevisiae*. The two strains have widely differing characteristics.

USM21 is known for its outstanding fermentative activity and excellent flavor production. However, it shows a very high fermentation speed, not always desired by the winemaking industry. BM45 is a widely used wine yeast strain known for its high efficiency, moderate fermentation speed, rather high nitrogen demand and

mannoprotein production, which is contributing to the mouth feel of the final wine.

3.3.2 Media

Strains were maintained on YPD plates (1.2 % yeast extract, 2.5 % peptone, 1.2 % dextrose and 2 % agar (Merck KGaA)). After growth for 2 - 3 days at 30 °C plates were stored at 4 °C. Liquid cultures were grown at 30 °C under aerobic conditions in YPD broth (1.2 % yeast extract, 2.5 % peptone and 1.2 % dextrose (Merck KGaA)).

A minimum medium (1 % potassium acetate, 0.1 % yeast extract, 0.05 % glucose (Merck KGaA) and 2 % agar (BD Biosciences)) was used for the sporulation of yeast cultures.

A synthetic grape must containing 10 % of the usually average concentrations of ammonium, aspartatic acid, glutamic acid, arginine and glutamine was used for the chemostat selection in limiting nitrogen conditions. Other amino acids were provided as described before (4). The medium contained (per liter) 50 g glucose, 50 g fructose, 6 g citric acid, 6 g malic acid, 0.75 g KH_2PO_4, 0.5 g K_2SO_4, 0.25 g $MgS0_4$ x $7H_2O$, 0.155 g $CaCl_2$ x $2H_2O$, 0.2 g NaCl, 0.09 g NH_4Cl, 4 mg $MnSO_4$ x H_2O, 4 mg $ZnSO_4$ x $7H_2O$, 1 mg $CuSO_4$ x $5H_2O$, 1 mg KI, 0,4 mg $CoCl_2$ x $6H_2O$, 1 mg H_3BO_3, 1 mg $(NH_4)_6Mo_7O_{24}$, 20 mg myo-Inositol, 1.5 mg pantothenic acid, 0.25 mg thiamine, 2 mg nicotinic acid, 0.25 mg pyridoxine, 0.003 mg biotin, 1.5 mg ergosterol, 0.5 mg oleic acid, 0.5 ml Tween 80, 18.3 mg tyrosine, 178.0 mg tryptophane, 31.4 mg isoleucine, 15.7 mg aspartatic acid, 41.9 mg glutamic acid, 125.7 mg arginine, 47.1 mg leucine, 78.5 mg threonine, 20.9 mg glycine, 167.6 mg glutamine, 146.6 mg alanine, 47.1 mg valine, 31.4 mg methionine, 36.7 mg phenylalanine, 78.5 mg serine, 31.4 mg histidine, 20.9 mg lysine, 612.6 mg proline. The pH of the medium was set to pH 3.3 – 3.5 before sterilization. 10 l batches of this medium were sterilized for 45 min at 121 °C before use. All substances used for the synthetic grape must were either from Merck KGaA or Sigma-Aldrich Co. The same medium was used for the chemostat selection in limiting nitrogen conditions and high ethanol concentrations except of the addition of 11 % ethanol (Merck KGaA) after the sterilization.

Evaluation of yeast populations that are representative of different stages of the selection process were done in laboratory scale in synthetic grape must not

providing any ammonium, aspartatic acid, glutamic acid, arginine and glutamine and only 10 % of the concentration of all other amino acids typically found in natural grape juice (4). Glucose and fructose were provided in concentrations of 100 g/l each.

Small scale fermentations for the assessment of single selected yeast strains for fermentative ability were done in a synthetic grape must containing 10 % of the concentrations for ammonia and amino acids as stated in the original protocol (4). This corresponds to a concentration of 30 mg/l of assimilable nitrogen. Glucose and fructose were supplied in a concentration of 100 g/l of medium each. All components except of the vitamins and amino acids were sterilized for 15 min at 121 °C. Stocks of amino acids and vitamins were filter sterilized and added in the desired amount at the time of inoculation. For the assessment of the fermentative performance in excess sugar and sufficient nitrogen concentration small scale fermentations were done in synthetic grape must containing a sugar concentration of 150 g/l of glucose and fructose each. The concentration of assimilable nitrogen was 300 mg/l (4).

3.3.3 Sporulation

Cells of yeast cultures to be sporulated were streaked out on a minimum medium agar plate directly from the YPD plate strains were maintained on. Sporulation took place at room temperature (RT) and over a period of 5 to 7 days. The ascus formation was confirmed microscopically at the end of the cultivation time.

3.3.4 Mass-Mating

Spores of both strains were harvested and the ascus were digested with zymolase (0.5 mg/ml zymolase (INC), 1 M sorbitol (Merck KGaA). The single spores were resuspended in dH_2O and an equal amount of both parental spores mixed. The mixture of spores were streaked out and cultivated on YPD agar plates for 3 days at 30 °C and harvested. An over night liquid culture in YPD was used to prepare freeze cultures of the culture yielded by mass-mating. Freeze cultures are kept at –80 °C in cryo vials (Greiner bio-one) and prepared to a final concentration of 15 % glycerol (Merck KGaA).

3.3.5 Chemostat selection

Continuous cultivations were done in a 1 l glass bioreactor unit (Bio Flo 110, New Brunswick Scientific Co INC, New Jersey). A six bladed Rushton-type Impeller (52 mm, New Brunswick Scientific Co INC, New Jersey) was used at a speed of 100 RPM to keep the culture equally distributed. The temperature was controlled at 25 °C. pH and the concentration of dissolved oxygen in the culture was monitored by appropriate sensors (Mettler Toledo, Switzerland) but not regulated. The medium was air saturated at the time of inoculation. No additional O_2 was supplied during the entire cultivation. The maintenance of a constant culture volume of 900 ml was realized by using a peristaltic pump (Minipuls 3, Gilson) equipped with a pump head allowing operating the pump with two tubes simultaneously. A set up allowing re-inoculation of the cells carried out of the fermentation culture was used for the continuous cultivation in high concentrations of ethanol. A reservoir prior to the waste container was used to allow the cells to saddle and being transferred back to the culture. This enabled the maintenance of a constant culture density in conditions not expected to allow growth of the culture population.

3.3.6 Number of colony forming units

The determination of the number of colony forming units (CFU) within a culture population was done by plating appropriate dilutions of the culture on YPD plates. Plates were incubated for 3 days at 30 °C and the number of colonies counted. All measures were done in duplicate.

3.3.7 Sugar and ethanol determinations

Glucose-, fructose- and ethanol concentrations in culture supernatants were determined by Fourier Transformed Infrared Spectroscopy (FTIR) on a Foss GrapeScan 2000 (Foss). The program originally used for the examination of natural grape musts under fermentation was used for this application. The verification for the applicability of this program for the examination of synthetic grape must was done by comparative studies of FTIR and the reference methods. Commercially available enzymatic assays for D-Glucose/D-Fructose and Ethanol (r-biopharm) were used as

reference methods.

3.3.8 Determination of karyotypes

The karyotypes of yeast strains were examined by Pulse Field Gel Electrophoresis. The preparation of the DNA was as described in (33). Yeast cultures were grown to stationary phase, harvested and washed with 10 mM EDTA (pH 7.5). The resuspended cell culture (50 mM EDTA, pH 7.5) was exposed to zymolase (INC) dissolved in 1 M sorbitol, 0.1 M sodium citrate, 60 mM EDTA (pH 5.8) and 2-mercapthoethanol. The mixture was solidified with 1 % agarose (Roche Diagnostics) in 125 mM EDTA (pH 7.5). After incubation at 37 °C for 24 h in 0.45 M EDTA (pH 9.0), 10 mM Tris-HCl (pH 8.0) and 7.5 % (vol/vol) 2-mercapthoethanol the buffer was changed (0.45 M EDTA (pH 9.0), 10 mM Tris-HCl (pH 8.0), 1 % (wt/vol) SDS and 1 mg/ml Proteinase K) and followed by incubation for 48 h at 50 °C. The solidified DNA extracts were stored in 0.5 M EDTA (pH 9.0) at 4 °C. The DNA extracts were separated on a 2 % agarose gel in a CHEF Mapper (BioRad) over a period of 42 h (6 V/cm; 13.5 °C) in 0.5 x TBE buffer (6.05 g/l Tris-HCl, 2.5 g/l boric acid, 0.18 g/l EDTA). Chromosomes were visualized by staining with ethidiumbromide. Chemicals used were either from Sigma-Aldrich Co or Merck KGaA if not different specified.

3.3.9 Small scale fermentations

The assessment of the fermentative activity of the selected hybrids in comparison to the parental strains was performed in fermentations in 250 ml Erlenmeyer flasks. The culture volume was 120 ml before inoculation and flasks were closed with an aluminum cap. It was assumed that anaerobic conditions will be present in the fermentations after depletion of the oxygen dissolved in the medium at inoculation. Yeast strains to be examined were grown up over night in YPD, re inoculated to YPD and grown to a culture density of approx. 2.0 OD_{600} aiming for an equal growth stage for all yeast strains used within one experiment. Cells were harvested and resuspended in YPD to a density of 20.0 OD_{600}. Synthetic grape must was inoculated at a culture density of 0.2 OD_{600}. Fermentations were performed at RT (20 – 22 °C). The culture was equally re-distributed for the determination of culture

density by gentle shaking. It is assumed that oxygen was supplied to the culture by this way. The fermentative activity was examined by recording the weight loss of the cultures. Determination of sugar and ethanol concentrations was done at selected days of the fermentation. Samples were done in triplicates. Fermentation was considered being finished when all samples showed a stable weight loss less than 0.1 g/100ml/24h and cultivation was stopped by that time.

3.3.10 Statistics

The confidence intervals for the percentages of hybrids within the different populations were calculated for the proportions of a binominal distribution.

3.4 Results

3.4.1 Mass-Mating

Spore formation found to be most efficient for both USM21 and BM45 when colonies were directly streaked out on agar plates of sporulation media from YPD rich media. During the incubation at room temperature the rate of sporulation was monitored microscopically. The plates were harvested by the time a sporulation rate of 90 % was detected. After mass-mating, the culture population was examined for the presence of hybrids produced by this method. For this purpose, 50 randomly selected colonies were examined by Pulse-Field-Gel Electrophoresis (PFGE). All of these colonies were of a diploid nature, as would be expected since the parents are homothallic strains. 6 % of the selected colonies showed a karyotype that was clearly characteristic of a hybrid of the two parental strains. The other randomly selected strains exhibited a karyotyping pattern that was similar to one of the parental strains in about equal proportions (Figure 3.1). The latter group of strains is most likely made up of non-sporulated parental cells, hybrids that are generated by the mating of two spores from the same parent, but could also present true hybrid. The 95 % confidence interval for this experiment is 0 % to 12 %.

Therefore, the mass-mating process and the generation of a culture population of highly diverse genetic background did only yield a limited percentage of true hybrids. This is not surprising considering the percentage of sporulation and the likelihood of

inter-strain mating in such a system. Considering the large number of cells in the total mass-mated population, collected from 3 densely populated sporulation plates for each of the strains, it is clear that a significant degree of genetic diversity has been generated in the process, and that this mass-mated culture can serve as the initial population for enrichment and directed evolution in the chemostat.

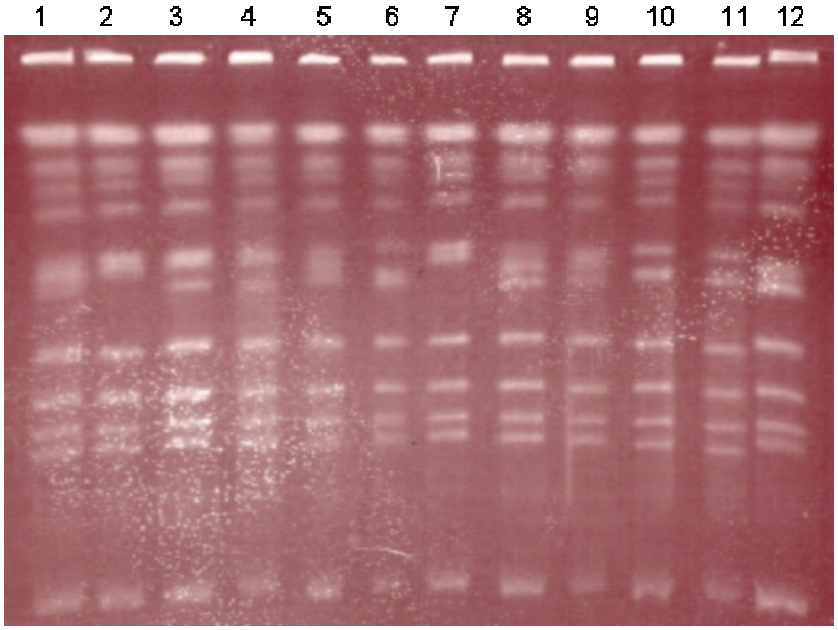


Figure 3.1: Determination of karyotypes of single colonies randomly selected from the culture population yielded by mass-mating of USM21 and BM45 (lanes 1-5, 8-12). Comparison of the banding pattern of the parental strains USM21 (lane 6) and BM45 (lane 7) shows the presence of three strains that are hybrids of these two strains (lanes 4, 5, 8). The other randomly selected strains show banding pattern similar to the parental strains or variations thereof.

3.4.2 Chemostat selection

Two separate selection steps were employed in order to yield yeast strains capable of strong fermentative activity in nitrogen limiting conditions and survival in high ethanol concentrations. The first step addressed the selection for fermentative activity in nitrogen limiting conditions. The media supplied during this procedure contained only 10 % of the average grape must concentrations of the preferentially used nitrogen sources ammonium, aspartatic acid, glutamic acid, arginine and

glutamine. Other amino acids usually found in grape must were supplied in the concentrations as described by Bely et al. (4). Therefore, yeast strains successfully maintaining themselves in these chemostat conditions can be expected to most efficiently use the supplied composition of nitrogen sources, including the less preferred ones.

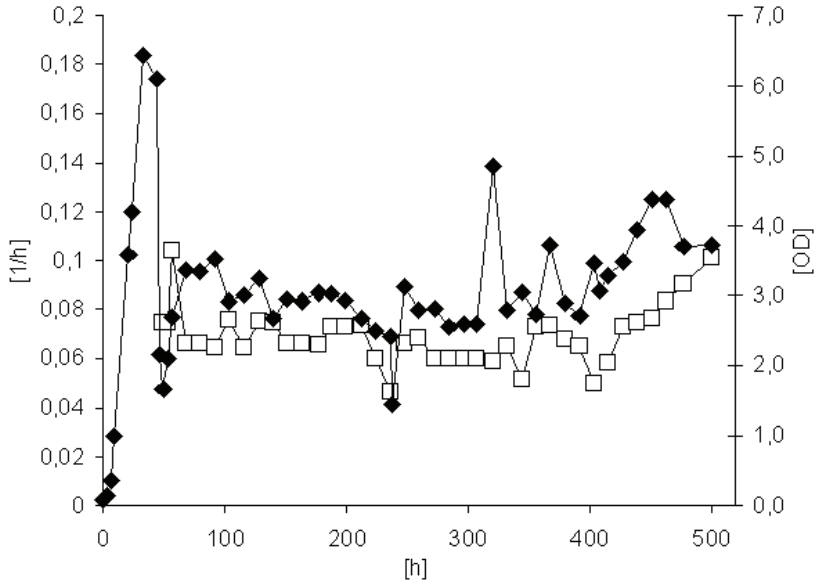


Figure 3.2: Continuous cultivation in nitrogen limiting conditions with a stable dilution rate was started at 44 h after inoculation. The culture density ([OD], ◆) stabilizes around OD 3.0. At the cultivation time of 370 h an increase in the culture density at a stable dilution rate can be observed. The growth rate ([1/h], □) reflects the progression of the culture population in the applied conditions over the cultivation time

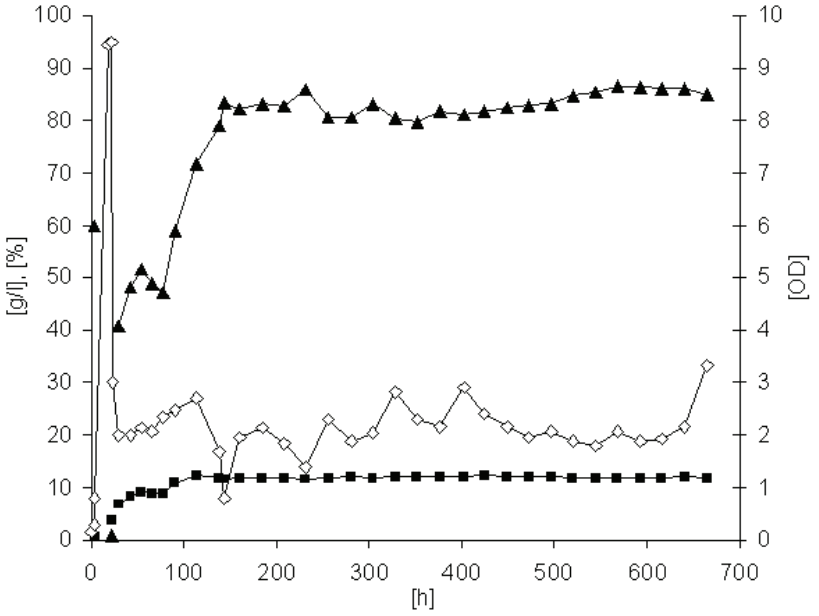
After inoculation, the population of mass-mated cells was cultivated for 44 h in batch conditions before feeding was initiated (Figure 3.2). Oxygen depletion was reached after 3 h of fermentation. The culture showed a growth rate of 0.1 1/h and a doubling time of 317 min calculated from start of inoculation to stationary phase at a culture density OD_{600} of approx. 6.2. The culture had therefore undergone 8 generations before the beginning of the continuous fermentation. The continuous fermentation conditions were kept for 455 h (Figure 3.2), corresponding to 49 generations with an average growth rate of 0.07 1/h (average doubling time of approx. 10 h). By maintaining a low dilution rate it was expected to ensure that the enrichment of yeast

strains would not favor a fast fermentation speed, but rather more efficient nitrogen utilization only. The residual sugar concentration stabilized during fermentation in a range of 20 to 28 g/l, indicating that the hexose supply was not a limiting factor in the cultivation. The ethanol concentration was found in the range of 4 to 4.8 %. It can be expected that yeast strains with low ethanol tolerance would be disadvantaged in these conditions, while efficient wine yeast strains are known to show a much higher ethanol resistance.

After 370 h of continuous cultivation in these conditions an increase in cell density in the continuous conditions was observed. This increase suggests that cells that are better adapted to the specific selective conditions were beginning to out-compete less well adapted cells (Figure 3.2). The increased biomass therefore suggests an improved efficiency of nutrient utilization by the yeast population.

The yeast population obtained in this selection was subjected to a second selection step. Since the first selection had been maintained at low ethanol concentrations, the second selection was designed to eliminate strains that would not be able to withstand the harsh conditions found towards the end of industrial wine fermentations, when ethanol levels are high. The aim of the second enrichment step was the selection of yeast strains showing survival and metabolic activity in a high ethanol concentration in addition to their ability to be fermentative active in nitrogen limiting conditions. The medium was prepared as described for the first selection but supplemented with 11 vol% of ethanol. After inoculation, the culture was grown for 22 h before reaching stationary phase. It showed a growth rate of 0.16 1/h and a doubling time of 258 min (Figure 3.3, A). This corresponds to 5 generations. The culture was diluted with 3 culture volumes of selection medium supplemented with ethanol to a concentration of 11 vol% prior to initializing the chemostat conditions. Simultaneously the culture in the bioreactor was also adjusted to 11 vol% of ethanol. During the first 19 h of continuous cultivation the culture density decreased by 30 %. The system was than modified to allow for re-inoculation of the cells washed out of the fermenter. The chemostat was maintained over 622 h. The culture density varied between 2.0 OD_{600} and 3.0 OD_{600} during cultivation (Figure 3.3, A). The concentration for glucose and fructose stabilized between 80 g/l and 86 g/l indicating continuous uptake and metabolization of the sugars in the high ethanol content, which stabilized at 12%. (Figure 3.3, A). After 622 h the number of colony forming

A



B

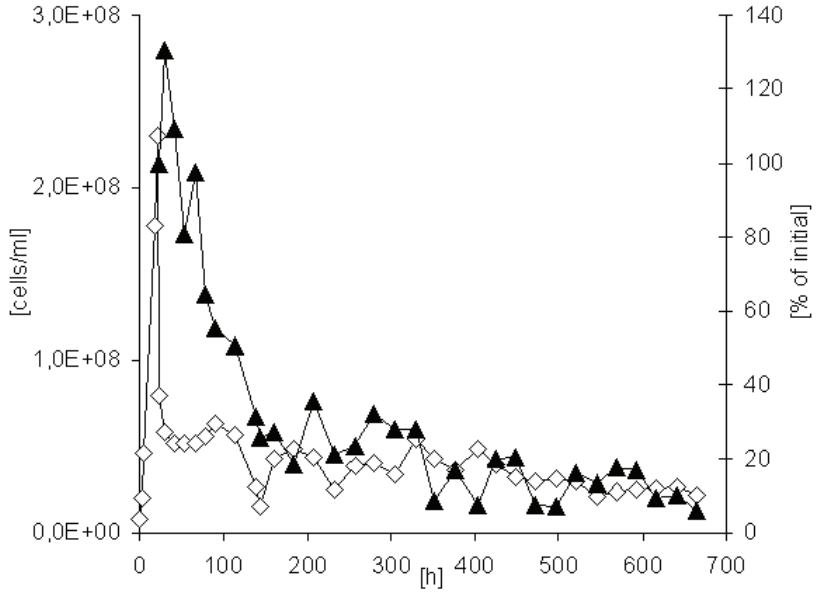


Figure 3.3: Second enrichment step was done in continuous cultivation under nitrogen limiting conditions and supplementation of 11 %(v/v) ethanol. A: Sugar concentration ([g/l], ▲), ethanol ([%], ■) concentration stabilized at 12 % throughout the entire cultivation time. The culture density ([OD], ◇) was found to fluctuate between OD 2.0 and OD 3.0. B: The number of cells/ml (▲) and the number of colony forming units/ml ([% of initial], ◇) decreased constantly over the cultivation time. The enrichment procedure was stopped when the number of colony forming units/ml decreased to less than 10 % of the initially present number.

units (CFU) reached 10 % of the number found initially at the beginning of the chemostat cultivation (23 h of cultivation) and the fermentation was stopped (Figure 3.3, B).

3.4.3 Fermentative performance of the yeast populations obtained at various stages of the selection procedure

To assess the impact of the selective conditions on the overall performance of the mixed cultures, the fermentative activity of the different populations obtained after mass-mating (A), after the selection in nitrogen limiting conditions (B) and after the second selection in nitrogen limiting and elevated ethanol conditions (C) were compared with the performance of the two parental strains USM21 and BM45. Small scale fermentations were done in a synthetic grape must containing 100 g/l glucose and fructose each. The concentration of assimilable nitrogen was adjusted in terms of not providing any ammonium, aspartatic acid, glutamic acid, arginine and glutamine. Only 10 % of the concentration of all other amino acids usually found in natural grape juice were provided (4). In this medium, all fermentations will become stuck because of the lack of nitrogen. However, the total amount of sugar consumed by the different populations at the end of fermentation would provide an indication of their nitrogen efficiency. After 13 days of fermentation, a significant improvement of the culture population B and C in their fermentative activity in comparison to the two parental strains and the culture population yielded by the mass-mating could be seen. At the end of cultivation, the cultures B and C had utilized 29 % more of the initial sugar than parental strain BM45 and 15 % more than the culture population yielded by the mass-mating (A). Parental strain USM21 showed similar sugar utilization as the culture populations B and C (Figure 3.4). The improved performance of the culture populations at various stages of the selection procedure in comparison to BM45 and their equal performance to USM21 were verified in a second experiment. Table 3.1 shows the results of two independent experiments of small scale fermentations in triplicates for each of the tested strains or culture populations.

3.4.4 Evaluation of karyotypes of single strains

57 strains were examined by Pulse-Field-Gelelectrophoresis for their karyotype status. 95 % of these strains proved to be hybrids of the two parental strains. 5 % of the selected strains showed a karyotype similar to BM45. None of the selected strains were found showing a karyotype similar to USM21. The 95 % confidence interval for this experiment is 95 % to 100 %. During the selection procedure the ratio of strains being hybrids of the two parental strains has therefore increased from 6 % to 95 %. The data clearly suggest a very significant enrichment for certain types of strains. The data also suggest that hybrids were better adapted to the specific selective conditions than either of the parental strains.

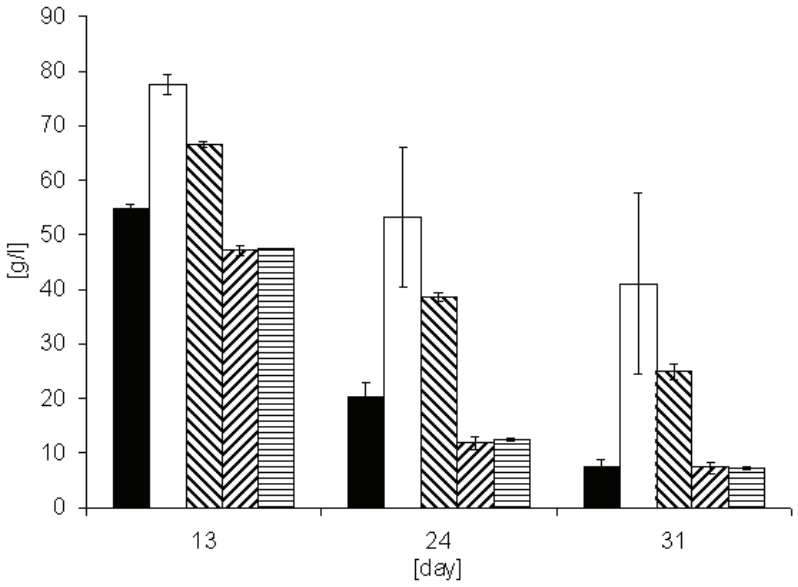


Figure 3.4: Residual sugar concentrations during fermentation. Assessment of the fermentative performance in nitrogen limiting conditions. Culture population after mass-mating (****), after enrichment in nitrogen limiting conditions (***//***) and after enrichment in nitrogen limitation and high ethanol conditions (=), USM21 (■) and BM45 (□). Initial sugar concentration: 100 g/l glucose and fructose each.

3.4.5 Fermentative activity in nitrogen limiting conditions

The medium used to assess the fermentative activity of the selected hybrids in comparison to the two parental strains in nitrogen limiting conditions contained only

Table 3.1: Fermentative performance in nitrogen limiting conditions

Yeast strain	Sugar fermented*	Difference to	
	[%]	USM21 [%]	BM45 [%]
	31 days of fermentation		
USM21	94	0	-29
BM45	65	29	0
A	79	15	-14
B	94	0	-29
C	94	0	-29
	33 days of fermentation		
USM21	96	0	-18
BM45	79	18	0
A	95	1	-17
B	97	-1	-18
C	97	-1	-19

Fermentative performance of culture populations at different stages of the selection process in comparison to the parental strains. Fermentations were done in nitrogen limiting conditions and moderate sugar concentration. A: culture population after mass-mating; B: culture population after enrichment in limiting nitrogen conditions; C: culture population after enrichment in limiting nitrogen and elevated ethanol conditions. * Initial sugar concentration: 100 g/l glucose and fructose each.

10 % of the usually used concentration of assimilable nitrogen and 100 g/l of glucose and fructose each. The sugar utilization and ethanol production determined at different stages of the fermentations showed an improved performance of some of the selected yeast strains especially in the later stage of fermentation (Figure 3.5). The increased sugar utilization and ethanol production becomes significant after 13 days of fermentation. After 16 days of fermentation the differences in remaining sugar between selected strain 167 and the parental strain BM45 is 2.9-fold. The ratio to the parental strain USM21 is 2.4-fold increased sugar utilization. Not all strains examined by this performance study proved to have an improved fermentative activity in the conditions of limited assimilable nitrogen concentration. Selected strain 12 did not show the same degree of sugar utilization as seen for the parental strains

Table 3.2: Fermentative performance of parental and selected strains in nitrogen limiting conditions

Yeast strain	Residual sugar *		Difference to	
	mean [g/l]	SD [g/l]	USM21 [%]	BM45 [%]
USM21	43.8	0.8	0	-5
BM45	53.4	4.1	5	0
167	18.2	1.5	-13	-18
182	20.2	0.7	-12	-17
USM21	42.4	2.8	0	-2
BM45	45.5	3.3	2	0
217	28.4	2.8	-7	-9
USM21	50.9	2.3	0	-6
BM45	62.3	3.1	6	0
241	46.5	1.6	-2	-8
243	44.2	1.5	-3	-9
246	15.7	1.8	-18	-23
249	11.8	1.0	-20	-25
253	33.0	4.2	-9	-15
262	27.0	5.5	-12	-18
263	27.8	2.6	-12	-17
270	40.8	3.4	-5	-11
277	35.3	2.6	-8	-13
USM21	14.8	8.0	0	-4
BM45	23.5	2.9	4	0
15	13.7	2.1	-1	-5
26	19.6	2.2	2	-2
31	7.7	4.7	-4	-8
70	21.8	1.8	3	-1
116	14.3	2.1	0	-5
145	18.7	1.0	2	-2
152	15.7	0.7	0	-4

Fermentative performance of selected strains in comparison to the parental strains. All of the selected strains show higher sugar utilization than BM45. Some of the selected strains show also improved fermentative capacity in comparison to USM21. The tested strains show a wide variety in degree of improvement. Experiments were done in triplicate for each strain. * Initial sugar concentration: 100 g/l glucose and fructose each

(Figure 3.5). 23 selected strains were examined and 52 % proved to show higher

fermentative activity in the selected conditions in comparison to both parental strains. Table 3.2 shows the residual sugar concentrations for all the examined yeast strains once fermentations had become stuck. The difference between the two parental strains was 2 – 6 % higher sugar utilization of USM21. The selected hybrids showed improved fermentative performance in nitrogen limiting conditions ranging from between 1 % and 25 % higher sugar utilization than BM45, a commercial yeast strain known to have a high nutrient demand. The difference in sugar utilization in comparison to USM21 is not as significant for all tested strains with a maximum of 20 %. Some of the selected hybrids showed only higher sugar utilization than BM45.

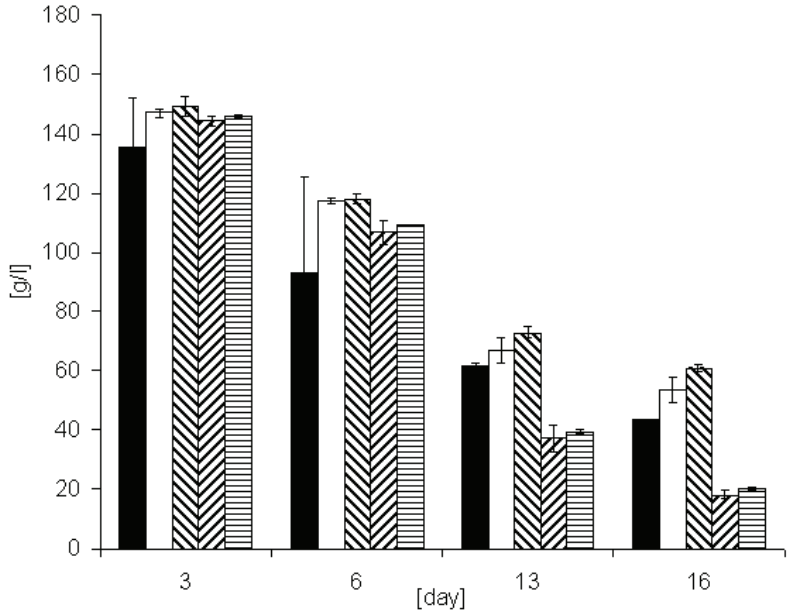


Figure 3.5: Residual sugar concentration in fermentations under nitrogen limiting conditions. Initial sugar concentration was 100 g/l glucose and fructose each. Comparison of the performance of selected colonies 12 (\\), 167 (*//*), 182 (=), parental strain USM21 (■) and parental strain BM45 (□). Colonies 167 and 182 show higher sugar utilization than the two parental strains, while colony 12 exhibits performance comparable to BM45.

3.4.6 Fermentative activity in excess sugar concentrations

17 of the selected strains were examined for their fermentative capacity in a synthetic grape juice containing 150 g/l of glucose and fructose each. Assimilable nitrogen was supplied in a sufficient concentration of 300 mg/l. Table 3.3 shows the remaining sugar concentration at the end of cultivation, when no further fermentative activity was detectable. None of the hybrids managed to show higher sugar utilization than

USM21, a yeast well known for its outstanding fermentative capacity. The strains all showed significant improvement when compared to the other parent, BM45. Sugar utilization was improved by a maximum of 9 %, while the majority of the tested yeast strains showed a 3 % to 4 % improvement

Table 3.3: Fermentative performance in excess sugar and sufficient nitrogen

Yeast strain	Sugar fermented*	Difference to	
	[%]	USM21 [%]	BM45 [%]
USM21	82	0	-12
BM45	70	12	0
15	76	6	-6
26	79	3	-9
31	76	6	-8
116	76	6	-6
145	72	10	-2
167	74	8	-4
182	72	10	-2
217	73	9	-3
241	73	9	-3
243	73	9	-3
246	72	10	-2
249	70	12	0
253	73	9	-3
262	73	9	-4
263	73	9	-3
270	74	8	-4
277	74	8	-4

Comparison of the fermentative performance of selected strains and the parental strains in high sugar and excess nitrogen concentrations as found in a high quality grape must. Results are mean values at the end of fermentation of two independent experiments each done in triplicates. Initial sugar concentration: 150 g/l of glucose and fructose each.

3.5 Conclusions

Our data show, that a large number of individually assessed strains present a significantly improved fermentative performance under nitrogen limiting conditions.

Around half of those strains performed better than both parents. Considering that both parents are well established and successful commercial strains, these data clearly demonstrate the potential of further improvement of such strains.

This is the first study showing the successful application of a strategy combining mass-mating and enrichment under nitrogen-limiting conditions and elevated ethanol concentrations for the generation of yeast strains optimized for their nitrogen utilization characteristic. Strains generated by the strategy show significantly higher sugar utilization under nitrogen limiting conditions in comparison to both parental strains when assessed in laboratory scale fermentations. Additionally, a high percentage of the improved strains displays increased fermentative performance in comparison to at least one of the parental strains under conditions of a high sugar synthetic must that contains sufficient nitrogen sources.

Experimental scale wine fermentations of Pinotage and Colombard must showed a significant potential of the selected strains for commercial winemaking. Sensorial evaluation of the produced wines revealed comparable ratings of the examined selected strains and the parental strains (data not shown).

We therefore suggest the strategy introduced in this study could become a successful methodology to generate new yeast strains of desired fermentative characteristics.

3.6 Acknowledgements

This work was supported by Lallemand Inc, Canada. The authors would like to thank Dr. M. Kidd for assistance in statistics.

3.7 Literature cited

1. **Ancin, C., B. Ayestaran, and J. Garrido.** 1996. Sedimentation clarification of Garnacha musts. Consumption of amino acids during fermentation and aging. Food Res. Int. **29:**345-353.

2. **Ayestaran, B., J. Garrido, and C. Ancin.** 1998. Sedimentation clarification of viura musts. Utilization of amino acids during fermentation. Food Chem. **63:**191-197.

3. **Bataillon, M., A. Rico, J.-M. Sablayrolles, J.-M. Salmon, and P. Barre.** 1996. Early thiamin assimilation by yeasts under enological conditions: Impact on alcoholic fermentation kinetics. J. Ferment. Bioeng. **82:**145-150.

4. **Bely, M., J.-M. Sablayrolles, and P. Barre.** 1990. Automatic detection of assimilable nitrogen deficiencies during alcoholic fermentation in oenological conditions. J. Ferment. Bioeng. **70:**246-252.

5. **Bisson, L. F., and C. E. Butzke.** 2000. Diagnosis and rectification of stuck and sluggish fermentations. Am. J. Enol. Vitic. **51:**168-175.

6. **Cooper, T. B.** 1982. Nitrogen metabolism in *Saccharomyces cerevisiae*, p. 39-99. *In* J. N. Strathern, E. W. Jones, and J. R. Broach (ed.), The molecular biology of the yeast *Saccharomyces*: Metabolism and gene expression. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

7. **Doignon, F., and N. Rozes.** 1992. Effect of triazole fungicides on lipid metabolism of *Saccharomyces cerevisiae*. Lett. Appl. Microbiol. **15:**172-174.

8. **Drake, J. W.** 1991. Spontaneous mutation. Annu. Rev. Genet. **25:**125-146.

9. **Dykhuizen, D. E.** 1993. Chemostats used for studying natural selection and adaptive evolution, p. 613-631, Methods Enzymol., vol. 224. Academic Press Inc.

10. **Dykhuizen, D. E., and D. L. Hartl.** 1983. Selection in chemostats. Microbiol. Rev. **47:**150-68.

11. **Fornairon-Bonnefond, C., V. Demaretz, E. Rosenfeld, and J.-M. Salmon.** 2002. Oxygen addition and sterol synthesis in *Saccharomyces cerevisiae* during enological fermentation. J. Biosci. Bioeng. **93:**176-182.

12. **Gafner, J., and M. Schütz.** 1996. Impact of glucose-fructose-ratio on stuck fermentations. Vitic. Enol. Sci. **51:**214-218.

13. **Heidenreich, E.** 2007. Adaptive Mutation in *Saccharomyces cerevisiae*. Crit. Rev. Biochem. Mol. Biol. **42:**285 - 311.

14. **Henick-Kling, T., and I. M. Larsson.** 1992. The effect of juice nitrogen composition and yeast strain on the formation of ethyl carbamate. Biolog. Oggi **6:**161-164.

15. **Herskowitz, I.** 1988. Life cycle of the budding yeast *Saccharomyces cerevisiae*. Microbiol. Rev. **52:**536-553.

16. **Higgins, V. J., P. J. L. Bell, I. W. Dawes, and P. V. Attfield.** 2001. Generation of a novel *Saccharomyces cerevisiae* strain that exhibits strong maltose utilization and hyperosmotic resistance using nonrecombinant techniques. Appl. Environ. Microbiol. **67:**4346-4348.

17. **Jiranek, V., P. Langridge, and P. A. Henschke.** 1991. Presented at the Proceedings of the International Symposium on Nitrogen in Grapes and Wines, Washington.

18. **Kunkee, R. E., and R. W. Goswell.** 1977. Table wine, p. 5-72. *In* A. H. Rose (ed.), Economic Microbiology. Academic Press, London.

19. **Lenski, R., and M. Travisano.** 1994. Dynamics of adaptation and diversification: A 10.000-generation experiment with bacterial populations. Proc. Nat. Acad. Sci. U.S.A. **91:**6808-6814.

20. **Lenski, R. E., J. A. Mongold, P. D. Sniegowski, M. Travisano, F. Vasi, P. J. Gerrish, and T. M. Schmidt.** 1998. Evolution of competitive fitness in

experimental populations of *E. coli*: What makes one genotype a better competitor than another? Antonie van Leeuwenhoek **73:**35 - 47.

21. **Lindegren, C. C., and G. Lindegren.** 1943. Selecting, inbreeding, recombining and hybridizing commercial yeasts. J. Bacteriol. **46:**405-419.

22. **Manginot, C., J. L. Roustan, and J. M. Sablayrolles.** 1998. Nitrogen demand of different yeast strains during alcoholic fermentation. Importance of the stationary phase. Enzyme Microb. Technol. **23:**511-517.

23. **Nakazawa, N., K. Okawa, T. Sato, H. Enei, and S. Harashima.** 1999. Mass mating method in combination with G418- and aureobasidin A-resistance markers for efficient selection fo hybrids from homothallic strains in *Saccharomyces cerevisiae*. J. Biosci. Bioeng. **88:**468-471.

24. **Nathmyth, K.** 1993. Regulating the *HO* endonuclease in yeast. Curr. Opin. Genet. Dev. **3:**361-368.

25. **Ough, C. F., and M. A. Amerine.** 1988. Nitrogen compounds, p. 172-195, Methods for Analysis of Must and Wines. Wiley-Interscience, New York.

26. **Pretorius, I. S.** 2000. Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. Yeast **16:**675-729.

27. **Pretorius, I. S., and F. F. Bauer.** 2002. Meeting the consumer challenge through genetically customized wine-yeast strains. Trends Biotechnol. **20:**426-432.

28. **Ribereau-Gayon, P.** 1985. New developments in wine microbiology. Am. J. Enol. Vitic. **36:**1-10.

29. **Sablayrolles, J.-M., C. Dubois, C. Manginot, J.-L. Roustan, and P. Barre.** 1996. Effectiveness of combined ammoniacal nitrogen and oxygen additions for completion of sluggish and stuck wine fermentations. J. Ferment. Bioeng. **82:**377-381.

30. **Sauer, U.** 2001. Evolutionary engineering of industrially important microbial phenotypes, p. 129-169. *In* T. Scheper (ed.), Advances in Biochemical Engineering/Biotechnology, vol. 73. Springer Verlag, Berlin Heidelberg.

31. **Schütz, M., and J. Gafner.** 1995. Lower fructose uptake capacity of genetically characterized strains of *Saccharomyces bayanus* compared to strains of *Saccharomyces cerevisiae*: A likely cause of reduced alcoholic fermentation activity. Am. J. Enol. Vitic. **46:**175-180.

32. **Schütz, M., and J. Gafner.** 1993. Sluggish alcoholic fermentation in relation to alterations of the glucose-fructose ratio. Chemie, Microbiologie, Technologie der Lebensmittel **15:**73-78.

33. **Schwartz, D. C., and C. R. Cantor.** 1984. Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. Cell **37:**67-75.

34. **Tegmo-Larsson, I. M., and T. Henick-Kling.** 1990. The effect of fermentation and extended lees contact on ethyl carbamate formation in wine. Am. J. Enol. Vitic. **41:**269-272.

35. **van Vuuren, H. J., and C. J. Jacobs.** 1992. Killer yeasts in the wine industry: a review. Am. J. Enol. Vitic. **43:**119-128.

36. **Viegas, C. A., M. F. Rosa, I. SA-Correia, and J. M. Novais.** 1989. Inhibition of yeast growth by octanoic and decanoic acids produced during ethanol fementation. Appl. Environ. Microbiol. **55:**21-28.

37. **Viviani-Nauer, A., P. Hoffmann-Boller, and J. Gafner.** 1997. In vitro detection of folpet and its degradation to phthalimide in aqueous solutions and in yeast suspensions. Am. J. Enol. Vitic. **48:**63-66.

38. **Viviani-Nauer, A., P. Hoffmann-Boller, and J. Gafner.** 1997. In vivo detection of folpet and its metabolite phthalimide in grape must and wine. Am. J. Enol. Vitic. **48:**67-70.

Chapter 4

RESEARCH RESULTS II

Evaluation of mutagenesis and mass-mating in combination with selection in continuous cultivation for the generation of wine yeast strains exhibiting reduced discrepancy in glucose and fructose utilization

4 Research Results II

Evaluation of mutagenesis and mass-mating in combination with selection in continuous cultivation for the generation of wine yeast strains exhibiting reduced discrepancy in glucose and fructose utilization

4.1 Abstract

In winemaking conditions, glucose and fructose are initially present in close to equimolar amounts. However, on-going fermentations are characterized by glucose concentrations that decrease faster than fructose concentrations, leading to a decrease in the glucose on fructose ratio (GFR). Furthermore, all wine yeast strains that have been evaluated show preference for glucose utilization under fermentative conditions. Previous studies have shown that low GFR ratios can be a causative factor of stuck and sluggish fermentations. Wine yeast strain that would utilize fructose with similar efficiency than glucose could therefore contribute significantly to reducing the likelihood of problem fermentations. In this work, an evolutionary engineering approach was attempted to generate such yeast. Genetically diverse populations were obtained either through mass-mating of two commercial wine yeast strains or through mutagenesis of the better performing of those two strains. These populations were exposed to continuous cultivation in a selective environment with a low GFR. The data show that the majority of strains that were present at the end of this selection procedure showed improved fructose utilization since they were able to out-compete the better performing parental strain in the specific conditions applied in the selection procedure. Assessment of the discrepancy in glucose and fructose utilization under conditions mimicking wine fermentation revealed a tendency of such strains to maintain a higher GFR ratio. This trend was observed in strains selected from both initial yeast populations.

4.2 Introduction

Stuck and sluggish fermentations are a severe problem and lead to significant financial losses to the global wine industry (25). Many comprehensively reviewed parameters negatively influence fermentation activity in winemaking conditions, and

may lead to such problem fermentations (1, 4). In industrial conditions, the data suggest that such problem fermentations are frequently due to the synergistic effect of two or more parameters.

One such identified parameter is the relative concentration of the two dominant hexoses that are found in grape must, glucose and fructose. This parameter is commonly expressed as the glucose on fructose ratio (GFR). Glucose and fructose are usually found in close to equimolar amounts in grape musts, with slight variations depending in particular on the maturity and on the health status of the grapes. Wine yeast strains, mostly of the species *Saccharomyces cerevisiae* (*S. cerevisiae*), however, display a preference for glucose utilization (3). While this preference has been described for all wine yeast strains investigated thus far, its extent appears strain and environmental condition dependent (3). Due to this preference, the GFR tends to diminish during the course of the fermentation process. Fermentations that reach a low GFR early in fermentation are prone to show declining fermentative activity, resulting in a stuck or sluggish process. Indeed, samples of random stuck fermentations examined for their GFR all showed values of below 0.1. In these cases, the occurrence of stuck and sluggish fermentations was mostly observed after 80 % of the sugars had been converted (9). Wucherpfennig et al. (33) determined the range of GFR found at various stages of successful wine fermentations and found values of 0.1 and below to normally only occur once at least 90 % of the initial sugars had been consumed. Therefore, the evolution of the GFR in these stuck fermentations can be considered to be significantly different from that of non-problem fermentations. Further studies confirmed that a low GFR can be a primary causative factor in stuck fermentations by showing that artificial decreases in GFR resulted in fermentations becoming stuck, and that many stuck fermentations could be cured by the addition of glucose alone to elevate the GFR above a threshold of 0.1 (28). As a consequence, a low GFR was discussed as a significant cause of stuck and sluggish fermentations by different authors (6, 9, 28, 33). A wine yeast strain that would exhibit a very low or even no discrepancy in glucose and fructose utilization would therefore present a significant advantage to the wine industry. Fermentations with such a strain would show similar or close to similar glucose and fructose utilization and eliminate a significant source of reduced fermentative activity.

Evolutionary engineering is a powerful tool for the generation of industrially important microorganisms exhibiting desired phenotypes. This strategy is based on

evolutionary principles, selecting strains that are best adapted for specific selective environmental conditions from a diverse and evolving population. It is different from metabolic engineering of single strains, where recombinant DNA technology is applied to introduce specific traits. Evolutionary engineering has the advantage that no prior knowledge of the molecular nature of the specific trait to be improved is required. Absence of such knowledge renders metabolic engineering approaches impossible to implement. Furthermore, many traits are governed by complex metabolic or signaling networks, and are not easily amenable to more directed interventions (27).

Recently, adaptive evolution was evaluated for its potential for the generation of wine yeast strains exhibiting generally improved fermentative performance (18). This strategy relies on the occurrence of advantageous mutations to generate strains that are better adapted to whatever selective conditions are applied. To be efficient, this method demands the maintenance of selective conditions over many generations and a suitable method for the detection of adaptive events that would emerge in the population (7). Contrary to an approach that would be solely based on adaptive evolution, evolutionary engineering offers the advantage of starting off with a highly diverse yeast population that may already contain the improved strains that are adapted to the selection criteria within the initial population (27).

Such genetically highly variable populations can be generated by exposure of yeast strains to procedures that cause recombination or direct alteration of DNA. Hybridization is the method of choice if the properties of two well adapted yeast strains are sought to be combined. Depending on the genetic properties of the strains to be combined various methods are available (25). Mass-mating was chosen in this study as a strategy for the generation of a varied yeast population. In a mass-mated culture, it is expected that the advantageous properties of the strains to be hybridized will be combined in at least some of the descendents. Descendants may also show new characteristics that were not phenotypically expressed in the parental strains. This method is particularly suitable if the strains to be mated sporulate efficiently. The mixture of the spores of both strains generates a potentially infinite number of new yeast strains that randomly combine the genetic background of the two parental strains.

Mutagenesis was chosen as the second method to generate genetic variability in this study. This method does not have an equally high potential to induce variability as hybridization, since it is based on changing the existing genetic blueprint of an existing single strain. It is nevertheless the method of choice for many strain development programs (25). Mutagenesis by exposure to ethyl methanesulfonate (EMS) is a well established method and has been extensively reviewed (23, 30). In *S. cerevisiae* 95 to 99 % of the genetic modifications introduced by EMS treatment are base-pair substitution mutations. The majority are GC to AT transitions. EMS causes more base-pair substitutions resulting in missense mutations than other mutagen agents. The introduction of mutations by EMS in *S. cerevisiae* was shown to be dependent on a functional repair system (30). EMS is together with UV-treatment and nitroso-methyl guanidine (NTG) one of the preferred mutagens for many applications, because it allows the introduction of a great variety of molecular alterations with no apparent specificity for genomic sub regions.

In the strategy applied here, the genetically altered populations were exposed to specific selection pressures in a chemostat. Continuous cultivation in a chemostat is a mean to provide a constant, homogeneous environment in which cells grow at a constant rate. Nutrients are added continually at a defined rate generating a stable nutrient limitation situation. Simultaneously, the medium, including yeast cells, is removed from the culture vessel at the same rate. At equilibrium, the growth rate of the culture population equals the dilution rate as fixed through the flux of the medium supply (8). Since the removal of the cells from the growth chamber by outflow is random, the growth rate of each individual cell will become the main factor deciding the continuous presence of any given strain within the culture population (27). Due to the selective character of the chemostat, disadvantageous mutations will be eliminated over time.

The aim of this study was the comparative evaluation of the two approaches, mass-mating and mutagenesis of two commercially successful wine yeast strains in combination with selection in chemostat continuous cultivation, for their potential to generate yeast strains of decreased discrepancy in glucose and fructose utilization under fermentative conditions.

The data show that the selection in continuous cultivation under fructose limitation yields yeast strains of improved fermentative performance in comparison to the

parental strain if cultivated in direct competition in the conditions applied in the selection procedure. The improvement was found for strains of both populations. The selected strains were found to exhibit decreased discrepancy in glucose and fructose utilization in comparison to W15 in laboratory scale conditions mimicking wine fermentation. Therefore, we are able to show the potential of the methodology for the generation of the desired phenotype, although the final results are less pronounced than desired.

To the best of our knowledge, this is the first study employing such a methodology to evolutionary engineer the fructose utilization rate of wine yeast strains.

4.3 Materials and Methods

4.3.1 Yeast strains

Lalvin W15 (W15, Lallemand Inc) is a wine yeast strain that is commercially available. This yeast strain was selected and isolated in the laboratory of J. Gafner. W15 was taxonomically classified as *S. cerevisiae*. The ploidy status of the strain is diploid and shows homozygosity for 10 different microsatellites located on 8 different chromosomes (R. C. Gardner, personal communication). Uvaferm 43 is also a commercially available wine yeast strain. It is classified as *Saccharomyces cerevisiae bayanus* (Lallemand Inc). The ploidy status of this yeast has been described as diploid, determined by propidium iodine fluorescence and flow cytometry (5). However, characterizing Uvaferm 43 by microsatellite-analysis shows three bands for two of the evaluated loci. This indicates either local amplifications or rearrangements of parts of its genome or that the strain is not truly diploid (5). Colonies of these strains were isolated from lyophilized samples of the yeast strain collection or from rehydrated dried yeast preparations. For preparation of freeze cultures as source for inoculation of further experiments a single colony of each strain was grown over night at 30 °C under aerobic conditions in 2 % YPD (10 % yeast extract, 20 % peptone (BD Biosciences) and 20 % glucose (Fluka)). Freeze cultures were prepared in 30 % glycerol and stored at –80 °C. Storage plates were prepared on 2 % YPD agar plates (10 % yeast extract, 20 % peptone (BD Biosciences), 20 % glucose (Fluka) and 20 % agar (Acros Chemicals)) which were kept at 4 °C.

A kanamycin resistant strain of the W15wt was generated (W15kanMX). The generation of the modified W15kanMX strain is object to confidentiality due to patent issues (F. Bauer, personal communication). W15kanMX is cultured in 2 % YPD and stored as freeze culture at -80 °C in 30 % glycerol. Additionally, single colonies of the strain are stored on 2 % YPD agar plates at 4 °C. Stability of integration of the kanamycin resistance was evaluated by cultivation in 2 %YPD over 100 generations.

Strains selected within this study after selection in continuous cultivation of the EMS treated W15 culture population are 15m2, 15m3, 15m37 and 15m38. Strains selected within this study after selection in continuous cultivation of the population yielded by mass-mating of W15 and Uvaferm 43 are 1543 2, 1543 3, 1543 4 and 1543 5.

4.3.2 Synthetic grape must (MS300)

The synthetic grape must MS300 was prepared as published by Bely M. et al. (2). All reagents were supplied by Sigma, Fluka or Acros Chemicals. The medium contained (per liter) glucose and fructose in the concentrations according to the needs of each experiment, 6 g citric acid, 6 g malic acid, 0.75 g KH_2PO_4, 0.5 g K_2SO_4, 0.25 g $MgS0_4 \times 7H_2O$, 0.155 g $CaCl_2 \times 2H_2O$, 0.2 g NaCl, 0.46 g NH_4Cl, 1 ml stock of minerals (4 mg/ml $MnSO_4 \times H_2O$, 4 mg/ml $ZnSO_4$, 1 mg/ml $CuSO_4 \times 5H_2O$, 1 mg/ml KI, 0.4 mg/ml $CoCl_2 \times 6H_2O$, 1 mg/ml H_3BO_3, 1 mg/ml $(NH_4)_6Mo_7O_{24}$; dissolved in dH_2O), 10 ml stock of vitamins (2 mg/ml myo-inositol, 0.15 mg/ml pantothenic acid, 0.025 mg/ml thiamine, 0.2 mg/ml nicotinic acid, 0.025 mg/ml pyridoxine, 3 µg/ml biotin; dissolved in dH_2O), 1 ml stock of anaerobic factors (15 mg/ml ergosterol, 5 mg/ml oleic acid (dissolved in 1:1 ethanol and Tween 80), 13 ml stock of amino acids (1.4 mg/ml tyrosine, 13.7 mg/ml tryptophane, 2.5 mg/ml isoleucine, 3.4 mg/ml aspartatic acid, 9.2 mg/ml glutamic acid, 28.6 mg/ml arginine, 3.7 mg/ml leucine, 5.8 mg/ml threonine, 1.4 mg/ml glycine, 38.6 mg/ml glutamine, 11.1 mg/ml alanine, 3.4 mg/ml valine, 2.4 mg/ml methionine, 2.9 mg/ml phenylalanine, 6.0 mg/ml serine, 2.5 mg/ml histidine, 1.3 mg/ml lysine, 1.0 mg/ml cysteine, 46.8 mg/ml proline; dissolved in 2% $NaHCO_3$). Glucose, fructose and salts were dissolved in dH_2O and the pH was set to pH 3.5 with 10 M NaOH. The solution was sterilized at 121 °C for 15 min. The stock of anaerobic factors was considered to be sterile due to the content of 50 % EtOH. Stocks of minerals, vitamins and amino acids were filter

sterilized before addition to the medium. All stocks were added in the appropriate amounts after sterilization.

4.3.3 Mutagenesis by ethyl methanesulfonate (EMS)

100 ml of 2 % YPD were inoculated by W15 and cultivated over night at 30 °C in aerobic conditions. Cell density of the over night culture was determined by haemocytometer. A culture volume containing $1x10^8$ cells in total was spun down at 3,000 RPM for 3 min. The supernatant was removed and cells were washed in 5 ml of sterile dH_2O. Cells were washed in 0.1 M sodiumphospate buffer pH 7.0 and resuspended in 1.7 ml 0.1 M sodiumphosphate buffer pH 7.0. The resuspended cells were transferred to a glass culture tube and 50 µl of EMS solution (9.4M, Sigma) were added. As control, the same amount of cells was harvested in the same way as described above but was not treated by EMS. The cultures were incubated at 30 °C for 20, 30, 40, 50 and 60 min. 8 ml of sterile 5 % sodium thiosulfate solution were added to stop the mutagenesis reaction. The cell suspension was spun down and the supernatant discarded. Cells were suspended in 9 ml of sterile dH_2O and streaked out in an appropriate dilution on 2 % YPD agar plates for determination of colony forming units. The incubation for 50 min led to a survival rate of the treated culture population of 60 % and was therefore further used. The treated cells were inoculated to 100 ml 2 % YPD and incubate over night at 30 °C in aerobic conditions. Freeze cultures of the over night cultures were prepared in 30 % glycerol and stored at -80 °C.

4.3.4 Mass-mating of W15 and Uvaferm 43

A single colony of each yeast strain was cultivated in 2 % YPD medium over night at 30 °C under aerobic conditions. Cultures were re-inoculated into 100 ml of pre-sporulation medium (0.8 % yeast extract (BD Bioscience), 0.3 % peptone (BD Bioscience) and 10 % glucose) at an OD_{600} of 0.05. Cultures were incubated for 5 h at 22 °C under aerobic conditions. Cell cultures were spun down at 4,000 RPM for 6 min and washed twice with sterile dH_2O. Cells were inoculated into 100 ml of sporulation medium (0.5 % potassium acetate, 0.1 % yeast extract and 0.05 % glucose) and incubated at 22 °C under aerobic conditions for 5 days. Sporulation efficiency was evaluated by microscopy. Ascospores were harvested at 4,000 RPM

for 8 min and washed with dH_2O. Addition of 6 ml lysis buffer (5 mg/ml Lyticase (980 U/mg) in 1 M D-sorbitol) was followed by 45 min of incubation at 30 °C. Samples were spun down at 4,000 RPM for 8 min and washed with dH_2O. The spores were resuspended in 500 µl dH_2O and mixed. The mixture was plated on PY plates (Phyton yeast extract agar (DB Biosciences)) in portions of 200 µl. Plates were incubated for 3 days at 30 °C. The culture population was harvested by resuspension in 1 ml dH_2O and inoculated to 2 % YPD medium. The culture was grown over night at 30 °C in aerobic conditions. Freeze cultures of the over night culture were prepared in 30 % glycerol and appropriate dilutions were plated on PY plates yielding single colonies for the evaluation of their genetic status.

4.3.5 Determination of glucose-, fructose- and ethanol concentration

Enzymatic determination of glucose-, fructose- and ethanol concentrations was done by a Konelab 20XT (Thermo Scientific). Determination of glucose was done using the Enzytec™ fluid D-Glucose reagents (Scil Diagnostics). Fructose was determined by Enzytec™ fluid Glucose/Fructose reagents. The Enzytec™ fluid Ethanol test kit was used for the determination of ethanol concentrations. Fermentation supernatants to be examined were collected, centrifuged (5 min at 13,000 RPM) and stored at -20 °C if not examined immediately. Samples were pre diluted in dH_2O according to the specific measuring range of the test kits.

4.3.6 Selection in continuous cultivation

The selection of the two newly generated culture populations was undertaken in continuous cultivation in a BBraun Biotech Bioreactor Quattro System. Agitation was maintained at 100 RPM. The bioreactor was closed by a sterile exhaust filter allowing pressure compensation. Active oxygen intake was not supplied throughout fermentation. Fermentation temperature was set at 20 °C. The working volume was 300 ml with a total vessel volume of 500 ml. The medium supplied for selection of fructophilic yeast strains was MS300 with adjusted sugar concentrations. The total sugar concentration was 25 g/l. The glucose / fructose ratio (GFR) was adjusted from 0.2 to 0.1, 0.05 and finally 0.02 during the selection procedure. After an initial batch phase to grow up the inoculated culture population, the dilution rate for continuous cultivation was maintained at a flow of 0.28 ml / min resulting in a doubling time of

12.4 h. Excess culture medium was continuously removed by overflow control. An amount of 2 ml of culture population were periodically withdrawn for determination of glucose-, fructose- and ethanol concentration. In addition, optical density (OD_{600}) and the number of colony forming units (CFU) were monitored. The pre culture for the selection process was prepared by inoculation of 100 ml 2 % YPF (10 % yeast extract (BD Biosciences), 20 % peptone (BD Biosciences), 20 % fructose (Fluka)) with freeze culture of the entire culture population obtained after mutagenesis of W15 or after mass-mating of W15 and Uvaferm 43. The pre-culture was grown at 30 °C over night in aerobic conditions. The culture volume to be inoculated was spun down at 3,000 RPM for 5 min. The supernatant was discarded and the cell pellet resuspended in MS300 (GFR=0.2) before inoculation.

4.3.7 Direct competition in continuous cultivation

The direct competition in continuous cultivation was conducted in the BBraun Quattro Bioreactor system. The technical set-up was identical to the set-up for the selection procedure in continuous cultivation. MS300 with a sugar concentration of 25 g/l and either a GFR of 0.02, or only glucose were used as feeding media. The dilution rate was set to 0.34 ml/min corresponding to a generation time of 10.2 h. The feeding of the medium started directly after inoculation of the two competing yeast strains. The reference strain in the competition in continuous cultivation was W15kanMX. Selected strains were not marked. This allowed the determination of the evolution of culture composition during fermentation time by replica plating on G418 containing agar plates. The strains were prepared by inoculation of 5 ml of 2 % YPF medium with freeze cultures. The inoculated samples were incubated over night at 30 °C in aerobic conditions. 500 µl of the over-night cultures were re-inoculated to 100 ml 2 % YPF medium, followed by over-night incubation at 30 °C. Cell density and OD_{600} of the pre cultures were determined and an appropriate volume was spun down at 3,000 RPM for 5 min. The supernatant was discarded and the cells were resuspended in the medium supplied during the direct competition cultivation. W15kanMX and the selected strain to be tested were simultaneously inoculated to the culture vessel in a concentration of $5x10^6$ cells/ml of each strain. Samples were taken periodically to determine OD_{600}, glucose-, fructose- and ethanol concentration.

Culture composition was monitored by replica plating. The continuous cultivation was conducted for at least 20, but no more than 30 generations.

4.3.8 Determination of culture composition by replica plating

Dilution series of the samples taken from direct competition cultivations was prepared in 0.9 % NaCl solution. 100 µl of the appropriate dilutions was streaked out on YD agar plates (5 % yeast extract (DB Bioscience), 20 % glucose (Fluka), 25 % agar (Acros Chemicals)). Plates were incubated at 30 °C for two days to generate single colonies. 60 colonies were picked and spotted on YD agar plates. Spotted colonies were replica plated on YD agar plates containing 75 mg/l G418 (5 % yeast extract (DB Bioscience), 20 % glucose (Fluka), 25 % agar (Acros Chemicals), 1.5 ml G418 solution (50 mg/ml in dH_2O)(Sigma)). The ratio of G418 resistant colonies to colonies lacking the resistance was determined after incubation of the plates for two days. The proportion of the two strains, present during cultivation, allowed the determination of their differential growth rate per hour (s), which is also a measure for the rate of displacement (8).

4.3.9 Evaluation of fermentative performance in laboratory scale

Yeast strains or culture populations to be evaluated were inoculated from a freeze culture into 5 ml 2 % YPF and incubated over-night at 30 °C under aerobic conditions. 100 µl of the over night culture was re inoculated to 5 ml of 2 % YPF and again incubated over night at 30 °C under aerobic conditions. Laboratory scale fermentations were conducted in “Müller-Thurgau” grape must, stuck fermentations of Pinot Noir, Dole Blanc and Pinot Blanc or MS300 containing the desired amounts and ratio of glucose and fructose. Inoculation was done at a cell density of $1x10^6$ cells/ml or at OD_{600} 0.2. Fermentations in 100 ml scale were done in 100 ml Erlenmeyer flasks closed by a cotton wool plug, without agitation at 20 °C. Fermentations in 300 ml scale were done in 500 ml bottles closed by a fermentation cap maintaining agitation at 100 RPM by magnetic stirrer. The temperature was kept at 20 °C throughout fermentation

4.3.10 DNA extraction

All reagents were supplied by Sigma or Fluka unless stated otherwise. A 5 ml culture of yeast was grown up in 2 % YPD over-night at 30 °C in aerobic conditions. 1.5 ml of the over-night culture were spun down for 5 min at 3,000 RPM at room temperature. The supernatant was discarded and cells resuspended in 0.5 ml dH_2O. The samples were centrifuged at high speed for 5 seconds at room temperature. The supernatant was discarded and the cell pellet disrupted by brief vortexing. The cells were resuspended in 200 µl breaking buffer (2 % (v/v) Triton X-100, 1 % (v/v) SDS, 100 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0), 0.3 g glass beads and 200 µl phenol/chloroform/isoamyl alcohol mixture (25/24/1) were added followed by vortexing at highest speed for 5 min. 200 µl TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM Na_2EDTA (pH 8), pH 8.0) were added and vortexed briefly. The samples were spun down at 13,000 RPM for 5 min at room temperature and the upper aqueous layer was transferred to a new tube. 1 ml of ethanol (≥99.8 %) was added and the tubes mixed by inversion. The samples were spun down at 13,000 RPM for 3 min at room temperature. The supernatant was removed and the pellet resuspended in 400 µl TE buffer (pH 8.0). 30 µl of 1 mg/ml RNase A were added. The sample was mixed by inversion and incubated for 5 min at 37 °C. 10 µl of 4 M NH_4OAc and 1 ml of ethanol (≥99.8 %) were added. The tube was mixed by inversion and spun down for at 13,000 RPM for 3 min at room temperature. The supernatant was discarded and the pellet dried for at least 2 h at 37 °C. DNA was resuspended in 100 µl TE buffer (pH 8.0).

4.3.11 PCR- and RFLP-analysis

PCR-analysis was done employing primers targeting δ elements in the genome of *S. cerevisiae* strains (d1: 5'-CAAAATTCACCTATATCTCA-3'; d2: 5'-GTGGATTTTTATTCCAACA-3') as published by Ness et al. (19). The PCR reaction mixture contained 0.1 U/µl *Taq* DNA polymerase (Fermentas; #EP0072), 2.5 mM $MgCl_2$, 0.2 mM for each dNTP (Fermentas), 1 µM of each primer and 1x reaction buffer (based on $(NH_4)_2SO_4$; Fermentas) and 10 ng of DNA template. Reactions were done in a volume of 25 µl. The amplification was carried out in a thermal cycler (Techne) and initiated by an incubation of 5 min at 95 °C, followed by 36 cycles of 95 °C/1 min, 45 °C/1 min, 72 °C/1.5 min and a finale extension of 5 min at 72 °C. The

amplification result was analyzed on a 1.5 % multi-purpose agarose gel by gel electrophoresis (BioRad).

The PCR product was further analyzed by RFLP-analysis. Digestions were carried out with the restriction enzymes *Rsa*I, *Hha*I and *Alu*I (Fermentas). Digestion reaction was 15 µl containing 1x enzyme buffer, 0.17 U/µl restriction enzyme and 5 µl PCR product. Incubation was for at least 2 h at 37 °C. The result of the digestion was evaluated by separation on a 1.5 % multi-purpose agarose gel by gel electrophoresis (BioRad).

4.3.12 Microsatellite-analysis

Table 4.1: Primer sequences used for genetic characterization of yeast strains by microsatellite analysis

Locus	Primer sequences	Repeat sequences	Concentration in PCR reaction
YLL049W	GCAACATAATGATTTTGAGGT	$(TA)_n$	0.75 µM
	GTGTCTTGTGTGAGCATAGTGGAGAA		0.75 µM
YFR028C	GTGTCTTGACACAATAGCAATGGCCTTCA	$(GT)_n$	0.1 µM
	GCAAGCGACTAGAACAACAATCACA		0.1 µM
YGL139W	GTGTCTCTTTTTATTTACGAGCGGGCCAT	$(CAA)_n$	0.1 µM
	AAATCTCATGCCTGTGAGGGGTAT		0.1 µM
YOL109W	GTGTCTAGGAGAAAAATGCTGTTTATTCTGACC	$(TAA)_n (TAG)_n$	0.75 µM
	TTTTCCTCCGGGACGTGAAATA		0.75 µM

YLL049W is located on chromosome XII, YFR028C on chromosome VI, YGL139W on chromosome VII and YOL109W on chromosome XV. Primer sequences are given in 5' to 3' as single strand.

Four microsatellite sequences located at four different chromosomes of the genome of *S. cerevisiae* were used for the identification of W15 by microsatellite-analysis. Reference data were provided by R. C. Gardner (personal communication). PCR reactions were performed on a Techne TC412 Thermal cycler. Table 4.1 shows the microsatellite primers used (5, 22) and the concentration of each primer in the reaction mix for PCRs. The forward primer of each primer set was either labeled by

Fam or Hex for detection of the amplified fragments with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). PCR reactions were carried out in a final volume of 10 µl containing 5 µl 2x Qiagen Multiplex PCR Master Mix, 1 µl 10 x primer stock 2 µl dH_2O and 20 ng DNA of the yeast strain to be evaluated. The amplification reaction included an initial step of 15 min at 95 °C followed by 35 cycles of 94 °C/30 sec, 54 °C/90 sec, 72 °C/60 sec and a final extension of 30 min at 60 °C. For fragment length analysis 1µl of pre-diluted PCR product (1:20 in dH_2O) was mixed with 15 µl formamide and 0.3 µl GeneScan™ 500 ROX DNA standard solution (Applied Biosystems). Samples were denatured for 2 min at 95 °C followed by rapidly cooling down before analysis by capillary electrophoresis. Data analysis was done by GeneMapper ® Software (Applied Biosystems).

4.3.13 Karyotyping

The chromosomal preparation for the evaluation of yeast strains by pulsed-field gel electrophoresis was carried out according a modified protocol of Schwartz and Cantor (29). Single colonies were suspended in 100 µl of 50 mM EDTA pH 8.0. Alternatively, 1 ml of liquid culture grown to mid-exponential phase in complete medium was used. The culture was centrifuged for 3 min at 5,000 RPM at room temperature. The supernatant was discarded and cells were washed in 1 ml of dH_2O. Cells were collected through centrifugation for 3 min at 5,000 RPM and the supernatant was discarded. The pellet was resuspended in 100 µl of 50 mM EDTA pH 8.0. 50 µl of freshly prepared Lyticase (Sigma) solution (1 mg/ml in SEC buffer; 1.2 M D-sorbitol, 40 mM EDTA pH 8.0, 20 mM citrate-buffer pH 5.6 (0.1 M Na_2HPO_4, 0.1 M citric acid)) were added; followed by an incubation of 45 min at 37 °C. 450 µl 1 % low melt grade agarose (BioRad, in 125 mM EDTA pH 7.5) were added. The samples were solidified in so called mould chambers (BioRad) at room temperature. 1 ml LET-buffer (0.5 M EDTA pH 8.0, 10 mM Tris-HCl pH 7.5, 7.5 % 2-mercaptoethanol) for one agarose block was added and incubated over night at 37 °C. The samples were washed three times with 10 ml of 50 mM EDTA pH 8.0. 1 ml of fresh Proteinase K (Sigma) solution (1 mg/ml in NDS-buffer (0.45 M EDTA pH 8.0, 10 mM Tris-HCl pH 7.5, 1 % N-lauroylsarcosine) was added for one agarose block and incubated over night at 50 °C. Samples were washed three times with 10 ml of 50 mM EDTA pH 8. Agarose blocks were stored in 50 mM EDTA pH 8.0

at 4 °C. Analysis of the chromosomal preparations was done by pulsed-field gel electrophoresis using a CHEF mapper (BioRad). Sections of approximately 3 mm of the chromosomal preparation were applied to a 1 % multipurpose agarose gel (Promega, in 05 x TBE-buffer) and sample slots were filled up with 1 % low melt grade agarose. Electrophoresis was done in 0.5 x TBE-buffer for 15 h at 120 ° angle and a switching time of 60 sec followed by 9 h at 120 ° angle and a switching time of 90 sec. The current voltage was set at 6 V/cm and the buffer temperature at 14 °C. Chromosomal pattern was detected after an incubation for 1.5 h in ethidiumbromide solution (1 µg/ml in 0.5 x TBE-buffer) at 4 °C.

4.3.14 Statistics

Statistic evaluation was carried out using the GraphPad Prism Software. Determinations of the fermentation time needed by the individual yeast strains to ferment 50 % of the glucose or fructose in the different laboratory scale fermentations (IC50 glucose or IC50 fructose) were established by nonlinear regression, employing a dose-response equation with variable slope and the constrain of a bottom value higher than zero. One-way ANOVA and Tukey's test were used for the evaluation of the statistical significance of the differences in the GFR of different strains during fermentation.

4.4 Results

4.4.1 Fermentation characteristics of W15 and Uvaferm 43

The aim of this study was to combine the desirable traits of two commercial strains while specifically improving the utilization of fructose during fermentation. More specifically, the focus was on improving the ability to consume fructose in the latter stages of fermentation, when the GFR tends to be very low. W15 was chosen as one of the parental strains due to its popularity within the commercial winemaking community in Europe, while Uvaferm 43 offers a significantly lower discrepancy in glucose and fructose utilization than W15. To verify the general traits of these two yeast strains, the fermentation characteristics of W15 and Uvaferm 43 were evaluated in 100 ml small scale cultures in "Müller-Thurgau" grape must in two independent experiments and using triplicate samples for each yeast strain.

Evaluation of the fermentation kinetics revealed a longer lag phase for Uvaferm 43 in both glucose and fructose utilization. However, 3 days after inoculation the fermentation rate of Uvaferm 43 exceeded the rate of W15. The difference in fermentation rate was found to be more pronounced when only fructose utilization was considered. While glucose was depleted by both strains at the same point in time, W15 samples showed some remaining fructose when Uvaferm 43 had completely fermented this sugar. Determination of the GFR values for the fermentation period revealed a higher value for Uvaferm 43 throughout fermentation. Figure 4.1 A shows the fermentation kinetics of W15 and Uvaferm 43 in these conditions.

The fermentation characteristics of the two strains were further evaluated in three different stuck fermentations of the grape varieties Pinot Blanc and Pinot Noir. The samples came from three different commercial wineries and could not be reactivated by temperature elevation or re-inoculation. 500 ml portions of the stuck wines were pasteurized before re-inoculation for laboratory scale experiments. The arrested fermentation of Pinot Blanc must contained 12 g/l residual fructose while glucose was completely fermented. The stuck fermentation of Pinot Noir still contained 23 g/l fructose and 1.7 g/l glucose. The third sample was a Dole Blanc, which is the official denomination for a Pinot Noir vinified as rosé, coming from the Canton Valais in Switzerland. This stuck wine contained 16 g/l residual fructose and 0.8 g/l glucose. Each strain was tested in triplicate in each of the stuck wines on a 100 ml laboratory scale. The evaluation revealed that W15 was unable to restart fermentative activity in these stuck fermentations. Uvaferm 43, however, was able to ferment all stuck wines to dryness within 32 days (Figure 4.1 B to D).

The data confirm that Uvaferm 43 is significantly better adapted to fermenting fructose than W15. In addition, the data show that Uvaferm 43 has the ability to restart stuck fermentations that show a very low GFR. However, Uvaferm 43 was selected as “fix-it” strain and is only recommended as the main fermentation yeast for musts of red varieties of high sugar content (Lallemand Inc). Therefore, the application range of this yeast is rather limited. By merging the genetic background of Uvaferm 43 and W15 by mass-mating, the aim is the generation of yeast strains combining the fructophilic trait of Uvaferm 45 with the high fermentative performance of W15 (Lallemand Inc, J. Gafner personal communication). The desired novel yeast

strain would ideally be able to efficiently ferment various grape varieties and to show reduced or even no discrepancy in glucose and fructose utilization.

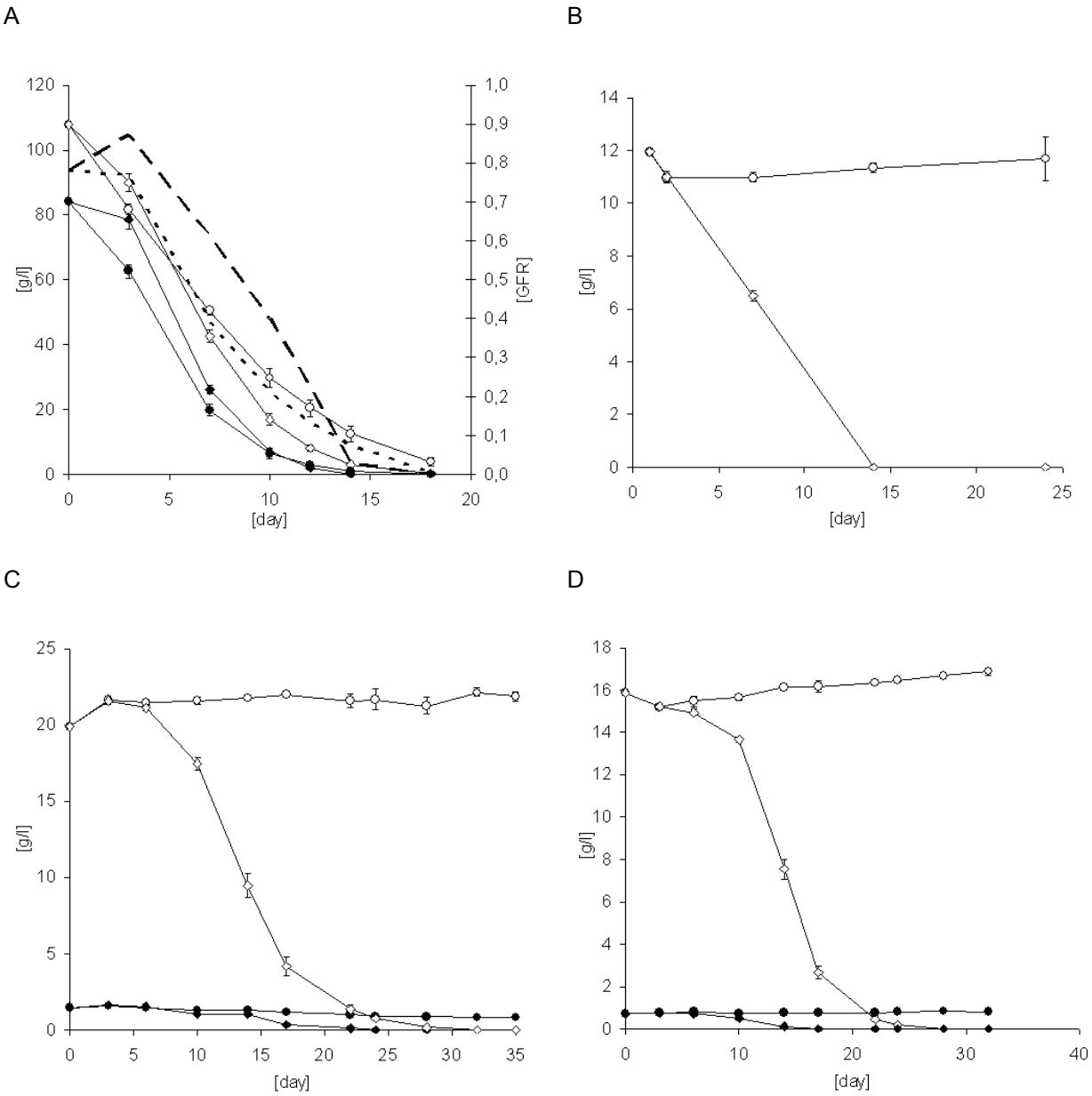


Figure 4.1: Fermentation characteristics of W15 and Uvaferm 43 in “Müller-Thurgau” grape must (A), Pinot blanc stuck fermentation containing only residual fructose (B), Pinot noir stuck fermentation (C), Dole blanc stuck fermentation (D). Error bars indicate standard division (SD) of the triplicates for W15 (circles) and Uvaferm 43 (rhombus). Glucose is indicated by filled symbols, while fructose is indicated by open symbols. The dotted line in graph A shows the progression of the GFR for W15 and the dashed line for Uvaferm 43 displaying the distinct discrepancy in glucose and fructose utilization. Uvaferm 43 was able to restart fermentative activity in all of the stuck fermentations, while W15 was not able to induce the fermentation of the residual sugars.

4.4.2 Mass-mating of W15 and Uvaferm 43

A possible drawback in choosing Uvaferm 43 as parental strain in a mass-mating approach is its uncertain ploidy status (5). This could indeed negatively influence the formation of hybrids in the mass-mating process.

To assess the likelihood of success, the sporulation efficiency of both strains was estimated by microscopic examination. Both strains showed an ascus formation of 70% in optimized sporulation conditions. This suggested a reasonably high chance of success for the mass mating strategy.

The isolated spores of both strains were therefore randomly mixed. After mass-mating of the spores, the culture was plated out on 2 % YPD plates to obtain single colonies for evaluation.

Initially, 60 colonies selected after the mass-mating of W15 and Uvaferm 43 were evaluated by PCR- and RFLP-analysis. W15 and Uvaferm 43 exhibited specific patterns for the amplification of the δ elements of their genome as well as after digestion of the PCR products by *Rsa*I, *Hha*I and *Alu*I. Therefore this method was used to characterize the genetic background and hybrid status of the single colonies. 28 % of the examined colonies were found to exhibit the same banding pattern in PCR- and RFLP-analysis as W15, 8 % were found with a pattern identical to Uvaferm 43. 28 % of the colonies could clearly be identified as hybrids of W15 and Uvaferm 43. The remaining 35 % of colonies showed some bands that were not present in either of the two parental strains, suggesting some recombination between DNA fragments. These results suggest that mass-mating has yielded a high number of hybrids.

In later experiments, which were initiated when the results of the enrichment and directed evolution process appeared difficult to interpret, this PCR-analysis was found to have been incorrect. Indeed, using a modified PCR protocol revealed that the band that allowed the characterization of the Uvaferm 43 genetic background in hybrids was not specific to the strain. When using the modified conditions, this band also appeared in the negative control and the W15 pattern, although not reproducibly. Due to these inconsistencies other methods were then employed in order to be able to characterize the parental strains as well as the genetic background of selected colonies.

4.4.3 Microsatellite-analysis and karyotyping

The 60 colonies, characterized by PCR- and RFLP-analysis before, were evaluated by microsatellite analysis and karyotyping. None of them could be identified as hybrid of the two parental strains by these methods. 55 colonies were found to exhibit the same fragment size pattern as W15 in the microsatellite-analysis. The remaining 5 strains were found to exhibit the fragment length pattern of Uvaferm 43. Since the possibility of a very low mating rate could not be excluded, 64 more selected colonies were evaluated. Therefore, a portion of the freeze culture of the mass-mated population was grown over-night and plated out in order to yield single colonies for examination. Microsatellite-analysis revealed one (1543ls48) of these tested colonies to exhibit a fragment length profile that would be characteristic for a hybrid of W15 and Uvaferm 43 (Table 4.2). All other colonies were found to exhibit a fragment length pattern identical to W15. The verification of the results was done by karyotyping (Figure 4.2).

Summarizing the assessment of 124 single colonies selected after mass-mating of W15 and Uvaferm 43, one strain was characterized as hybrid of the two parental strains and 4 % with Uvaferm 43-like genetic background. The vast majority of the colonies, however, were found to exhibit a W15-like genetic background. It has to be taken into consideration that the additional growth phase, necessary for the generation of single colonies for the assessment of the second set of strains, does have an influence on the culture composition, since differences in growth rate will be amplified during this step. Strains of higher growth rate will therefore increase in proportion. However, the data show that we were able to generate hybrids of the two parental strains which can be detected by karyotyping and microsatellite analysis employing four loci distinct for each of the parental strains and located on chromosomes, which are not distinguishable for the parental strains in the karyotyping. Although these results indicate a very low mating-rate, the hybrids have the same potential as all other strains for being enriched in the selection process if they are sufficiently well adapted to the specific selective conditions.

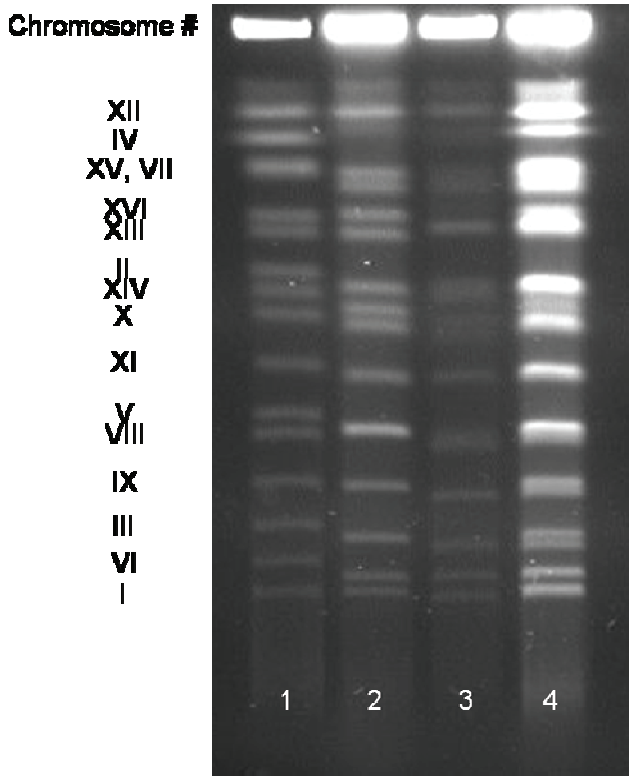


Figure 4.2: Karyotyping of *Saccharomyces cerevisiae* standard (strain YNN295; 1), W15 (2), Uvaferm 43 (3), 1543ls48 (4).

4.4.4 Fermentative performance of populations obtained after mass-mating or EMS treatment

W15, Uvaferm 43 and the two populations, obtained either through the mass-mating or EMS treatment of W15, were grown in 2 % YPF medium. The populations were inoculated into synthetic grape must with preset GFRs of 1, 0.2, 0.1, 0.05 and 0.02. Initial total sugar concentrations were 100 g/l and 25 g/l. Cultures were inoculated at an initial OD_{600} of 0.05 in triplicate. Fermentation was conducted at 20 °C, corresponding to a frequently used fermentation temperature in wineries. By the time OD_{600} was found to reach its plateau, the concentrations of glucose, fructose and ethanol were determined. The results showed that all samples were able to ferment to dryness. None of the conditions led to stuck or sluggish fermentation for any of the samples.

4.4.5 Selection in continuous cultivation

The various populations were subjected to the selective conditions in a continuous fermentation setup. The culture vessels were fed from the same medium reservoir, while all in- and out-fluxes were controlled by a single pump. This technical set-up guaranteed similar treatment of the two populations. The continuous cultivation was conducted for 162 generations. For 39 generations, the feeding medium MS300 exhibited a GFR of 0.2. Development of elongated cells and occurrence of some cell clusters in the culture populations at around generation 35 suggested some adaptive processes. The selection pressure was increased by decreasing the GFR of the fresh medium to 0.1 for the following 45 generations. The size of the cell clusters increased within the following 96 h of fermentation. The proportion of elongated cells stabilized. Within the next 96 h of fermentation the proportion of cell clusters decreased constantly until not detectable any longer, while the proportion of elongated cells started to increase again. After that period cell clusters were again detected in the culture population. For the following 30 generations of cultivation the medium composition was changed to a GFR of 0.05. The proportion of ellipsoid shaped yeast cells was increasing and became the majority within the culture population. The composition of the medium fed during the last 48 generations of cultivation was again decreased to a GFR of 0.02. No cell clusters were present in the culture population within the last 168 h of cultivation. The development of the morphological changes in the culture population was seen for both samples and varied only little in value. Determination of glucose and fructose concentrations showed sugar depletion throughout cultivation duration in continuous cultivation. The ethanol concentration remained stable at approx. 10 g/l. No oxygen was detected in the cultures throughout cultivation.

4.4.6 Genotypic characterization during fermentation time

The culture population obtained after the mass-mating of W15 and Uvaferm 43 was subjected to genotypic characterization at various stages of the selection process, corresponding to generations 39, 61, 75, 105, 137 and 153. In each case, twenty single colonies were selected and examined by microsatellite-analysis for their genetic background. All but one colony (F2 5 14) were found to exhibit a profile identical to W15. Colony F2 5 14, which was found to be distinct from the others,

showed two different fragment lengths for one of the examined loci. One of the amplified fragments was equal to the length found for W15, while the other was equal to the length found for Uvaferm 43 (Table 4.2). At generation 162, the end of the selection procedure, 32 colonies were examined for their microsatellite profile. All of the colonies show a profile identical to W15. The data suggest that none of the hybrid strains or Uvaferm 43 parental strains had been able to compete in the selection procedure with yeast strains exhibiting the W15 genetic background. It can therefore be argued that the strains are entirely a result of a directed evolution process, and that the mass mating process that acted on the W15 wild type strain, and that strains derived from Uvaferm 43 or hybrid strains had been eliminated.

Table 4.2: Microsatellite profile of parental strains and selected strains

Yeast Strain	**Microsatellite locus**							
	YLL049W		**YFR028C**		**YGL139W**		**YOL109W**	
	Allele 1	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**
W15	294		123		124		259	
U43	277		132		121	127	304	
1543ls48	277	294	123	132	124	127	259	304
F2 5 14	294		123		124	127	259	

W15 and U43 (Uvaferm 43) are the two parental strains used in the mass-mating. 1543ls48 was the only selected colony out of 124 colonies picked after mass-mating exhibiting a microsatellite profile characteristic for a hybrid of the two parental strains. F2 5 14 was selected at generation 153 of the selection procedure for genotypic characterization of the culture population during fermentation time.

Four colonies (1543 2 to 1543 5) where selected for further assessment and phenotypic characterization, and were in addition characterized by karyotyping. The results of the karyotyping show identical band pattern to W15 and therefore verify the findings of the microsatellite-analysis.

Similarly, four colonies selected at the end of the enrichment of the mutated W15 culture population (15m2, 15m3, 15m37, 15m38) were also examined by microsatellite-analysis and karyotyping, and were confirmed to be of a W15 genetic background (data not shown).

4.4.7 Direct competition in continuous cultivation

To assess whether the process had selected strains that were better able to grow in the low GFRs used during the selection procedure, a direct competition assay was used. This methodology became necessary since all of the selected strains de facto presented a W15 genetic background. In the direct competition assay, the relative performance of a selected strain can be assessed in comparison to the initial strain before genetic manipulation and selection treatment (7). To differentiate between the selected strains and the WT W15 strain, the WT strain was transformed with a kanamycin resistance gene (W15kanMX). This allows the differentiation between WT and the selected strains directly by replica plating on G418 containing agar plates, and thus to determine the percentage of the two competing strains in the culture population.

To verify that the transformation procedure and the presence of the kanMX gene did not affect the performance of the W15 strain, three independent direct competition assays between the transformed and untransformed W15 strains were conducted. The relative percentage of the two strains varied by less than 10 % over 23 generations and 210 h of fermentation. This result suggests that the transformed strain behaved identically to the untransformed parent in these conditions.

Table 4.3 shows the results of all the strains selected after the chemostat cultures when evaluated in this direct competition assay in synthetic must MS300 containing 25 g/l total sugar at a GFR of 0.02. The conditions of these direct competition cultivations were identical to the conditions applied during the last step of the selection procedure.

The strains selected after mutagenesis of W15 and selection in continuous cultivation showed two distinct phenotypes. Strains 15m2 and 15m3 were able to out-compete W15kanMX. Strain 15m2 represented 100 % and 99 % of all cells after 25 generations of competitive culture, while 15m3 represented 96 % and 66 % of all cells after the same number of generations. Both strains showed an elongated cell shape after some generations in these conditions.

However, the two other randomly selected strains, 15m37 and 15m38 were not able to out-compete W15kanMX. Two independent fermentations were conducted for each strain over 22 and 27 generations. The percentage of 15m37 dropped to a minimum of 1 % and 2 % even if the competing strains were present in equal

numbers at the start of fermentation (50 % and 43 % of 15m37). Strain 15m38 was also out-competed by W15kanMX and its percentage dropped to 4 % in both fermentations. Interestingly, these strains did not show elongated cell morphologies during fermentation. The colony morphology of these strains was also found to be distinct. Strains 15m2 and 15m3 showed smooth colony morphology, if grown on 2 % YPF agar plates, whereas rough colony morphology was observed for strains 15m37 and 15m38. When cultivating these strains in rich medium, the altered colony morphology disappeared.

Table 4.3: Direct competition assay in continuous cultivation

Strain	Fermentation 1			Fermentation 2		
	s [1/h]	[%]	Change [%]	s [1/h]	[%]	Change [%]
W15	-0.0014	46	-1	0.0032	58	10
W15 $^{+}$	0.0013	43	7	0.0025	52	13
15m2	0.0195	99	60	*	100	53
15m2 $^{+}$	0.0141	95	47	0.0150	93	56
15m3	0.0198	96	55	0.0169	66	32
15m37	-0.0186	1	-49	-0.0165	2	-41
15m38	-0.0153	4	-46	-0.0143	4	-36
15m38 $^{+}$	-0.0161	3	-40	°	0	-60
1543 2	0.0169	100	48	*	100	48
1543 2 $^{+}$	0.0147	95	42	0.0120	93	35
1543 3	*	100	50	0.0211	97	54
1543 4	0.0163	98	43	0.0135	92	40
1543 4 $^{+}$	0.0090	87	39	0.0080	85	35
1543 5	0.0149	93	41	0.0218	98	46

Selective difference s displaying the rate of displacement per hour of cultivation (8), percentage of the wild type strain and selected strains at the end of cultivation in direct competition to W15kanMX and percentage of change within the entire fermentation. MS300 containing 25 g/l sugar and GFR=0.02; $^{+}$ MS300 containing 25 g/l glucose as sole carbon source; * strain took over culture population; ° strain was washed out of culture population negative values indicate inferiority and positive values superiority of the selected strain in competition to W15kanMX.

Strains selected from the “mass-mated “culture, 1543 2, 1543 3, 1543 4 and 1543 5, were similarly evaluated. All were found to be able to out-compete the W15kanMX strain. The least efficient strain was 1543 4, which still represented 92 % of all cells at

the end of the competition assay. Interestingly, 1543 4 did not develop elongated cells during cultivation, whereas 1543 2, 1543 3 and 1543 5 showed this specific morphological feature that is similar to the morphology observed for strains 15m2 and 15m3. Elongated cells were first observed at generation 8 of the cultivation. This clear difference in phenotype shows that at least some of the four selected strains 1543 2 to 1543 5, are not identical to each other.

In order to evaluate if the improved performance of the selected strains is independent of the carbon source, the direct competition cultivations were repeated with a feed-medium containing 25 g/l of glucose as sole carbon source (Table 4.3). All strains tested in these conditions showed the same relative performance to W15kanMX as seen in the competition cultivations done in fructose containing medium. Even the development of the cell shape during cultivation was identical. The data are summarized in Table 4.3

4.4.8 Evaluation of fermentative performance in laboratory scale fermentations

The first set of experiments was conducted in 100 ml small scale fermentations. The fermentative performance was evaluated in conditions close to winemaking conditions, in MS300 containing 100 g/l glucose and fructose each. All other known ingredients of a grape must were supplied as described in the “Materials and Methods” section. After inoculation of the yeast strains, the Erlenmeyer flasks, closed by cotton wool plug, were incubated without agitation at 20 °C. Periodic determination of glucose and fructose concentration enabled the determination of the yeast strain specific fermentation performance and the evolution of GFR during fermentation. The time period needed for the fermentation of 50 % of the initial glucose (IC50 glucose) or fructose (IC50 fructose) was determined by nonlinear regression, employing dose-response modeling. The statistic evaluation was done separately for the two experiments, since fermentation progress varied. Fermentations were stopped when less than 1 g/l of glucose was present. In these conditions, the total fermentation durations varied between 335 h and 431 h.

The fructose concentration at the end of fermentation varied among the selected yeast strains. The parental strains W15 and Uvaferm 43 always successfully completed fermentation, and remaining fructose concentrations were below 2 g/l.

Strains 1543 2 and 15m2 were found to exhibit the highest residual fructose concentrations in both experiments.

Figure 4.3 A shows the IC50 glucose values for each yeast strain. The data confirm the initially slow fermentation rate by Uvaferm 43 when compared to W15. The differences in the IC50 fructose values between the two strains were as expected less pronounced (Figure 4.3 B). The yeast strains selected from the mass-mated culture, 1543 2-5, generally showed intermediate fermentative performances. With regard to IC50 glucose values, strain 1543 2 was found to be closest to Uvaferm 43, while 1543 5 exhibited a performance more similar to W15, in one case out-performing this yeast. For the fermentation of fructose, the differences between strains were found to be less pronounced.

The data for strains 15m2, 15m3, 15m37 and 15m38 generally suggested fermentation characteristics that are more similar Uvaferm 43 than W15. The IC50s for glucose and fructose for all four strains are indeed more similar to Uvaferm 43, with the IC50 for fructose however being slightly higher. This is interesting considering that the genetic background of these strains is similar to W15.

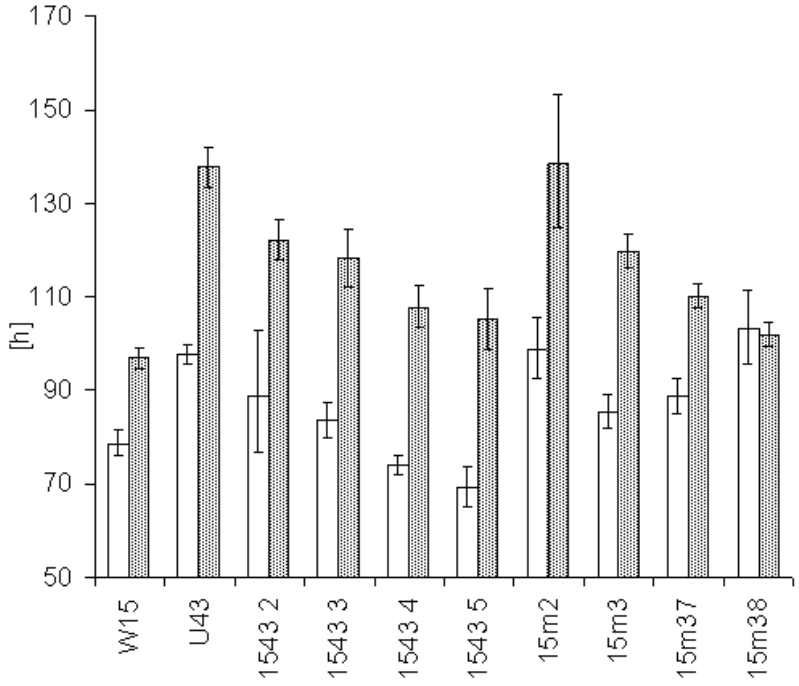
The results for the evaluation of the fermentation progress by nonlinear regression modeling show a specific profile for each of the tested strains in these conditions. None of the selected strains exhibits fermentation characteristics identical to the parental strains W15 and Uvaferm 43.

Determination of the GFR values showed that Uvaferm 43 continued to exhibit the lowest discrepancy in glucose and fructose utilization, while W15 exhibited the highest discrepancy (Figure 4.4). This was consistent in both experiments. The GFR of the selected strains generally showed intermediate values between the two reference strains. However, the data were less consistent than for the two parents.

Compared to the progress of Uvaferm 43, none of the selected strains revealed a lower GFR. Statistically, no significant ($p<0.05$) difference in GFR during fermentation could be seen when the selected strains were compared to W15 because of the significant variation between the two experiments. Nevertheless, and as can be seen in Figure 4.4, several strains consistently showed higher GFRs than W15.

The same experimental set-up was chosen for the evaluation of the fermentative performance in a medium that was similar to the one supplied during the last period of the selection process in continuous cultivation. The synthetic grape must MS300

A



B

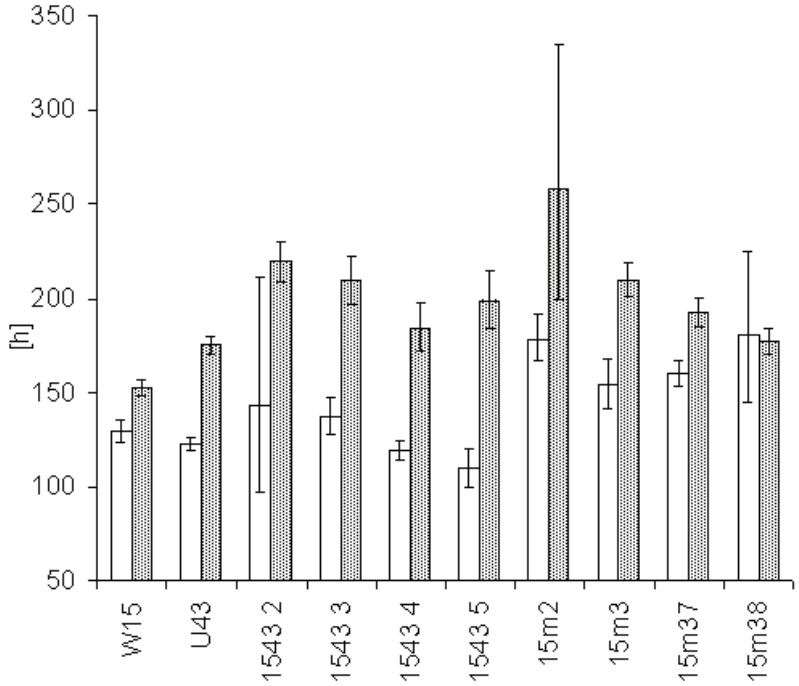
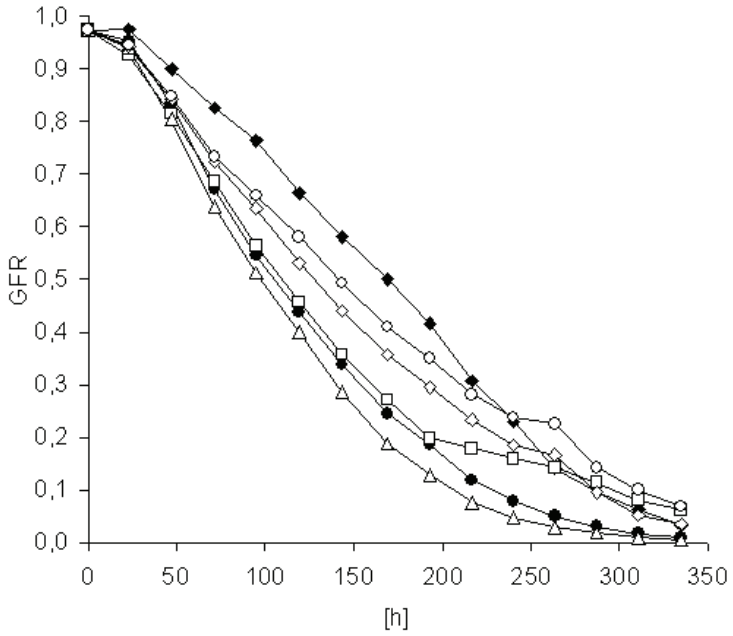


Figure 4.3: IC50 for glucose (A) and fructose (B) fermentation in two independent experiments. Fermentation 1 (white bars); fermentation 2 (grey bars); error margins indicate maximum and minimum values. Since the overall fermentation time for fermentation 2 was higher than for fermentation 1 the IC50 values are found to be generally higher for fermentation 2.

A



B

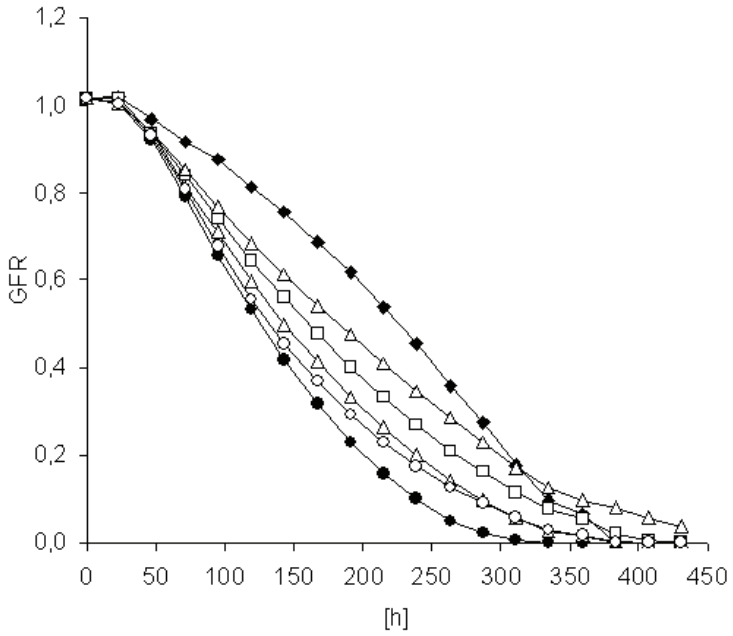


Figure 4.4: GFR during fermentation in two independent fermentations (A and B). W15 ●, Uvaferm 43 ◆, 1543 2 □, 1543 4 △, 15m2 ◇, 15m38 ○. Fermentation times varied significantly (A: 335 h; B: 431 h). Specific progress of GFR for the selected strains during fermentation time was not reproducible compared to W15; compared to Uvaferm 43 all strains exhibited higher discrepancy in glucose and fructose fermentation. Within the last third of fermentation time GFR equalizes due to glucose depletion. Display of error margins was abstained in order to enhance clarity of the figure.

contained a total sugar concentration of 25 g/l glucose and fructose in a GFR of 0.02. The overall fermentation was 120 h for both independently conducted experiments. Therefore, the data were combined for statistic evaluation. Determination of the IC50 values for glucose and fructose utilization revealed a significant longer time period for Uvaferm 43 to ferment 50 % of initial glucose and fructose. The performance of the selected strains was found to be very close to W15 and significantly different to Uvaferm 43. Table 4.4 shows the results of evaluation by nonlinear regression.

Table 4.4: IC50 for fructose utilization

Strain	**IC50 fructose**		
	mean [h]	**min [h]**	**max [h]**
W15	37	32	44
U43	55	47	64
15m2	34	28	42
15m3	34	28	41
15m37	36	32	41
15m38	35	32	38
1543 2	43	38	48
1543 3	39	36	43
1543 4	41	34	50
1543 5	39	33	45

IC50 for fructose utilization determined by nonlinear regression employing a dose-response equation with variable slope and bottom value of higher than zero. IC50 values indicate the fermentation time needed to ferment 50 % of the initially present fructose. W15 and U43 (Uvaferm 43) are the two parental strains; other strains were selected after enrichment procedure conducted to the culture populations after EMS treatment of W15 culture population (15m2, 15m3, 15m37, 15m38) and mass-mating of W15 and Uvaferm43 (1543 2 to 1543 5).

Similar to the IC50 values, the overall fermentation progress of the selected strains for glucose and fructose utilization was found to be very similar to W15, with Uvaferm 43 showing a significantly different pattern.

Determination of the GFR values during fermentation showed similarity between W15 and the selected strains. Uvaferm 43 is the only strain exhibiting a statistically

significant ($p<0.05$) different GFR at a fermentation time of 24 h and 48 h, compared to W15. Glucose was depleted within 71 h of fermentation and therefore, GFR values could not be determined later in fermentation. Fructose was depleted within 120 h of fermentation by all strains.

The differences in fermentation progress of the selected yeast strains in comparison to W15, seen in MS300 containing 100 g/l glucose and fructose each, were not detectable in the conditions supplied in this second set of laboratory scale experiments.

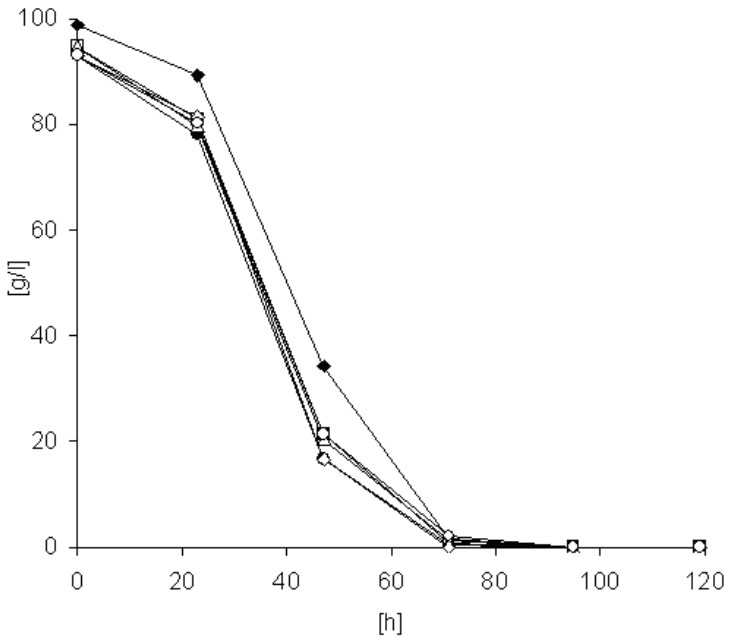
4.4.9 Laboratory scale fermentation under agitation

The duplicate experiments in the previous section were characterized by high variability which resulted in data that, although indicating some trends, do not allow drawing statistically relevant conclusions. Such variations are commonly observed in small scale laboratory fermentations. In particular, in these conditions the fermenting yeast settled rapidly (within 24 hours) to the bottom of the fermentation vessel. Since settling affects fermentation efficiency, and since sampling can lead to various extend of resuspension, this may be a significant cause for poor reproducibility.

For this reason, the experiments were repeated using an altered experimental design. The culture volume was increased to 300 ml, agitation was set to 100 RPM, bottles were closed by a fermentation cap and the incubation temperature was maintained at 20 °C. The medium (MS300) contained 100 g/l glucose and fructose each. Since the bottles are closed by fermentation cap, the process can be considered to take place under anaerobic conditions, after the initially present oxygen is depleted and CO_2 is produced. The experiment was done three times in triplicates for each strain to be evaluated.

Fermentation progress was found to be reproducible for all tested strains by employing this experimental set-up. Figure 4.5 (A and B) shows the fermentation progress of the tested strains for glucose (A) and fructose (B). The selected strains 1543 2, 1543 4, 15m2 and 15m38 exhibit a similar fermentation performance for glucose as W15. Uvaferm 43 shows a lower fermentation rate during the first 23 h of fermentation, followed by significant increase in fermentative activity, with glucose

A



B

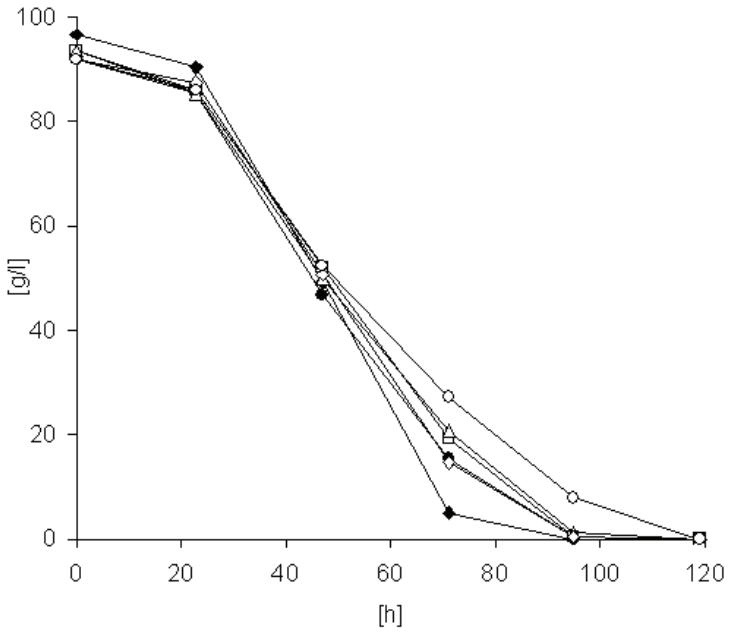


Figure 4.5: Glucose utilization (A) and fructose utilization (B) in laboratory scale fermentation with agitation. W15 ●, Uvaferm 43 ◆, 1543 2 □, 1543 4 △, 15m2 ◇, 15m38 ○. Error margins are abstained for clarity of the figures. Uvaferm 43 exhibits a distinct glucose and fructose fermentation rate compared to W15 and selected strains. Glucose utilization characteristics of selected strains are similar to W15. Differences in fructose utilization characteristics are most pronounced for 15m38, while 15m2 exhibits identical fermentation progress as W15.

depletion being achieved at the same time as for the other strains. The fructose fermentation performance of Uvaferm 43 is different from all other strains. The fermentation rate of fructose during the first 23 h of fermentation was found to be similar for all tested strains. Within the fermentation period of 23 h to 71 h, Uvaferm 43 shows a significantly higher fermentation rate for fructose. The selected strain 15m2 exhibits the same fructose utilization as W15, while strains 1543 2 and 1543 4 show slightly lower fermentation rates than W15. In comparison to the fermentation rate of W15, the fructose utilization rate of 15m38 declines during fermentation time.

The GFR values determined at the various sampling times (23 h, 47 h and 71 h) show a very significantly lower discrepancy in glucose and fructose utilization for Uvaferm 43. The slightly higher GFR values (23 h and 47 h) for the strains 1543 2, 1543 4 and 15m38, compared to W15, were found to be statistically significant ($p<0.05$). The GFR values determined for strain 15m2 do not differ from GFR values for W15. GFR values could only be determined for 71 h of fermentation, since glucose depletion was reached for the majority of the evaluated strains by a fermentation time of 95 h. These results show that the conditions in the selection procedure indeed enriched the treated culture populations for yeast strains exhibiting a higher GFR, although general fermentation performance was decreased.

Evaluation of the fermentation progress by nonlinear regression modeling revealed the lowest IC50 glucose value for W15. However, IC50 glucose values determined for the selected strain do not vary significantly. In contrast, Uvaferm 43 exhibits a much higher time demand in order to ferment 50 % of the initial glucose concentration. Looking at the IC50 fructose values, the differences between the evaluated strains are not very pronounced. Uvaferm 43 does not exhibit significantly differences in comparison to W15 (Figure 4.6). In these conditions, the significantly lower discrepancy of glucose and fructose utilization in Uvaferm 43 in comparison to W15 is due to slower glucose utilization and not caused by higher fructose utilization.

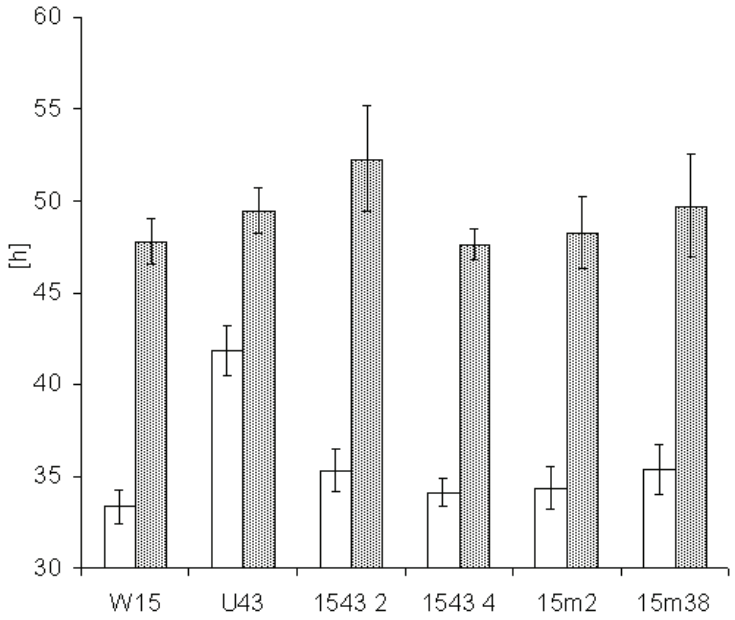


Figure 4.6: IC50 glucose (white bars) and IC50 fructose (grey bars) for fermentation under agitation; error margins display minimum and maximum values, displaying a high reproducibility within the fermentations. Uvaferm 43 exhibits a significantly higher time demand in order to ferment 50 % of the initial glucose concentration when compared to W15 and the selected strains. The differences in fructose utilization are not as pronounced within the evaluated strains.

4.5 Discussion

Evaluation of colonies selected after mass-mating of W15 and Uvaferm 43 showed a low percentage of hybrids within the population. In many cases, hybridization techniques for the generation of wine yeast strains have proven to be difficult due to the homothallic character of the strains (24, 31). However, mass-matings of *S. cerevisiae* strains (11) and *S. cerevisiae* and *S. bayanus* (26) were successfully implemented. In these papers, however, the authors fail to report on the proportion of hybrids within the generated populations. To improve the rate of hybridization between wine yeast strains, a heterothallic parental strain, generated by genetic manipulation, was employed in other studies (17). Due to the low acceptance of organisms with GM-status in the wine production process, this strategy was not applied in this study (24, 25). In addition, direct spore to spore mating by micromanipulator increases the possibility of hybridization events and is therefore frequently used for this purpose. This strategy, however, limits the genetic variability

and number of strains that can be generated in comparison to the random mass-mating strategy. Marinoni et al. (16) reported the formation of 20 to 40 % zygotes by mass-matings of *Saccharomyces* yeasts, dependent on the mating type and the species of the crossed strains. Several of the employed yeast strains were heterothallic and apparently also haploid and therefore a high rate of hybridization could be expected.

In our study, the mass mating strategy itself was of a rather limited success. In this case, several indicators suggest that Uvaferm 43 was probably not a good choice as a parental strain. Firstly, while mass matings will always produce a large proportion of strains with parental karyotypes, the proportion of such strains should be similar for both parents. In our study, a large number of W15 compared to Uvaferm 43 parental strains was obtained. Considering that the sporulation ability of Uvaferm 43 did not differ much from W15, the data suggest that Uvaferm 43 spores were significantly less well suited for - or efficient in - the mating process itself. A large number rapidly formed W15 diploids would then easily out-compete the Uvaferm 43 spores or inefficiently formed diploids. Secondly, the proportion of true hybrids was low. Indeed, since only a single hybrid was identified out of 124 analyzed strains, no true statistical evaluation of the proportion of hybrids can be derived. Nevertheless, it is likely that the overall population contained a significant total number of hybrid strains, since even a hybrid rate of 1% would correspond to several 100,000 individual hybrid cells in the total population. Furthermore, the generation of genetic variability introduced by mass-mating is not limited to the formation of hybrids between the two parental strains. Genetic recombination takes place during meiosis of individual parental strains and therefore their descendants will exhibit an altered genetic background, even if generated by self-diploidization (15, 31).

In summary, we therefore consider that the mass-mated population was suitable for the purpose of this project. The same is valid for the population generated by EMS treatment of the homozygous, diploid wine yeast W15. Klein et al. (13) showed the high potential of EMS treatment for the introduction of lesions to the genetic material in *S. cerevisiae*. Due to the diploid character of W15 it is difficult to evaluate the rate of mutations, since the development of auxotrophies could only be facilitated by the simultaneous mutation of both homologous alleles. The conditions for the EMS

treatment were therefore determined by the evaluation of a suitable dose and incubation time (12).

Our data related to the evaluation of the composition of the population during the selection process in continuous cultivation reveal the almost exclusive presence of strains exhibiting fragment length patterns identical to W15. Suppression of the hybrid strains and strains exhibiting Uvaferm 43-like genetic background indicates their mal-adaptation to the applied selection conditions in competition to the apparently better adapted W15-like strains. Therefore, the selection process of the population yielded by mass-mating turned out to be mainly characterized by adaptive evolution of the W15-like strains. This process is, however, not identical to the selection of the mutagenised W15 culture population, since genetic variation within the two initial culture populations was brought about by two distinct methods causing different types of alterations within the genome, and both are of unknown potential for the purpose of this study.

Finally, the marked shifts in cell morphologies during the selection process suggest that different strains or consortia of cooperating strains swept through the population during the process, with more efficient strains emerging and becoming dominant in the population. However, while this view of a continuously improving culture was certainly the aim here, it has also been shown that such clonal replacements may not always lead to an overall improved phenotype over time. Firstly, the advantage of a given clone over another in the context of the chemostat may be linked to factors other than the selective pressure alone. Cooperation between different strains in chemostats has been observed, with none of the individual strains performing well when in isolation. It is therefore possible that better performing individual strains where present at various stages of the selection process, and where replaced by consortia of cooperating strains (21, 27). It would however go beyond the scope of this work to evaluate strains throughout the selection process.

Evaluation of the finally and randomly selected strains in direct competition to W15, in the cultivation conditions applied during the selection procedure, revealed strain dependent and carbon source independent changes in the selected strains when compared to the parental strains. Selection of more than one specialized strain can be expected, since different molecular changes might lead to improved performance in the selection conditions (7). While six of the eight randomly selected strains were

able to out-compete the parental strain W15 in these conditions, two of the eight strains were unable to do so.

Strains 15m37 and 15m38 were present at the end of the selection process, although they were not able to out-compete the parental strain in the direct competition assay. Such a phenomenon was already reported by Paquin and Adams (21) for a study with haploid and diploid cultures of *S. cerevisiae*, subjected to glucose-limiting conditions over 300 generations. A possible explanation could be the strictly sequential appearance of adaptive events and therefore competition of newly appearing variants only with their immediate predecessors and not to possibly counter-selected earlier variants. Thus, combination of adaptive events may lead to mal-adapted clones if compared to progenitors (27).

In addition, it was already shown that long term cultivation of a single clone of *E. coli* under glucose-limited conditions may yield coexisting mutant strains of the desired phenotype and strains successfully surviving by co-metabolism in such conditions (10). Co-metabolism within culture populations was described to manifest itself by physiological and often morphological polymorphisms as was also seen for the culture populations in our study (10, 32). While the distinct colony morphology of the strains 15m37 and 15m38 was only of transient nature, the absence of the development of elongated cells during continuous cultivation of strain 1543 4 was reproducible and unique. These polymorphisms indicate that the presence of the selected strains in the culture populations at the end of the selection procedure is based on various distinct mechanisms.

Despite previous reports that extended cultivation in carbon-source limited conditions is selective for the limiting nutrient due to the development of specifically increased affinity, the performance of all evaluated selected strains in the direct competition assay is independent of the supplied carbon source, although fructose was supplied in the vast majority (8, 14). Since *S. cerevisiae* does not comprise a fructose specific transport or phosphorylation system, it could be hypothesized that the affinity of the pivotal metabolic mechanisms were generally improved for all of their substrates (20).

The evaluation of the glucose and fructose utilization rate of the selected strains in comparison to the parental strains in small scale fermentations revealed a generally

lower fermentative capacity of the selected strains, especially for fructose, independent of their performance in the direct competition assay. In contrast, the fermentative capacity of the selected strains proved to be comparable to the parental strains if evaluated in an experimental set-up similar to the technical conditions in the selection procedure. The most important difference was the agitation of the culture in order to keep the cells equally distributed. A possible explanation could therefore be the inhibition of a pivotal mechanism which is dependent on an equal distribution of the cells within the culture and consequently the loss of the selective advantage in a differing experimental set-up.

Assessment of the desired phenotype of reduced discrepancy in glucose and fructose utilization under winemaking conditions on the single strain level revealed minor improvements of the selected strains compared to W15. This is also valid for strain 15m38, which is inferior to W15 in the direct competition assay. This may be indicative of a distinct strategy of this strain to maintain itself in the population to the end of the selection procedure that triggers a similar phenotype under laboratory scale winemaking conditions.

Considering the clearly improved performance of several of the selected strains when evaluated in direct competition to W15kanMX, the improvements observed under winemaking conditions can be considered minor. Fitness in continuous cultivation is determined by the ability to compete with all strains present at a given time in specific selective conditions. In our case, the chemostat conditions were maintained to enable strains to compete at low GFR ratios and low sugar concentrations. This specific environment only corresponds to a short phase of the real winemaking process, which in addition is characterized by much higher ethanol levels, than found in our experimental set-up. In such a case, selection conditions should be adjusted in order to increase the selective pressure according to the needs of the desired phenotype (27). Indeed, changes within the selection criteria were reported to be advantageous for the success of a selection procedure by applying increasing selection pressure (7). We adjusted the GFR downward throughout the process, but this single parameter change may not provide sufficient variability to avoid the emergence of less suited strains.

Prolonged cultivation time would also support success if the desired phenotype is determined by the occurrence of multiple adaptive events. However, the possibility

for introducing various advantageous adaptive mutations into one strain is generally considered to be a rather unlikely process. The success for the generation of a yeast strains exhibiting a desired phenotype is strongly dependent on the number of evolutionary events necessary to be aggregated and therefore, the success rate is decreasing the more adaptations are required (27).

4.6 Conclusions

Our study shows that the strategy employed here can be used for the generation of yeast with improved GFR under fermentative conditions. The hybrids of W15 and Uvaferm 43 and yeast strains of Uvaferm 43-like genetic background could not successfully compete with yeast strains exhibiting W15-like genetic background, in the conditions applied during the selection procedure. Uvaferm 43 genetic traits were clearly of no or very limited advantage in these conditions. It is also clear that the prolonged exposure to selective conditions did not yield strains that showed very significant improvements for general wine fermentations. However, it is possible that our strains would perform significantly better than W15 in conditions that are close to stuck fermentations with a low GFR. This hypothesis still needs to be tested.

4.7 Acknowledgments

We thank Prof. Richard C. Gardner for supplying the protocol for the microsatellite-analysis and reference data for the fragment length pattern of W15 and Uvaferm 43. We also want to thank the department of Life Sciences and Facility Management of the ZHAW in Wädenswil for kindly providing the BBraun Quattro fermenter system used in this study. We are also thankful for the assistance in statistics by Dr. M. Kidd and Dr. D. Baumgartner.

4.8 Literature cited

1. **Alexandre, H., and C. Charpentier.** 1998. Biochemical aspects of stuck and sluggish fermentation in grape must. J. Ind. Microbiol. Biotechnol. **20:**20-27.
2. **Bely, M., J.-M. Sablayrolles, and P. Barre.** 1990. Automatic detection of assimilable nitrogen deficiencies during alcoholic fermentation in oenological conditions. J. Ferment. Bioeng. **70:**246-252.

3. **Berthels, N. J., R. R. Cordero Otero, F. F. Bauer, J. M. Thevelein, and I. S. Pretorius.** 2004. Discrepancy in glucose and fructose utilisation during fermentation by *Saccharomyces cerevisiae* wine yeast strains. FEMS Yeast Res. **4:**683-689.

4. **Bisson, L. F.** 1999. Stuck and sluggish fermentations. Am. J. Enol. Vitic. **50:**107-119.

5. **Bradbury, J., K. Richards, H. Niederer, S. Lee, P. R. Dunbar, and R. Gardner.** 2006. A homozygous diploid subset of commercial wine yeast strains. Antonie van Leeuwenhoek **89:**27-37.

6. **Dittrich, H. H., and M. Grossmann.** 2005. Mikrobiologie des Weines, 3 ed. Verlag Eugen Ulmer, Stuttgart.

7. **Dykhuizen, D. E.** 1993. Chemostats used for studying natural selection and adaptive evolution, p. 613-631, Methods Enzymol., vol. 224. Academic Press Inc.

8. **Dykhuizen, D. E., and D. L. Hartl.** 1983. Selection in chemostats. Microbiol. Rev. **47:**150-68.

9. **Gafner, J., and M. Schütz.** 1996. Impact of glucose-fructose-ratio on stuck fermentations. Vitic. Enol. Sci. **51:**214-218.

10. **Helling, R. B., C. N. Vargas, and J. Adams.** 1987. Evolution of *Escherichia coli* during growth in a constant environment. Genetics **116:**349-358.

11. **Higgins, V. J., P. J. L. Bell, I. W. Dawes, and P. V. Attfield.** 2001. Generation of a novel *Saccharomyces cerevisiae* strain that exhibits strong maltose utilization and hyperosmotic resistance using nonrecombinant techniques. Appl. Environ. Microbiol. **67:**4346-4348.

12. **Kaplan, N. L., R. R. Hudson, and C. H. Langley.** 1989. The ``hitchhiking effect'' revisited. Genetics **123:**887-899.

13. **Klein, F., A. Karwan, and U. Wintersberger.** 1989. After a single treatment with EMS the number of non-colony-forming cells increases for many generations in yeast populations. Mutat. Res. **210:**157-164.

14. **Kurland, C. G.** 1992. Translational accuracy and the fitness of bacteria. Annu. Rev. Genet. **26:**29-50.

15. **Lindegren, C. C., and G. Lindegren.** 1943. Selecting, inbreeding, recombining and hybridizing commercial yeasts. J. Bacteriol. **46:**405-419.

16. **Marinonie, G., M. Manuel, R. F. Petersen, J. Hvidtfeldt, P. Sulo, and J. Piskur.** 1999. Horizontal transfer of genetic material among *Saccharomyces* yeasts. J. Bacteriol. **181:**6488-6496.

17. **Marullo, P., M. Bely, I. Masneuf-Pomarede, M. Aigle, and D. Dubourdieu.** 2004. Inheritable nature of enological quantitative traits is demonstrated by meiotic segregation of industrial wine yeast strains. FEMS Yeast Res. **4:**711-719.

18. **Mc Bryde, C., J. M. Gardner, M. de Barros Lopes, and V. Jiranek.** 2006. Generation of novel wine yeast strains by adaptive evolution. Am. J. Enol. Vitic. **57:**423-430.

19. **Ness, F., F. Lavallée, D. Dubourdieu, M. Aigle, and L. Dulau.** 1993. Identification of yeast strains using the polymerase chain reaction. J. Sci. Food Agric. **62:**89-94.

20. **Özcan, S., and M. Johnston.** 1999. Function and regulation of yeast hexose transporters. Microbiol. Mol. Biol. Rev. **63:**554-69.

21. **Paquin, D., and J. Adams.** 1983. Frequency of fixation of adaptive mutations is higher in evolving diploid than haploid yeast populations. Nature **302:**495-500.

22. **Perez, M. A., F. J. Gallego, I. Martinez, and P. Hidalgo.** 2001. Detection, distribution and selection of microsatellites (SSRs) in the genome of the yeast *Saccharomyces cerevisiae* as molecular markers. Lett. Appl. Microbiol. **33:**461-466.

23. **Prakash, L., and D. Higgins.** 1982. Role of DNA repair in ethyl methanesulfonate-induced mutagenesis in *Saccharomyces cerevisiae*. Carcinogenesis **3:**439-444.

24. **Pretorius, I. S.** 2000. Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. Yeast **16:**675-729.

25. **Pretorius, I. S., and F. F. Bauer.** 2002. Meeting the consumer challenge through genetically customized wine-yeast strains. Trends Biotechnol. **20:**426-432.

26. **Sato, M., M. Kishimoto, J. Watari, and M. Takashio.** 2002. Breeding of brewer's yeast by hybridization between a top-fermenting yeast *Saccharomyces cerevisiae* and a cryophilic yeast *Saccharomyces bayanus*. J. Biosci. Bioeng. **93:**509 - 511.

27. **Sauer, U.** 2001. Evolutionary engineering of industrially important microbial phenotypes, p. 129-169. *In* T. Scheper (ed.), Advances in Biochemical Engineering/Biotechnology, vol. 73. Springer Verlag, Berlin Heidelberg.

28. **Schütz, M., and J. Gafner.** 1993. Sluggish alcoholic fermentation in relation to alterations of the glucose-fructose ratio. Chemie, Microbiologie, Technologie der Lebensmittel **15:**73-78.

29. **Schwartz, D. C., and C. R. Cantor.** 1984. Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. Cell **37:**67-75.

30. **Sega, G. A.** 1984. A review of the genetic effects of ethyl methanesulfonate. Mutat. Res. **134:**113-142.

31. **Snow, R.** 1983. Genetic improvement of wine yeast, p. 439-460. *In* J. F. T. Spencer, D. M. Spencer, and A. R. W. Smith (ed.), Yeast genetics: Fundamental and applied aspects. Springer-Verlag, New York.

32. **Weikert, C., U. Sauer, and J. E. Bailey.** 1997. Use of a glycerol-limited, long-term chemostat for isolation of *Escherichia coli* mutants with improved physiological properties. Microbiology **143:**1567 - 1574.

33. **Wucherpfennig, K., K. Otto, and Y.-C. Huang.** 1986. Aussagekraft des Glucose-Fructose-Verhältnisses. Die Weinwirtschaft-Technik **122:**254-260.

Chapter 5

RESEARCH RESULTS III

Selection of wine yeast strains exhibiting reduced discrepancy in glucose and fructose utilization in sequential batch cultivation

5 Research Results III

Selection of wine yeast strains exhibiting reduced discrepancy in glucose and fructose utilization in sequential batch cultivations

5.1 Abstract

A low ratio of glucose and fructose concentration (GFR) in wine fermentation is known to be a causative factor of stuck and sluggish fermentations. GFR values of 0.1 and lower were shown to have a negative influence on the fermentative performance of wine yeast strains. The observed discrepancy in glucose and fructose utilization during wine fermentations is due to the generally glucophilic character of all common wine yeast strains. Yeast strains exhibiting a reduced discrepancy in glucose and fructose utilization would therefore prevent the evolution of unfavorable GFR values and diminish for the likelihood of stuck or sluggish wine fermentations. Here we describe an approach based on evolutionary engineering by mutagenesis and selection in sequential batch cultivation. Through this approach, we were able to evolutionarily engineer yeast strains that exhibit improved utilization of fructose when compared to the parental strains, as demonstrated through direct competition experiments of selected strains and their untreated parental strain. The improved phenotype was also detected in laboratory scale wine fermentations, where the discrepancy in glucose und fructose utilization exhibited by the newly generated yeast strains was found to be statistically significantly reduced in comparison to the parental strains. Such yeast may lead to new industrial starter cultures that are less likely to be involved in stuck or sluggish fermentations.

5.2 Introduction

Despite the common practice in commercial winemaking to employ specialized, selected dried yeast cultures for wine fermentations, stuck and sluggish fermentations continue to present a serious problem for the global wine industry. Stuck fermentations exhibiting a high concentration of residual sugar are very susceptible to microbiological spoilage and can not be bottled before the stability of

the wine is ensured. Sluggish fermentations are characterized by a low fermentative activity causing the wine fermentation to last much longer than the usually expected duration of 7 to 10 days. Such fermentations might require special treatment in terms of antioxidation operations due to the loss of the protective carbon dioxide blanket. Additionally, fermenter space becomes limiting due to expanding remaining times of the fermenting wines. Finally, the intended wine-style might not be achievable and such wines often have to be blended. Therefore, the problem of stuck and sluggish fermentations is a serious threat to the wineries world wide (7, 29, 30).

Many factors that affect wine fermentations in a negative ways have been elucidated. Indeed, the occurrence of a stuck or sluggish fermentation is commonly assumed to be caused by the synergistic effect of several of the known negative factors (for review see (1, 7)). Besides nitrogen deficiency (5), lack of oxygen (12, 33), excessive clarification of the grape juice (2, 3) and other factors, the ratio of glucose and fructose that is present during wine fermentation has been shown to contribute to the problem (8, 13, 36, 40). Indeed, a low glucose on fructose ratio (GFR) appears characteristic for sluggish and stuck fermentations.

The majority of specialized winemaking yeast strains belong to the species S*accharomyces cerevisiae* (*S. cerevisiae*), which is known to preferably metabolize glucose. The normally close to equimolar amounts of glucose and fructose that are present in grape juice are therefore utilized at different rates during fermentation, causing a higher residual fructose than glucose concentration. This discrepancy in glucose and fructose utilization has been described for all wine yeast strains tested thus far. The data however also suggest that there are significant strain-dependent differences regarding the degree of this trait (6).

Data indicate that most sluggish and stuck fermentations occur when approximately 80 % of the initially present sugars are metabolized (13). In all cases, it was found that the GFRs of the problematic fermentations were below a value of 0.1 (13). Such low GFR values were only detected when at least 90 % of the initial sugar concentration has been metabolized in normally proceeding wine fermentations (40).

A wine yeast strain showing a very low or even no discrepancy in glucose and fructose utilization would therefore be of considerable interest to the winemaking industry, since low GFR values during fermentation could be prevented.

Most wine yeast strains currently on the market have been selected through time and labor-intensive procedures that involve the individual assessment of large numbers of strains, which may either be natural isolates or hybrids obtained through breeding (31). Recently, adaptive evolution was evaluated as a procedure for a more efficient way to select wine yeast strains exhibiting generally improved properties. This method involves the exposure of individual yeast strains or of populations of different yeast strains to specific selection pressures over large numbers of generations (24). Evolutionary adaptation of microbiological populations to a given selection pressure through accumulation of spontaneous mutations has been described in the past (16, 19, 24, 38, 41). The rate of spontaneous mutagenesis is usually rather low. The mutation rate for *S. cerevisiae* was reported to be about $2.2x10^{-7}$ /kb /replication (0.0027 /genome /replication) (10). However, under certain circumstances is may rise considerably and is modulated by environmental conditions such as metabolic stress or nutrient limitation (9).

In the present work, a population containing a large number of genetically variable yeast strains was generated through mutagenesis of a strain, W15, with known superior industrial properties. This population was than subjected to specific selection pressure. While cultivation for up to 20 generations is employed for studying the physiological characteristics of culture populations or single strains qualitatively, longer cultivation duration will significantly influence the culture population in an evolutionary way depending on the specific selective pressure(s) exerted by the specific cultivation conditions (35). These conditions are designed in order to direct the evolutionary adaptation towards the desired biotechnological phenotypic traits (11, 35).

In this study, sequential batch cultivation was chosen as the experimental set-up for the selection procedure. Sequential batch cultivation is characterized by the fact that the culture population is repeatedly going through all cycles of a fermentation process. This includes lag phase, exponential phase and stationary phase of growth but also variable concentrations of nutrients and substrates as well as exposure to increasing ethanol concentrations (25, 35). Each of these phases and conditions will exert different types of selection pressures on the yeast population, with different individual strains responding differently to each phase. However, by sequentially repeating the batch fermentation numerous times, selection should favor strains that

overall are best adapted to the specific fermentative environment, in this case an environment with a strongly biased fructose to glucose ratio and an initially high sugar concentration. Since fructose is provided in a 20 times higher concentration than glucose, fructose will be the main carbon source during the majority of the fermentation time. Successful strains therefore need to ferment fructose efficiently throughout the process to sugar depletion.

For both, the wine industry and consumers, mutagenesis is an accepted technique for the generation of new wine yeast populations (30). Exposing a wine yeast strain to a mutagen causes the genetic background of this strain to be changed in a non-recombinant but also non-directed, random way. The generally successfully used alkylating agent ethyl methanesulfonate (EMS) was chosen for mutagenesis. Introduction of mutations by EMS in yeast and especially the species *S. cerevisiae* was studied extensively and therefore the underlying mechanisms are well known (17, 18, 21, 37). In yeast, EMS was found to induce almost exclusively base-pair substitution mutations (95-99 %). These transition mutations are mainly of G/C to A/T transitions (28). Additionally, a percentage of mosaic mutations and leaky mutants were observed to occur after EMS treatment of *Schizosaccharomyces pombe* (*S. pombe*) (21, 22). Besides the nucleotides, the phosphates present in DNA are also the target for alkylation. 15 % of the total DNA ethylation by EMS accounts to ethylation of phosphates (4). The induction of mutations by EMS treatment was found to be dependent on a functional DNA repair system, despite the prevailing opinion that mutations caused by ethylated nucleotides are induced by misreplication of these bases (28).

Exposure of the wine yeast strain W15 used for this study, to EMS allows the generation of a highly diverse culture population of new yeast strains as source for the selection of yeast strains highly adapted to the conditions applied in the selection procedure. Our data show that the selection in sequential batch cultivations in excess fructose concentrations has the potential to select for yeast strains of a fructose specific improved phenotype. This could be verified by direct competition of selected strains to the wild type strain W15 in the conditions they were selected in. In addition, under laboratory scale winemaking conditions, the selected strains proved to exhibit reduced discrepancy in glucose and fructose utilization in the intermediate stage of the fermentation.

To our knowledge, this is the first study evaluating the potential of mutagenesis combined with selection in sequential batch cultivation in order to generate novel yeast strains exhibiting reduced discrepancy in glucose and fructose utilization under fermentative conditions.

5.3 Materials and Methods

In this section, only those techniques not previously described in Chapter 4, are reported. Following materials and methods have been outlined in Chapter 4 and were also employed for this study.

- Description of the yeast strain Lalvin W15 (W15, Lallemand Inc)
- Medium composition of MS300 synthetic grape must
- Mutagenesis by ethyl methanesulfonate (EMS)
- Determination of glucose-, fructose- and ethanol concentration
- Direct competition in continuous cultivation
- Determination of culture composition in direct competition cultivations by replica plating
- DNA extraction
- Microsatellite-analysis
- Karyotyping
- Statistics

5.3.1 Yeast strains

The strain Lalvin W15 (W15, Lallemand Inc) used in this study and its handling has been described in Chapter 4.

The W15 wild-type strain was modified by integration of a kanamycin resistance gene (W15kanMX). The generation of the modified W15kanMX strain is object to confidentiality due to patent issues (F. Bauer, personal communication). W15kanMX is cultured in 2 % YPD (10 % yeast extract, 20 % peptone (BD Biosciences) and

20 % glucose (Fluka)) and stored as freeze culture in 30 % glycerol at -80 °C. Stability of integration of the kanamycin resistance was evaluated by cultivation in non-selective medium (2 %YPD) for 100 generations.

Strains selected within this study after selection in sequential batch cultivation of EMS treated W15 culture population are 15mA1, 15mA5, 15mA10, 15mA15, 15mA20, 15mA25, 15mA30, 15mA35, 15mA40 and 15mA45.

Strains isolated in a previous study after mutagenesis of a W15 culture population and selection in continuous cultivation (Horsch et al., in preparation) are 15m2 and 15m38.

5.3.2 Selection in sequential batch cultivation

Sequential batch cultivations were conducted in 350 ml scale in 500 ml glass bottles closed by a fermentation cap, allowing pressure compensation. The culture was agitated at 100 RPM by magnetic stirrer bar throughout the fermentation at 20 °C. The medium MS300 contained a total sugar concentration of 250 g/l at a GFR of 0.05. Inoculation of sequential batch cultivations was done at $3x10^6$ cells/ml. The initial culture population was prepared by inoculation of 500 µl of the freeze culture, prepared after mutagenesis of the W15 culture population, to 5 ml 2 % YPF medium (10 % yeast extract, 20 % peptone (BD Biosciences), 20 % fructose (Fluka)) and grown over-night at 30 °C in aerobic conditions. 500 µl of the over-night culture were re-inoculated to 100 ml 2 % YPF medium followed by over-night incubation at 30 °C in aerobic conditions. Cell density and OD_{600} of the pre culture were determined. Inoculation of the sequential batch cultivation was done after spinning down the culture volume containing $1x10^9$ cells for 5 min at 3,000 RPM, elimination of the supernatant and re-suspension of the cells in the selection medium. Re-inoculation of the culture population to a new batch occurred after sugar depletion. Before every third re-inoculation the culture population was cultivated over-night in 2 % YPF at 30 °C in aerobic conditions to allow the population to regain fermentative vigor. After 29 generations (5 batches) of cultivation in such conditions, the composition for the selection medium was changed by addition of 10 % “Müller-Thurgau” grape juice to the MS300 protocol. Periodical samples were taken to determine cell density, OD_{600}, CFU, glucose-, fructose- and ethanol concentrations.

5.3.3 Direct competition in sequential batch cultivation

The reference strain in the direct competition cultivation was W15kanMX. Selected strains were not marked by a metabolic marker. This allowed the determination of the evolution of culture composition during fermentation time by replica plating on G418 containing agar plates. The technical set-up for the direct competition in sequential batch cultivation is identical to the set-up used for the selection procedure in sequential batch cultivation. MS300 contained 250 g/l total sugars at a GFR of 0.05 or 250 g/l of glucose solely. The preparation of pre-cultures was done as described for the sequential batch cultivations. Inoculation was done at $5x10^6$ cells/ml of each competing strain. Direct competition in sequential batch culture was done in two different ways. Cultures were re-inoculated either by the time biomass production reached stationary phase or at depletion of the provided sugars. Both cultivation schemes were conducted for at least 20 and a maximum of 29 generations. Samples were taken periodically and OD_{600}, glucose-, fructose- and ethanol concentrations were determined.

5.3.4 Evaluation of fermentative performance in laboratory scale

Pre-cultures of yeast strains were prepared by inoculating of 5 ml 2% YPF medium from freeze cultures, followed by over-night incubation at 30 °C in aerobic conditions. 100 µl of the over-night culture was re-inoculated to 5 ml of 2 % YPF and re-incubated over night at 30 °C in aerobic conditions. Small scale fermentations were inoculated at $1x10^6$ cells/ml. Evaluations in 100 ml scale were conducted in 100 ml Erlenmeyer flasks, closed by a cotton wool plug, without agitation at 20 °C. MS300 was used as medium containing 100 g/l of glucose and fructose each or 25 g/l of glucose and fructose at a GFR of 0.02.

Evaluations in 300 ml scale were done in 500 ml bottles closed by a fermentation cap. Agitation was maintained at 100 RPM by magnetic stirrer bar. The temperature was kept at 20 °C throughout fermentation. Synthetic grape must MS300 containing 100 g/l of glucose and fructose each was used as medium.

5.4 Results

5.4.1 Mutagenesis of W15

W15 was chosen for this study since it is known as very successful yeast strain in commercial winemaking. It exhibits a strong and reliable fermentative activity, accompanied by the development of fruity aroma. A side aim of the present study would be to seek new improved strains that would preserve these positive traits.

Due to the diploid and homozygous character of W15 the verification of the rate of mutagenesis introduced by the EMS treatment by evaluation of the generation of auxotrophy is not a suitable option. Therefore, only survival rate was used as a parameter to assess the efficiency of the mutagenic treatment. Previous data by Sonderegger and Sauer (38) had shown that a value of 60% survival led to significant mutagenic events. EMS dosage and exposure times were thus optimized to achieve 60% survival of the W15 strain.

5.4.2 Evaluation of the culture population after EMS treatment of W15

The evaluation of the fermentation performance of the culture population yielded by EMS treatment of W15 has been outlined in Chapter 4.

Summarizing the results of this experiment it can be said that all samples showed complete sugar depletion, and none of the conditions led to a stuck or sluggish fermentation. The data suggest that the mutagenised culture population continues to show a good fermentative performance, and that the extend of DNA damage inflicted by using a 60% survival rate is not affecting excessively overall performance of the strains.

5.4.3 Selection in sequential batch cultivation

The mutagenised W15 culture population was subjected to the sequential batch cultivations in medium containing 250 g/l total sugars at a GFR of 0.05 as described in the Material and Methods. Each batch was cultured to sugar depletion before re-inoculation into fresh medium. The culture was maintained for a total of 17 sequential batch cultivations. Biomass monitoring indicated that each batch corresponded to

6.1 ± 1.2 cell replication cycles or generations, adding up to a total of approximately 104 generations. Maximum cell densities for the different batches varied between $1.3x10^8$ cells/ml and $2.6x10^8$ cells/ml. A statistically significant increase ($p<0.05$) in fructose utilization per hour, within the different batches during fermentation could be detected. The elevation of the fructose utilization rate is a general, however not strictly linear, trend over the entire selection procedure. The fructose utilization rates ranged from 1.2 g/lh and 1.5 g/lh. The glucose utilization rates per hour ranged between 0.04 g/lh and 0.16 g/lh. Since glucose makes up only 5 % of the total sugar, the much smaller utilization rate could be caused by preferred utilization of fructose in these conditions, which represented 95 %. The differences in glucose utilization within the sequential batches were however not statistically significant ($p>0.05$). Microscopic evaluation of the culture population showed an ellipsoid shape of the cells throughout fermentation. No changes of cell morphology were detected throughout the selection procedure. The number of budding cells changed according to the growth phase within each batch. At the end of a batch and during stationary phase of biomass production there were almost no budding yeast cells present, while after re-inoculation and during exponential growth phase the number of budding yeast cells represented the majority of the culture population.

5.4.4 Genetic characterization by microsatellite-analysis and karyotyping

The genetic characterization of W15 by microsatellite-analysis showed the presence of only one allele for each tested locus. This result is in accordance with results of R. C. Gardner (personal communication). The evaluation of the strains that were randomly isolated for further characterization after the selection procedure in sequential batch cultivation, 15mA10, 15mA15, 15mA25 and 15mA40, showed that all strains possessed the same profile as W15 (Table 5.1). All strains evaluated by microsatellite-analysis were also assessed by karyotyping. These results confirmed the findings of the microsatellite-analysis (results not shown). Therefore, the randomly selected strains derive from the original W15 strain and no contaminants were selected.

Table 5.1: Genetic characterization of W15 and selected strains by microsatellite-analysis

Yeast Strain	Microsatellite locus							
	YLL049W		YFR028C		YGL139W		YOL109W	
	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2
W15	294		123		124		259	
15mA10	294		123		124		259	
15mA15	294		123		124		259	
15mA25	294		123		124		259	
15mA40	294		123		124		259	

Fragment lengths found for W15, before mutagenesis by EMS treatment, are in accordance with the reference data supplied by R. C. Gardner (personal communication). Yeast strains selected after selection in sequential batch cultivation, 15mA10, 15mA15, 15mA25 and 15mA40, show identical profiles to W15.

5.4.5 Direct competition in sequential batch cultivation

To asses the success of the selection procedure, the randomly selected strains were subjected to a direct competition assay with the parental strain using a sequential batch cultivation system with conditions that were similar to those applied throughout the selection process. To reduce time constraints, the only difference was that re-inoculation between new batches was undertaken at the time when biomass formation had reached a plateau and not after full sugar depletion as had been the case during selection. In order to conduct cultivation for at least 20 generations, four sequential batches were undertaken in each case. To be able to follow the evolution of the competition cultures, the W15 strain was transformed with the dominant marker gene kanMX.

To assess whether this transformed strain could serve as wild type control, a series of competition assays using the W15kanMX strain and the untransformed W15 strain were conducted. These strains were inoculated in a 50 / 50 ratio into the initial batch culture and grown in four sequential cultures in triplicate for a total of 24 generations. In such conditions, even a small difference in generation time between the transformed and untransformed strains would lead to a significant shift in the proportion of the two strains. At the end of these cultures, the proportion of the two simultaneously cultivated strains had shifted by a maximum of 8 % and 9 % in favor

of W15kanMX, indicating that the transformed strain was not significantly different from the WT strain.

All strains isolated after the selection procedure were assessed in an identical manner. The results are summarized in Table 5.2.

Table 5.2: Direct competition in sequential batch cultivations

Yeast strain	Fermentation 1		Fermentation 2	
	[%]	Change [%]	[%]	Change [%]
W15	58	-9	45	-8
W15 $^{+}$	48	-4	48	0
W15 $^{++}$	62	2	50	-2
10mA25	77	12	77	29
10mA25 $^{+}$	80	38	77	34
10mA25 $^{++}$	23	-24	18	-25
10mA40	53	-4	63	21
10mA40 $^{+}$	77	22	83	20
10mA40 $^{++}$	25	-22	17	-33
15m2	28	-33	22	-38
15m38	35	-13	38	-19

Percentage of the wild type strain and selected strains at the end of cultivation in direct competition to W15kanMX and percentage of change within the entire fermentation. MS300 containing 250 g/l sugar and GFR=0.05, re inoculation at plateau of biomass formation; $^{+}$ MS 300 containing 250 g/l sugar, GFR=0.05, re inoculation at sugar depletion; $^{++}$ MS300 containing 250 g/l glucose as sole carbon source.

Strain 15mA25 showed an increase in proportion of 12 % and 29 % in the two independent fermentations. The increase did not proceed in a linear manner. In fact, during the first batch of cultivation a decrease of the selected strain was observed. Only after re-inoculation for the second to fourth batch was an increase in percentage of 15mA25 detectable (Table 5.3). The data suggest that the strain was selectively improved for the specific conditions used during the sequential batches, but that it was less well able to implant itself when transferred from the conditions applied during the pre culturing of the cells.

Table 5.3: Percentage of selected strains over fermentation time in direct competition in sequential batch cultivation

Yeast strain	Batch No	Fermentation 1	Fermentation 2
		Colonies [%]	Colonies [%]
15mA25	Initial	65	48
	1	48	38
	2	55	63
	3	75	73
	4	77	77
15mA40	Initial	57	42
	1	35	50
	2	48	52
	3	45	57
	4	53	63
15mA25^{+}	Initial	42	43
	1	47	47
	2	63	62
	3	73	70
	4	80	77
15mA40^{+}	Initial	55	63
	1	72	63
	2	68	62
	3	60	73
	4	77	83

Development of the percentage of selected strains in direct competition to W15kanMX in sequential batch cultivation. Cultivations were done in MS300 containing 250 g/l sugar and GFR=0.05, re-inoculation at plateau of biomass formation; $^{+}$ cultivation in MS 300 containing 250 g/l sugar, GFR=0.05, re-inoculation at sugar depletion.

The results for strain 15mA40 show that in the first fermentation, the percentage of the tested strain varied by only 4 % within the 24 generations of the four sequential batch fermentations. However, as for 15mA25, a 21% decline was observed during the first batch. Within the following three batches the number of colonies for the two competing strains equalized again. In the second experiment an increase of 21 % of 15mA40 was found. The increase developed steadily during all four batch

fermentations (Table 5.3).

These results were obtained for direct competition in sequential batch cultivation with re-inoculation when biomass formation reached its plateau. During the selection procedure, however, re-inoculation was initiated only after sugar completion. Therefore, strains 15mA25 and 15mA40 were also subjected to direct competition with W15kanMX in sequential batch cultures that were re-inoculated at sugar depletion (Table 5.2). In two independent cultivations of 24 generations, an increase of 34 % and 38 % in number of colonies for 15mA25 was found (Table 5.3). In these cultivations the tested yeast strain showed a continuous increase in proportion throughout the sequential batch fermentations. These results strengthen the findings of the first set of competition assays in sequential batch cultivation.

The evaluation of strain 15mA40 showed reproducible results for the two independent fermentations with re-inoculation at sugar depletion. An overall increase of the proportion of 15mA40 was calculated to be 22 % and 20 % in competition with W15kanMX (Table 5.3).

The fermentative performances of 15mA25 and 15mA40 in direct competition to W15kanMX were also evaluated in sequential batch cultivation in medium containing glucose as the sole carbon source (Table 5.2). Both selected strains showed a decrease as percentage of the culture population during 29 generations. Strain 15mA25 showed a 24 % and 25 % continuous decrease during the fermentation. The percentage of strain 15mA40 decreased by 22 % and 33 %. The cultivation of the parental strain W15 and the reference strain W15kanMX in direct competition in these conditions showed a non-significant difference after 29 generations. Therefore, the performance of strains 15mA25 and 15mA40 in competitive fermentations proved to be carbon source dependent. Indeed, the selected strains show a selective advantage in competition to W15kanMX only if the supplied medium contains fructose.

5.4.6 Comparison of two selection strategies

To further assess the nature of the improvements achieved with the various selective strategies, two strains from the fructose-specific improvement strategy, based on continuous fermentations of fructose-rich synthetic must, described in Chapter 4 of

this thesis, were also evaluated for there ability to compete in sequential batch fermentations. The strains 15m2 and 15m38 were selected after mutagenesis of the W15 culture population but using selection in continuous cultivation in chemostats under carbon, and in particular fructose, limiting conditions. The selection procedure in continuous cultivation was also aiming at the selection of yeast strains that would exhibit reduced discrepancy in glucose and fructose utilization under fermentative conditions.

These strains were also subjected to competition in sequential batches in order to assess their selective potential under conditions that were different from those under which they had been selected, but that were more representative of fermentations in high sugar concentrations as found in winemaking conditions. Both strains, 15m2 and 15m38, showed significantly reduced ability to compete with the W15kanMX strain in conditions of direct competition in sequential batch fermentation, but were however not completely displaced by W15kanMX (Table 5.2). Strain 15m2 had been able to out-compete W15kanMX strain in the conditions under which it had been selected, demonstrating the highly specialized nature of the adaptation. Indeed, if the selective pressure had led to a generally improved ability to ferment fructose, it would have been expected that this strain was able to out-compete W15kanMX in the sequential batch conditions as well. Strain 15m38, on the other hand had not been able to out-compete W15kanMX in the conditions it had been selected in. The same performance was seen in the sequential batch cultivations. Therefore, our data suggest a generally decreased fermentative capacity of 15m38 compared to W15kanMX. The presence of this strain in the culture population at the end of the selection procedure could be explained by a transiently improved fermentative capacity which is lost by cultivation of the strain in rich growth medium. Additionally one could discuss an improved metabolic mechanism dependent on synergistic effects within the culture population 15m38 was isolated from.

5.4.7 Direct competition in continuous cultivation

The selective potential of the strains 15mA25 and 15mA40 was also evaluated by direct competition to W15kanMX in continuous cultivation characterized by carbon limitation, the conditions in which strains 15m2 and 15m38 were selected in. The

evaluation was done by feeding media containing mainly fructose (GFR=0.02) and secondly also by feeding media containing glucose as the sole carbon source. Strain 15mA25 was reproducibly found with increasing proportion in all conditions, while strain 15mA40 was unable to out-compete the wild type strain in both conditions (Table 5.4). The ability of strain 15mA25 to successfully compete with the reference strain in carbon limiting continuous cultivation conditions is independent of the carbon source. This is in contrast to the finding of a fructose specific competitive advantage of this strain in sequential batch cultivation. These results show the generation of selection procedure specific phenotypes, as it was expected.

Table 5.4: Direct competition in continuous cultivation

Strain	Fermentation 1			Fermentation 2		
	s [1/h]	[%]	Change [%]	s [1/h]	[%]	Change [%]
W15	-0.0014	46	-1	0.0032	58	10
W15 +	0.0013	43	7	0.0025	52	13
15mA25	0.0086	83	40	0.0025	73	12
15mA25 +	0.0061	88	18	0.0057	88	17
15mA40	-0.0087	15	-32	-0.0059	22	-28
15mA40 +	-0.0048	23	-20	-0.0048	30	-22

Selective difference s displaying the rate of displacement per hour of cultivation (11), percentage of the wild type strain and selected strains at the end of cultivation in direct competition to W15kanMX and percentage of their change within the entire fermentation. MS300 containing 25 g/l sugar and GFR=0.02; $^{+}$ MS300 containing 25 g/l glucose as sole carbon source.

5.4.8 Evaluation in laboratory scale fermentation

The laboratory scale fermentations in 100ml scale Erlenmeyer flasks did not show reproducible fermentation durations. Two independent fermentations of synthetic grape must containing 100 g/l glucose and fructose each showed fermentation times of 360 h and 430 h. Statistical analysis of the data was therefore evaluated separately for each experiment. Determination of the IC50 values for glucose and fructose utilization revealed small differences of the selected strains in comparison to W15. However, the fermentative performance of the selected strains when compared to the parental strain W15 was not identical in the two independent experiments. In

general, the IC50 value for glucose utilization of the selected strains was equal to the value determined for W15 or slightly more elevated. The same is valid for the IC50 values for fructose utilization (Figure 5.1).

Statistic evaluation of the differences in GFR values of the selected strains in comparison to W15 at various sampling times did not show any statistical significance (α=0.5). Therefore differences in fermentative performance do not indicate a general decrease in the discrepancy of glucose and fructose utilization of the selected strains in comparison to the parental strain W15 in these conditions.

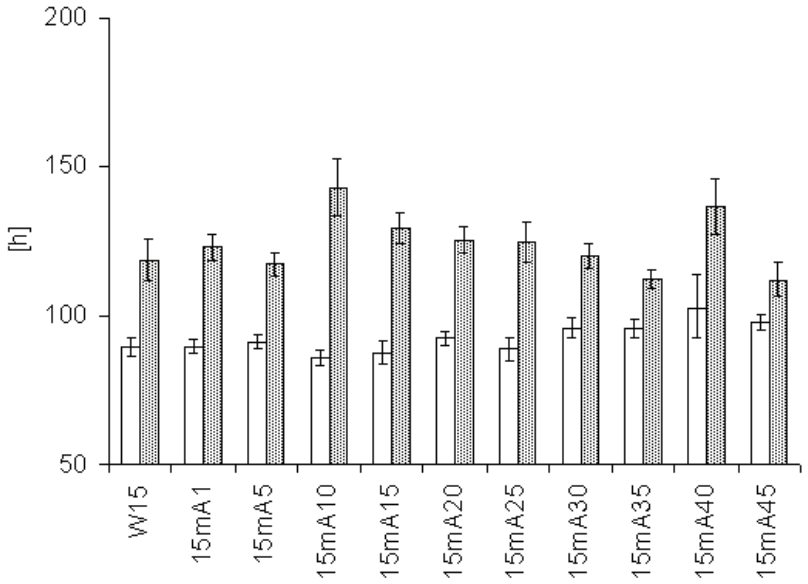
Four of the selected strains and the parental strain W15 were as also evaluated for their fermentative performance in a medium containing only 25 g/l glucose and fructose at a GFR of 0.02. This medium mimics the conditions of the glucose and fructose concentrations found in the majority of stuck and sluggish fermentations. The medium was not adjusted to an ethanol concentration that would be found in a wine fermentation at the corresponding stage in order to be able to judge the effect of the sugar composition independently of a possible inhibiting influence of ethanol. Table 5.5 shows the detailed results for the evaluation of the IC50 values for fructose utilization, determined by nonlinear regression. All selected strains are found to exhibit values within the same range as determined for W15. However all strains are able to ferment the medium to dryness and are not inhibited by the unfavorable initial ratio of glucose to fructose concentration.

Table 5.5: IC50 for fructose utilization

Yeast Strain	Mean	Min	Max
	[h]	[h]	[h]
W15	55	60	50
15mA10	57	62	53
15mA15	52	55	49
15mA25	51	54	48
15mA40	53	56	49

MS300 containing 25 g/l total sugars and GFR = 0.02 was supplied, mimicking the ratio of glucose and fructose found in the majority of stuck and sluggish fermentations.

A



B

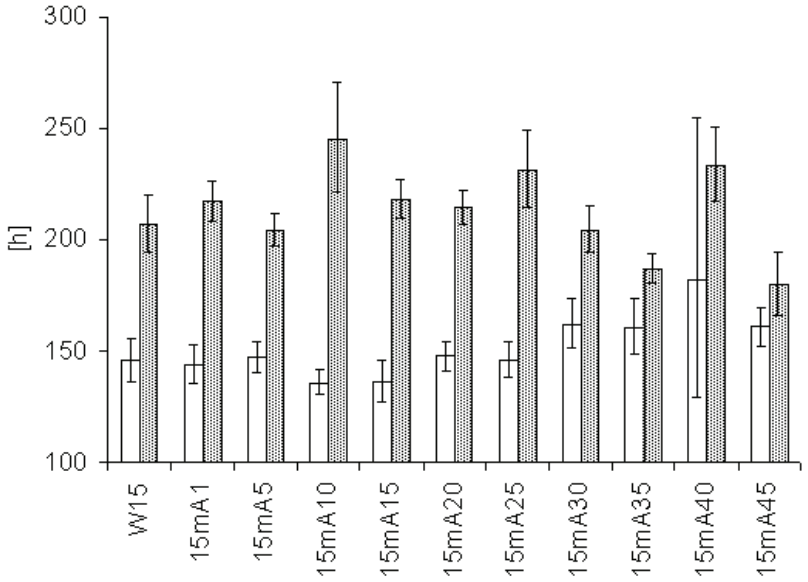


Figure 5.1: IC50 glucose (A) and IC50 fructose (B) determined for various strains selected by sequential batch cultivation of the mutagenised W15 culture population. Two independent fermentations in duplicates were conducted (white and grey bars) in laboratory scale of 100 ml without agitation (200 g/l glucose and fructose; GFR=1). The error margins indicate minimum and maximum values.

5.4.9 Evaluation in laboratory scale fermentation under agitation

Evaluation of fermentative performance in an increased volume of 300 ml and agitation at 100 RPM proved to be more reproducible and did not show the high variance in fermentation time as seen for the fermentations in 100 ml scale. Agitation at 100 RPM ensures an equal distribution of the culture population in the fermentation volume. Settling of the cells is prevented and therefore equal excess of nutrients and substrate to all cells is facilitated. Since fermentation bottles were closed by fermentation cap, anaerobic conditions can be assumed after depletion of the initially available oxygen. Three independent fermentations in triplicates were conducted for strains W15, 15mA25 and 15mA40. Sugar depletion was found to be reached within all fermentations by 119 h. Figure 5.2 shows the results of the evaluation of the fermentation progress by nonlinear regression with a dose-response model.

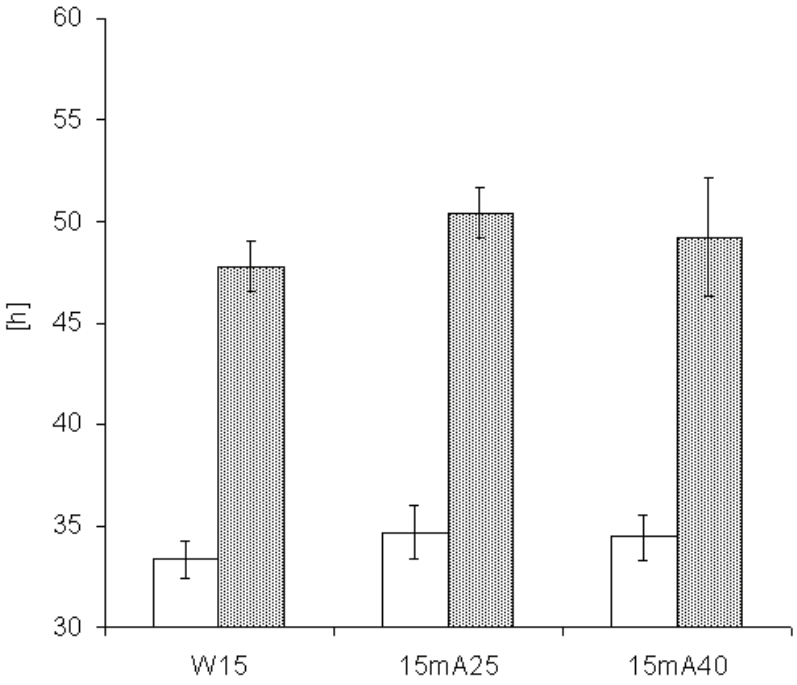


Figure 5.2: IC50 glucose (white bars) and IC50 fructose (grey bars) determined in laboratory scale fermentations done in 300 ml scale and agitation at 100 RPM (200 g/l glucose and fructose; GFR=1). The error margins indicate minimum and maximum values found within three independent fermentations, each done in triplicate for each tested strain.

Both selected strains required slightly more time to ferment 50 % of the initially available amount of glucose or fructose. However, the ranges of minimum and maximum values for the selected strains overlap with the values of W15. Therefore the strains can be considered to exhibit comparable fermentation performance in these conditions.

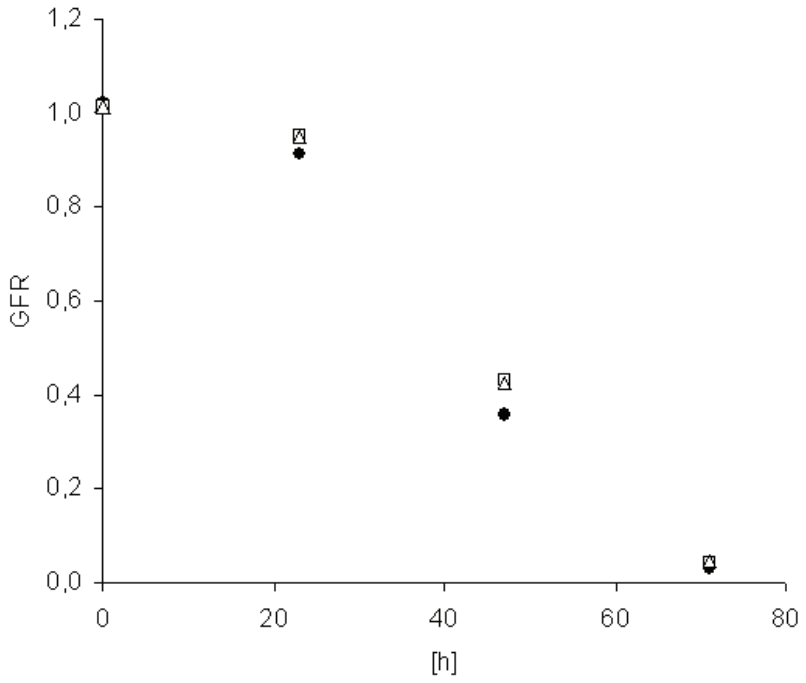


Figure 5.3: GFR values during fermentation time in laboratory scale fermentations in 300 ml scale and agitation at 100 RPM (200 g/l glucose and fructose; GFR=1). Depletion of all supplied sugar was for all samples found at 119 h of fermentation. Glucose was depleted for the majority of the samples at 95 h of fermentation. Calculation of GFR is therefore only applicable up to 71 h of fermentation. Parental strain W15 (●) and the selected strains 15mA25 (□) and 15mA40 (△) were evaluated in these conditions. Error margins are abstained for reasons of clarity of the figure.

Evaluation of the GFR values during fermentation time reveals increased values for the selected strains compared to the parental strain W15 at 47 h of fermentation. This corresponds to the stage of fermentation when approximately half of the initially present glucose has been converted (Figure 5.3). The differences seem to be low. However, statistical analysis by Tukey's multiple comparison tests indicates statistical significance for the observed differences in GFR values ($p<0.05$). GFR values calculated for other sampling times do not show statistically significant differences

between the evaluated strains.

These results indicate a tendency of the selected strains to exhibit a reduced discrepancy in glucose and fructose utilization compared to W15.

5.5 Discussion

Our data show a fructose specific improved fermentative performance of the selected strains 15mA25 and 15mA40 in direct competition to the parental strain W15 in the cultivation conditions applied during the selection process in sequential batch cultivation. This result contrasts with the findings of a study in *Escherichia coli* (*E. coli*) which used sequential batch cultivation over 10,000 generations. In this case, the improved phenotypes of the descendants where found to consist in a reduced lag phase and higher maximum growth rate, and present when alternative carbon sources taken up by the same transport system were provided as substrates. Therefore, the authors suggested that enhanced carbon transport had been the specific target of their strategy (20). However, carbon-source dependent selection in *E. coli* was reported for experiments carried out in nutrient-limiting chemostat cultivation (15). In our study, the improved phenotype was found to be fructose specific although no fructose specific transport system is present in *S. cerevisiae* and glucose and fructose are transported by the same proteins (39).

Evaluation of the selected strains 15mA25 and 15mA40 in continuous cultivation under fructose or glucose-limiting conditions showed that their improved fructose specific phenotype is not transferable to those conditions. In addition, our data also showed that the strains 15m2 and 15m38 selected under carbon-limitation in continuous cultivation are not able to successfully compete with the parental strain in the conditions of sequential batch fermentation. These results indicate that different metabolic mechanisms were addressed in the selection processes of continuous and sequential batch cultivation, although fructose was the major carbon source supplied in both trials. It is likely that the improved phenotype obtained after sequential batch cultivation corresponds at least in part to adaptations to earlier stages of the fermentation process in a high sugar environment, and is not linked to metabolic mechanisms involved in conditions of carbon-source limitation.

It is generally believed that the discrepancy in glucose and fructose utilization in *S. cerevisiae* is due to differences in hexose transport or hexose phosphorylation (6,

14). For the fructose-specific improvement observed in our conditions, both an improved affinity for fructose and an overall increased fructose specific transport capacity might contribute significantly to the phenotype. In this context, it is well possible that transport proteins such as Hxt1p to Hxt4p, Hxt6p and Hxt7p, were targets of the adaptation process by elevation of their fructose specificity in regard to affinity and/or transport capacity. In addition, one of the less well characterized hexose transporters (Hxt5p and Hxt8p to Hxt17p) could possibly contribute to the fructose specific phenotype.

Since the improved phenotype of the strains 15mA25 and 15mA40 is even more pronounced if the cultivation of the competing strains is extended to sugar depletion (as opposed to the end of biomass production), improvement with regard to lag phase and growth rate, as seen in the study done with *E. coli*, is unlikely to be the improved target in our selected strains. Indeed, no indication of a shortened lag phase or improved fermentation kinetics is seen in our data. Improved fructose specific metabolic activity in the stationary phase of the fermentation process is therefore suggested to significantly contribute to the phenotype.

The results of Rossignol et al. (32), Perez et al. (27) and Luyten et al. (23) show that the different hexose transporters are expressed at various stages to different degrees during the fermentation under winemaking conditions. Therefore, it can be speculated that more than one transporter may be involved in the improved fructose specific performance, contributing at various stages of the fermentation process.

An involvement of the hexose phosphorylation system in the development of the improved phenotype can be considered as well (6, 14). Increased fructose specific affinity of the hexokinases (Hxk1p and Hxk2p) could facilitate such an improvement. Rather unlikely is the involvement of a so far unknown hexokinase or unknown functions of Hxk1p or Hxk2p, since those proteins have been studied intensively (26, 34).

In contrast, only 9 proteins belonging to the hexose transporter family were comprehensively studied so far, while 11 await further characterization (26, 39).

Evaluation of the fermentative performance of the selected strains 15mA25 and 15mA40 in winemaking conditions showed that the improved phenotype was transferable to those conditions. Indeed, the newly generated yeast strains were found to exhibit a reduced discrepancy in glucose and fructose utilization in the

intermediate phase of the fermentation process when compared to the parental strain W15.

Successful application of sequential batch cultivation for the generation of novel wine yeast strains by adaptive evolution of a well accepted commercial yeast strain was reported by McBryde et al. (24). Their study was, however, aiming to generally improve the fermentative characteristics and not a more specific phenotype. Mutants of the commercial wine yeast were isolated after 350 generations of applying selective pressure. This is an approximately 3-fold higher number of generations than used in our study. Our approach to start off with a culture population of high genetic variability induced by chemical mutagenesis is certainly shortening the length of selection time that is required in order to isolate yeast strains exhibiting an improved phenotype. Indeed, a certain number of strains will carry mutations that pre-adapt those strains to the selective conditions.

5.6 Conclusions

According to our knowledge this is the first report about an evolutionary engineering approach by sequential batch cultivation for the generation of yeast strains exhibiting reduced discrepancy in glucose and fructose utilization. This study shows the significant potential of this strategy for the generation of desired phenotypes. From a scientific point of view, the availability of a yeast strain carrying the desired trait of very low discrepancy in glucose and fructose utilization could be useful for the elucidation of the factors determining this specific phenotype. This knowledge would contribute to our general understanding of the mechanisms underlying the differences of glucose and fructose utilization by *S. cerevisiae*.

5.7 Acknowledgements

We are thankful to R. Gardner for supplying the protocol and reference data for microsatellite-analysis employed in this study. We also want to thank the department of Life Sciences and Facility Management of the “Zürcher Hochschule für Angewandte Wissenschaften” in Wädenswil for kindly providing the BBraun fermenter system used in this study.

5.8 Literature cited

1. **Alexandre, H., and C. Charpentier.** 1998. Biochemical aspects of stuck and sluggish fermentation in grape must. J. Ind. Microbiol. Biotechnol. **20:**20-27.
2. **Ancin, C., B. Ayestaran, and J. Garrido.** 1996. Sedimentation clarification of Garnacha musts. Consumption of amino acids during fermentation and aging. Food Res. Int. **29:**345-353.
3. **Ayestaran, B., J. Garrido, and C. Ancin.** 1998. Sedimentation clarification of viura musts. Utilization of amino acids during fermentation. Food Chem. **63:**191-197.
4. **Bannon, P., and W. Verly.** 1972. Alkylation of phosphates and stability of phosphate triesters in DNA. Eur. J. Biochem. **31:**103-111.
5. **Bely, M., J.-M. Sablayrolles, and P. Barre.** 1990. Automatic detection of assimilable nitrogen deficiencies during alcoholic fermentation in oenological conditions. J. Ferment. Bioeng. **70:**246-252.
6. **Berthels, N. J., R. R. Cordero Otero, F. F. Bauer, J. M. Thevelein, and I. S. Pretorius.** 2004. Discrepancy in glucose and fructose utilisation during fermentation by *Saccharomyces cerevisiae* wine yeast strains. FEMS Yeast Res. **4:**683-689.
7. **Bisson, L. F.** 1999. Stuck and sluggish fermentations. Am. J. Enol. Vitic. **50:**107-119.
8. **Dittrich, H. H., and M. Grossmann.** 2005. Mikrobiologie des Weines, 3 ed. Verlag Eugen Ulmer, Stuttgart.
9. **Drake, J. W.** 1991. Spontaneous mutation. Annu. Rev. Genet. **25:**125-146.
10. **Drake, J. W., B. Charlesworth, D. Charlesworth, and J. F. Crow.** 1998. Rates of spontaneous mutation. Genetics **148:**1667-1686.
11. **Dykhuizen, D. E., and D. L. Hartl.** 1983. Selection in chemostats. Microbiol. Rev. **47:**150-68.
12. **Fornairon-Bonnefond, C., V. Demaretz, E. Rosenfeld, and J.-M. Salmon.** 2002. Oxygen addition and sterol synthesis in *Saccharomyces cerevisiae* during enological fermentation. J. Biosci. Bioeng. **93:**176-182.
13. **Gafner, J., and M. Schütz.** 1996. Impact of glucose-fructose-ratio on stuck fermentations. Vitic. Enol. Sci. **51:**214-218.
14. **Guillaume, C., P. Delobel, J.-M. Sablayrolles, and B. Blondin.** 2007. Molecular basis of fructose utilization by the wine yeast *Saccharomyces cerevisiae*: a mutated HXT3 allele enhances fructose fermentation. Appl. Environ. Microbiol. **73:**2432-2439.
15. **Helling, R. B., T. Kinney, and J. Adams.** 1981. The maintenance of plasmid-containing organisms in populations of *Escherichia coli*. J. Gen. Microbiol. **123:**129-141.
16. **Helling, R. B., C. N. Vargas, and J. Adams.** 1987. Evolution of *Escherichia coli* during growth in a constant environment. Genetics **116:**349-358.
17. **Kilbey, B., and F. Hunter.** 1983. Factors affecting mutational yield from EMS exposure of yeast (*S. cerevisiae*). Mutat. Res. Lett. **122:**35-38.

18. **Klein, F., A. Karwan, and U. Wintersberger.** 1989. After a single treatment with EMS the number of non-colony-forming cells increases for many generations in yeast populations. Mutat. Res. **210:**157-164.

19. **Lenski, R., and M. Travisano.** 1994. Dynamics of adaptation and diversification: A 10.000-generation experiment with bacterial populations. Proc. Nat. Acad. Sci. U.S.A. **91:**6808-6814.

20. **Lenski, R. E., J. A. Mongold, P. D. Sniegowski, M. Travisano, F. Vasi, P. J. Gerrish, and T. M. Schmidt.** 1998. Evolution of competitive fitness in experimental populations of *E. coli*: What makes one genotype a better competitor than another? Antonie van Leeuwenhoek **73:**35 - 47.

21. **Loprieno, N.** 1966. Differential response of *Schizosaccharomyces pombe* to ethyl emthanesulfonate and methyl methanesulfonate. Mutat. Res. **3:**486-493.

22. **Loprieno, N., R. Guglielminetti, S. Bonatti, and A. Abbondandolo.** 1969. Evaluation of the genetic alterations induced by chemical mutagens in *Schizosaccharomyces pombe*. Mutat. Res. **8:**65-71.

23. **Luyten, K., C. Riou, and B. Blondin.** 2002. The hexose transporters of *Saccharomyces cerevisiae* play different roles during enological fermentation. Yeast **19:**713-726.

24. **Mc Bryde, C., J. M. Gardner, M. de Barros Lopes, and V. Jiranek.** 2006. Generation of novel wine yeast strains by adaptive evolution. Am. J. Enol. Vitic. **57:**423-430.

25. **Moreno-Arribas, M. V., and M. C. Polo.** 2005. Winemaking biochemistry and microbiology: Current knowledge and future trends. Crit. Rev. Food Sci. **45:**265-286.

26. **Özcan, S., and M. Johnston.** 1999. Function and regulation of yeast hexose transporters. Microbiol. Mol. Biol. Rev. **63:**554-69.

27. **Perez, M., K. Luyten, R. Michel, C. Riou, and B. Blondin.** 2005. Analysis of *Saccharomyces cerevisiae* hexose carrier expression during wine fermentation: both low- and high-affinity Hxt transporters are expressed. FEMS Yeast Res. **5:**351-361.

28. **Prakash, L., and D. Higgins.** 1982. Role of DNA repair in ethyl methanesulfonate-induced mutagenesis in *Saccharomyces cerevisiae*. Carcinogenesis **3:**439-444.

29. **Pretorius, I. S.** 2000. Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. Yeast **16:**675-729.

30. **Pretorius, I. S., and F. F. Bauer.** 2002. Meeting the consumer challenge through genetically customized wine-yeast strains. Trends Biotechnol. **20:**426-432.

31. **Ribereau-Gayon, P.** 1985. New developments in wine microbiology. Am. J. Enol. Vitic. **36:**1-10.

32. **Rossignol, T., L. Dulau, A. Julien, and B. Blondin.** 2003. Genome-wide monitoring of wine yeast gene expression during alcoholic fermentation. Yeast **20:**1369-1385.

33. **Sablayrolles, J.-M., C. Dubois, C. Manginot, J.-L. Roustan, and P. Barre.** 1996. Effectiveness of combined ammoniacal nitrogen and oxygen additions

for completion of sluggish and stuck wine fermentations. J. Ferment. Bioeng. **82:**377-381.

34. **Santangelo, G. M.** 2006. Glucose signaling in *Saccharomyces cerevisiae*. Microbiol. Mol. Biol. Rev. **70:**253-282.

35. **Sauer, U.** 2001. Evolutionary engineering of industrially important microbial phenotypes, p. 129-169. *In* T. Scheper (ed.), Advances in Biochemical Engineering/Biotechnology, vol. 73. Springer Verlag, Berlin Heidelberg.

36. **Schütz, M., and J. Gafner.** 1993. Sluggish alcoholic fermentation in relation to alterations of the glucose-fructose ratio. Chemie, Microbiologie, Technologie der Lebensmittel **15:**73-78.

37. **Sega, G. A.** 1984. A review of the genetic effects of ethyl methanesulfonate. Mutat. Res. **134:**113-142.

38. **Sonderegger, M., and U. Sauer.** 2003. Evolutionary engineering of *Saccharomyces cerevisiae* for anaerobic growth on xylose. Appl. Environ. Microbiol. **69:**1990-1998.

39. **Wieczorke, R., S. Krampe, T. Weierstall, K. Freidel, C. P. Hollenberg, and E. Boles.** 1999. Concurrent knock-out of at least 20 transporter genes is required to block uptake of hexoses in *Saccharomyces cerevisiae*. FEBS Lett. **464:**123-128.

40. **Wucherpfennig, K., K. Otto, and Y.-C. Huang.** 1986. Aussagekraft des Glucose-Fructose-Verhältnisses. Die Weinwirtschaft-Technik **122:**254-260.

41. **Zelder, O., and B. Hauer.** 2000. Environmentally directed mutations and their impact on industrial biotranformation and fermentation processes. Curr. Opin. Microbiol. **3:**248-251.

Chapter 6

Concluding remarks and future prospects

6 Concluding remarks and future prospects

6.1 Concluding remarks

6.1.1 Evolutionary engineering for the generation of microorganisms of improved phenotypes

Evolutionary engineering is a powerful strategy for the generation of novel industrially important microbial phenotypes (11, 18, 34). It is based on genetic variation and selection. In comparison to metabolic engineering, it has the advantage that the genetic or molecular mechanisms underlying the desired phenotype must not be necessarily known. This broadens the field of application tremendously (34). We successfully implemented such an approach, generating novel yeast strains of improved fermentative capacity in nitrogen limiting conditions (Chapter 3). We then applied several strategies for the generation of yeast strains that would exhibit a reduced discrepancy in glucose and fructose utilization. Indeed, although sugar metabolism in *S. cerevisiae* was studied intensively, the mechanism responsible for glucose preference in *S. cerevisiae* is not yet fully understood (for review see (6, 39)). In this conclusion, the reasons for achieving or not achieving certain aims will be discussed, and the different strategies of evolutionary engineering will be compared.

6.1.1.1 Generation of genetic variability

Our strategy relied on the generation of a very large number of genetic variants of the parental strain(s) all commercial wine yeast strains presenting desirable traits for specific applications. To achieve the target, two approaches were used in this work, mutagenesis and hybridization through mass-mating. While hybridization through mass-mating, which randomly recombines the genomes of two parental wine yeast strains, proved highly successful in the first approach to generate nitrogen efficient strains, it failed in the second approach.

Although the percentage of 6 % of hybrid strains after mass-mating in the first approach was lower than theoretically possible, the properties of some of those hybrids were obviously highly suitable for the specific selection conditions, since hybrids were strongly enriched, representing the vast majority of strains at the end of

the selection process in limiting nitrogen conditions. Most of those hybrids showed the desired trait of improved fermentative activity under nitrogen limiting conditions.

When applied in the fructose project, mass mating failed to produce a satisfactory number of hybrids, since such strains were found to represent only about 1 % of the mass-mated population. The initial proportion of 28 %, suggested by data generated through PCR employing δ elements, and which led us to use this population as a starting point for our selection, had to be corrected, when PCR results were at a later stage found to be unreliable. At the end of the selection procedure none of the examined strains exhibited Uvaferm 43-based or hybrid genetic background. Therefore it can be assumed that the properties of such strains were not advantageous in the selection process. Independently of the initial proportion of a subpopulation within a population, if the phenotype of an individual cell is best adapted to the selective conditions, its percentage can be expected to increase due to the selective advantage within the population (8, 34). Nevertheless, it has to be taken into consideration that a higher initial proportion of hybrid strains or strains of Uvaferm 43 genetic backgrounds could have supported their maintenance in the population under these conditions.

The reason for the low mating rate of W15 and Uvaferm 43 can probably be found in the genetic background of the parental strains. In particular, there are some inconsistencies regarding the ploidy status of Uvaferm 43 (7) and recent data appear to indicate a low mating ability (R. Gardner, personal communication). Our data would support such findings, since the sporulation rate of Uvaferm 43 was similar to W15, both strains achieving at least 70 %. Usually, zymolase treatment of a sporulating population should not only facilitate the disintegration of the ascus to release the single spores, but also contribute to the inactivation of vegetative, non-sporulating yeast cells. However, many yeast strains show a high resistance to the zymolase treatment. It is therefore possible that a significant number of the W15-like strains detected after the mass-mating originate from surviving spheroplasts. Additionnal treatments could have prevented such an outcome (15). However, we were initially misled by the data generated through PCR.

The low percentage of Uvaferm 43-like strains found after mass-mating could be an indication of reduced spore viability compared to W15. Therefore, an improved mating rate could be brought about by the assessment of the germination proficiency

before mass-mating, followed by the mixture of Uvaferm 43 spores in increasing proportions in order to support inter-strain mating events.

Microsatellite analysis recently suggested that Uvaferm 43 might not be a true diploid, which could be a contributing factor for low mating efficiency (7). However, other wine yeast strains of comparable genetic background were found to show sufficient sporulation efficiency and spore viability, but these characteristics are yeast strain dependent (4, 30).

An alternative methodology to increase the proportion of inter-strain hybrids could be the direct inoculation of spores into the selective media. The successful generation of inter-strain hybrids by direct inoculation of tetrads into laboratory scale winemaking conditions was recently reported by Ambrona and Ramirez (2). Indeed, hybrid strains were repeatedly sporulated and their fermentative performance was found to be increased. The low hybridization rate of 1.5 % under those conditions was explained by the inoculation of tetrads and could therefore be improved by the inoculation of single spore preparations.

Mutagenesis was the second approach used in our study to generate genetic variability. The success of this approach is difficult to assess on a molecular level, since all strains are of the same genetic background, and the diploid status renders a phenotypic assessment of mutational events rather difficult. In general, mutagenesis does not have the same potential of generating genetic diversity than hybridization, since only a single parental background is modified. Considering that the populations were maintained for a very large number of generations in the selective environment, it could be questioned whether the initial mutagenesis contributed significantly to the specific phenotypes of the strains selected at the end of the process. Indeed, it is possible that the adaptations shown by these strains are due to mutation that occured during the adaptive evolution phase. Nevertheless, mutagenesis would have generated many mutations in each individual genome, providing more diverse starting material with strains that in some cases may harbor complementary mutational events. Furthermore, mutagenesis of a single parental background has the advantage that improved strains can be used for the study of the molecular events that led to the successful adaptation and provide a tool for further analysis.

6.1.1.2 Selection in continuous cultivation

The selection procedure in continuous cultivation was characterized by very low growth rates under anaerobic conditions and carbon source limitation. The medium supplied throughout cultivation contained fructose in increasing proportions. These conditions are somewhat representative of the late stages of the fermentation process which is characterized by metabolically active cells in stationary phase and a high proportion of fructose. This strategy was expected to yield yeast strains exhibiting fructose utilization specific improved traits, since selection in continuous cultivation under carbon limiting conditions was reported to yield a carbon source specific increased maximum growth rate and reduction in the Monod constant (K_s) (1, 9, 10, 17). Selection in aerobic, glucose-limited chemostat cultivation of *S. cerevisiae* was recently described to yield yeast strains exhibiting higher affinity and velocity for glucose (16). In addition, chemostat cultivation of *S. cerevisiae* in anaerobic and glucose-limiting conditions was shown to cause an increase in high-affinity uptake (32). Unfortunately, both studies did not comment on the carbon source specificity of the adaptations in hexose uptake.

The yeast strains selected in continuous cultivation in this study showed a carbon source independent improved fermentative performance which was verified in continuous cultivation in direct competition to the parental strain W15. Therefore it can be suggested, that selection in continuous cultivation in carbon limiting, anaerobic conditions does not trigger a carbon source dependent improvement in *S. cerevisiae*. A generally elevated capacity of the high-affinity hexose transport system could be suggested and would be in agreement with the recently published study of Rintala et al. (32).

The selection of strains exhibiting a lower level of fitness compared to the parental strain, as seen for the selection in continuous cultivation in our study, has been reported in previous studies using evolutionary engineering approaches. In some cases, a polymorphic population was reported to harbor different subpopulations surviving in the specific selection conditions. The survival strategies of each sub-population might be dependent on each others metabolic products (13). The evolution of such polymorphic populations is complicating selection strategies, since only the entire population may express the desired phenotype or appear well adapted to specific conditions. But on the single strain level, the phenotype might not be

expressed and therefore the aim of the selection procedure may not be fulfilled (34).

Strains selected by continuous cultivation that did not out-compete the parental strain in the direct competition assays were characterized by a distinctive colony morphology when plated on rich growth medium immediately after withdrawal from the selective culture. This colony morphology was however reversible and disappeared after growing the strains in rich liquid medium followed by plating. This morphological change could be an indication of the loss of a transient, advantageous phenotype maintained during the selection process. Colony morphology is known to be in part controlled through epigenetic regulation of cell wall proteins, and such proteins may well impact on fermentation performance as well (36). Therefore, it could be speculated that the selected strains, which appear inferior in direct competition to W15, lost an improved phenotype through cultivation in rich growth conditions.

Nevertheless, the selection strategy in continuous cultivation can be considered at least in part successful, since most of the selected strains clearly out-competed the wild type in the selective environment.

6.1.1.3 Selection in sequential batch cultivation

The wine fermentation process can be best described as batch cultivation. Therefore selection in sequential batch cultivations was one of the approaches used in this study. The specific cultivation condition in batch cultivation is characterized by continuously changing environmental and physiological conditions (34). This involves the presence of very high initial sugar and nutrient concentrations, many of which decrease rapidly and may be depleted at various stages of fermentation. In addition, the ethanol concentration is constantly increasing and the microbial population is continuously changing, with *S. cerevisiae* usually being the most dominant species in the latter stages.

The change of the competitive fitness within a culture population exposed repeatedly to those conditions follows a steady but hyperbolic evolution (20). In a recent study of Mc Bryde et al. (25) adaptive evolution in sequential batch cultivation was employed for the first time in order to improve the general fermentative performance of a commercial wine yeast strain. These authors were able to isolate a mutant after 350 generations of cultivation under selective conditions, which exhibited generally

improved fermentative performance.

We were able to generate a fructose specific improved phenotype by exposing the mutagenised W15 culture population to sequential batch cultivation over 100 generations. The selected strains showed the potential to out-compete the parental strain W15 by cultivation in direct competition in the conditions applied during the selection process. If the same strains were cultivated under the same conditions in medium containing glucose as the sole carbon source the selected strains were not able to take over majority in the culture population.

Since it was shown that the members of the hexose transporter family are expressed at various stages and to a specific extent under winemaking fermentation conditions, it can be speculated that the fructose specific improved phenotype is facilitated by several of those transporters (21, 29, 33). Hexose phosphorylation, the second step in the glycolysis pathway, was also discussed to be a possible trigger responsible for the discrepancy in glucose and fructose utilization and can therefore also be hypothesized to contribute to the expression of the improved phenotype (5, 35).

6.1.2 Transferability of selected phenotypes to winemaking conditions

The improved phenotype of the novel yeast strains selected by continuous cultivation was not fully transferable to winemaking conditions. The evaluation of the improved phenotype under winemaking conditions can be done by the determination of the GFR during the fermentation process. A reduced discrepancy in glucose and fructose utilization is characterized by higher GFR values. Repeated fermentations in laboratory scale revealed a tendency of the selected strains to show higher GFR values than W15 in the same conditions. However, the degree of improvement was statistically questionable and different for each yeast strain.

Evaluation of the novel yeast strains selected by sequential batch cultivation showed statistically significant higher GFR values for the selected strain in the intermediate fermentation stage. This was valid for all of the examined selected strains.

Our results suggest that sequential batch cultivation may be a more successful strategy when trying to improve general fermentation aspects than continuous fermentation, which has by definition to focus selection on a narrow window of specific selective conditions. Strains improved through this technology may therefore

not show generally improved winemaking ability. On the other hand, we were not able to fully asess whether the selected strains would not display a significantly improved phenotype in conditions were the parental strains would become stuck due to a low GFR.

Meijer et al. (26) found a 2 to 3-fold difference in glucose and fructose uptake rates of a culture population maintained in aerobic continuous cultivation and the in-vitro uptake in batch cultivation. The authors speculate that a general component or regulator of the uptake system is inactivated between continuous cultivation and exposing a sample of the culture population to the in-vitro uptake assay. Such a phenomenon could also impede the transferability of the improved fermentative activity of the strains selected in continuous cultivation to winemaking conditions. At the same time our findings support the notion that an improved phenotype generated in sequential batch cultivation is easier to transfer to batch industrial conditions (34).

A study by Rossignol et al. (33) monitoring genome wide gene expression of wine yeast during alcoholic fermentation shows, that the switch from the replicative to the non-replicative state of the culture population is a key physiological event in the wine fermentation process. Many genes responding at this stage were found to encode proteins of unknown function. In this regard it could be hypothesized, that the selection in continuous cultivation is less suitable for the generation of wine yeast strains altered in their hexose utilization profile due to the lack of this distinct physiological mechanism.

In addition, the data of Luyten et al. (21) and Perez et al. (29) show, that the hexose transport proteins Hxt1p to Hxt7p are not expressed and functional in the same way under winemaking conditions as under laboratory conditions. These findings are also indicating that the characteristic expression profile of the hexose transporter under winemaking conditions can not be triggered by focusing on a specific part of the process, as it is done in the selection in continuous cultivation.

This might be valid especially for Hxt3p, which is expressed throughout the wine fermentation process, although it is a low affinity transporter and only expressed at high sugar concentrations in laboratory scale conditions (21, 29, 31). Therefore, it could be the target of evolutionary improvement due to its continuing presence. Indeed, Guillaume et al. (12) found the genetic background of a mutated *HXT3* allele to be the reason for enhanced fructose fermentation of a wine yeast strain and

therefore suggested Hxt3p to play a key role in the determination of the glucose to fructose utilization ratio.

Hxt6p and Hxt7p, known to show high affinity for fructose, are expressed and active under winemaking conditions already after entry into stationary phase in the presence of high hexose concentrations, although they are defined as high-affinity hexose transport proteins and appear to only be active in low hexose concentrations (21, 29, 31). This could make them a possible target of evolutionary improvement in the later stage of fermentation. Our data suggest the importance of the fermentative activity during stationary phase for the expression of the fructose improved phenotype, since the strains showed pronounced improvements in the direct competition assays when re-inoculation was only implemented after sugar depletion instead of at entry into stationary phase.

The fructose specific improvement by selection in sequential batch cultivation is triggered by the availability of this nutrient throughout the fermentation process. An evolutionary advantage can therefore be gained for improvements at all stages of the process. Our data suggest that the evolutionary adaptations of the successful strains selected under these conditions are fructose specific and can, to a certain extend, be transferred to the biotechnological application. This is contrary to the situation in the continuous fermentation, were fructose limitation was the driving force for adaptation. Such conditions are expected to trigger the selection of yeast strains exhibiting improved affinity of the hexose transporters facilitating the most efficient access to the very low fructose concentrations (9, 34). Strains selected under these conditions however showed carbon source independent improvement, as further supported by the insignificantly altered GFR values of the selected yeast strains during winemaking conditions.

The success of the selection of yeast strains exhibiting improved utilization of less preferred nitrogen sources in continuous fermentation however also shows that such selection conditions can yield strains that show significant improvement in winemaking conditions. Nitrogen metabolism and its regulation are highly complex, and offer many theoretical genetic targets for strain improvements. Rossignol et al. (33) for example showed that the induction of genes involved in the utilization of poor nitrogen sources only occurs at the onset of stationary phase, which is in part triggered by the depletion of preferably used nitrogen sources (14). Modification to

this regulation may allow yeast to better use less preferred nitrogen sources as were used in our selective conditions. The potential of continuous cultivation to maintain such specific cultivation conditions over a long time could therefore facilitate the selection of the improved phenotype. Indeed, our data show that the improved fermentative capacity of the selected strains is most pronounced in nitrogen limiting conditions during the stationary phase of the fermentation process.

Our studies show that the combination of mutagenesis of a commercial yeast strain of desirable enological traits and selection in sequential batch cultivation characterized by supplying excess concentrations of fructose yields yeast strains exhibiting reduced discrepancy in glucose and fructose utilization. However, the dimension of improvement can not be considered to be sufficient for the application of the strains in the winemaking industry, since Uvaferm 43 still exhibits higher GFR values under winemaking conditions than any of the newly selected yeast strains. However, since wine yeast strains were selected over centuries of cellar operations, all of the strains have already a significant capacity of fructose utilization. Therefore the potential for improvement and the likelihood of success of the selection process may be limited.

We therefore suggest our studies to be an initial evaluation of different evolutionary engineering approaches, high-lightening the potential of these methodologies and allowing further evaluation of the most promising strategies for future investigations.

6.2 Future prospects

In order to further improve the fructose specific phenotype of the selected strains several additional strategies can be thought of.

By exposing the generated populations to further generations of selection, additional adaptive events can be triggered. Since sequential batch cultivation was found to be characterized by a steady gain in fitness of the population and the cultivation conditions applied in our study were shown to trigger fructose specificity, additional advantageous adaptive mutations can be expected (19).

As an alternative a single selected strain could be further developed in a similar selection strategy. Although this approach minimizes the possible variability in comparison to the treatment of a whole culture population, a specific follow up of the

adaptive changes occurring under extended selective pressure would be simplified due to the limited number of possible changes.

In addition to extended exposure to the selection condition, slight modifications of the selection scheme were previously found to have a positive influence to the success of a selection procedure (20, 27, 28). However, in order to define modifications to the selection procedure, it would be advantageous to know more about the mechanisms triggering the desired phenotype.

First insights into understanding the improved phenotypes in our case could be gained by a comparison of the transcriptome or proteome of W15 and a selected strain, which proved to out-compete the parental strain if cultured in direct competition. Such results would enable to more specifically define alterations in the selection conditions, adjusting the selective pressure to better target the desired biotechnological phenotype.

For longer-term prospects, the availability of a selected strain exhibiting a clearly reduced discrepancy in glucose and fructose utilization under winemaking conditions could reveal all related major quantitative trait loci (QTL) and therefore lead to the definition of specific genetic markers that may be useful in future breeding programs for the characterization of newly generated yeast strains (22-24, 37, 38).

The knowledge about the pivotal factors triggering the discrepancy in glucose and fructose utilization could be used for an inverse metabolic engineering approach in a strictly scientific sense (3). Integration of the desired phenotype into a laboratory strain by DNA recombination would enable the study of these traits.

6.3 Literature cited

1. **Adams, J., C. Paquin, P. W. Oeller, and L. W. Lee.** 1985. Physiological characterization of adaptive clones in evolving populations of the yeast, *Saccharomyces cerevisiae*. Genetics **110:**173-185.
2. **Ambrona, J., and M. Ramirez.** 2007. Analysis of homothallic *Saccharomyces cerevisiae* strain mating during must fermentation. Appl. Environ. Microbiol. **73:**2486-2490.
3. **Bailey, J. E., A. Sburlati, V. Hatzimanikatis, K. Lee, W. A. Renner, and P. S. Tsai.** 1996. Inverse metabolic engineering: A strategy for directed genetic engineering of useful phenotypes. Biotechnol. Bioeng. **52:**109-121.

4. **Barre, P., F. Vézinhet, S. Dequin, and B. Blondin.** 1993. Genetic improvement of wine yeast, p. 55-75. *In* G. H. Fleet (ed.), Wine Microbiology and Biotechnology, vol. 1. Harwood Academic Publishers, Philadelphia, PA.

5. **Berthels, N. J., R. R. Cordero Otero, F. F. Bauer, I. S. Pretorius, and J. M. Thevelein.** 2008. Correlation between glucose/fructose discrepancy and hexokinase kinetic properties in different *Saccharomyces cerevisiae* wine yeast. Appl. Environ. Microbiol. **77:**1083-1091.

6. **Bisson, L. F., D. M. Coons, A. L. Kruckeberg, and D. A. Lewis.** 1993. Yeast sugar transport. Crit. Rev. Biochem. Mol. Biol. **28:**259-308.

7. **Bradbury, J., K. Richards, H. Niederer, S. Lee, P. R. Dunbar, and R. Gardner.** 2006. A homozygous diploid subset of commercial wine yeast strains. Antonie van Leeuwenhoek **89:**27-37.

8. **Dykhuizen, D. E.** 1993. Chemostats used for studying natural selection and adaptive evolution, p. 613-631, Methods Enzymol., vol. 224. Academic Press Inc.

9. **Dykhuizen, D. E., and D. L. Hartl.** 1983. Selection in chemostats. Microbiol. Rev. **47:**150-68.

10. **Ferea, T. L., D. Botstein, P. O. Brown, and R. F. Rosenzweig.** 1999. Systematic changes in gene expression patterns following adaptive evolution in yeast. Proc. Nat. Acad. Sci. U.S.A. **96:**9721-9726.

11. **Floras, N., J. Xiao, A. Berry, F. Bolivar, and F. Valle.** 1996. Pathway engineering for the production of aromatic compounds in *Escherichia coli*. Nat. Biotechnol. **14:**620-623.

12. **Guillaume, C., P. Delobel, J.-M. Sablayrolles, and B. Blondin.** 2007. Molecular basis of fructose utilization by the wine yeast *Saccharomyces cerevisiae*: a mutated HXT3 allele enhances fructose fermentation. Appl. Environ. Microbiol. **73:**2432-2439.

13. **Helling, R. B., C. N. Vargas, and J. Adams.** 1987. Evolution of *Escherichia coli* during growth in a constant environment. Genetics **116:**349-358.

14. **Henschke, P. A., and V. Jiranek.** 1993. Yeast metabolism of nitrogen compounds, p. 77-163. *In* G. H. Fleet (ed.), Wine Microbiology and Biotechnology. Harwood Academic Publishers, Chur.

15. **Herman, P. K., and J. Rine.** 1997. Yeast spore germination: a requirement for Ras protein activity during re-entry into the cell cycle. The EMBO Journal **16:**6171-6181.

16. **Jansen, M. L. A., J. A. Diderich, M. Mashego, A. Hassane, J. H. de Winde, P. Daran-Lapujade, and J. T. Pronk.** 2005. Prolonged selection in aerobic, glucose-limited chemostat cultures of *Saccharomyces cerevisiae* causes a partial loss of glycolytic capacity. Microbiology **151:**1657-1669.

17. **Kurland, C. G.** 1992. Translational accuracy and the fitness of bacteria. Annu. Rev. Genet. **26:**29-50.

18. **Kuyper, M., A. A. Winkler, J. P. Dijken, and J. T. Pronk.** 2004. Minimal metabolic engineering of *Saccharomyces cerevisiae* for efficient anaerobic xylose fermentation: a proof of principle. FEMS Yeast Res. **4:**655-664.

19. **Lenski, R., and M. Travisano.** 1994. Dynamics of adaptation and diversification: A 10.000-generation experiment with bacterial populations. Proc. Nat. Acad. Sci. U.S.A. **91:**6808-6814.

20. **Lenski, R. E., J. A. Mongold, P. D. Sniegowski, M. Travisano, F. Vasi, P. J. Gerrish, and T. M. Schmidt.** 1998. Evolution of competitive fitness in experimental populations of *E. coli*: What makes one genotype a better competitor than another? Antonie van Leeuwenhoek **73:**35 - 47.

21. **Luyten, K., C. Riou, and B. Blondin.** 2002. The hexose transporters of *Saccharomyces cerevisiae* play different roles during enological fermentation. Yeast **19:**713-726.

22. **Marullo, P., M. Aigle, M. Bely, I. Masneuf-Pomarede, P. Durrens, D. Dubourdieu, and G. Yvert.** 2007. Single QTL mapping and nucleotide-level resolution of a physiologic trait in wine *Saccharomyces cerevisiae* strains. FEMS Yeast Res. **7:**941-952.

23. **Marullo, P., M. Bely, I. Masneuf-Pomarede, M. Aigle, and D. Dubourdieu.** 2004. Inheritable nature of enological quantitative traits is demonstrated by meiotic segregation of industrial wine yeast strains. FEMS Yeast Res. **4:**711-719.

24. **Marullo, P., G. Yvert, M. Bely, M. Aigle, and D. Dubourdieu.** 2007. Efficient use of DNA molecular markers to construct industrial yeast strains. FEMS Yeast Research **7:**1295-1306.

25. **Mc Bryde, C., J. M. Gardner, M. de Barros Lopes, and V. Jiranek.** 2006. Generation of novel wine yeast strains by adaptive evolution. Am. J. Enol. Vitic. **57:**423-430.

26. **Meijer, M. M. C., J. Boonstra, A. J. Verkleij, and C. T. Verrips.** 1996. Kinetic analysis of hexose uptake in *Saccharomyces cerevisiae* cultivated in continuous culture. Biochim. Biophys. Acta **1277:**209-216.

27. **Orr, H. A.** 1999. The evolutionary genetics of adaptation: a simulation study. Genet. Res. **74:**207-214.

28. **Orr, H. A.** 2005. The genetic theory of adaptation: a brief history. Nat. Rev. Genet. **6:**119-127.

29. **Perez, M., K. Luyten, R. Michel, C. Riou, and B. Blondin.** 2005. Analysis of *Saccharomyces cerevisiae* hexose carrier expression during wine fermentation: both low- and high-affinity Hxt transporters are expressed. FEMS Yeast Res. **5:**351-361.

30. **Puig, S., A. Querol, E. Barrio, and J. E. Perez-Ortin.** 2000. Mitotic recombination and genetic changes in *Saccharomyces cerevisiae* during wine fermentation. Appl. Environ. Microbiol. **66:**2057-2061.

31. **Reifenberger, E., E. Boles, and M. Ciriacy.** 1997. Kinetic characterization of individual hexose transporters of *Saccharomyces cerevisiae* and their relation to the triggering mechanisms of glucose repression. Eur. J. Biochem. **245:**324-333.

32. **Rintala, E., M. G. Wiebe, A. Tamminen, L. Ruohonen, and M. Penttila.** 2008. Transcription of hexose transporters of S*accharomyces cerevisiae* is affected by change in oxygen provision. BMC Microbiology **8**.

33. **Rossignol, T., L. Dulau, A. Julien, and B. Blondin.** 2003. Genome-wide monitoring of wine yeast gene expression during alcoholic fermentation. Yeast **20:**1369-1385.

34. **Sauer, U.** 2001. Evolutionary engineering of industrially important microbial phenotypes, p. 129-169. *In* T. Scheper (ed.), Advances in Biochemical Engineering/Biotechnology, vol. 73. Springer Verlag, Berlin Heidelberg.

35. **Schütz, M., and J. Gafner.** 1995. Lower fructose uptake capacity of genetically characterized strains of *Saccharomyces bayanus* compared to strains of *Saccharomyces cerevisiae*: A likely cause of reduced alcoholic fermentation activity. Am. J. Enol. Vitic. **46:**175-180.

36. **Verstrepen, K. J., and F. M. Klis.** 2006. Flocculation, adhesion and biofilm formation in yeasts. Mol. Microbiol. **60:**5-15.

37. **Winzeler, E. A., C. I. Castillo-Davis, G. Oshiro, D. Liang, D. R. Richards, Y. Zhou, and D. L. Hartl.** 2003. Genetic diversity in yeast assessed with whole-genome oligonucleotide arrays. Genetics **163:**79-89.

38. **Winzeler, E. A., D. R. Richards, A. R. Conway, A. L. Goldstein, S. Kalman, M. J. McCullough, J. H. McCusker, D. A. Stevens, L. Wodicka, D. J. Lockhart, and R. W. Davis.** 1998. Direct allelic variation scanning of the yeast genome. Science **281:**1194-1197.

39. **Zimmermann, F. K., and K.-D. Entian.** 1997. Yeast sugar metabolism. Technomic Publishing, Lancaster, PA.

Chapter 7

Appendix

The fructophilic yeast *Zygosaccharomyces bailii* in its application in curing industrial stuck fermentations

7 Appendix

The fructophilic yeast *Zygosaccharomyces bailii* in its application in curing industrial stuck fermentations

7.1 Abstract

Despite the availability of various yeast nutrients and adjuvants to enhance yeast's ability to conduct wine fermentation, stuck fermentations continue to be a severe problem in wineries all over the world. It has been established that a glucose-fructose-ratio (GFR) of below 0.1 has a negative influence on the activity of traditional wine yeast strains, mostly of the species *Saccharomyces cerevisiae* (*S. cerevisiae*). Data collected in wineries in Switzerland by our laboratory suggest that wine fermentations tend to become stuck when the GFR falls below 0.1 before approximately 80 % of the initial sugar has been depleted. Here we show that by employing a strain of the fructophilic yeast species *Zygosaccharomyces bailii* (*Z. bailii*) the fructose concentration in a stuck wine fermentation can be specifically reduced, while the glucose concentration remains stable, leading to an increase in the GFR. The data show that this shift enables the remaining active wine yeast population to restart the fermentation. In this study, the impact of *Z. bailii* in curing industry-scale stuck fermentations in Swiss wineries is shown. A standard procedure for the evaluation and treatment of problematic fermentations is established. The selected *Z. bailii* strain reduces fructose efficiently in these conditions without adverse influence on the sensory properties of the treated wines.

7.1 Introduction

Wine fermentations are nowadays mostly conducted by inoculation of specialized wine yeast strains that are supplied by various manufacturers as active dried wine yeast (ADWY) products. When compared to spontaneous fermentations, which is facilitated by the yeast flora that is present on the grape skins, this practice has improved the reproducibility and control of wine fermentations. Indeed, spontaneous fermentations are considered to be rather unpredictable in terms of fermentation progress, and lead to wiser variations in the sensory properties of the products.

Although specialized yeast strains are generally selected for their strong fermentative performance, the occurrence of stuck and sluggish fermentations has not been eradicated, and such fermentations continue to be a severe problem in the wine industry (16).

Investigations by many research groups have revealed numerous causes for a reduction or arrest in fermentative activity and these causes were reviewed in several publications (2). However, the specific reason that is responsible for individual cases of stuck fermentations in industrial settings is in most cases not easily defined. This is due to the fact that many factors act synergistically. In addition, each of the commercially available wine yeast strains exhibits specific properties and demands in terms of fermentation conditions. In order to facilitate successful fermentation, the knowledge about the specific needs of a wine yeast strain is therefore crucial (3).

The most commonly accepted strategy for re-initiation of stuck fermentations is based on the removal of the settled yeast biomass and re-inoculation of a starter culture that has been pre-adapted to the conditions of the stuck fermentation to be treated. The adaptation process of the starter culture is facilitated by gradual increase in volume of the arrested fermentation to the inoculum. By this process the newly re-hydrated yeast population is gradually exposed to the adverse conditions in a sluggish fermentation like high ethanol and low nutrient concentrations and probably several other inhibitory substances. In addition, knowledge about possible causes of a specific problem fermentation may lead to supporting actions. For example, inhibitory substances may be removed specifically before re-inoculation. However, even if these strategies are implemented with care, many attempts are met with failure (4).

The procedure for curing stuck fermentations described here is based on a different strategy. The mechanism of this strategy is the increase of the glucose to fructose ratio (GFR), which was found to inhibit fermentative activity of wine yeast strains. The majorities of stuck fermentations investigated by our group show a characteristic GFR value of 0.1 or lower (7). In laboratory scale experiments, addition of glucose to stuck fermentations in order to elevate the GFR to a value of at least 0.1 led to a reactivation of the fermentative activity and to complete depletion of the residual sugars (17). Glucose addition is however not a legal strategy for commercial winemaking. In addition, by increasing the sugar concentration in a wine

fermentation, the resulting ethanol concentration of the finished wine will also be elevated. If the ethanol concentration reaches the resistance limit of the yeast strain, it might again lead to an inhibition of fermentative activity (2). Elevated ethanol concentrations are generally not desirable for most wine styles and glucose addition would therefore not be a suitable measure (16). Therefore, the specific fermentation of excess fructose by a fructophilic yeast species was found to be a strategy to suit the needs of the winemaking industry.

Zygosaccharomyces bailii (*Z. bailii*) is known to prefer fructose as carbon source, even in the presence of glucose. Although it is regarded as a wine spoilage yeast, its ability to be metabolically active in the harsh conditions of finished wine, combined with its resistance to various acids and ethanol, makes this species an interesting candidate for this task (6, 13). *Z. bailii* strains, isolated from wines from the Eastern part of Switzerland and from grape leaves in a vineyard of Wädenswil, were evaluated for this purpose and found to exhibit the desired fructose specific fermentative activity in conditions of stuck fermentations. It was shown that the selected strains did not spoil wine and were well suited for curing stuck fermentations (Sütterlin et al., in preparation). The results gained by laboratory scale treatments of stuck fermentations were convincing and here we report on the implementation of this strategy in industrial wineries.

7.3 Materials and Methods

7.3.1 Yeast strains

The *Z. bailii* strains 3a (*Z. bailii* 3a) and 210 (*Z. bailii* 210) were isolated either from a wine produced in the Eastern part of Switzerland or from grapevine leafs in a vineyard in Wädenswil in Switzerland. Both strains were characterized in our laboratory and confirmed by the Centraalbureau voor Schimmelcultures (CBS) and included in the yeast strain collection at the Research Station Agroscope Changins-Wädenswil ACW (ACW).

Lalvin W15 was selected at ACW and is a commercially available wine yeast strain (Lallemand Inc). Rehydration was done according to the recommendations of the producer.

7.3.2 Propagation of *Z. bailii* strains

Z. bailii 3a was maintained on 2 % YPF agar plates (2 % fructose (Fluka), 2 % peptone (DB Biosciences), 1 % yeast extract (DB Biosciences), 2 % agar (Acros Organics). For the propagation of *Z. bailii* 3a a single colony of the agar plate culture was inoculated into 100 ml of 2 % YPF (2 % fructose, 2 % peptone, 1 % yeast extract), incubated at 30 °C under aerobic conditions until stationary phase of biomass production was reached. For propagation of higher volumes of *Z. bailii* culture, a laboratory scale bioreactor (InforsHT) 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 sterilized in the bioreactor at 121 °C for 20 min. After cooling down the medium to the desired growth temperature of 30 °C, approximately 10 ml of the pre-culture grown in 100 ml Erlenmeyers was inoculated into the bioreactor. Alternatively, a small volume of a previously produced culture population was used to inoculate the new batch. Filter sterilized air was used for aeration of the fermentation culture. Agitation was done at 700 RPM with a 3 bladed impeller. The temperature was maintained at 30 °C. “SIHA anti-foam” (Begerow) was used to avoid excessive foam formation in the culture vessel. Cells were harvested after at least 36 hr of cultivation. The harvested population was used as wet biomass.

In addition to the laboratory scale production batches, two industrial scale productions of *Z. bailii* 210 were conducted (Danstar Ferment) and also used for the treatment of stuck fermentations.

7.3.3 Quality control of propagation batches of *Z. bailii* 3a

The fructose utilization rate of the produced biomass of *Z. bailii* 3a was determined by inoculation of $1x10^8$ cells/ml 2 % or 3 % YPF (2 % or 3 % fructose, 2 % peptone, 1 % yeast extract), incubation at 25 °C, shaking at 160 RPM for at least 4 h. The remaining fructose and the acetic acid concentration after incubation was determined by HPLC-analysis as described for the evaluation of the wine samples (see following chapter). The fructose utilization rate per hour of incubation was calculated and used for comparison of the different production batches.

The production batches were also evaluated for bacterial or yeast contaminations. Therefore a dilution of the harvested biomass was streaked out on MRS^+ plates (MRS agar containing 0.4 g/l potassium sorbate and 3 mg/l actidione; Fluka), PY plates (DB BBL™ Phyton™ Yeast Extract Agar; DB Biosciences) and YPF plates (2 % fructose, 2 % peptone, 1 % yeast extract, 2 % agar). One colony of each phenotype grown on either of these plates was examined by PCR-analysis and RFLP-analysis in order to confirm the strain identity.

7.3.4 PCR-analysis and RFLP-analysis for strain identification

Colonies grown on agar plates were picked and genomic DNA was extracted according to the protocol (Part #TM050) of Promega. However, the isolated DNA was not treated by RNAse. Dried DNA samples were resuspended in 50 µl TE buffer (pH 8.0). Primers amplifying the region of the Internal-Transcribed-Spacers (ITS) were used for the PCR reaction (ITS 1 – 5´-TCCGTAGGTGAACCTGCGG-3´, ITS 4 – 5´-TCCTCCGCTTATTGATATGC-3´; synthesis by Microsynth). PCR reactions were done in a volume of 50 µl. The reaction mix for the PCR is composed of 1.5 U HotStart Taq, 1.5 mM $MgCl_2$, 200 µM dNTP (Qiagen; Microsynth), 0.4 µM primer ITS 1, 0.4 µM primer ITS 4 and 3 µl of DNA template. For amplification of the PCR product the following steps were employed: 1 cycle at 95 °C of 15 min followed by 30 cycles of the three steps 1 min at 95 °C, 1 min at 56 °C and 2 min at 72 °C. A final DNA extension step is done for 7 min at 72 °C. After completion of the amplification procedure, samples are kept at 6 °C until further use. Evaluation of the PCR product is done by gel-electrophoresis in a 1 % agarose gel (Multi Purpose agarose; Promega).

In order to be able to identify the *Z. bailii* strain 3a and 210 specifically, the PCR product was digested by the restriction enzyme *Tru*9I (Qbiogene). The digestion was done over night at 65 °C in a volume of 15 µl according to the instructions of the manufacturer. After completion of the digestion, the evaluation of the digested PCR product was done by gel-electrophoresis in a 1.8 % agarose gel (Multi Purpose agarose; Promega).

7.3.5 HPLC-analysis of wine samples at various stages of fermentation

The HPLC analysis was done according to the method by Kaufmann (11). This method allows the determination of the most important wine components, including 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 H_2SO_4 (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 cycler. 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 H_2SO_4.

7.3.6 Evaluation of problematic fermentations

A sample of each wine showing a problematic fermentation was evaluated by HPLC for its content of glucose, fructose, tartaric acid, malic acid, lactic acid, acetic acid, glycerol and ethanol. In addition, the wine was evaluated by light microscope to assess the microbiological flora. In short, 10 ml of the wine sample were centrifuged for 10 min. at 13,000 RPM. The supernatant was discarded. The remaining wet pellet was resuspended and evaluated for yeast cells and the presence of undesired microorganisms such as lactic acid bacteria. If undesired microorganisms were detected, the wine was filter-sterilized in order to avoid production of undesired compounds by these microorganisms.

7.3.7 Strategy for the treatment of sluggish fermentations

All wines were treated following the same strategy to restart stuck fermentation. As a first step the fermentation temperature was raised to at least 22 °C in order to support the fermentative activity of the active wine yeast population. In such a case, an increase in fermentative activity should be detected within two weeks. If this measure did not show success, the wine was re-inoculated with 0.5 g/l of Lalvin W15. The fermentation temperature was kept at least at 22 °C to support the activity of the newly inoculated yeast population. If no reactivation of the fermentative activity was observed the problematic fermentation was considered to be a stuck fermentation and could only be restarted by employing *Z. bailii*. According to the needs of each

wine, re-inoculations of *Z. bailii* in several steps to a final inoculation cell density of $1x10^6$ cells/ml were done. In addition, Lalvin W15 was also inoculated at 0.2 g/l if the original yeast population was judged not to be in a good condition at this stage. The temperature was kept at a minimum of 22 °C. Periodical samples were examined for their microbiological composition by light microscopy in order to avoid growth of undesired microorganisms, as well as for establishing residual sugar and other wine parameters for optimal quality. At depletion of all residual sugars or at desired residual sugar concentration, the finished wine was separated from the yeast lees and stabilized by sulphur dioxide (35 mg/l free SO_2).

7.4 Results

7.4.1 General observations in industrial problem fermentations

Industrial wine fermentations that had become stuck were treated by the strategy described above in 2003 and 2004. A comparison of the treatments of sluggish or stuck fermentations in these two vintages reveals a significant difference between the characteristics of the problematic wines. In 2003, 90 % of about 120,000 l of problematic fermentations from 18 different wineries had to be inoculated with *Z. bailii* to be able to restart fermentations. The majority of these wines were of the Pinot Noir variety. Only 10 % of the wines were cured by the traditional methods such as temperature elevation to at least 22 °C and re-inoculation of dry yeast Lalvin W15 in a concentration of 0.5 g/l (19). In 2004 only 41 % of 68 wines showing a problematic fermentation had to be treated by *Z. bailii*. The varieties affected in 2004 were mostly white varieties of various origins. Pinot Gris (14 %), Pinot Blanc (14 %) and Chardonnay (23 %) were most affected (Table 7.1). Of these problem fermentations, 59 % were characterized as sluggish fermentations, allowing to be reactivated by temperature elevation and re-inoculation by dry yeast Lalvin W15. In total, 94 % of all problematic fermentations in 2004 were cured successfully either by the traditional methods or by employing *Z. bailii*. 24 of all stuck fermentations treated by *Z. bailii* achieved complete sugar depletion or the desired residual sugar concentration. However, 4 stuck fermentations did not respond to any treatment.

These data suggest that the characteristics and probably the causes of stuck and sluggish wine fermentations vary significantly from vintage to vintage. It is also not possible to point out a specific grape variety generally most affected. The two

vintages 2003 and 2004 were characterized by very different climatic conditions. While 2003 was unusually hot and dry, the climate in 2004 can be considered to be more representative of the region. However, the problem of stuck and sluggish fermentations was present in both vintages.

Table 7.1: Grape varieties and total volumes of stuck fermentations treated in 2004

Grape variety	**Volume [l]**
Pinot Noir Rosé	2,000
Pinot Noir	2,600
Chardonell	100
Chardonnay	8,400
Pinot Gris	4,300
Kerner	4,500
Oeil de Perdrix	3,000
Pinot Blanc	10,190
Garanoir Rosé	1,000
Riesling x Sylvaner	1,500
Sauvignon Blanc	2,150
Schiller	5,000
Granacha	800

Treatments were done according to the specific needs of each problematic fermentation by *Z. bailii* and the established standard procedure

7.4.2 Evaluation of the quality of laboratory scale produced batches of *Z. bailii* 3a

The propagation of *Z. bailii* was done in medium containing fructose as the sole carbon source in order to support the fructophilic character of this yeast species (18).In addition, it was shown that *Z. bailii* yeast strains exhibit a higher specific consumption rate for fructose in aerobic batch cultures. However, even under anaerobic condition, *Z. bailii* was found to show growth in complex media containing fructose (14).

In order to be able to judge the quality of the various laboratory scale batches of *Z. bailii* 3a, the fructose utilization rate of each batch was determined in YPF in

Table 7.2: Laboratory scale production batches of *Z. bailii* 3a

Batch No	Mean activity	Mean acetic acid
	[g fructose/h]	[g/l]
1	1,0	0,3
2	0,8	0,3
3	0,8	0,3
4	0,9	0,3
5	0,7	0,2
6	1,1	0,3
7	1,1	0,3
8	1,2	0,2
9	1,2	0,2
10	1,2	0,2
11	2,0	0,2
12	2,1	0,2
13	2,1	0,3
14	2,0	0,3
15	2,4	0,2
16	1,4	0,1
17	2,5	0,2
18	2,5	0,2
19	1,9	0,2
20	2,5	0,3
21	3,1	0,3
22	2,5	0,3
23	2,7	0,4
24	1,5	0,2
25	1,6	0,1
26	2,4	0,4

Mean values of double determination. The CV% for the mean values for the fructose utilization activity was < 8 % (but for batch 16: 20 %) and the CV% for the mean values of the produced acetic acid did not exceed 25 %.

aerobic conditions. The determination of the fructose utilization rate in respiratory conditions served as basis for the comparison of the batches before they were used to cure stuck fermentations. The minimum rate of fructose utilization found within 26 laboratory scale batches, was 0.8 g/h, while the highest activity was 3.1 g/h (Table 7.2). These values indicate the fructose depletion activity of the tested populations of

Z. bailii 3a in respiratory growth conditions. An anaerobic experimental set-up would have had the draw back of being very time-consuming since the fermentative activity of *Z. bailii* 3a is very low (15). The wet biomass was intended to be inoculated as freshly as possible, since it is expected that the number of active yeast cells decreases over storage time. Therefore, a fast and reproducible method in order to assess a key parameter for quality control of the produced *Z. bailii* batches had to be established.

Acetic acid production by *Z. bailii* 3a under respiratory conditions is insignificant. A maximum concentration of 0.4 g/l of acetic acid was found at the end of the cultivation for the quality assessment (Table 7.2). Laboratory scale batches before cells were harvested showed a maximum acetic acid concentration of 1.6 g/l. Since cells were washed in saline solution after harvesting and before inoculation to the stuck fermentation, it can be assumed that no detectable concentration of acetic acid was added to the treated wine.

Colonies grown on YPF, PY or MRS^{+} plates from the batch of *Z. bailii* for detection of bacterial or yeast contaminations were also evaluated for their morphology and growth. Only one colony morphology was found and growth of colonies was found to be uniform. A colony from each plate was picked and characterized by PCR employing primers amplifying the ITS region and digestion of the PCR products by *Tru*9I. All colonies were found to exhibit the same pattern in PCR and RFLP as the original sample of the *Z. bailii* strain 3a (Sütterlin et al., in preparation). Therefore, no contaminations were detected within the batches of *Z. bailii* 3a culture populations used for the treatment of stuck fermentations.

All *Z. bailii* 3a culture populations showed fructose utilizing ability in respiratory conditions, however of various capacity. Since all of the production batches were free of contaminating organisms, the metabolization of fructose in the treated wine fermentations can be considered to be the result of the inoculation of *Z. bailii* 3a.

7.4.3 Treatment of stuck wine fermentations with *Z. bailii* inoculation

All treatments of sluggish or stuck industrial wine fermentations by *Z. bailii* were done as described in the chapter “Materials and Methods”. The practical handling at individual winery was always carried out by the resident winemaker. After inoculation of *Z. bailii*, samples of the treated fermentations were sent periodically to our

laboratory. The analysis of the initial sample of the problematic fermentation, the determination of fermentation kinetics after re-inoculation by Lalvin W15 and the evaluation of the success of the strategy after inoculation of *Z. bailii* were evaluated by our laboratory. In this way, it was possible to conduct large-scale treatments in several regions of Switzerland under true industrial conditions, without involvement of members of our laboratory in the winery work. A drawback of this situation was that we depended on the information provided by the winemakers regarding several crucial fermentation parameters.

In the vintage season 2004, stuck and sluggish fermentations of 13 different grape varieties were treated (Table 7.1). The progress of the different treatments was different for each of the problematic fermentation and independent of grape variety and fermentation volume. Differences were experienced in particular regarding the number of *Z. bailii* cells that needed to be inoculated in order to detect a significant decrease in fructose concentration. The majority of stuck fermentations had to be inoculated more than once by *Z. bailii*, each time to a concentration of $1x10^6$ cells/ml. The decision for additional inoculation of *Z. bailii* was taken either when no decline in fructose concentration was observed or when fructose fermentation declined after initial success.

Figure 7.1 shows the kinetics of a treatment of a stuck Chardonnay fermentation by *Z. bailii* 210 in a 2,000 L tank. By the time the winemaker requested advice for the treatment of the problematic fermentation, the wine had already been re-inoculated with Lalvin W15. The remaining fructose concentration was found to be 27.5 g/l, while glucose was present in a concentration of 3.4 g/l. Re inoculation of Lalvin W15 was not followed by reactivation of fermentative activity. Therefore, the problematic fermentation was classified as stuck fermentation and treatment by *Z. bailii* 210 was initiated. According to the information of the winemaker, fermentation temperature was constantly maintained at a temperature of at least 22 °C throughout the period of treatment. Fructose was found to be constantly decreasing after inoculation of *Z. bailii* 210 at a rate of 0.2 g/day to 1.5 g/day. The maximum fermentation rate of 1.5 g fructose/day was detected between days 18 to 25 of the treatment. Glucose was metabolized at a rate of maximum 0.2 g/day. The total time demand for this treatment was 45 days.

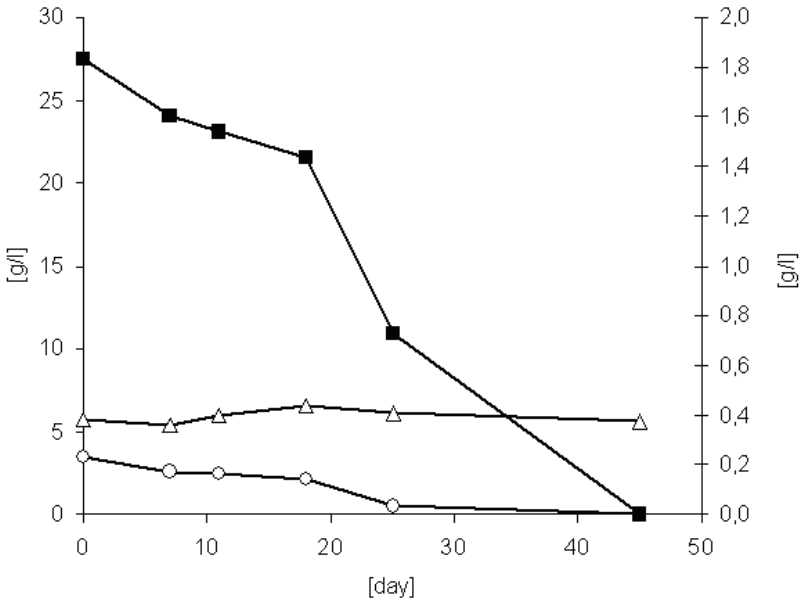


Figure 7.1: Progress of a treatment by *Z. bailii* of a stuck Chardonnay fermentation (2,000 l). Fructose (●) is metabolized significantly immediately after inoculation of the yeast population. Glucose concentration (○) is decreasing at a lower rate. Eventually, both sugars are depleted and the treatment is completed within 45 days of fermentation. Acetic acid concentration (△) is stable over fermentation time.

In contrast to this very straight forward treatment, another treatment required four inoculations of *Z. bailii* for successful sugar depletion (Figure 7.2). In this case, 4,000 L of a problematic fermentation of Pinot blanc were treated. Each addition of *Z. bailii* was done at a concentration of $1x10^6$ cells/ml. The four additions of *Z. bailii* were done at the beginning of the treatment and at days 41, 97 and 131 after the first inoculation. The whole treatment took 140 days. Glucose concentration was only 0.9 g/l, while fructose was still present in a concentration of 14.3 g/l by the time of first addition of *Z. bailii*. A significant fructose utilization rate of 0.15 g fructose/day was only detectable at 106 days after the first inoculation of *Z. bailii*. The maximum depletion rate of 0.65 g fructose/day was reached towards the end of the treatment, after day 131. Fermentation temperature was kept at constantly 22 °C, according to the information by the winemaker. Fermentation was stopped at a residual fructose concentration of 4.3 g/l since this supported the wine style the winemaker was aiming for. The evaluation of the sample by light microscopy after 140 days of treatment showed no conspicuous microorganisms. Therefore the inoculated *Z. bailii* and Lalvin

W15 were considered to have been the fermentatively active yeast strains that reduced the residual fructose concentration.

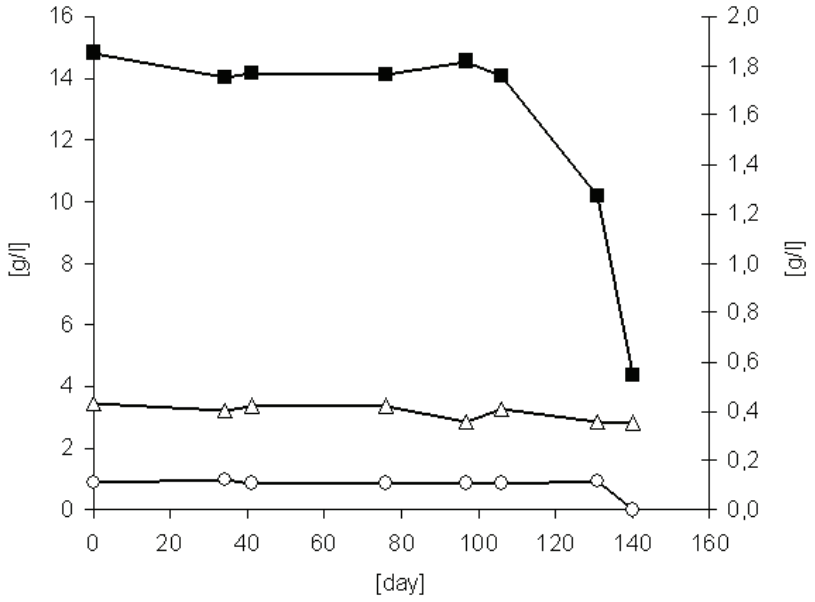


Figure 7.2: The treatment of a stuck Pinot Blanc fermentation showed fructose (■) metabolization only after 97 days of fermentation and a third inoculation of *Z. bailii*. Glucose was only converted within the last 9 days of fermentation. The treatment was stopped at a remaining fructose concentration of around 4 g/l since this was according to the wine style the winemaker was aiming for. Even though the curing of this stuck fermentation took 140 days, the acetic acid concentration (△) was found to be stable throughout fermentation duration at approximately 0.4 g/l.

The reason for the delayed onset of fructose metabolization by *Z. bailii* in the problematic Pinot Blanc fermentation could not be further investigated. Since fermentation temperature, found to be a crucial parameter for the success of the strategy, was kept at a minimum at 22 °C throughout the period of treatment, an inhibitory effect by a low fermentation temperature can be excluded. A possible explanation is the lower fructose utilizing rate of *Z. bailii* 210, which was used for the first and second inoculation (Sütterlin et al., in preparation). The fructose utilization rate of *Z. bailii* 3a, used for the third and fourth inoculation, was found to be higher. However, the very efficient treatment of the stuck Chardonnay fermentation was done with the same *Z. bailii* 210 production batch as used for the first and second inoculation for the treatment of the Pinot Blanc. Therefore, the lower fructose utilization rate of *Z. bailii* 210 can not be considered to be the sole reason for the

delay in fructose utilization in the treatment of the stuck Pinot Blanc treatment. Additional factors inhibiting the fructose metabolization of *Z. bailii* had to be present during the first stages of the treatment. Due to the complexity of the composition of a grape must and ongoing fermentation, it was not possible to conduct an in depth analysis about possible inhibitory factors and their evolution over the period of treatment.

Although *Z. bailii* is a yeast species known to be resistant to very inhospitable conditions in food and beverages, it can not be excluded that inhibitory substances, like for example small concentrations of fungicides, also negatively affect the metabolic activity of this yeast species, as it was shown for *S. cerevisiae* and *Zygosaccharomyces rouxii* (*Z. rouxii*) (5, 6, 13, 20, 21).

Analysis of the acids present in the treated Chardonnay and Pinot Blanc fermentations reveals that malolactic fermentation had already taken place before the treatment of the Pinot Blanc fermentation started, while in the Chardonnay fermentation no lactic acid was detectable. The inhibitory potential of lactic acid present in wine on different yeasts has already been investigated (1, 4, 8, 12). The authors found *S. cerevisiae* and *Z. bailii* to be the yeast species showing the highest resistance to inhibition by lactic acid. In addition, it was found that the concentrations necessary to generate a significant inhibition were much higher than those usually found in wine (10). Therefore, the presence of lactic acid in the treated Pinot Blanc can not be considered to negatively affect the fructose degradation of the inoculated *Z. bailii* culture population.

Figure 7.3 shows an example of an unsuccessful treatment of a stuck Pinot Gris fermentation. After a first inoculation of *Z. bailii* 3a, a decline in residual fructose concentration was observed. This initially positive development was followed by a decline in fructose utilization rate. Evaluation of the evolution of malic acid and lactic acid concentrations within the period of treatment indicated an onset of malolactic fermentation after day 25 of the treatment. MLF occurred in parallel to the observed decline in fructose degradation. This correlation suggests that the condition of active malolactic fermentation is unfavorable to the fermentative activity of *Z. bailii*. Similar observations were made in other treatments. Practically, a microscopic evaluation of the wine to be treated is therefore recommended in order to verify the absence of *O. oeni*. Quantitative RT-PCR-analysis allows the detection of less than 100 cells/ml of

O. oeni in a sample of the wine to be treated. Employing such analysis increases the precision of the prediction of a developing malolactic fermentation. If *O. oeni* are detected in the stuck fermentation to be treated, it is advisable to wait for the malolactic fermentation to occur and to be finalized before inoculation of *Z. bailii* 3a.

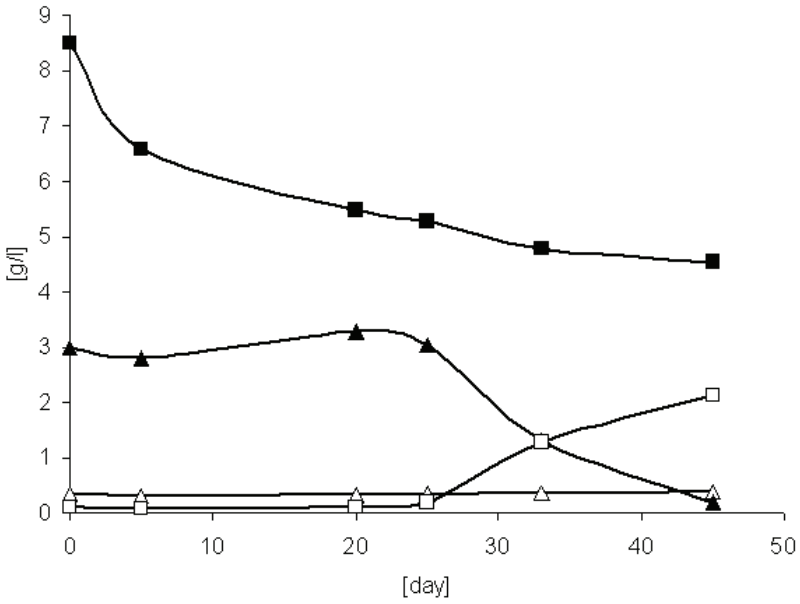


Figure 7.3: The stuck fermentation of a Pinot Gris did not contain any residual glucose concentration. The residual fructose (■) concentration could not be depleted by the inoculated culture population of *Z. bailii*. A possible explanation for not successful fructose depletion could be the onset of malolactic fermentation, detected by decreasing malic acid concentration (▲) and increasing lactic acid concentration (□). Acetic acid concentration (△) was stable over fermentation time at a value of approximately 0.4 g/l.

The interaction of *O. oeni* and *S. cerevisiae* during alcoholic fermentation has already been intensively studied and various impacts have been observed (1, 4, 8, 12). The evaluation of different strains of *O. oeni* and their interaction with different strains of *S. cerevisiae* revealed a strain dependent potential of growth inhibition and fermentative activity. In particular, it was shown that *S. cerevisiae* inhibits the initial growth of *O. oeni*, whereas a significant presence of *O. oeni* led to a higher rate of cell death in the *S. cerevisiae* population (8, 12). Nevertheless, simultaneous alcoholic and malolactic fermentations were reported to occur successfully, with no adverse effects being observed (4). In addition, current winemaking practices tend towards simultaneous inoculation of grape musts by wine yeast strains and *O. oeni*,

as recently reviewed by Krieger and Arnink (Krieger and Arnink 2005, dspace.library.cornell.edu) and Jussier et al. (9).

The onset of malolactic fermentation can not have been the inhibiting factor in all of the unsuccessful treatments. In several cases, no probable reason for these problems could be identified.

All wines treated by this strategy were reported to exhibit the acceptable sensory properties. Indeed, none of the winemakers reported a negative impact of the treatment by *Z. bailii* 3a on the organoleptic properties of the treated wines.

7.4.4 Development of acetic acid in treated problematic fermentations

Z. bailii is known as a spoilage yeast (6, 13) and winemakers mostly fear an undesired increase in acetic acid. In order to show the non spoiling properties of the selected *Z. bailii* strains 3a and 210, the acetic acid concentration was also monitored during fermentation. As the data show (Table 7.3) acetic acid concentrations increased insignificantly during the treatments of stuck wine fermentations under various conditions. Even when the treatment took 140 days, no increase in acetic acid concentration could be detected. In fact, during 6 out of 15 treatments the acetic acid concentration decreased. In cases showing an increase in acetic acid concentration, the maximum increase observed was 0.26 g/l resulting in a final acetic acid concentration of 0.68 g/l. This concentration is still below the legal limit of 1.2 g/l of Switzerland and below the sensorial detection limit (Verordnung des EDI über Fremd- und Inhaltsstoffe in Lebensmitteln; SR 817.021.23).

7.5 Conclusions

In this paper the successful implementation of a novel strategy for the treatment of stuck wine fermentations is described. This strategy, based on a selected strain of the fructophilic yeast species *Z. bailii*, enables reactivation of the fermentative activity by decreasing the excess fructose, allowing the wine yeast strain, commonly of the species *S. cerevisiae*, to restart alcoholic fermentation again. Due to the convincing results of this study, the strain *Z. bailii* 3a is now commercially available as a dried yeast product under the product name “Fructoferm W3” (Lallemand Inc) and recommended to be used according to the strategy presented here.

Table 7.3: Treatments of stuck fermentations by *Z. bailii* in 2004

N°	Duration [day]	Glucose [g/l]	Fructose [g/l]	Malic acid [g/l]	Lactic acid [g/l]	Acetic acid [g/l]
1		0.0	8.5	3.0	0.1	0.35
	47	0.0	5.5	0.1	2.1	0.42
1a		0.0	14.9	3.3	0.1	0.35
	47	0.5	13.7	1.7	1.2	0.42
2		0.3	17.3	0.0	2.4	1.31
	63	0.0	5.3	0.0	2.4	1.36
3		0.0	13.9	5.2	0.0	0.48
	54	0.0	4.3	4.4	0.3	0.54
4		0.0	16.2	3.6	0.2	0.29
	54	0.0	8.8	3.3	0.1	0.25
5		0.9	14.8	0.0	2.4	0.43
	140	0.0	4.3	0.0	2.0	0.35
6		0.0	14.3	4.3	0.1	0.33
	62	0.0	1.4	1.3	2.3	0.39
7		5.4	31.8	4.5	0.0	0.42
	67	0.0	1.1	0.0	3.0	0.68
8		3.4	27.5	4.1	0.0	0.38
	45	0.0	0.0	3.7	0.0	0.37
9		2.5	17.9	0.1	2.1	0.65
	28	2.3	16.2	0.1	1.9	0.57
10		3.7	23.9	0.0	2.7	0.92
	28	3.5	22.1	0.0	2.4	0.77
11		0.0	8.2	3.8	0.1	0.31
	23	0.0	5.4	3.8	0.2	0.28
12		0.0	12.8	3.8	0.1	0.30
	23	0.0	5.6	3.7	0.1	0.36
13		0.6	16.8	3.9	0.1	0.30
	23	0.0	8.4	3.7	0.1	0.37
14		2.6	26.7	4.1	0.1	0.47
	23	0.0	0.0	3.7	0.1	0.49
15		4.1	31.3	4.1	0.1	0.40
	23	0.0	0.0	3.7	0.1	0.42

For each treatment the initial values before inoculation of *Z. bailii* and the values at the end of treatment are given. Treatments No 1 and 1a are two fermentations of one must distributed to two different tanks before the first inoculation of a commercial wine yeast.

The experiences gained while implementing the strategy led to the definition of the following crucial factors to ensure the success of the procedure:

- The fermentation temperature has to be maintained at a minimum of 22 °C in order to support the fermentative activity of *Z. bailii*. Since the fermentation rate of *Z. bailii* is low in comparison to the known commercial wine yeast strains of the species *S. cerevisiae*, a lower fermentation temperature would result in extended fermentation times or even inhibition of the fermentative activity.
- An onset of malolactic fermentation during treatment by *Z. bailii* has to be avoided, since the fructose degradation was found to be inhibited in these conditions.
- The recommended amount for the inoculation of *Z. bailii* ensures a sufficient number of active cells for the process. Since the conditions found in a stuck fermentation do not allow growth of the inoculated population, only cells, inoculated in metabolic active state are able to facilitate fructose degradation. Therefore, the process of rehydration has to be done carefully and according to the instructions supplied by the manufacturer.
- It was also found to be advantageous to start treatments of stuck fermentations for volumes exceeding 4,000 l in a subset of 500 l. As soon as fructose utilization is observed in the smaller volume, it is recombined with the larger volume in order to transfer the active *Z. bailii* culture population to the whole volume to be treated.
- Throughout the treatment, periodic microscopic evaluation of the treated fermentation is recommended in order to detect the appearance of undesired microorganisms and if necessary to apply conventional measures to avoid spoilage of the treated wine.

Although this novel strategy for the cure of stuck and sluggish fermentations is very powerful, it requires significant efforts, time and money. Therefore, further investigations to improve the presented strategy should be undertaken. Strategies could be facilitated by the development of wine yeast strains of the species *S. cerevisiae* that would exhibit a low or even no discrepancy in glucose and fructose utilization. Conducting wine fermentations with wine yeast strains displaying such

properties would diminish the development of an unfavorable GFR and therefore reduce the occurrence of stuck and sluggish fermentations.

7.6 Acknowledgements

The authors would like to thank Prof. Daniel Gygax at the “Fachhochschule beider Basel” (FHBB) in Muttenz, Switzerland for kindly providing us with the InforsHT bioreactor used for the propagation of the laboratory scale cultures of the strain *Zygosaccharomyces bailii* 3a. We also would like to thank the Danstar Ferment AG for providing industrial scale production batches of the strain *Zygosaccharomyces bailii* 210.

7.7 Literature cited

1. **Alexandre, H., P. J. Costello, F. Remize, J. Guzzo, and M. Guilloux-Benatier.** 2004. *Saccharomyces cerevisiae-Oenococcus oeni* interactions in wine: current knowledge and perspectives. Int. J. Food Microbiol. **93:**141-154.
2. **Bisson, L. F.** 1999. Stuck and sluggish fermentations. Am. J. Enol. Vitic. **50:**107-119.
3. **Bisson, L. F., and C. E. Butzke.** 2000. Diagnosis and rectification of stuck and sluggish fermentations. Am. J. Enol. Vitic. **51:**168-175.
4. **Boulton, R. B., V. L. Singleton, L. F. Bisson, and R. E. Kunkee.** 1996. Principles and practices of winemaking. Chapman Hall, New York.
5. **Calhelha, R. C., J. V. Andrade, I. C. Ferreira, and L. M. Estevinho.** 2006. Toxicity effects of fungicide residues on the wine-producing process. Food Microbiol. **23:**393-398.
6. **Fleet, G.** 1992. Spoilage yeasts. Crit. Rev. Biotechnol. **12:**1-44.
7. **Gafner, J., and M. Schütz.** 1996. Impact of glucose-fructose-ratio on stuck fermentations. Vitic. Enol. Sci. **51:**214-218.
8. **Huang, Y.-C., C. G. Edwards, J. C. Peterson, and K. M. Haag.** 1996. Relationship between sluggish fermentations and the antagonism of yeast by lactic acid bacteria. Am. J. Enol. Vitic. **47:**1-10.
9. **Jussier, D., A. Dube Morneau, and R. Mira de Orduna.** 2006. Effect of simultaneous inoculation with yeast and bacteria on fermentation kinetics and key wine parameters of cool-climate Chardonnay. Appl. Environ. Microbiol. **72:**221-227.
10. **Kalathenos, P., J. P. Sutherland, and T. A. Roberts.** 1995. Resistance of some wine spoilage yeasts to combinations of ethanol and acids present in wine. J. Appl. Bacteriol. **78:**245-250.

11. **Kaufmann, A.** 1993. Übersicht und Anwendung der HPLC-Analytik von Säuren und Zucker in Wein. Mitt. Geb. Lebensmittelunters. Hyg. **84:**311-333.

12. **King, S. W., and R. B. Beelman.** 1986. Metabolic interactions between *Saccharomyces cerevisiae* and *Leuconostoc oenos* in a model grape juice/wine system. Am. J. Enol. Vitic. **37:**53-60.

13. **Loureiro, V. B., and M. Malfeito-Ferreira.** 2003. Spoilage yeasts in the wine industry. Int. J. Food Microbiol. **86:**23-50.

14. **Merico, A., D. Capitanio, I. Vigentini, B. M. Ranzi, and C. Compagno.** 2003. Aerobic sugar metabilism in the spoilage yeast *Zygosaccharomyces bailii*. FEMS Yeast Res. **4:**277-283.

15. **Porret, N. A., P. Coreth, P. Hoffmann-Boller, D. Baumgartner, and J. Gafner.** 2003. Reperaturhefen zur Behebung von Gärstockungen. Schweizerische Zeitschrift für Obst- und Weinbau **22:**4-7.

16. **Pretorius, I. S., and F. F. Bauer.** 2002. Meeting the consumer challenge through genetically customized wine-yeast strains. Trends Biotechnol. **20:**426-432.

17. **Schütz, M., and J. Gafner.** 1993. Sluggish alcoholic fermentation in relation to alterations of the glucose-fructose ratio. Chemie, Microbiologie, Technologie der Lebensmittel **15:**73-78.

18. **Sousa-Dias, S., T. Gancalves, J. S. Leyva, J. M. Peinada, and M. C. Loureiro-Dias.** 1996. Kinetics and regulation of fructose and glucose transport systems are responsible for fructophily in *Zygosaccharomyces bailii*. Microbiology **142:**1733-1738.

19. **Sütterlin, K. A., H. Gut, P. Hoffmann-Boller, and J. Gafner.** 2005. Presented at the 14. Internationales Oenologisches Symposium, Konferenzzentrum Porto Carras, Chalkidiki, Griechenland, 08.05.2005 - 12.05.2005.

20. **Thomas, D. S., and R. R. Davenport.** 1985. *Zygosaccharomyces bailii* — a profile of characteristics and spoilage activities. Food Microbiol. **2:**157-169.

21. **Viviani-Nauer, A., P. Hoffmann-Boller, and J. Gafner.** 1997. In vivo detection of folpet and its metabolite phthalimide in grape must and wine. Am. J. Enol. Vitic. **48:**67-70.