Enhancing xylose utilisation during fermentation by engineering recombinant *Saccharomyces cerevisiae* strains

by

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Vasudevan Thanvanthri Gururajan **28 August 2007**

SUMMARY

Xylose is the second most abundant sugar present in plant biomass. Plant biomass is the only potential renewable and sustainable source of energy available to mankind at present, especially in the production of transportation fuels. Transportation fuels such as gasoline can be blended with or completely replaced by ethanol produced exclusively from plant biomass, known as bio-ethanol. Bio-ethanol has the potential to reduce carbon emissions and also the dependence on foreign oil (mostly from the Middle East and Africa) for many countries.

Bio-ethanol can be produced from both starch and cellulose present in plants, even though cellulosic ethanol has been suggested to be the more feasible option. Lignocellulose can be broken down to cellulose and hemicellulose by the hydrolytic action of acids or enzymes, which can, in turn, be broken down to monosaccharides such as hexoses and pentoses. These simple sugars can then be fermented to ethanol by microorganisms. Among the innumerable microorganisms present in nature, the yeast *Saccharomyces cerevisiae* is the most efficient ethanol producer on an industrial scale. Its unique ability to efficiently synthesise and tolerate alcohol has made it the 'workhorse' of the alcohol industry.

Although *S. cerevisiae* has arguably a relatively wide substrate utilisation range, it cannot assimilate pentose sugars such as xylose and arabinose. Since xylose constitutes at least one-third of the sugars present in lignocellulose, the ethanol yield from fermentation using *S. cerevisiae* would be inefficient due to the non-utilisation of this sugar. Thus, several attempts towards xylose fermentation by *S. cerevisiae* have been made. Through molecular cloning methods, xylose pathway genes from the natural xylose-utilising yeast *Pichia stipitis* and an anaerobic fungus, *Piromyces*, have been cloned and expressed separately in various *S. cerevisiae* strains. However, recombinant *S. cerevisiae* strains expressing *P. stipitis* genes encoding xylose reductase (*XYL1*) and xylitol dehydrogenase (*XYL2*) had poor growth on xylose and fermented this pentose sugar to xylitol.

The main focus of this study was to improve xylose utilisation by a recombinant *S. cerevisiae* expressing the *P. stipitis XYL1* and *XYL2* genes under anaerobic fermentation conditions. This has been approached at three different levels: (i) by creating constitutive carbon catabolite repression mutants in the recombinant *S. cerevisiae* background so that a glucose-like environment is mimicked for the yeast cells during xylose fermentation; (ii) by isolating and cloning a novel xylose reductase gene from the natural xylose-degrading fungus *Neurospora crassa* through functional complementation in *S. cerevisiae*; and (iii) by random mutagenesis of a recombinant *XYL1* and *XYL2* expressing *S. cerevisiae* strain to create haploid xylose-fermenting mutant that showed an altered product profile after anaerobic xylose fermentation. From the data obtained, it has been shown that it is possible to improve the anaerobic xylose

utilisation of recombinant *S. cerevisiae* to varying degrees using the strategies followed, although ethanol formation appears to be a highly regulated process in the cell.

In summary, this work exposits three different methods of improving xylose utilisation under anaerobic conditions through manipulations at the molecular level and metabolic level. The novel *S. cerevisiae* strains developed and described in this study show improved xylose utilisation. These strains, in turn, could be developed further to encompass other polysaccharide degradation properties to be used in the so-called consolidated bioprocess.

OPSOMMING

Xilose is die tweede volopste suiker wat in plantbiomassa teenwoordig is. Plantbiomassa is die enigste potensiële hernubare en volhoubare bron van energie wat tans vir die mensdom beskikbaar is, veral vir die produksie van vervoerbrandstowwe. Vervoerbrandstowwe soos petrol kan vermeng word met etanol wat uitsluitlik van plantbiomassa vervaardig is, bekend as bio-etanol, of heeltemal daardeur vervang word. Bio-etanol het die potensiaal om koolstofuitlatings te verminder en vir baie lande ook afhanklikheid op buitelandse olie (hoofsaaklik afkomstig van die Midde-Ooste en Afrika) te verminder.

Bio-etanol kan vanaf beide die stysel en sellulose in plante vervaardig word, maar sellulosiese etanol word as die meer praktiese opsie beskou. Lignosellulose kan deur die hidrolitiese aksie van sure of ensieme in sellulose en hemisellulose afgebreek word en dit kan op hulle beurt weer in monosakkariede soos heksoses en pentoses afgebreek word. Hierdie eenvoudige suikers kan dan deur mikro-organismes tot etanol gegis word. Onder die tallose mikro-organismes wat in die natuur teenwoordig is, is die gis *Saccharomyces cerevisiae* die doeltreffendste etanolprodusent in die bedryf. Sy unieke vermoë om alkohol te vervaardig en te weerstaan het dit die werksperd van die alkoholbedryf gemaak.

Hoewel *S. cerevisiae* ‘n taamlike breë spektrum van substrate kan benut, kan dit nie pentosesuikers soos xilose en arabinose assimileer nie. Aangesien xilose ten minste ‘n derde van die suikers wat in lignosellulose teenwoordig is, uitmaak, sou die etanolopbrengs uit gisting met *S. cerevisiae* onvoldoende wees omdat hierdie suiker nie benut word nie. Verskeie pogings is dus aangewend om xilosegisting deur *S. cerevisiae* te bewerkstellig. Deur middel van molekulêre kloneringsmetodes is gene van die xilose-weg uit ‘n gis wat xilose natuurlik benut, *Pichia stipitis*, en ‘n anaërobiese swam, *Piromyces*, afsonderlik in *S. cerevisiae*-rasse gekloneer en uitgedruk. ‘n Rekombinante ras wat *P. stipitis*- se *XYL1*-xilosereduktase- en *XYL2*-xilitoldehidrogenase gene uitdruk, het egter swak groei op xilose getoon en het dié pentosesuiker tot xilitol gegis.

Die hooffokus van hierdie ondersoek was om die benutting van xilose deur ‘n rekombinante *S. cerevisiae*-ras wat *P. stipitis* se *XYL1 en XYL2*-gene uitdruk onder anaërobiese gistingstoestande te verbeter. Dit is op drie verskillende vlakke benader: (i) deur konstitutiewe koolstofkataboliet-onderdrukkende mutante in die rekombinante *S. cerevisiae*-agtergrond te skep sodat ‘n glukose-agtige omgewing tydens xilosegisting vir die gisselle nageboots word; (ii) deur ‘n nuwe xilose-reduktasegeen uit die natuurlike xilose-afbrekende swam *Neurospora crassa* te isoleer en deur funksionele komplementasie in *S. cerevisiae* te kloneer; en (iii) deur willekeurige mutagenese van die rekombinante *S. cerevisiae*-ras ‘n haploïede xilose-gistende mutant te skep wat ‘n gewysigde produkprofiel ná anaërobiese xilosegisting vertoon. Deur hierdie drieledige benadering te volg, is dit bewys dat dit moontlik is om die anaërobiese xilosebenutting van rekombinante *S. cerevisiae-rasse* in wisselende mate deur die aangepaste

metodes te verbeter, hoewel etanolvorming ‘n hoogs gereguleerde proses in die sel blyk te wees.

Opsommend kan gesê word dat hierdie werk drie verskillende metodes uiteensit om xilosebenutting onder anaërobiese toestande te verbeter deur manipulasies op die molekulêre en metaboliese vlak. Die nuwe *S. cerevisiae*-rasse wat in hierdie studie ontwikkel en beskryf word, toon verbeterde xilosebenutting. Hierdie rasse kan op hulle beurt verder ontwikkel word om ander polisakkariedafbrekende eienskappe in te sluit wat in die sogenaamde gekonsolideerde bioproses gebruik kan word.

"***INDRIYEBHYAH PARAM MANO MANASAH SATTVAM UTTAMAM***
SATTVAD ADHI MAHAN ATMA MAHATO VYAKTAM UTTAMAM"

--Katha Upanishad 6.7

[Beyond the senses is the mind, and beyond the mind is reason, its essence.
Beyond reason is the Spirit in man, and beyond this, is the Spirit of the Universe, the evolver of all]

To

Amma, Appa, Akka
& **family**

"***ASATO MA SADH GAMAYA; TAMASO MA JYOTIR GAMAYA; MRTYOR MAMRTAM GAMAYA***"

--Brhadaranyaka Upanishad 1.3.28

[From delusion lead me to truth
From darkness lead me to light
From death lead me to immortality]

BIOGRAPHICAL SKETCH

Vasudevan Thanvanthri Gururajan was born on 4 November 1976 in the quaint town of Chidambaram in southern state of Tamil Nadu, India. He has been living in nearby Cuddalore since then, which he considers as hometown. He matriculated in 1994 from Baba Matriculation Higher Secondary School, Cuddalore, India. He completed his Bachelor's degree in Microbiology in 1997 at J.J. College of Arts and Science, Pudukkottai, India, which is affiliated to Bharathidasan University, Tiruchirapalli, India. He continued his Master's degree in Microbiology at the same institution during 1998-2000. He enrolled for his PhD at the Institute for Wine Biotechnology, Stellenbosch University in the year 2002.

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PREFACE

This dissertation is presented as a compilation of six chapters. Each chapter is introduced separately and is written according to the style of the journal **Annals of Microbiology** to which Chapters **3**, **4** and **5** were submitted for publication. The two appendices are publications worked on as a contributing author during the course of the PhD.

Chapter 1 **General Introduction and Project Aims**

Chapter 2 **Literature Review**
Engineering recombinant *Saccharomyces cerevisiae* towards improved xylose fermentation

Chapter 3 **Research Results I**
A constitutive catabolite repression mutant of a recombinant *Saccharomyces cerevisiae* strain improves xylose consumption during fermentation

Chapter 4 **Research Results II**
Molecular cloning and functional expression of a novel *Neurospora crassa* xylose reductase in *Saccharomyces cerevisiae* in the development of a xylose fermenting strain

Chapter 5 **Research Results III**
Development and characterisation of a *Saccharomyces cerevisiae* recombinant strain with enhanced xylose fermentation properties

Chapter 6 **General Discussion and Conclusions**

Appendix A Evaluation of polygalacturonase activity in *Saccharomyces cerevisiae* wine strains

Appendix B Engineering of an oenological *Saccharomyces cerevisiae* strain with pectinolytic activity and its effect on wine

CONTENTS

Chapter 1

INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

Global warming as a result of increased emissions of greenhouse gases, especially carbon dioxide (CO_2), has been increasing during the past two decades and has led to intense debate among the nations of the world. The main purported culprits are the fuels used for transportation, chiefly those derived from fossil resources. Although at the dawn of the mass production of cars in 1908, Henry Ford offered the Model T Ford in both ethanol and petrol powered models, humankind has depended on cheaper fossil fuel sources since the mid-20^{th} century. So, the present interest in running cars on ethanol is actually an old idea, although the need is quite new and perhaps urgent.

The use of fossil fuels has been subjected to politico-economic factors over the years. With the forecast precipitous decline in fossil fuel reserves, there is an urgent need for alternative renewable energy sources such as biofuels, hydrogen, electricity, solar energy, etc. Such alternative energy sources would also lead to the lower dependence of many countries on the Middle East or Africa for their energy needs.

Plant biomass has been put forward as one of the potential alternative, renewable energy resources for the production of biofuels (Sheehan and Himmel 1999). Bioethanol is the most common product obtained from plant biomass and is the same alcohol that is present in alcoholic beverages. While the desire for alcoholic beverages continues unabated, there is increasing interest in using biologically produced ethanol as automotive fuel and to slow the growth of atmospheric CO_2 levels. The attraction of ethanol as a fuel is that green plants combine CO_2 from the atmosphere with water through solar-powered photosynthesis to produce carbohydrates. Yeasts ferment these to produce CO_2 and ethanol. Ethanol, when burned in an internal combustion engine, produces water and CO_2, which can then be photosynthesised back to carbohydrates in the next plant crop. In this way, CO_2 is continuously recycled between the air, carbohydrates and ethanol without adding to the total atmospheric level of CO_2. So why doesn't the world switch from petrol/gasoline to ethanol as a fuel? The reason is that there is a catch. Enthusiasts for this concept must recognise that energy is required for the cultivation of green plants, fertiliser and pesticide production, harvesting, conversion to ethanol fuel and transportation. So, present production methods relying on conversion of carbohydrates to ethanol would take up far more land than is available if food production and wilderness areas are to be maintained at present levels (Pearce 2006). Therefore, the ethanol production by yeasts needs to be more efficient to be viable.

To date, the most efficient ethanol-producing yeast is *Saccharomyces cerevisiae*. It converts fermentable sugars present in crops such as sugarcane, wheat, maize (corn), etc. into ethanol. Upon distillation, 95-96% ethanol is obtained and this can be further passed through a molecular sieve to obtain 99% pure or anhydrous ethanol (Swain 1999). For 95% ethanol to be used as fuel, special types of engine are required, but 99% ethanol could be blended with gasoline/petrol and used without any

modifications to existing engines. The use of biofuels such as bioethanol has huge ecological benefits because the time taken for complete recycling of the carbon released into the atmosphere as CO_2 is four to five times less than that for fossil fuels. This would help to reduce the level of greenhouse gases in the atmosphere. If all the energy for bioethanol production also came from non-fossil sources, the use of bioethanol as a fuel would add no greenhouse gases to the environment. Although the values have been disputed (Pimentel and Patzek 2005), bioethanol usage has also been shown to provide about 30% more energy than the energy used for its production (USA Department of Energy 2006).

While most industrially developed and developing countries around the world were (and some still are) debating the pros and cons of global warming, Brazil was one of the first countries to realise the potential of bioethanol. It produces vast quantities through sugar cane fermentation, and is now self-sufficient in fuel usage (Marris 2006). In 2005, the Brazilians produced 15 billion litres of ethanol from sugar cane and the energy used was exceeded by 35% in energy contained in the ethanol manufactured. This gave a surplus of 5 billion litres of liquid solar energy. In the United States of America (USA), where ethanol is mainly produced from maize, the energy balance is less favourable. The Americans mix ethanol with gasoline for use in vehicles in place of methyl tertiary butyl ether (MTBE) as an additive, reducing the amount of hydrocarbon pollutants released. The ethanol mix can be either 10% (E10) or 20% (E20) of the gasoline. The USA Energy Policy Act of 2005 (http://www.ferc.gov) requires the oil industry to blend 7.5 billion gallons of renewable fuels into gasoline by 2012. However, the reliance of ethanol producers on corn (maize) has led to an unfortunate debate about ‘food versus fuel’ because more and more of the corn grown is used by the ethanol industry, resulting in an increase in the price of corn for food. Recently, Sanderson (2006) had presented the reasons why corn-based ethanol production in the USA could not be sustainable in the near future. In 2000, the European Union (EU) had already taken a stand by issuing a directive to replace 20% of fossil fuels with biofuels by the year 2020. Both the EU and the USA have sanctioned subsidies for the bioethanol industry. In spite of these subsidies, there is widespread fear that government targets might not be achieved because of limitations in the production of biofuels, especially bioethanol. Thus, several aspects of bioethanol production have been the subject of continuing research, especially the aspect of using biomass resources other than food stocks (for a recent review, see Gray *et al*. 2006 and Pearce 2006).

Traditionally, ethanol has been produced from several plants used for food and animal feed. However, increasing demand for food and feed could affect use of these stocks for ethanol production. Thus, there is a need to look for other natural carbon-rich resources. Various plant biomass resources such as sugar cane bagasse, corn stover, and lignocellulose could be used to produce ethanol. Ethanol produced from lignocellulose and other agricultural waste products might provide a relief to the ‘food versus fuel’ debate because these are usually not considered as food or feed stocks,

and are also completely sustainable and renewable. Most of these substrates are rich in various polysaccharides, especially cellulose. The polysaccharides need to be broken down into simpler monosaccharides such as hexoses and pentoses through chemical or enzymatic hydrolysis before these sugars can be fermented into ethanol, preferably by a single microorganism (in a process known as *consolidated bioprocessing*; Lynd 1996). Several microorganisms exist in nature that are capable of degrading complex polysaccharides. *S. cerevisiae* is the preferred microorganism for producing ethanol because of its versatility and industrial robustness in terms of ethanol tolerance, rapid anaerobic growth etc. However, this yeast is unable to hydrolyse polysaccharides and utilise the pentose sugars, xylose and arabinose, which constitute at least 28% of the sugars present in the hydrolysates. Several yeast, bacteria and filamentous fungi present in nature are capable of utilising the pentose sugars but none capable of anaerobic ethanol fermentation has been identified. Hence, research has usually been focussed in two areas: (i) to engineer ethanol-producing strains that ferment xylose (e.g. *S. cerevisiae*, *Zymomonas mobilis*), and (ii) to engineer xylose-utilising strains that exclusively produce ethanol (e.g. *Pichia stipitis*, *Escherichia coli*). Most of the research and improvements have occurred with recombinant *S. cerevisiae* containing xylose pathway genes from *P. stipitis*. Xylose is the second most abundant sugar present in the plant biomass and effective utilisation of this substrate would help to increase the yield and cost effectiveness of the ethanol production process. In the early 1990s, xylose pathway genes from *P. stipitis* were cloned into *S. cerevisiae*. The recombinant yeast was able to grow on xylose but its fermentation properties were sub-optimal (Kötter and Ciriacy 1993).

Through advances in molecular biology techniques, metabolic engineering and the application of mathematical modelling, the fermentation performance of recombinant *S. cerevisiae* in xylose medium has been improved over the years through systematic identification of factors limiting fermentation, and overcoming such factors through genetic or metabolic manipulations. Introduction of mutations – random or directed – has also been used to generate strains showing improved fermentation. Xylose, like glucose, is transported mainly by the hexose transporters and is metabolised by the pentose phosphate pathway (PPP) and converted to ethanol via glycolysis. However, with the low flux of the PPP and the lack of energy production associated with it, yields and productivity results from xylose fermentation have been lower than those from glucose. In addition, one of the main limiting factors for anaerobic xylose fermentation has been determined to be the redox imbalance arising out of the cofactor differences between the two initial xylose pathway enzymes of *P. stipitis*. However, with the successful cloning and expression of the xylose isomerase gene from *Piromyces sp*. in *S. cerevisiae*, Kuyper *et al*. (2003) have been able to overcome the redox cofactor imbalance arising in the isomerisation step. Recent research has shown the presence of endogenous xylose-metabolising genes in *S. cerevisiae*, although with low expression levels. This has led to a fascinating hypothesis – ironically, just as humankind replaced ethanol with fossil fuels as automobile fuel, the rigorous domestication process of the

yeast carried out over the centuries since the first bread and wine were made might have caused it to lose its ability to assimilate the sugar.

1.2 SCOPE OF THE DISSERTATION

The main scope of this study is to improve xylose utilisation by recombinant *S. cerevisiae* during anaerobic fermentation. While a recombinant *S. cerevisiae* strain has been constructed to aid growth on xylose, it has been beset with several limiting factors, which are detailed in **Chapter 2**. However, as a result of continued research during the past two decades using recombinant DNA technology with metabolic and evolutionary engineering, many engineered strains have been developed that show improved fermentation properties. It has become essential to consider factors that might be of importance in the further application of these strains in an industrial situation. Of major concern for industrial use is the issue of repression of other sugars by glucose. Several hexose and pentose sugars are present in the lignocellulose hydrolysate but utilisation of all sugars except glucose will be repressed until the glucose levels are low or depleted. This leads to an increase in fermentation time and cost, and subsequently affects the cost effectiveness of the process as well as the product. Because both glucose and xylose share the same transporters and part of the metabolic pathway for their metabolism, it is hypothesised that mimicking a glucose-like scenario, by creating constitutive carbon catabolite repression mutants, would aid in xylose utilisation by the yeast. The results are outlined in **Chapter 3**.

Xylose reductase (XR) is the first enzyme involved in xylose metabolism and it has been one of the most extensively researched enzymes because of its applications in the fermentation industry and in medicine. While a considerable amount of data is available for yeast and fungal aldose reductases, there are still many untapped sources of xylose-utilising organisms present in nature. *Neurospora crassa* is one such filamentous fungus capable of growth on xylose and other plant matter. Although XR activity has been reported in this fungus, no XR-encoding gene had been identified until recently (Woodyer *et al*. 2005). However, screening of a cDNA library of *N. crassa* resulted in transformants containing a gene encoding aldose reductase. The cloning and characterisation of this second xylose reductase of *N. crassa* are outlined in **Chapter 4**.

Random mutagenesis of a recombinant *S. cerevisiae* haploid strain was carried out to improve its characteristic of slow growth on xylose. From the several strains screened, the fastest growing mutant was isolated and evaluated in anaerobic batch fermentation. The mutant showed an altered fermentation profile from the parent and produced more glycerol and less xylitol. The characterisation details of this mutant are reported in **Chapter 5**.

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Chapter 2

LITERATURE REVIEW

Engineering recombinant *Saccharomyces cerevisiae* towards improved xylose fermentation

2.1 INTRODUCTION

The use and effects of fossil fuels have created economical, political and ecological issues since the latter half of the twentieth century. With the potential depletion of fossil fuel resources looming, a fuel energy crisis could cripple the world economy. Ever-increasing environmental awareness of the various hydrocarbons and greenhouse gas emissions from fossil fuel usage has led to the search for alternative renewable energy sources, with the keyword being renewable. Transportation is the major industry dependent on fossil fuels and is one of the major culprits in so-called 'global warming'. The rationale for continued usage of fossil fuels or petroleum products is varied, with the main reasons being: (i) availability in large amounts in nature; (ii) limited downstream processing (refining) before the product reaches the market; (iii) by-products with other applications; and, more importantly, (iv) cost-effectiveness. However, there are ecological disadvantages with these products. Thus, an alternative energy resource for use as fuel would have to be ecologically advantageous as well as fulfilling most, if not all, the advantages of fossil fuels. Ethanol has been suggested as a viable and perhaps better alternative, especially in the transportation industry. Its advantages include complete combustion, reduced release of hydrocarbons, especially CO_2, into the atmosphere, and hence eco-friendliness, and the ability to be produced chemically or through a biological process. At present, ethanol is not as cost-effective as petroleum products. However, the emergence of technology to use plant matter (biomass) to produce ethanol has opened the possibility of it becoming a long-term solution in the alternative renewable energy debate (McMillan 1997; Claassen *et al.* 1999; Wyman 1999; Kheshgi *et al.* 2000). Ethanol is currently produced from starchy products such as maize (corn), sugarcane and wheat. Lignocellulosic materials are cheaper than starch-based raw materials (Dumitriu 1998) and can help to reduce the cost of the raw material, which forms a substantial part of the final cost of the product – ethanol (Wingren *et al.* 2003). Recent articles (Farrell *et al.* 2006; Hammerschlag 2006; Sanderson 2006) highlight the environmental advantages of using cellulosic ethanol. Ethanol, which needs to be distilled for recovery, also affects the energy demand of this process when it is present at concentrations of less than 4% (Zacchi and Axelson 1989). Hence, for the process to be cost effective there should be a higher ethanol concentration in the feed for distillation (Wingren *et al.* 2003).

2.2 LIGNOCELLULOSE

Lignocellulosic crop residues comprise more than half of the world's agricultural phytomass (Smil 1999) and significant fractions of the total can be recovered without competing with other uses (Lynd 1996; Wyman 1999). Until recently, lignocellulosic biomass (plant matter) had been used sparingly as food and in industry. Lignocellulose is composed of mainly cellulose, hemicellulose and lignin (Fig. 2.1). Cellulose, the major component, is a linear crystalline polymer of D-glucose. Hemicellulose is made up of a

diverse group of heterogeneous polymers with branched chains consisting mainly of the hexoses D-glucose, D-mannose and D-galactose, and pentoses such as D-xylose and D-arabinose. In addition, acetyl and methyl groups are attached to the sugars of the backbone polymer. Depending on the plant material, the composition of the sugars may vary in proportion. An efficient procedure for ethanol fermentation would be possible only by using both cellulose and hemicellulose fractions. Lignin is composed of polymers of aromatic compounds and not carbohydrates and thus cannot be fermented to ethanol. Prior to fermentation, lignocellulose has to be degraded to metabolisable sugars by physical, chemical or biological processes such as milling, steam treatment, acid or alkaline hydrolysis, and enzymatic treatment. Sugar composition in lignocellulose hydrolysate can also vary depending on the treatment procedure employed.

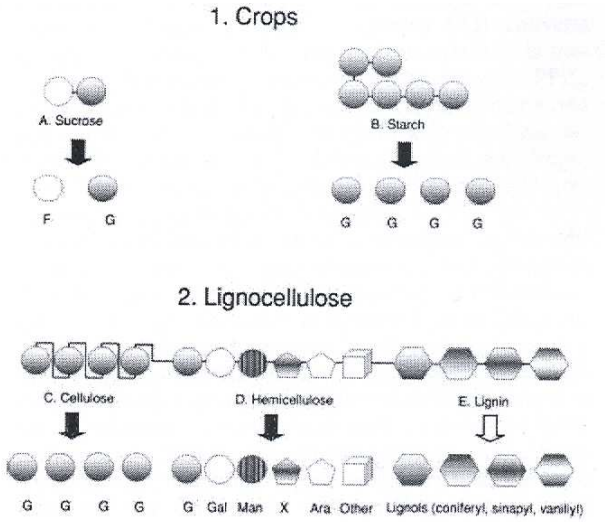

FIG. 2.1 Sources of sugars for ethanol production. Arrows represent hydrolysis. Dark arrows represent the monomers generated from hydrolysis, which are fermentable. *G* Glucose, *F* Fructose, *Gal* Galactose, *Man* Mannose, *X* Xylose, *Ara* Arabinose, *Other* L-rhamnose, L-fucose, uronic acids. © Zaldivar *et al*. (2001)

In general, hardwoods and herbaceous plant material have a high xylan content (11-23%) and a low mannan content (0-2%), while softwoods have a lower xylan content (<6%) and a higher mannan content (7-14%) (Hayn *et al*. 1993). Based on the hydrolysis treatment applied, between 50% and 95% of hexoses and 60% and 90% of pentoses are released (Eklund *et al.* 1995, Von Sivers and Zacchi, 1995). Apart from the sugars, the lignocellulose hydrolysate also contains several inhibitory compounds, such as acids, alcohols, terpenes, furfurals and tannins, which are formed during hydrolysis and depend on the type of hydrolysis. Because of the presence of these compounds, one important criterion for lignocellulosic fermentation to ethanol is the inhibitor tolerance of the microorganism. In relation to the performance of ethanol-producing microorganisms in lignocellulose hydrolysates that have been compared in the literature (Bjorling and Lindman 1989; Olsson *et al.* 1992; Olsson and Hahn-

Hägerdal 1993, 1996), *Saccharomyces cerevisiae* is the best performing yeast in all but one criterion – xylose fermentation.

2.2.1 Xylose

Xylose constitutes at least 30% of the total biomass (Pettersen, 1984; Hespell 1998; Lee 1997) and is the major constituent of xylan. It is the second most abundant sugar in nature, comprising more than 25% of woody angiosperms (Pettersen, 1984), and is easily recovered in various hydrolytic treatments (Jeffries and Kurtzman 1994 and the references therein). For instance, depending on the substrate and reaction conditions, dilute acid pre-treatments of lignocellulosic residues can recover 80% to 95% of xylose from the feedstock (Chen *et al.* 1998; Kim *et al*. 2001; Aguilar *et al.* 2002). Xylose is present in many waste streams, such as those of sulfite and dissolving pulp mills, and fibreboard and hardboard manufacturing plants (Sell *et al.* 1984). However, because of the lack of a fermenting organism, most of the xylose fraction is left unutilised during fermentation. This subsequently affects the product yield and the cost of the process as well as the product.

2.3 XYLOSE METABOLISM

Xylose metabolism in microorganisms generally differs between eukaryotes and prokaryotes, with a few exceptions. The major difference lies in the initial isomerisation step of xylose to xylulose. Prokaryotes (bacteria) convert xylose to xylulose in a single step catalysed by the enzyme, xylose isomerase (XI). Xylulose is then phosphorylated before entering the Pentose Phosphate Pathway (PPP) or the Entner-Doudoroff (ED) pathway. Eukaryotes, such as some yeasts and filamentous fungi, convert xylose to xylulose in a two-step process through the intermediate formation of xylitol. The initial reduction of xylose to xylitol is catalysed by the enzyme xylose reductase (XR). Almost all known xylose reductases are dependent on either NADPH or NAD(P)H. For enzymes with dual cofactor dependency, the NADPH-affinity is generally stronger than that for NADH. The enzyme, xylitol dehydrogenase (XDH), carries out the oxidation of xylitol to xylulose, and is predominantly NAD^+-specific. Thus, in fungi and yeasts, the isomerisation of xylose to xylulose results in the production of $NADP^+$ and NADH, and these two cofactors need to be efficiently utilised by the cells to maintain the redox balance. The xylulose is then phosphorylated by the xylulokinase (XK) enzyme to xylulose-5-phosphate, which can then be metabolised further through the Pentose Phosphate, Embden-Mayerhof-Parnas or Phosphoketolase pathways (Evans and Ratledge, 1984; Skoog and Hahn-Hägerdal, 1988; Sonderegger *et al*. 2004b) (Fig. 2.2). In the PPP, non-oxidative reactions convert xylulose-5-phosphate to glyceraldehyde-3-phosphate and fructose-6-phosphate, which link the PPP to glycolysis. The non-oxidative PPP is a sequence of many reversible reactions that operate close to equilibrium. Thus it lacks irreversible reactions, such as those involving kinases with large differences in Gibbs free energies, which would drive the reactions efficiently in the forward direction (Jeffries, 1990).

2.4 MICROORGANISMS AND XYLOSE

Many microorganisms found in nature are capable of utilising and growing on xylose by any of the pathways mentioned above. These include several prokaryotes, yeasts and filamentous fungi. Karczewska (1959) has been credited as one of the first to report on the fermentation of xylose to ethanol.

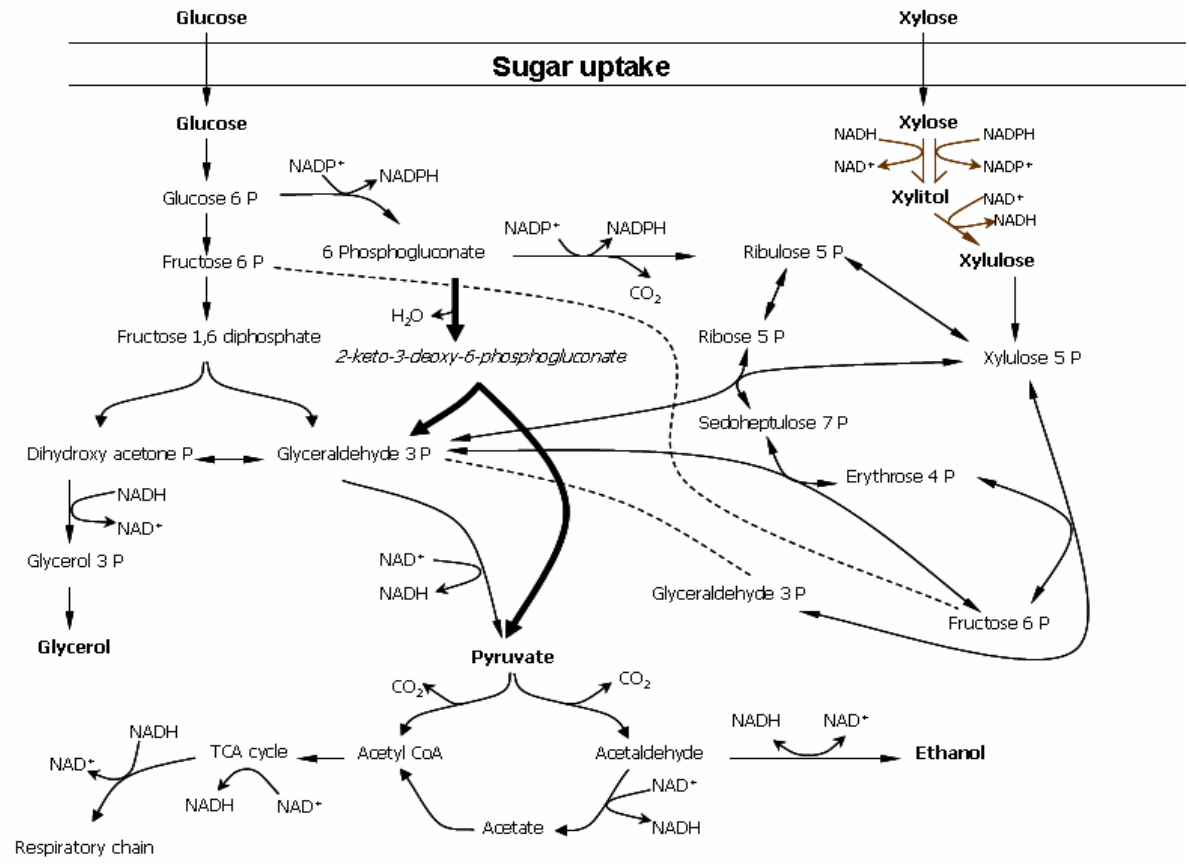

FIG. 2.2 Metabolic pathways for glucose and xylose fermentation through the **Embden-Meyerhof-Parnas (EMP) pathway** and the **Entner-Doudoroff (ED) pathway** (denoted by **bold arrows**). The major metabolites are represented in **bold** letters. Adapted from Hahn-Hägerdal *et al*. (1994).

2.4.1 Yeasts

Barnett (1976) recorded that almost half the known yeast species would assimilate D-xylose but none would ferment it. Several authors have published a wide variety of screening studies for xylose-utilising yeasts (Toivola *et al.* 1984; Du Preez and Prior 1985; Baraniak 1988) and obtained more or less similar findings pertaining to the yeasts identified, although Van der Walt *et al.* (1987) identified a novel xylose-fermenting yeast, *Candida lyxosophila*, from woodland soil isolates. *Pachysolen tannophilus* was one of the first yeasts to have been shown to possess significant capacity to utilise and convert xylose to ethanol (Schneider *et al*. 1981; Slininger *et al*. 1982). Since this breakthrough, several yeasts have been reported to grow effectively on xylose and some of them, namely *Candida shehatae*, *Candida tenuis*, *Pa. tannophilus*, *Pichia stipitis*, *Pichia*

segobiensis, *Kluyveromyces cellobiovorus*, *Kluyveromyces marxianus*, *Candida guilliermondii* and *Candida tropicalis*, have been shown to ferment D-xylose to ethanol (Jeffries 1981; Margaritis and Bajpai 1982; Gong *et al*. 1983; Du Preez and Van der Walt 1983; Dellweg *et al*. 1984; Toivola *et al*. 1984; Slininger *et al*. 1985; Du Preez *et al*. 1984; Jeffries 1985a; Du Preez *et al*. 1986; Sreenath *et al*. 1986). Among the frequently-studied yeasts, *K. cellobiovorus* has been reported to produce up to 27 g/L of ethanol and almost the same quantity of xylitol (Morikawa *et al.* 1985). *C. shehatae* has been reported to produce 24 g/L of ethanol and less xylitol (Slininger *et al.* 1985). However, none of these yeasts was able to perform better in lignocellulose hydrolysate (Olsson and Hahn-Hägerdal 1993). These yeasts also required a carefully controlled, low oxygen level for optimal performance (Ligthelm *et al.* 1988; Skoog and Hahn-Hägerdal 1988, 1990; Skoog *et al.* 1992) and, as mentioned above, produced an ethanol/xylitol mixture, in which the ratio depended on the strain and oxygenation conditions (Watson *et al.* 1984; Furlan *et al.* 1994).

Several contributing factors to the inefficiency of xylose fermentation and the methods to overcome these limitations to improve xylose fermentation by yeasts have been reported in the literature. Oxygen availability was shown to be one of the well-known factors, and various studies on different yeasts have been performed to elucidate the effect of oxygen on xylose metabolism in yeasts. Skoog and Hahn-Hägerdal (1990) studied the role of oxygenation in *P. stipitis* and concluded that it was essential for growth and/or mitochondrial function, in addition to energy generation for xylose transport, but found that it was not primarily essential for the redox balance. This report agreed strongly with earlier studies by Ligthelm *et al.* (1988), who studied the effect of oxygen on *Pa. tannophilus*, *C. shehatae* and *P. stipitis*. Several discrepancies were observed among the various reports pertaining to *Pa. tannophilus*, *C. shehatae* and *P. stipitis* (Schneider *et al*. 1981; Slininger *et al*. 1982; Jeffries 1982; Debus *et al*. 1983; Dellweg *et al*. 1984; Du Preez *et al*. 1984; Jeffries 1985a), but these might have been due to the medium or pH conditions used for cell growth by the different authors. Bruinenberg *et al*. (1984) presented a biochemical explanation for the inability of yeasts to ferment xylose, suggesting this was due to a redox imbalance under anaerobic conditions. Using *Candida utilis* as a model, they showed that the cofactor dependency of the two enzymes in xylose isomerisation – XR and XDH – was different in yeasts. While XR had an NADPH dependency in general, XDH was NAD^+ dependent, thereby necessitating dependence on the oxidative PPP for NADPH production and/or on the glycerol pathway for NADH utilisation. The mechanism by which this occurs can be described as follows: the conversion of xylose to xylulose via xylitol in yeasts will result in the net production of $NADP^+$ and NADH. While the $NADP^+$ can be recycled by the oxidative PPP through the formation of fructose-6-phosphate, the NADH needs to be siphoned through an electron acceptor such as oxygen (under aerobic conditions). Under anaerobic conditions, however, the ability to produce NADPH and consume NADH becomes critical, limiting fermentation of xylose to ethanol. Some yeasts, such as *P. stipitis*, possess xylose reductases that are dual-cofactor dependent, thus making

it possible for the yeast to metabolise xylose to ethanol with no xylitol formation under controlled oxygen conditions. However, in the majority of studies, yeast xylose fermentation led to ethanol and xylitol formation, with occasional glycerol formation.

Thus, the ideal solution for effective xylose fermentation to ethanol would be via a closed redox balance in the xylose to xylulose conversion steps. Bruinenberg *et al.* (1984) proposed three different solutions for this conundrum: (i) finding NADH-dependent XR and cloning it into yeasts, or engineering the known XRs to use NADH exclusively as the cofactor; (ii) finding an $NADP^+$-dependent XDH or engineering one from the known XDHs; or (iii) cloning a xylose isomerase gene that can sidestep the above issues and result in the direct conversion of xylose to xylulose with no xylitol formation. Some XRs, such as that from *P. stipitis*, show dual cofactor dependency but have been found to be predominantly NADPH dependent during fermentation. Jeffries (1985b) reviewed the factors that affect xylose fermentation by natural xylose-utilising yeasts. Other methods to overcome the oxygen dependency by yeasts have been to screen for respiratory deficient strains, which will then predominantly channel the sugar into ethanol. Such mutants do not grow as large as the normal respiratory cells and are consequently referred to as *petite* mutants. Jeffries (1984) reported on unstable *petite* mutants of *C. shehatae* and their fermentative abilities. However, some yeasts, such as *Schizosaccharomyces pombe* and *Pa. tannophilus*, are *petite*-negative by nature and will not survive a lack of respiration.

The discovery by Wang and Schneider (1980) that some yeasts, such as *S. cerevisiae* and *Sch. pombe,* could ferment the keto-isomer of xylose – xylulose – opened a new avenue of research that has continued for more than two decades. It meant that, by using isomerisation processes *in vitro*, adding xylose isomerase to the medium (Gong *et al*. 1981a; Lastick *et al.* 1989), or *in vivo*, expressing an XI-encoding gene in *Sch. pombe* (Chan *et al.* 1989), xylose fermentation by these yeasts could be accomplished. However, most of these efforts did not succeed, either due to the incompatible pH and temperature of the expressed XI enzyme or because of the cost of externally added XI enzyme. Until recently, about 200-odd xylose-utilising yeasts were known. But Suh *et al*. (2005) identified more than treble the amount of yeasts from the hindgut of beetles. Among the several yeasts isolated, *Enteroramus dimorbhus* is of particular interest because it supposedly belongs to the same clade as *P. stipitis*, and its host, *Odontaenius disjunctus*, feeds on fungi in white-rotted hardwood (Suh *et al*. 2004). Thus, it seems that nature might still produce more surprises regarding xylose utilisation and fermentation by yeasts.

2.4.2 Fungi

Filamentous fungi play a most important part in the decomposition of complex organic matter in nature. Chiang and Knight (1960) were among the earliest to report xylose utilisation by fungi. However, none of the reported fungi was shown to convert xylose to ethanol under complete anaerobic conditions. Although filamentous fungi consume xylose and generate ethanol concentrations and yields comparable to those obtained in

hexose fermentation with yeast, the productivity is too low and hence not economically feasible (Gong *et al*. 1981b; Suihko and Enari 1981; Ueng and Gong 1982). Among the filamentous fungi tested for xylose growth have been *Fusarium*, *Mucor* (Ueng and Gong 1982) and *Monilia* (Gong *et al*. 1981b). *Fusarium oxysporum* was shown to possess cellulase and xylanase activities (Christakopoulos *et al.* 1989; 1995; 1996a; 1996b; 1997) and was able to ferment lignocellulose directly to ethanol if other metabolic limitations were overcome. Recently, Panagiotou and Christakopoulos (2004) isolated and characterised two aldose reductases from *F. oxysporum* and performed xylose fermentation under oxygen-limited as well as anaerobic conditions. However, *F. oxysporum* could not ferment xylose to xylitol under anaerobic conditions unless an external electron acceptor, such as acetoin, was added, when both ethanol and xylitol were formed. Even though the two xylose reductases possess NADPH and NADH activity, NADPH was the predominant cofactor. These results were similar to the findings of Singh *et al*. (1992). Banerjee *et al*. (1994) have reported on the xylose metabolism of the thermophilic fungus *Malbranchea pulchella* var. *sulfurea*. Harhangi *et al*. (2003) identified an XI gene from the anaerobic fungus *Piromyces* sp., isolated from the dung of an Indian elephant. Although this gene has been cloned and expressed in *S. cerevisiae* successfully, very little information is available on the xylose capabilities of this fungus itself. The only other fungus that has been characterised considerably is *Neurospora crassa*.

N. crassa has been reported to grow on cellulose biomass and produce ethanol (Rao *et al.* 1983; Deshpande *et al.* 1986), although it also could not grow under complete anaerobic conditions. The organism showed an ethanol conversion rate of between 60% and 70% with cellulose substrate. It also fermented D-xylose to ethanol with a 60% conversion rate (Deshpande *et al*. 1986). D-xylose-metabolising enzymes have been reported to be present in this organism (Rawat *et al.* 1993; Rawat and Rao, 1996; Phadtare *et al*. 1997), although no XR gene had been characterised until recently (Woodyer *et al.* 2005). With the availability of the *N. crassa* genome database, Woodyer *et al.* (2005) identified and isolated a xylose reductase gene using a homology search with other known xylose reductases. Filamentous fungi and some yeasts have been shown to possess several isozymes in one species (Yokoyama *et al.* 1995b; Mayr *et al.* 2000; Nidetzky *et al.* 2003). Knowledge about as many isozymes as possible would help in our understanding of their function and could lead to novel characterisations.

2.4.3 Bacteria

Several bacteria have been shown to be able to grow on a wide range of substrates, especially pentoses such as xylose and arabinose. Because they convert xylose to xylulose by a one-step isomerisation process, they are thought not to have the redox balance issues that plague most yeasts and fungi under anaerobic conditions. This would make bacteria suitable hosts for xylose fermentation and, in turn, for lignocellulose fermentation. But there are other disadvantages with the bacterial system that make it unsuitable for industrial applications. Of the various organisms tested for

growth on lignocellulosic substrates (Olsson and Hahn-Hägerdal, 1993), yeasts such as *S. cerevisiae* were found to be the most suitable. Also, bacteria are known to produce byproducts such as organic acids instead of ethanol, thereby reducing the product yield and, in turn, the cost effectiveness of the process and of the product. Most bacteria have a lower ethanol tolerance than yeasts and moulds, and an optimum pH range in the neutral region, which can increase the chance of contamination during the process, and not many bacteria fit the GRAS (Generally Regarded As Safe) label. However, there are a few bacteria that have shown promise for pentose fermentation, such as *Zymomonas mobilis*, *Escherichia coli*, and *Klebsiella oxytoca*.

Z. mobilis is an exception to most other bacteria because it has a high ethanol yield, high productivity and high tolerance to ethanol. It ferments at pH 5.0 and temperatures between 30℃ and 40℃. Since it is the only organis m that follows the ED pathway (Fig. 2.2) for anaerobic glucose metabolism, it is thought to possess accelerated levels of glycolytic and ethanologenic enzymes and thus has an exceptional ethanol yield (97% of theoretical yield; Zhang *et al*. 1995a) and higher productivity (2.5 times) than *S. cerevisiae* (Rogers *et al.* 1982). The ED pathway yields only half as much ATP per mole of glucose as the more common EMP pathway, thereby producing less biomass and more fermentation products such as ethanol. However, *Z. mobilis* is not well suited for biomass conversion because of its limited substrate range – it ferments only glucose, fructose and sucrose.

To overcome the substrate limitation of *Z. mobilis*, Feldmann *et al*. (1992) inserted the genes coding for xylose isomerase (*xylA*) and xylulokinase (*xylB*) from *Xanthomonas compestris* and *Klebsiella pneumoniae*, respectively, into this organism, but the recombinant strain was unable to grow on xylose as sole carbon source. This was due to the inefficient activation of the PPP enzymes in the recombinant strain. To overcome this limitation, *xylA*, *xylB*, *tal* (transaldolase) and *tktA* (tranketolase) from *E. coli* were cloned in the host (Zhang *et al*. 1995a). The resultant recombinant *Z. mobilis* strain CP4 (pZB5) grew on xylose as sole carbon source and attained 86% of the theoretical ethanol yield. With a mixture of sugars under fermentative conditions, the ethanol yield from this strain increased to 95% of the theoretical yield (Zhang *et al*. 1995b). Since the publication of this work, several other improved *Z. mobilis* strains have been developed. One strain, ZM4(pZB5), showed increased ethanol tolerance (Joachimsthal and Rogers 2000). Another strain, ATCC39767, which had high sensitivity to microbial inhibitors present in hydrolysates such as acetic acid, was adapted by Lawford and Rousseau (1999) to tolerate acetic acid and produce ethanol by continuous culture in hydrolysates. This strain was subjected to fermentation with steam-treated wood hydrolysate and, after seven days of cultivation, produced 30 g/L of ethanol with a yield of 54% based on the total initial carbohydrates (McMillan *et al*. 1999). However, such a Simultaneous Saccharification and Fermentation (SSF) process is still uneconomical compared to use of *S. cerevisiae*. Another approach that has been well developed with *Z. mobilis* was to integrate both xylose and arabinose metabolism in the same organism. This strain, AX101, fermented glucose and xylose to

completion and 75% of arabinose in 50 h (Lawford and Rousseau 2002; Mohagheghi *et al*. 2002). Ethanol yields were 0.43 g/g to 0.46 g/g, with minor quantities of xylitol, lactic acid and acetic acid. However, acetic acid tolerance still affected the performance of recombinant *Z. mobilis* strains.

In contrast to *Z. mobilis*, *E. coli* was one of the first organisms to be metabolically engineered to selectively produce ethanol (Ingram *et al.* 1987). *E. coli* can ferment a wide spectrum of sugars and has been used in the industry for recombinant protein production. However, the other disadvantages associated with bacteria for lignocellulosic fermentation are also true for this organism. Hence, efforts to engineer *E. coli* for xylose fermentation have been focussed on increasing the ethanol yield and productivity by reducing the levels of other metabolites, especially acetic acid, and increasing the ethanol tolerance of the bacteria. One early approach to increase the ethanol production was to clone the pyruvate decarboxylase gene in *E. coli*. Ingram *et al*. (1987) managed to insert the required alcohol dehydrogenase gene (*ADH11*) from *Z. mobilis* and the pyruvate decarboxylase gene (*PDC*), under the control of a native *LAC* promoter (known as the PET operon, for production of ethanol), to produce a strain that produced ethanol almost exclusively. Later, to overcome the genetic instability of the genes being carried in plasmids, they were integrated and selected for high gene expression. One transformant, which showed high resistance to chloramphenicol selection, was disrupted of its fumarate reductase (*FRD*) gene to eliminate succinate production. The final strain (KO11) fermented glucose and xylose to ethanol at yields higher than the theoretical. While this strain has been shown to perform well in most laboratories, instabilities have been reported (Lawford and Rousseau 1995; 1996). Dumsday *et al*. (1999) suggested that *E. coli* KO11 might be unstable only during continuous cultivation processes. Yomano *et al*. (1998) have adapted the strain for increased ethanol tolerance by 10%. This strain, LY01, also tolerated hydrolysate inhibitors such as aldehydes, alcohols and organic acids better than KO11.

In addition to the two bacteria mentioned above, research has also been carried out on *Klebsiella oxytoca* and *Erwina chrysanthemi*. By transforming the PET operon in *K. oxytoca*, Ohta *et al*. (1991) showed a 90% conversion of fermentation products to ethanol. This strain fermented xylose and glucose rapidly and twice as fast as KO11. Screening and selection on chloramphenicol, as performed for *E. coli*, resulted in mutant P2, which showed 45% conversion of glucose and cellobiose, although no data for xylose has been reported (Wood and Ingram 1992). Apart from the bacteria mentioned so far, reports on xylose metabolism and fermentation have also been published for *Thermoanaerobacter ethanolicus* (Carreira *et al*. 1983), *Bacteroides polypragmatus* (Patel, 1984), *Zymomonas anaerobia* and *Clostridium saccharolyticum* (Asther and Khan, 1985), and *Bacillus marceans* (Williams and Withers, 1985). Also, almost all bacterial work relating to xylose fermentation has been carried out in gram-negative bacteria. Efforts to express the PET operon or just the *PDC* gene in gram-positive bacteria have not been successful. It would be of interest to consider this anomaly more intensively.

2.5 RECOMBINANT *S. CEREVISIAE* AND XYLOSE METABOLISM

Xylose utilisation by *S. cerevisiae* was initially thought to be non-existent because cells did not show growth in xylose medium (Barnett 1976). The yeast grew on xylulose, although at a growth rate one-tenth of that seen in glucose medium (Chiang *et al.* 1981; Senac and Hahn-Hägerdal, 1990). This was thought to be due to the generally lower expression in and flux through the PPP, through which the sugar is metabolised. Not more than 10% of the carbon was metabolised through the PPP when glucose was used as the substrate (Gancedo and Lagunas 1973). Because *S. cerevisiae* had been continuously cultivated and adapted for growth in hexose sugars over the course of time, it is possible that there was no requirement for the yeast to possess machinery for pentose metabolism.

However, the need for an efficient ethanol producer on an industrial scale under anaerobic conditions, coupled with the advances in recombinant DNA technology, made it possible to engineer and use *S. cerevisiae* for pentose fermentation. This also augured well for the so-called consolidated bioprocess (CBP) technology, wherein a single organism would be able to break down all complex carbohydrates such as cellulose, hemicellulose etc. into simpler units and metabolise them to ethanol and other by-products (Lynd *et al*. 1999). This involves the SSF process (Takagi *et al*. 1977), in which complex substrates such as lignocellulose are broken down into component sugars by the enzymatic hydrolysis of microorganisms such as yeasts, which then ferment the obtained sugars into ethanol. *S. cerevisiae* has been widely suggested as the organism best suited for this process because it can ferment most sugars obtained by the hydrolysis of lignocellulose, except for the pentose sugars, and in particular xylose. Because *S. cerevisiae* can utilise xylulose (Wang and Schneider 1980; Ueng *et al*. 1981; Gong *et al*. 1981a) and ferment the sugar (Yu *et al*. 1995; Jeppsson *et al*. 1996), it was thought feasible to introduce the isomerisation step to make the yeast metabolise xylose, and several research attempts were made in the early 1990s. Kötter *et al*. (1990) cloned the *P. stipitis XYL1* gene, coding for xylose reductase (Rizzi *et al*. 1988; Verduyn *et al*. 1985), and the *XYL2* gene, coding for xylitol dehydrogenase, into *S. cerevisiae*. This recombinant yeast was able to grow on xylose at a very low rate, although the growth was only oxidative. But the advent of this yeast opened the floodgates of recombinant yeast development for efficient xylose utilisation, and in the next decade almost every possible avenue for improving xylose utilisation and fermentation was researched (reviewed by Hahn-Hägerdal *et al*. 2001). Although the results obtained so far are impressive, there is still much work to be done to make the process economically feasible. In the following sections, the different stages of xylose metabolism in *S. cerevisiae* are outlined, together with the problems and limitations they pose for effective anaerobic xylose fermentation and the various engineering approaches carried out to overcome these and other limitations.

2.5.1 Xylose transport

Xylose, like glucose, is taken up into the yeast cell by facilitated diffusion (Kotyk 1967; Busturia and Lagunas 1986), predominantly by the action of hexose transporters, which are thought to transport the sugar concomitantly with glucose (Hamacher *et al*. 2002; Lee *et al*. 2002; Gardonyi *et al*. 2003a; Sedlak and Ho 2004). Kötter and Ciriacy (1993) varied the concentrations of the two sugars in a growth medium and showed that xylose was transported into the cell at a much lower rate than glucose because the transporters showed a 200-fold lower affinity for the pentose sugar than for the hexose. They determined the K_m for xylose to be 190 mM to 1.5 M, leading to the suggestion that there are high-affinity and low-affinity systems for xylose uptake. This suggested the K_m, when compared to that of glucose (1.5 mM to 35 mM), showed that the monosaccharide transport system had a 200-fold lower affinity for xylose than for glucose. Kötter and Ciriacy (1993) proposed that, in order for the cell to overcome the transport limitation, the xylose concentration in the medium should be higher than the K_m but suggested the initial concentration of 133 mM (20 g/L) used in their study, did not limit xylose utilisation. Hamacher *et al*. (2002) studied the effect of each individual transporter during xylose transport by using a mutant strain with all the hexose transporters deleted (Wieczorke *et al*. 1999). They showed that *HXT4, HXT5* and *HXT7* were involved in xylose transport, with *HXT7* being the most active. They found that the *GAL2* gene coding for galactose permease (Kou *et al*. 1970) also had xylose-transporting ability. This correlated with the findings of Lindén *et al*. (1992), who reported that cells grown on galactose and xylose together had a better growth rate in a sulfite liquor plant. With the apparent similarities in structure, it is surprising to note that no further studies have been published in relation to the effect of galactose in xylose fermentation. Sedlak and Ho (2004) also carried out a characterisation study of hexose transporters during xylose utilisation and postulated that *HXT5* was the major xylose transporter. Xylose transport in *S. cerevisiae* has been thought to be one of the limiting steps for fermentation, especially at low concentrations in the medium. Even though there are several limitations downstream (as discussed in the following sections), it might become crucial at a later stage to increase the transport rate of xylose in *S. cerevisiae* either by altering the native transporter or by cloning and expressing xylose-specific transporter(s) from other organisms.

Many natural xylose-utilising yeasts have been thought to possess transporters that can transport xylose, probably even at low concentrations (<1 g/L) (Alcorn and Griffin 1978; Lucas and Van Uden 1986; Kilian and Van Uden 1988; Does and Bisson 1989; Kilian *et al*. 1993; Nobre *et al*. 1999; Weierstall *et al*. 1999). While no xylose-specific transport gene has been isolated so far, the mechanism by which xylose transport occurs differs among organisms. For instance, *P. stipitis* is thought to transport xylose through two proton symport systems, one of high and the other of low affinity (Kilian and Van Uden 1988; Does and Bisson 1989). In most cases, two kinetically distinct xylose transporting systems have been reported (Fig. 2.3). The low-affinity transporter is generally shared with glucose and the high-affinity transporter is specific

for xylose. The low-affinity system transports xylose through facilitated diffusion, while the high-affinity system is thought to symport xylose with a proton using proton motive force. *C. shehatae*, when grown on either D-glucose or D-xylose, produced a facilitated diffusion system that could transport D-glucose (high affinity/low capacity), D-xylose (low affinity/high capacity), or D-mannose (Lucas and Van Uden, 1986). Thus, all these three

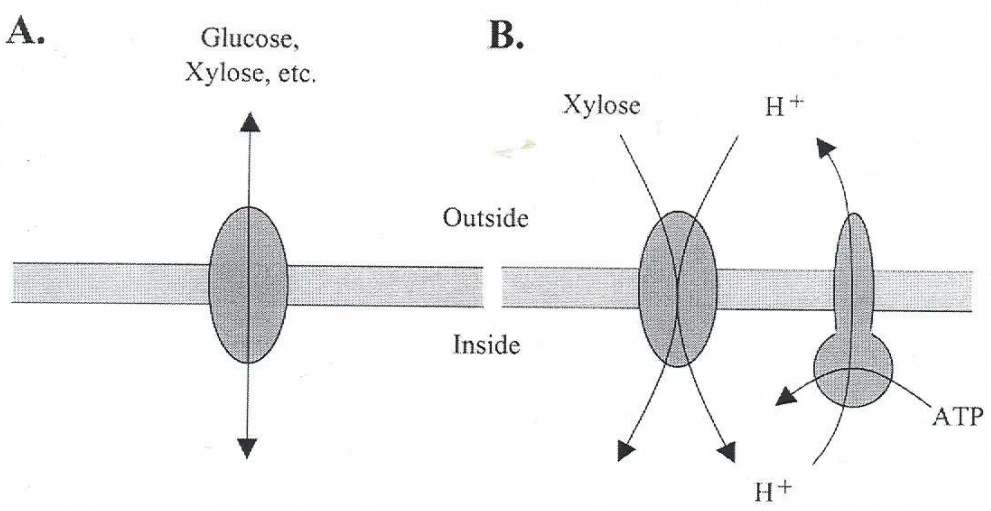

FIG. 2.3 A, B. The two mechanisms of xylose uptake by yeast: A. facilitated diffusion – the concentration gradient is the driving force – these transporters have a broader substrate range; **B. proton-xylose symport** – driving force is the proton motive force, maintained by the plasma membrane proton-ATPase. Adapted from Weusthuis *et al*. (1994).

sugars were competitive inhibitors of each other. Due to the high capacity of the system, D-xylose was taken up even in the presence of D-glucose. Another transport system that has been reported is that of the presence of glucose-repressible, high-affinity and constitutive facilitated diffusion xylose uptake systems in *Candida intermedia* (Gardonyi *et al.* 2003a; 2003b). Leandro *et al*. (2006) reported on the characterisation of the two transporters from *C. intermedia*. One was a facilitator for glucose/xylose called *GXF1* (K_m for xylose 0.4 mM), while the other was a glucose/xylose symporter named *GXS1*. It was thought that *GXF1* would be more efficient under oxygen-limited or anaerobic fermentation due to its lower energy needs for activation in contrast to the symporter system. *P. stipitis* is supposed to possess three different transporters, each having different levels of affinities for the sugar. Three genes (*SUT1, SUT2* and *SUT3*) were cloned and sequenced (Weierstall *et al*. 1999). *SUT1* had a K_m of 145 mM for xylose and seemed to be the major contributor to the low-affinity component of the glucose and xylose transport, which was evident from the lack of a low-affinity component in the *SUT1* disruption strain grown on glucose. *SUT2* and *SUT3* had higher affinities for xylose and were expressed under aerobic conditions only. They had a substrate-concentration-modulated affinity for glucose, similar to that observed for *HXT2* in *S. cerevisiae* (Reifenberger *et al*. 1997). The Michaelis constant for the low-affinity xylose transport in *P. stipitis* was different from that for *SUT1* determined in an *HXT1-7* deletion

strain in *S. cerevisiae* (Weierstall *et al*. 1999). This suggested that the low-affinity component could in fact be the superposition of several individual transporters. Therefore, it would be necessary to refine the kinetic constants for xylose transporters from various species once all the corresponding genes were cloned and expressed in a model system, such as the *S. cerevisiae hxt1-7* strain.

2.5.2 Xylose isomerase

The initial step towards developing recombinant xylose-utilising yeast was to introduce the xylose pathway genes. It had been shown that natural xylose-utilising yeasts and filamentous fungi carry out the isomerisation of xylose into xylulose in two steps, involving the intermittent formation of xylitol and its conversion to xylulose, rather than the single-step process featured in bacteria. Because the eukaryotic two-step conversion has its own pitfalls, various studies were carried out to clone and express the bacterial XI – the enzyme responsible for the single-step conversion process. Of the several bacteria screened, *E. coli* (Ho *et al*. 1984; Sarthy *et al*. 1987)*, Actinoplanes missouriensis* (Amore *et al*. 1989)*, Bacillus subtilis* (Amore *et al*. 1989)*, Lactobacillus pentosus* (Hallborn 1995)*, Clostridium thermosulfurogenes* (Moes *et al*. 1996), *Thermus thermophilus* (Walfridsson *et al*. 1996) and *Streptomyces rubiginosus* (Gardonyi and Hahn-Hägerdal 2003c) had XI enzymes that were cloned in *S. cerevisiae*. However, only the *T. thermophilus* XI was actively expressed in the host. It was suggested that the non-expression could be due to protein misfolding, post-translational modifications, disulfide bridge formation or the internal pH of the heterologous host. But the enzyme from *T. thermophilus* had a higher temperature optimum (85℃) than *S. cerevisiae* and lower activity at 30℃, which is the optimal temperature for *S. cerevisiae*. Hence, random PCR mutagenesis was performed on the XI gene to increase its activity at lower temperatures. Such a mutated XI strain still performed below its optimum (Lönn *et al*. 2002). When this mutated gene was expressed in *S. cerevisiae,* either as a single copy or in multi-copy plasmids (Lönn *et al*. 2003), there was an increase in ethanol yield with the multi-copy strain. However, the activity of XI was much lower (0.7-45 mU/mg protein), probably due to misfolding of the protein. Xylitol was also formed, probably due to the action of native non-specific aldose reductases, especially *GRE3* (Träff *et al*. 2002). Fungi have also been reported to contain the XI-encoding gene (Rawat and Rao, 1996), although not many reports are available about its cloning and expression in *S. cerevisiae*. However, Harhangi *et al*. (2003) reported the presence of XI in the anaerobic fungus *Piromyces* sp. When cloned and expressed in *S. cerevisiae* (Kuyper *et al*. 2003), the transformed strain showed a low growth rate on xylose but a high XI activity. However, when grown in xylose for prolonged periods, a mutant strain showing a higher growth rate was obtained. This strain had increased ethanol and glycerol yield, while very little xylitol was formed. The authors metabolically engineered this strain further by deleting *GRE3* (coding for a non-specific aldose reductase) (Träff-Bjerre *et al*. 2004) and overexpressing five PPP genes (Kuyper *et al*. 2005a). The resultant strain grew anaerobically on xylose at a faster growth rate than the parent and produced

negligible amounts of xylitol. However, this strain was still limited by the kinetics of xylose uptake under anaerobic conditions, and was hence subjected to prolonged adaptation in sequential anaerobic fermentation with xylose as sole carbon source. During successive cultivations, the kinetics of fermentation improved and the resultant strain was able to grow at a faster rate and produce ethanol at close to theoretical yields (Kuyper *et al*. 2005b). This strain expressing the functional XI has not yet been tested in lignocellulosic hydrolysates and it would be of great interest to evaluate its performance under those conditions. Recently Karhumaa *et al*. (2007) compared isogenic *S. cerevisiae* strains expressing the XI pathway and XR-XDH pathway. Their results suggest that although expressing fungal XI in *S. cerevisiae* resulted in higher ethanol yield, the XR-XDH strain had faster production rate and produced no xylitol in lignocellulosic hydrolysates.

2.5.3 Xylose Reductase and Xylitol Dehydrogenase

Due to the difficulties posed in cloning the XI gene in *S. cerevisiae*, several advances have been made in the 'conservative' approach to the two-step isomerisation process. Among the various XR and XDH genes tested, the *XYL1* (Verduyn *et al*. 1985) and *XYL2* (Kötter *et al*. 1990) genes from *P. stipitis* were shown to be active in *S. cerevisiae* (Kötter *et al*. 1990; Kötter and Ciriacy 1993; Tantirungkij *et al*. 1993; Walfridsson *et al*. 1997). Although many reports exist for the expression of *P. stipitis* XR in *S. cerevisiae*, Govinden *et al.* (2001) expressed XR from *C. shehatae* in the yeast and showed improved activity to the results with *P. stipitis*. It has been suggested that expressing both *XYL1* and *XYL2* from *C. shehatae* in *S. cerevisiae* should be explored, but no reports have yet been published. The major disadvantage of the two-step isomerisation process was found to be the apparent redox cofactor imbalance, especially under anaerobic conditions. Almost all known XRs have been shown to have a preference for NADPH as cofactor (Bruinenberg *et al*. 1983), with the exception of some (Verduyn *et al*. 1985; Rizzi *et al*. 1988; Neuhauser *et al*. 1997; Govinden *et al*. 2001) that have dual cofactor dependence (NAD(P)H). Even among these, the NADH dependence was lower than that for NADPH and was displayed only under certain conditions. The K_m of XR for xylose differed between the two cofactors. According to Kostrzynska *et al*. (1998), the K_m for NADPH was 11 μM, while the K_m for NADH was 28 μM. These values were similar to the values reported earlier – 9 μM and 21 μM respectively – by Verduyn *et al*. (1985). Thus the kinetics of this enzyme favoured NADPH utilisation. This contrasted with the cofactor requirement of the XDH enzyme in the next step of xylose utilisation. This enzyme was almost exclusively NAD^+ dependent (Kötter *et al*. 1990) and hence released NADH. Cloning and expressing these two genes, *XYL1* and *XYL2,* in *S. cerevisiae*, where ethanol production under fermentative conditions was redox neutral, created an imbalance in redox potential. This usually resulted in the exclusive production of xylitol from xylose or, in some cases, in the production of more glycerol because the cell adapted itself to correct the redox imbalance. This scenario was different from that of *P. stipitis*, where xylose was converted to ethanol and xylitol under

anaerobic conditions (Ligthelm *et al*. 1988), but produced no xylitol under oxygen-limited conditions. This led to the suggestion that *P. stipitis* maintained its redox balance through other mechanisms, which was proven by the studies of Jeppsson *et al*. (1995) and Hallborn (1995). They found two redox sinks that were thought to prevent xylitol formation in *P. stipitis*. They were: (i) an alternative cyanide-insensitive oxidase believed to regenerate the NAD^+ required for xylitol oxidation; and (ii) a D-arabinitol dehydrogenase that may act as redox balancer by reducing D-ribulose to D-arabinitol. These activities were not present in native *S. cerevisiae* strains and therefore could explain the xylitol produced during xylose growth in *S. cerevisiae* and not *P. stipitis*. On the other hand, it was also suggested that *P. stipitis* produced both ethanol and xylitol under anaerobic conditions due to the dual cofactor dependency of the XR (Prior *et al*. 1989; Dellweg *et al.* 1990; Skoog and Hahn-Hägerdal 1990; Kötter and Ciriacy 1993). It is intriguing that the same enzyme when present in *P. stipitis* showed dual cofactor dependency but, when expressed in *S. cerevisiae,* was almost exclusively NADPH dependent, as observed from the exclusive xylitol production (Skoog and Hahn-Hägerdal 1990; Hallborn *et al*. 1991). However, Ligthelm *et al*. (1988) showed that very little xylose carbon was channelled through the oxidative PPP in *P. stipitis*. Bruinenberg *et al.* (1984) showed the importance of NADH as well as that of the redox couple (Bruinenberg *et al*. 1983) in xylose fermentation in yeasts by using a *C. utilis* model.

It was proposed and shown that the expression levels and ratio of the two enzymes – XR and XDH – were very important for better xylose utilisation and conversion. Walfridsson *et al*. (1997) studied the effect of differentially expressed *XYL1* and *XYL2* genes on xylose fermentation in recombinant yeast by cloning the two genes under two different promoters, phosphoglycerate kinase (*PGK1*) and alcohol dehydrogenase (*ADH1*), under different orientations in the plasmid. Analysing the resultant XR and XDH activities in the various recombinant strains led them to conclude that a ratio of 0.06 between XR and XDH was crucial for better xylose fermentation to yield products other than xylitol.

Jeppsson *et al*. (2006) expressed a mutated *P. stipitis* XR-encoding *XYL1* gene (Kostrzynska *et al*. 1998) that showed altered K_m in a recombinant *S. cerevisiae* strain at two different levels together with the native *P. stipitis XYL2* gene and the overexpressed endogenous *XKS1* gene. The resultant strain displayed a decrease in xylitol yield, accompanied by enhanced ethanol yields. Flux analysis showed that the mutated XR utilised a larger fraction of NADH for xylose reduction. Petschacher *et al*. (2005) reported the kinetics of a similar mutant XR in *Candida tenuis*. The mutated XR (K270M) had an increased catalytic constant (k_{cat}) with both NADH and NADPH as cofactors, with the fold change being significantly higher for NADPH (2.8) than for NADH (1.7). This result suggested that, apart from reducing the *in vitro* affinity for NADPH, the mutation may also have enhanced the catalytic rate of the similarly mutated *P. stipitis* XR. However, the fermentation properties of this enzyme have not yet been described. Metzger and Hollenberg (1995) had earlier reported on direct mutagenesis on the coenzyme binding sites of *P. stipitis* XDH enzyme, with the

resultant mutant showing decreased NAD^+ affinity. They also introduced the proposed NADP-recognition sequence (GSRPVC) of alcohol dehydrogenase from *Thermoanaerobium brockii* into the XDH sequence. This increased the NADP-dependent activity but, of greater significance, was the reduction in NAD^+ affinity. Woodyer *et al*. (2003) reported on relaxing the nicotinamide specificity of phosphite dehydrogenase, while Watanabe *et al*. (2005) performed multiple site-directed mutageneses on amino acids from the coenzyme-binding domain of XDH. However, the effect of expressing the mutant XDH in *S. cerevisiae* has not yet been reported.

2.5.4 Xylulokinase

Xylulokinase (XK), as the name suggests, phosphorylates xylulose so that xylulose-5-phosphate can enter the PPP. This phosphorylation step consumes energy in the form of ATP. The apparent growth of *S. cerevisiae* on D-xylulose is made possible by this enzyme. However, its growth on this substrate is so slow that its improved growth on D-xylulose was used as a screen to identify a xylulokinase gene (Ho and Chang 1989; Deng and Ho 1990). Ho and Tsao (1995) published the sequence of this gene first, although Rodriguez-Pena *et al*. (1998) reported an almost identical sequence of the open reading frame YGR194c in the *S. cerevisiae* genome and this was renamed *XKS1*. Various views have been aired about the importance of *XKS1* in fermentation (Ho *et al*. 1998; Eliasson *et al*. 2000; Johansson *et al*. 2001; Toivari *et al*. 2001; Jin *et al*. 2003). While most of the reports suggested that *XKS1* should be overexpressed so that more xylulose could go through the PPP, other reports (Rodriguez-Pena *et al*. 1998; Jin *et al*. 2003) argued that, because of the high ATP requirement, an uncontrolled overexpression of this gene might lead to energy depletion, resulting in cessation of fermentation and cell toxicity. The authors suggested such a condition would be similar to the substrate-accelerated cell death observed with an *S. cerevisiae TPS1* mutant during glucose metabolism. Thus, it seemed that an optimal/moderated level of *XKS1* expression was needed (Jin *et al*. 2003) for better xylose fermentation, although several strains that have overexpressed *XKS1* have shown better performance through modification of other steps in xylose metabolism (Jeppsson *et al*. 2003b; Karhumaa *et al*. 2005). Richard *et al*. (2000) explored the kinetics of this enzyme in *S. cerevisiae* and reported that the overexpression of xylulokinase did not increase the growth rate of the cell on xylulose to a level comparable to that of glucose, which meant that other factors such as activities of other enzymes may be limiting, e.g. transaldolase, because sedoheptulose-7-phosphate has been shown to accumulate in D-xylulose fermentation (Senac and Hahn-Hägerdal 1990). These authors also concluded that the activities of the xylulokinase from *S. cerevisiae* resembled those of *P. stipitis* (Jin *et al.* 2002). Thus, the expression level of xylulokinase was very crucial in xylose and xylulose fermentation, although this might in turn be shown to be dependent on other factors such as downstream pathway enzymes. Eliasson *et al.* (2001) proposed a kinetic model, which implied that, under simplified simulation conditions, a 1:≥10:≥4 relationship of the XR/XDH/XK ratio was optimal in minimising xylitol formation during

xylose utilisation in yeast. Overexpression of XK was found to be necessary for ethanol formation from xylose. Xylitol formation decreased with a decreasing XR/XDH ratio, while ethanol formation increased. A recombinant *S. cerevisiae* strain (TMB3004) with a XR/XDH/XK ratio corresponding to the theoretical optimal ratio fermented xylose to ethanol efficiently (Eliasson *et al*. 2001).

2.5.5 Pentose phosphate pathway enzymes

Two enzymes acting downstream of *XKS1*, transaldolase (TAL) (EC 2.2.1.2, encoded by *TAL1*) and transketolase (TKL) (EC 2.2.1.1, encoded by *TKL1*), have an important role in xylose metabolism and fermentation. Overexpression of these two genes, individually and in conjunction, led to changes in the xylose consumption pattern and metabolite production (Walfridsson *et al*. 1997). Overexpression of *TAL1* alone resulted in enhanced growth on xylose. Overexpression of the two genes simultaneously resulted in a similar growth pattern, showing that TAL overproduction enhanced the growth of recombinant *S. cerevisiae*, irrespective of the activities of XR, XDH and TKL. This finding correlated well with that of Senac and Hahn-Hägerdal (1990) that TAL expression was limiting in xylose growth. The TAL enzyme converted sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate into erythrose-4-phosphate and fructose-6-phosphate. *TKL1* overexpression did not result in any visible changes in xylose growth when expressed under a different promoter (Metzger and Hollenberg 1994). Johansson and Hahn-Hägerdal (2002) overexpressed four genes in the non-oxidative PPP, viz. ribulose-5-phosphate epimerase (EC 5.1.3.1), ribose-5-phosphate ketol isomerase (EC 5.3.1.6), TAL and TKL, simultaneously as well as individually. Overproduction of the non-oxidative PPP enzymes did not influence the xylose fermentation rate. However, the specific growth rate on xylulose increased in the *TAL1* overexpressed strain as well as in the strain with all four genes overexpressed. Thus, it was suggested that the non-oxidative PPP controlled xylulose growth and fermentation, while control of xylose growth and fermentation lay elsewhere. The gene *ZWF1*, encoding glucose-6-phosphate dehydrogenase (G6PDH), has been disrupted in a recombinant strain to decrease NADPH levels during xylose fermentation (Jeppsson *et al*. 2003b). Although this strain showed increased ethanol yield (0.41 g/g xylose) and decreased xylitol yield (0.05 g/g xylose), it did so at the expense of a six-fold lower xylose consumption rate. To increase the xylose consumption rate, these authors increased the production of XR (Jeppsson *et al*. 2003a) and varied the expression levels of G6PDH to generate a range of intracellular NADPH levels (Jeppsson *et al*. 2003b). They reported a trade-off between a high xylose consumption rate and a low xylitol yield when *ZWF1* levels were modulated. Thus, with a 6% G6PDH level, the xylose consumption rate was only 77% of the control strain.

2.5.6 Engineering byproduct formation during xylose fermentation

One of the major metabolites produced by anaerobic xylose fermentation is glycerol. It is thought to be produced in large quantities due to the necessity for redox balancing.

The availability of NADH, produced by the action of XDH during anaerobic fermentation, might lead to cell toxicity if it is not converted back to NAD^+. Because mitochondrial respiration is ruled out during anaerobic conditions, the only way in which the cell can process the excess NADH is through the glycerol pathway by the action of two enzymes – glycerol phosphatase (GPP) and glycerol phosphate dehydrogenase (GPD). Each of these two enzymes is encoded by two homologous genes, viz. *GPP1* and *GPP2,* and *GPD1* and *GPD2*. Each of these genes has specific actions and is expressed individually under certain conditions only, although their actions can overlap. *GDH1* encodes NADPH-dependent glutamate dehydrogenase activity and can be complemented with *GDH2*, which is NAD^+ dependent. With the help of metabolic engineering, Nissen *et al.* (2000) have shown that, by deleting the *GDH1* gene coding for NADPH-dependent glutamate dehydrogenase and overexpressing the *GLN1* gene, encoding glutamine synthetase, and *GLT1*, encoding glutamate synthase genes, glycerol production can be reduced. Their engineered strain had 10% more ethanol yield and 38% reduced glycerol yield from glucose. However, the strain had a lower growth rate. Roca *et al*. (2003) evaluated the performance of recombinant strains that had *GDH1* deleted and either *GDH2* overexpressed or *GLN1* and *GLT1* overexpressed together. The former strain produced 44% less xylitol, while the ethanol yield showed a marginal increase. The latter strain showed a 16% increase in ethanol yield during carbon-limited chemostat at a low dilution rate. Grotkjær *et al*. (2005) developed metabolic models to compare the effect of deleting *GDH1* in xylose recombinant strains. This approach increased Xyl1p activity with NADH and increased ethanol production by 25%, but it needed efficient cell growth. The presence of amino acids in the medium could repress the function of the enzyme in a mutant strain (Nissen *et al*. 2000). Bro *et al*. (2006) also developed a genome-wide metabolic flux model to decrease glycerol formation and one of the best strategies was to express NADP-dependent non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase (GAPN), which converts 3-phosphoglyceraldehyde into 3-phosphoglycerate with NADPH formation. However, it bypassed *PGK1*, thereby reducing the ATP yield but increasing the biomass via NADPH. This strain produced 50% less glycerol, but showed only a 3% increase in ethanol yield during glucose fermentation. When the xylose pathway genes (*XYL1, XYL2, XKS1*) were introduced into this *gapN* background and the recombinant strain was grown anaerobically in a glucose-xylose mix, the glycerol formation decreased by 58% and xylitol formation decreased by 33%, while the ethanol yield increased by 24% (Bro *et al.* 2006). Thus, the use of metabolic flux analysis and genome-wide metabolic modelling has helped to improve the ethanol yield by changing the flux from glycerol towards ethanol through the manipulation of genes not involved in that pathway.

Transhydrogenases are enzymes that facilitate the reversible conversion of NADPH to NADH. No known yeasts or fungi possess these enzymes, although several bacteria and prokaryotes have been shown to have one of them. The lack of transhydrogenases has been thought to be one of the reasons why redox balance is so critical for xylose fermentation in many yeasts and fungi, especially in recombinant

S. cerevisiae. Anderlund *et al.* (1999) cloned and overexpressed the *pntA* and *pntB* genes coding for transhydrogenase from *E. coli* into *S. cerevisiae*. Although the genes were successfully cloned and expressed, they did not function in a reversible manner and converted only NADPH into NADH, thereby defeating the purpose (and producing more glycerol). Jeun *et al*. (2003) expressed a transhydrogenase from *Azotobacter vinelandii* in a recombinant *S. cerevisiae* expressing the *PsXYL1* gene. The resultant recombinant strain produced more ethanol than xylitol during fed-batch cultivation. The authors suggested that the soluble transhydrogenase from *A. vinelandii* mediated NADH and $NADP^+$ formation, instead of the desired NADPH and NAD^+ formation. Their work also showed that the NADH preference of *P. stipitis* XR was lower than its NADPH preference. Expression of the transhydrogenase increased the production of 2-oxoglutarate and glycerol and shifted the intracellular ratio of ($NADPH/NADP^+$):($NADH/NAD^+$) from 35 to 17 (Nissen *et al*. 1997). These results indicated that the thermodynamic equilibrium for the transhydrogenase reaction lay in the direction of NADH formation. It therefore seems unlikely that this approach will be useful in the absence of other energy-consuming reactions. No other transhydrogenase expression studies have been published so far, although the presence of transhydrogenase-like cycles have been reported in *S. cerevisiae* and it would be interesting to screen other prokaryotes or try to mutate the already-cloned ones. Expressing a functional transhydrogenase that can interconvert NADPH and NADH without apparent growth defects would have enormous potential in industrial applications as well as in xylose fermentation.

2.5.7 Endogenous xylose-metabolising genes in *S. cerevisiae*

With the availability of the whole genome sequence of the yeast, it was possible to search for sequences similar to other known genes, such as *XYL1* and *XYL2*. Träff *et al*. (2002) reported on five putative aldose reductase-encoding genes obtained from the *S. cerevisiae* genome database based on sequence homology. Whereas all five genes showed a residual level of xylose reduction, *GRE3* was the most active, while *YPR1, GCY1* and the protein encoded by *YJR096W* showed lower levels of XR activity. This means *S. cerevisiae* was capable of endogenous xylose reduction, although with low specificity and activity. Richard *et al*. (1999) found that the gene *YLR070C* encoded a xylitol dehydrogenase on the basis of its sequence homology to other enzymes. This, together with the presence of an endogenous xylulokinase gene (Ho and Chang 1989; Rodriguez-Pena *et al*. 1998), led most researchers to conclude that *S. cerevisiae* possessed an endogenous xylose pathway. Batt *et al*. (1986) and Toivari *et al*. (2004) published reports supporting the same. Toivari *et al*. (2004) overexpressed the aforementioned genes in a native *S. cerevisiae* strain and evaluated its growth and byproduct formation under aerobic and anaerobic conditions. This strain was able to grow on xylose, although at a much slower rate than a recombinant *S. cerevisiae* strain that contained the *P. stipitis XYL1-2* genes. The major byproduct was xylitol, probably because of the exclusive NADPH-dependence of the aldose reductase *GRE3* and the

scarcity of NAD^+ for the XDH reaction, thereby creating a severe redox imbalance. All this suggested *S. cerevisiae* could initially have had both glucose- and xylose-utilising capabilities, but that it could have lost its xylose-utilising character during the course of its evolution into domesticated yeast. This would fit well with the low endogenous expression of its xylose pathway genes and enzymes. This scenario was recently boosted when Attfield and Bell (2006) derived a non-recombinant *S. cerevisiae* (without any heterologous genes) using population genetics, by which they successively mated and generated yeasts that grew on xylose at a comparatively faster growth rate. After 22 successive mating and regeneration cycles and about 1300 days, their resultant xylose-growing strain showed increased XR and XDH activities. It would be interesting to know how this non-recombinant strain performs under anaerobic fermentation in xylose.

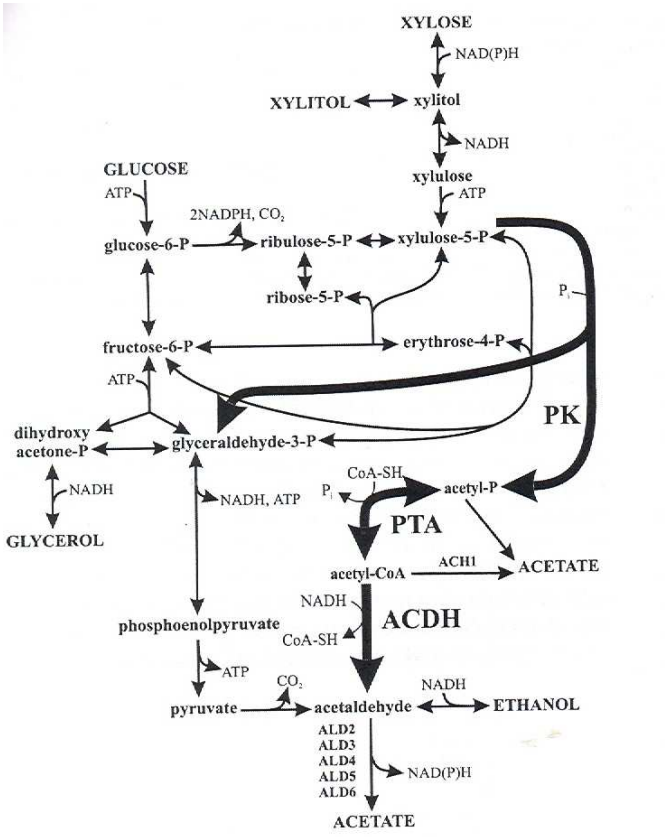

FIG. 2.4 Cytosolic bioreaction network of *S. cerevisiae*. The **bold arrows** indicate the recombinant phosphoketolase pathway. Abbreviations: PK, phosphoketolase; PTA, phosphotransacetylase; ACDH, acetaldehyde dehydrogenase (acylating); ACH1, acetyl-CoA hydrolase; ALDx, aldehyde dehydrogenase isoenzymes. © Sonderegger *et al*. (2004b)

2.5.8 Phosphoketolase pathway in recombinant *S. cerevisiae*

Sonderegger *et al*. (2004b) engineered the presence of a phosphoketolase pathway in *S. cerevisiae* (Fig. 2.4). Phosphoketolases (EC 4.1.2.9) are the key catabolic enzymes of many lactic acid and bifidobacteria (Gottschalk 1986) that convert xylulose-5-phosphate to acetyl-phosphate and glyceraldehyde-3-phosphate and/or convert

fructose-6-phosphate to acetyl-phosphate and erythrose-4-phosphate (Meile *et al*. 2001; Posthuma *et al*. 2002). This pathway leads to net reoxidation of one molecule of NADH per molecule of xylose converted to ethanol, and would potentially allow the achievement of the maximum theoretical 0.51 g/g ethanol yield on xylose without affecting the NADPH/NADH ratio of the xylose reductase reaction. However, such an engineered strain yielded less xylitol and 25% more ethanol (Sonderegger *et al*. 2004b). Overexpression of phosphoketolase resulted in acetate accumulation. The authors consequently created an *ald6* mutant, which reduced acetate formation while increasing the ethanol yield by 20% and it had a 40% higher fermentation rate than its parent strain. This approach differs considerably from the many other approaches and the further adaptation of such a strain might result in strains with improved fermentation rates and ethanol yields.

2.5.9 Global analysis of recombinant *S. cerevisiae* during xylose utilisation

It is important to determine the limiting steps in xylose utilisation, but they are all different in the way they affect xylose metabolism. Xylose uptake and the PPP affect the xylose consumption rate because of their poor kinetic properties at the level of single reactions. In contrast, limitations caused by cofactor imbalances are related to the overall availability of cofactors such as NADPH and NAD^+. Therefore, investigating the limitations requires a whole-cell or global approach, which can be carried out by Metabolic Flux Analysis (MFA) or through genome-wide transcriptome and/or proteome analysis.

MFA has been applied extensively, including in the quantification of metabolic fluxes in glucose-consuming yeast (Nissen *et al*. 1997), genetically engineered *S. cerevisiae* (Wahlbom *et al*. 2001; Wahlbom and Hahn-Hägerdal 2002), and the natural xylose-utilising yeast *Candida milleri* (Granström *et al*. 2000). MFA has also been used to calculate theoretical yields for ethanol production from xylose-utilising recombinant *S. cerevisiae* (Lee *et al*. 2001). Pitkänen *et al*. (2003) analysed the fluxes in recombinant *S. cerevisiae* under aerobic conditions with low glucose and high xylose concentrations in the medium, focussing on cofactor dependencies. They showed that although NADPH regeneration was not a problem, NAD^+ generation was a major bottleneck, more so under anaerobic conditions. Grotkjær *et al*. (2005) calculated the flux in a *gdh1* mutant (CPB.CR4) with a modulated redox metabolism under xylose fermentation conditions and showed that there was a shift in XR activity towards the use of NADH as a cofactor rather than NADPH. The strain also showed glyoxylate cycle induction and a 25% increase in ethanol yield. Jin and Jeffries (2004a) performed a Flux Balance Analysis (FBA) and calculated extreme pathways using a stoichiometric model that described the biochemistry of yeast cell growth. FBA predicted that the ethanol yield from xylose was at a maximum under oxygen-limited conditions. On the basis of the fermentation details, the authors concluded that oxygen (or some other electron-accepting system) was required to resolve the redox imbalance caused by the cofactor differences between the two enzymes of the xylose pathway.

The advent of array-based technology to analyse global changes in genomes under varying conditions has also helped in analysing the metabolic changes in recombinant xylose-fermenting *S. cerevisiae* at the transcriptome and proteome levels. Wahlbom *et al*. (2003b) were the first to perform microarray analysis of a diploid, industrial random mutant that was able to show a faster rate of growth on xylose (Wahlbom *et al*. 2003a). This strain, TMB3400, had higher expression levels of *HXT5, XKS1* and PPP genes such as *SOL3, GND1, TAL1* and *TKL1*. Genes involved in galactose metabolism were also up regulated, along with two genes in gluconeogenesis and the glyoxylate shunt (*PCK1, ICL1*). The levels of expression of genes encoding glycolytic enzymes, such as *HXK2* and *MIG1*, were down regulated when this strain was grown on xylose alone. Jin *et al*. (2004b) also analysed the transcript levels of a recombinant *S. cerevisiae* strain (FPS-YSX3) expressing the *P. stipitis* genes coding for XR, XDH and XK enzymes in glucose and xylose, under both aerobic and oxygen-limited conditions. Their analysis showed increased expression levels of genes encoding the tricarboxylic acid (TCA) cycle, while expression levels of genes encoding respiration enzymes (*HXK1, ADH2, COX3, NDI1* and *NDE1*) and regulatory proteins (*HAP4, MTH1*) increased significantly when this strain was grown on xylose. The levels were increased even more under oxygen limitation. These authors also found up-regulation of the genes involved in gluconeogenesis and the glyoxylate shunt. Thus they suggested that xylose was recognised by the cell as a non-fermentable carbon source and that the respiratory proteins increased in response to the cytosolic redox imbalance. The increase in production of respiratory transcripts may also have been in response to low levels of sugar uptake, as has been shown for *Kluyveromyces lactis* (Goffrini *et al*. 2002) and *S. cerevisiae* with an altered regulatory network in galactose medium (Østergaard *et al*. 2000). The expression levels of genes involved in redox balancing, such as *GDH2* and *LYS12*, have also shown an increase when they were grown on xylose.

In another study with an evolutionary-engineered mutant C1 (Sonderegger and Sauer 2003), Sonderegger *et al*. (2004a) reported that the transcript levels throughout central carbon metabolism, specifically those of the xylose-specific, pentose phosphate and glycerol pathways, were significantly higher for the mutant strain C1. Similar to the studies mentioned above, the levels of genes involved in galactose metabolism were higher, as were the levels of *SOL3, GPD2, ADH5, PYK2*, etc. The hexose transporter gene *HXT2* was down-regulated, while many glycerol pathway genes and minor isozymes in the central carbon metabolism pathway were down-regulated only under aerobic conditions on xylose. However, the genes involved in redox balancing, especially those associated with NADPH formation (*ZWF1*) and NADH consumption (*ADH4, ADH5, GPD2*), were up regulated, while genes linked to NADH formation (*ALD3* and *GUT2*) were down-regulated. Thus, it was suggested that the mutations in C1 were regulatory rather than xylose-specific. Bro *et al*. (2004) performed a similar analysis on an *S. cerevisiae* strain deleted in *GDH1* and encoding an $NADP^+$-dependent glutamate dehydrogenase in glucose medium under anaerobic conditions. From the 16 transcripts

showing significant differences, only one gene with a direct link to redox balancing – *GND1*, encoding phosphogluconate dehydrogenase – was observed. In a subsequent analysis for genes coding enzymes that use NAD^+ or $NADP^+$, the authors obtained a subset of genes that included *GND1, ZWF1* and *ALD6*, which were down-regulated, together with eight other genes encoding NADP(H)-dependent enzymes. They also found conserved motifs in the promoter regions of genes involved in redox balancing, suggesting a common regulation of genes involved in NADPH regeneration in *S. cerevisiae*. Kuyper *et al*. (2005a) overexpressed PPP genes in an XI mutant strain and the resultant strain was able to grow at a faster rate on xylose under anaerobic conditions, producing negligible amounts of xylitol but higher amounts of ethanol and glycerol. When a microarray was performed on the transcripts of the overexpressed PPP genes, it showed a 1.7 to 8.5-fold increase in all the mRNA levels. This suggested that xylose flux through the PPP was one of the limiting steps even when the XR/XDH system, which creates a redox imbalance, was overcome in recombinant *S. cerevisiae*. A genome-wide transcription analysis of an evolutionarily adapted mutant strain with XI expression could shed more light on efficient anaerobic xylose fermentation.

Salusjärvi *et al*. (2003) were the first to report the global profile of their recombinant xylose-utilising *S. cerevisiae* strain (H2490) at the proteome level. Protein levels in this strain differed significantly between growth on xylose and glucose, as well as between the aerobic and anaerobic states. Proteins for alcohol dehydrogenase 2 (Adh2p), acetaldehyde dehydrogenases (Ald4p and Ald6p) and DL-glycerol 3-phosphatase (Gpp1p) were the major enzymes, with increased levels on xylose under all conditions. Glycerol-3-phosphate dehydrogenase 1 (Gpd1p) showed decreased levels on xylose under anaerobic conditions, but Gpd2p levels were not found to differ significantly. The major changes were seen with the glycerol and acetate pathways, probably as a measure of redox balancing by the cell.

As an offshoot of the global analysis of the transcriptome and proteome of the mutant, recombinant *S. cerevisiae*, increased expression levels of previously uncharacterised genes/proteins have been reported. The list included the following genes: *YDL124W, YMR315W, YCR020C, YBR083W, YPR199C, YNL260C* and *YLR022C*. While some of these have been shown to have no direct influence on xylose metabolism (Wahlbom *et al*. 2003b), it would be useful to determine if these genes do play any significant role.

2.6 CARBON CATABOLITE REPRESSION AND XYLOSE

When grown in mixed sugar medium, most microorganisms exhibit a preference for one sugar over another until it is exhausted. This phenomenon is called carbon catabolite repression, where the presence of one sugar in the medium inhibits the uptake and utilisation of another. Because glucose is the most common carbon source and is predominantly preferred by organisms, it is also referred to as glucose catabolite repression (Magasanik 1961; Ullmann 1996), or simply glucose repression (Gancedo, 1998). It is one of the well-characterised mechanisms in yeast (reviewed by Gancedo,

1998). The genetic and metabolic regulation of the several genes involved in glucose repression has been studied in detail. There are various levels in which glucose repression occurs in yeasts, as seen in Fig. 2.5, so that other sugars such as sucrose, maltose and galactose, are repressed until glucose exhaustion in the medium.

MIG1 is one of the well-characterised genes in the glucose repression pathway and was first isolated as a multi-copy inhibitor of galactose genes (Nehlin and Ronne 1990). It acts by binding to the promoter regions of *SUC*, *MAL*, *GAL*, etc., thereby blocking their transcription in the presence of high glucose concentrations (Nehlin and Ronne 1990; Nehlin *et al*. 1991; Hu *et al*. 1995; Klein *et al*. 1996). *MIG2* is a homologue of *MIG1* and is found to be active in a *mig1* mutant. The *MIG1* gene product is thought to act in concert with the Ssn6-Tup1 complex (Treitel and Carlson 1995) and is most probably inhibited by Snf1 kinase, which acts upstream of MIG1 and under low glucose conditions (Treitel *et al*. 1998). It has been proposed (Treitel and Carlson 1995) and shown (DeVit *et al*. 1997; Treitel *et al*. 1998) that, under high glucose conditions, MIG1 is localised in the nucleus, where it can bind to the target genes, while under low glucose conditions the Mig1 is phosphorylated by Snf1 and is localised in the cytoplasm. Thus, the target genes would be derepressed. However, certain deletions in the regulatory domains of Mig1 (Östling *et al*. 1996) resulted in a probable loss of phosphorylation sites for Snf1, forcing Mig1 to be localised in the nucleus, even when glucose levels were low (DeVit *et al*. 1997). Thus, a glucose-like environment is maintained even in the absence of or at low levels of glucose due to constitutive catabolite repression.

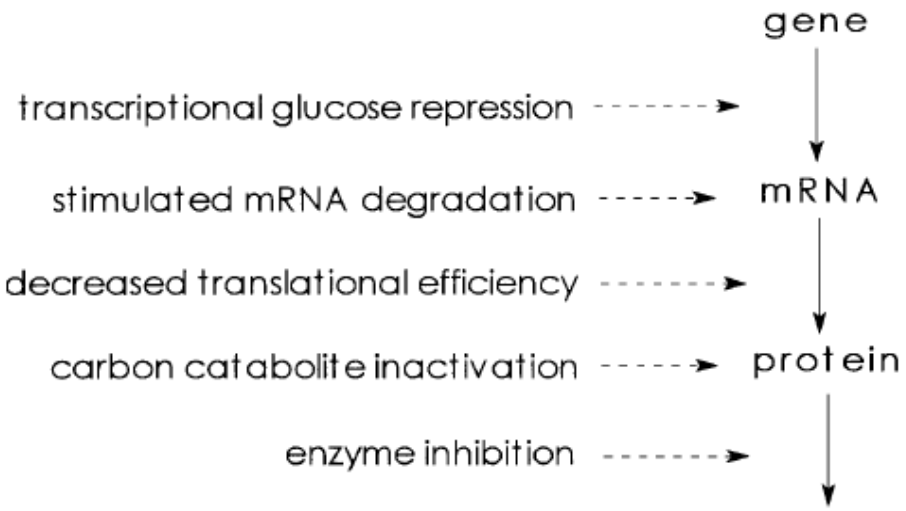

FIG. 2.5 Mechanisms of carbon (glucose) control in *S. cerevisiae*. Adapted from Klein *et al*. (1998).

Glucose and xylose are transported into the yeast cell by the same transport machinery and it was of interest to know the effect of glucose repression on xylose. Earlier studies showed that glucose consumption was faster by at least an order of magnitude than that of xylose and, as a result, xylose consumption usually started only when most of the glucose in the medium had been consumed (Meinander and Hahn-

Hägerdal, 1997). This led to the conclusion that xylose utilisation was repressed. Roca *et al*. (2004) undertook an extensive study on the effect of *MIG1* and *MIG2* deletions in a recombinant xylose-utilising strain and found that a *mig1* strain showed improved xylose consumption during fermentation and also in carbon-limited chemostat. However, they also showed that, when glucose was not present in the medium, xylose itself could be a repressible sugar because they found *SUC2* repression. Belinchon and Gancedo (2003) reported that xylose caused repression, suggesting that, under anaerobic conditions, xylose was being recognised as a non-fermentable sugar by the yeast. Recently, Öhgren *et al*. (2006) have shown that xylose consumption was higher during fermentation when residual glucose was close to 4 g/L. It is generally thought that the presence of low amounts of glucose helps in maintaining the higher levels of glycolytic enzymes needed for increased flux to ethanol. These authors used fed-batch cultivations in tandem with SSF to maintain low amounts of glucose throughout the fermentation, and thereby prevented the competitive inhibition of xylose transport by glucose (Meinander *et al*. 1999). All the above factors led to our hypothesis, namely that xylose consumption could be improved by constitutively repressing the recombinant yeast for glucose. In a constitutively repressed strain, the genes involved in glucose transport and utilisation would be active irrespective of the levels of glucose and would probably sense and consume the other sugar (xylose), thereby increasing xylose consumption.

2.7 XYLOSE REDUCTASES

Xylose reductase (XR) belongs to the family of aldo-keto reductases (AKR) (EC 1.1.1.21) and is responsible for the conversion of xylose to xylitol in the presence of NAD(P)H. It has gained importance in the fermentation industry due to its role in xylose conversion from lignocellulose. It also has applications in the food industry in the production of xylitol, which is added as a dental-caries preventing, low-calorie sweetener, and in the field of medicine, where xylitol has been suggested as a sugar substitute for diabetic patients. This is because xylitol, which has the same order of sweetness as fructose or sucrose (Hyvõnen *et al*. 1982), does not involve insulin synthesis during its breakdown (Emodi, 1978). Aldose reductases have been found in a variety of organisms from mammals, birds, amphibians, reptiles, fish and insects to yeasts and fungi. Among these, the XRs from yeasts and fungi are most involved in lignocellulose fermentation, while the mammalian enzymes are usually used in treatment of diabetic complications.

2.7.1 Yeast and fungal XR

The yeast and fungal enzymes are among the well-characterised XRs (reviewed by Lee 1998), although there are several unidentified or non-characterised enzymes in nature. Over the years, several novel XRs have been identified, characterised (Ditzelmuller *et al*. 1984; Ho *et al*. 1990; Billard *et al*. 1995; Bolen *et al*. 1996; Neuhauser *et al*. 1997; Panagiotou and Christakopoulos 2004), and cloned into yeasts for evaluation (Amore *et*

al. 1991; Takuma *et al*. 1991; Walfridsson *et al.* 1997; Handumrongkul *et al.* 1998; Hacker *et al.* 1999; Kang *et al.* 2003). Yeast XRs exhibited Michaelis-Menten kinetics with respect to both the substrate and the cofactor (Webb and Lee, 1991; 1992). Most of the yeast and fungal XRs identified and expressed so far were found to be predominantly dependent on NADPH (Rizzi *et al*. 1988; Amore *et al*. 1991; Billard *et al*. 1995; Yokoyama *et al*. 1995a; Panagiotou and Christakopoulos 2004), although some enzymes have been reported that were more NADH- than NADPH-specific, such as those of *Candida parapsilosis* (Neuhauser *et al*. 1997; Lee *et al*. 2003). This dual cofactor specificity is of particular importance in xylose fermentation by *S. cerevisiae* because it leads to a closed redox balance and therefore less glycerol and more ethanol formation. XR activity has been shown to be essential for xylose utilisation in yeasts and fungi. XR-deficient mutants of *Pa. tannophilus* (Schneider *et al*. 1989), *P. stipitis* (Hagedorn and Ciriacy 1989) and *K. lactis* (Billard *et al.* 1995) showed more reduced growth rates on xylose than their wild types.

2.7.2 Catalytically important residues

The majority of enzymes of the AKR family are monomeric, but most XRs function as non-cooperative, tightly associated dimers with a subunit molecular mass of 33 to 40 kDa (Rizzi *et al*. 1988; Yokoyama *et al.* 1995a). The enzymes from *P. stipitis, N. crassa,* and *C. tropicalis* are made up of two subunits (Rizzi *et al*. 1988; Yokoyama *et al*. 1995a; Rawat *et al*. 1996; Woodyer *et al*. 2005), while the enzyme from *S. cerevisiae* (Kuhn *et al*. 1995) is monomeric. While different XRs may differ in their structure, there are a number of conserved domains and amino acids, especially in the active site of the enzyme. Knowledge about the essential residues in the enzymes has been obtained through various methods. Webb and Lee (1991) used chemical modification by group-specific *ρ*-chloromercuriphenyl sulfonate and diethylpyrocarbonate to identify a cysteine and a histidine residue as being critical for enzyme binding. Zhang and Lee (1997) followed this study by individually mutating three cysteine residues to serine. The variants were functional but had lower catalytic activity, leading to the conclusion that the cysteine residues were not directly involved in cofactor binding. Rawat and Rao (1996) identified an essential tryptophan near the coenzyme-binding region of *N. crassa* XR using the chemical modifier, N-bromosuccinide. In a subsequent chemical modification study involving the lysine-specific modifier 2,4,6-trinitrobenzenesulfonate, the authors suggested the presence of an essential lysine in the catalytic site not involved in substrate or cofactor binding (Rawat *et al*. 1996). Apart from these chemical modification studies, site-directed mutagenesis has also helped in identification of essential residues. Kostrzynska *et al.* (1998) used a combined approach of chemical modification and site-directed mutagenesis to show that a lysine 270 was essential for the coenzyme binding of the XR from *P. stipitis*. Klimacek *et al.* (2001) performed site-directed mutagenesis of the lysine and tyrosine residues at the active site and proved that lysine 55 was not involved in enzymatic action, although lysine 80 and tyrosine 51 were involved in the catalytic action. The crystallographic structure of xylose/aldose

reductases from human (Khurana *et al*. 1998) and yeast (Hur and Wilson 2000; Kavanagh *et al*. 2002) sources has made it possible to gain more insight into the structure and function of XRs. It has been shown that, like most of the AKR family enzymes, XRs also possess the $(\alpha/\beta)_8$ TIM barrel structure and conserved active site residues.

2.7.3 Mechanism of catalytic action

Structural information has also revealed important details about the catalytic mechanism of XRs that involves a tetrad of amino acids (tyrosine, histidine, aspartic acid and lysine). Early studies based on initial velocity and product inhibition, using *P. stipitis* and *N. crassa* XRs, have shown the forward reaction towards xylitol to be an ordered bi-bi mechanism, with initial cofactor binding followed by binding of the substrate to the enzyme. Based on crystallographic evidence, a reaction mechanism for human aldose reductase was proposed and it has been thought to be the same for many XRs. The reaction begins with the protonation of the carbonyl group in the aldehyde substrate by an acid-base catalyst, followed by a stereospecific transfer of a hydride ion from the C4 of the nicotinamide ring in the coenzyme to the carbonyl group (Fig. 2.6). Whereas the active site pocket of human aldose reductase was found to be lined with several aromatic and non-polar residues (Urzhumstev *et al*. 1997), the corresponding region of the yeast XRs was not conserved. This probably led to the differences in cofactor specificities observed. However, among the polar residues seen in the active site pocket of human aldose reductase, tyrosine 49 (based on *P. stipitis* XR numbering) was found to donate the proton to the substrate, thereby facilitating the hydride transfer from the cofactor NADPH to the carbonyl carbon of the aldehyde substrate (Bohren *et al*. 1994). To facilitate proton transfer and lower the potentially higher p*K* of tyrosine 49, a spatially proximal lysine 78 forms a hydrogen bond through its ε-NH_2 group with the carboxyl group of tyrosine 49. Lysine 78 was itself stabilised through a salt bridge to aspartic acid 44 (Bohren *et al.* 1994). Histidine 111 was postulated to direct the orientation of substrate in the active site pocket (Bohren *et al*. 1994). Interestingly, it is proposed that the short-chain dehydrogenases/reductases (SDR) family of enzymes has a similar mechanism of action (Jörnwall *et al*. 1995).

The SDR family of enzymes is relevant in the case of XRs, because most of the known yeast XRs, although not the human and other XRs, possess the unique coenzyme-binding and catalytic motifs of this enzyme family. The coenzyme-binding SDR family motif – GXXXGXG – is found in almost all the known yeast XRs. The catalytic motif of the SDR enzymes, characterised by YXXXK residues, is also present twice in the yeast XRs, although its significance is not known. It should be noted, however, that the SDR motif in yeast XRs is present in addition to the coenzyme-binding and catalytic motifs of the AKR family enzymes. The coenzyme-binding motif of the AKRs is the IPKS motif with a conserved arginine five residues downstream (present between residues 268 and 271 in *P. stipitis* XR). Among these four residues, lysine 270 most likely binds to the 2'-phosphate of NADPH (Kostrzynska *et al*. 1998).

The arginine is thought to contribute to tighter binding (Kubiseski and Flynn 1995). The presence of both the IPKS and GXXXGXG coenzyme-binding motifs in yeast XRs raises the question of their relevance in xylose utilisation.

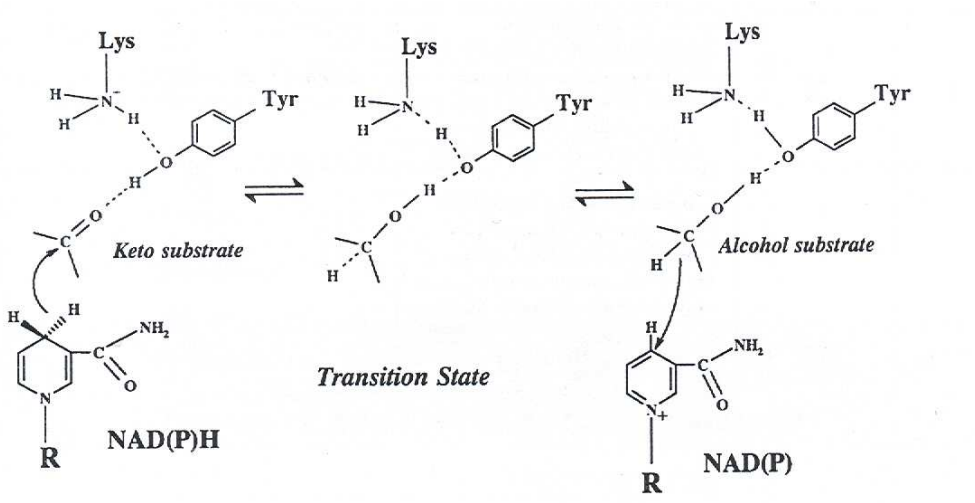

FIG. 2.6 Proposed reaction mechanism for human aldose reductase. Adapted from Jörnwall *et al*. (1995).

While Kostrzynska *et al*. (1998) showed that lysine 270 was essential for coenzyme binding, Chu and Lee (2006) used mutational studies to show that the GXXXGXG motif was not critical for XR activity in *S. cerevisiae*. Similarly, the role of tyrosine 49 in catalysis was proved by mutational studies (Jeong *et al*. 2001), where the Tyr49Phe mutant (*sic*) showed a 98% loss of activity. In a later study, the same authors showed that lysine 78 and not lysine 53 is the essential residue that H-bonds to tyrosine 49 (Jeong *et al*. 2002), thereby proving that the yeast XR functions in a similar way to that of other AKRs in spite of the presence of SDR motifs. This finding correlated with the findings of Klimacek *et al.* (2001). Thus, although the presence of SDR motifs in yeast XRs confers on them a unique position among the aldose reductase enzymes, these motifs have not been associated with any of the critical activities of the enzyme.

2.8 CONCLUSION

Lignocellulosic biomass is the major foreseeable alternative energy resource available in mankind's search for renewable energy sources. Because of its high energy content and lower cost, it is likely to become the primary raw material for production of ethanol, which will be used as a fuel to replace fossil fuels. Using ethanol as fuel provides several advantages, especially in reducing greenhouse gas emissions. However the industrial production of ethanol from lignocellulosic biomass is not yet cost effective because of the lack of microorganisms that can consume all the sugars present in lignocellulose hydrolysate. Xylose is the major sugar present in abundance, but the best-known industrial ethanol-producing organism, *S. cerevisiae*, is unable to utilise xylose. Thus, efforts have been made for more than two decades to engineer this yeast to utilise and ferment xylose.

Using recombinant DNA technology, the genes coding for the isomerisation of xylose into xylulose were cloned and expressed in *S. cerevisiae*. This included cloning of genes involved in the two-step process (Kötter *et al*. 1990) or the one-step conversion process (Kuyper *et al*. 2003). While both strategies have resulted in recombinant *S. cerevisiae* strains that are capable of ethanol production under anaerobic conditions, they have also aided in unravelling the metabolic regulations involved. The major limitations of anaerobic xylose fermentation to ethanol by *S. cerevisiae* were initially thought to be: (i) the redox imbalance created as a result of the cofactor dependencies of the two initial enzymes in the xylose pathway; (ii) the low flux through the PPP in general, but this is the only pathway for xylose metabolism in yeast; (iii) the energy limitations arising due to ATP depletion during anaerobic conditions; and (iv) xylose transport under low xylose conditions. Among these, the redox imbalance issue has been researched the most and solutions to this problem have been varied. Cloning and expression of the fungal xylose isomerase, altering the cofactor specificities of XR and XDH through metabolic engineering, and mutating the XR and XDH enzymes to show altered cofactor preferences were only a few of the solutions. Deleting and overexpressing enzymes not directly involved in xylose metabolism as such have also been used to address the redox imbalance issue. This has resulted in an increased xylose fermentation rate, as well as in altered product formation (less xylitol and more ethanol). The PPP flux has been increased through overexpression of the genes in the PPP and this has improved the fermentation characteristics of the yeast. Alternative pathways, such as the phosphoketolase pathway, have also been introduced. Apart from these solutions, random mutants and evolutionary mutants have been developed with xylose fermentative capabilities. These mutant strains have been globally analysed through the use of microarrays, but the analyses have not shown any one particular mutation that was directly involved in xylose fermentation. Rather, there were many system-wide changes in the cell, leading to the conclusion that the key to effective xylose fermentation in *S. cerevisiae* lies within the regulatory network of the cell and involves a number of genes and pathways. While the absolute understanding of xylose metabolism may be possible through more research, it is pleasing to note that there are now recombinant *S. cerevisiae* strains that show an efficient xylose consumption and fermentation rate. However, not all xylose-fermenting yeast strains have been tested on lignocellulose hydrolysates. This will be the ultimate test for their effectiveness.

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Chapter 3

RESEARCH RESULTS I

A constitutive catabolite repression mutant of a recombinant *Saccharomyces cerevisiae* strain improves xylose consumption during fermentation

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A constitutive catabolite repression mutant of a recombinant *Saccharomyces cerevisiae* strain improves xylose consumption during fermentation

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3.1 ABSTRACT

Efficient xylose utilisation by microorganisms is of importance to the lignocellulose fermentation industry. The aim of this work was to develop constitutive catabolite repression mutants in a xylose-utilising recombinant *Saccharomyces cerevisiae* strain and evaluate the differences in xylose consumption under fermentation conditions. *S. cerevisiae* YUSM was constitutively catabolite repressed through specific disruptions within the *MIG1* gene. The strains were grown aerobically in synthetic complete medium with xylose as the sole carbon source. Constitutive catabolite repressed strain YCR17 grew four-fold better on xylose in aerobic conditions than the control strain YUSM. Anaerobic batch fermentation in minimal medium with glucose-xylose mixtures and N-limited chemostat with varying sugar concentrations were performed. Sugar utilisation and metabolite production during fermentation were monitored. YCR17 exhibited a faster xylose consumption rate than YUSM under high glucose conditions in nitrogen-limited chemostat cultivations. This study shows that a constitutive catabolite repressed mutant could be used to enhance the xylose consumption rate even in the presence of high glucose in the fermentation medium. This could help in reducing fermentation time and cost in mixed sugar fermentation.

Key words: xylose fermentation, xylitol, *MIG1*, constitutive catabolite repression, *Saccharomyces cerevisiae*.

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3.2 INTRODUCTION

Xylose is the second major monosaccharide available in nature, constituting about 30% of plant biomass (Hayn *et al*., 1993; Lee, 1997). Many bacteria, yeast and filamentous fungi are capable of xylose utilisation (Chiang and Knight, 1960; Gong *et al*., 1983; Hahn-Hägerdal *et al*., 1993). *Saccharomyces cerevisiae*, the well-characterised industrial fermentation yeast, is unable to utilise xylose. It can, however, grow slowly on xylulose, which is an isomer of xylose, with a growth rate one-tenth lower than on glucose (Chiang *et al*., 1981; Senac and Hahn-Hägerdal, 1990). Availability of a xylose-fermenting *S*. *cerevisiae* is of importance to the fermentation industry that uses agricultural waste as raw material.

The inability of *S*. *cerevisiae* to grow on xylose is due to the lack of active enzymes that convert xylose to xylulose. In natural xylose-utilising fungi and yeasts this conversion is brought about by two enzymes – xylose reductase (XR) and xylitol dehydrogenase (XDH) (Chakravorty *et al*., 1962; Bruinenberg *et al*., 1983) – while in bacteria, a single enzyme, namely xylose isomerase, carries out the isomerisation step (Hochster and Watson, 1954). Xylulose is then metabolised through the Pentose Phosphate Pathway (PPP) into either biomass or other fermentation products. When the genes coding for the two enzymes XR and XDH from the natural, xylose-utilising yeast *Pichia stipitis* were introduced into *S*. *cerevisiae*, growth on xylose was observed, when xylulokinase (XK) was overexpressed. Since then, several attempts have been made to develop a *S*. *cerevisiae* strain capable of showing as efficient growth and yield on xylose as on glucose (reviewed by Hahn-Hägerdal *et al*., 2001; Jeffries and Jin, 2004).

Xylose, however, being a pentose sugar metabolised through the non-oxidative PPP, which by itself is a low-flux pathway under normal (glucose-grown) conditions (Fiaux *et al*., 2003), limits the efficacy of growth and yield on xylose. Recombinant *S*. *cerevisiae* strains, in which XR, XDH and XK encoding genes were expressed, have also been reported to be limited by: (*i*) lower affinity for xylose by *S*. *cerevisiae*'s hexose transporters (Kotyk, 1967; Busturia and Lagunas, 1986; Kötter and Ciriacy, 1993); (*ii*) its low activity pentose phosphate pathway (PPP) (Senac and Hahn-Hägerdal, 1990; Fiaux *et al*., 2003); (*iii*) the inability to oxidize reduced cofactors produced in the initial xylose metabolism (Bruinenberg *et al*., 1983); and (*iv*) reduced energy (ATP) recovery during xylose metabolism (Sonderegger *et al*., 2004). In order to improve xylose utilisation by the aforementioned recombinant *S*. *cerevisiae* strains, genetic and metabolic engineering approaches (Meinander and Hahn-Hägerdal, 1997; Jin *et al*., 2002; Johansson and Hahn-Hägerdal, 2002; Jeppsson *et al*., 2003; Verho *et al*., 2003) have been combined with a mutational approach (Wahlbom *et al*., 2003a) as well as evolutionary engineering (Sonderegger and Sauer, 2003), resulting in mutants that are able to grow on and ferment xylose. Genome-wide transcription analysis of a random mutant (Wahlbom *et al*., 2003b) and an evolutionary mutant (Sonderegger *et al*., 2004) showed the induction of genes involved in transport, redox metabolism, and the lower part of glycolysis.

It has been shown (Meinander and Hahn-Hägerdal, 1997) that xylose utilisation is facilitated by the presence of low amounts of glucose in the medium. Xylose utilisation was impeded either when glucose was depleted or when there was excess. It is difficult to determine the optimal

concentration of glucose that can aid efficient xylose utilisation, although a recent report by Öhgren *et al*. (2006) has shown xylose to be co-fermented at glucose levels below 4 g/l in a simultaneous saccharification and fermentation (SSF) process. In general, *S. cerevisiae* prefers glucose to most other sugars in the medium, and metabolises it efficiently. Thus, in the presence of glucose other sugar-metabolising pathways are inhibited, in a process known as carbon catabolite repression or, simply, glucose repression (reviewed by Gancedo, 1998). Therefore, in mixed sugar fermentation involving glucose, the utilisation of other sugars is delayed affecting the efficacy of the process.

We hypothesized that by mimicking a glucose-like environment in xylose-rich conditions, xylose utilisation could be improved. In order to prolong/mimic the glucose-like condition we created constitutive catabolite repression mutants through in-frame deletions within the *MIG1* gene. Östling *et al*. (1996) have shown that deleting one or either of the two regulatory domains in *MIG1* conferred constitutive repression to the strain. In such strain, the glycolytic genes remain induced irrespective of the presence of glucose in the medium. Since xylose shares transporter genes as well as several glycolytic pathway genes with glucose, this condition might prove conducive for improved xylose utilisation. Previously, Roca *et al*. (2004) studied the role of glucose catabolite repression on xylose fermentation through *MIG1* disruption and found a derepressed strain showing improved xylose consumption during mixed sugar fermentation. They also suggested that in a recombinant strain, xylose itself could be a repressive sugar. Belinchon and Gancedo (2003) also reported that xylose causes repression, suggesting that xylose might be recognized as a non-fermentable sugar.

Here we report on the effect of a constitutive glucose-repressed recombinant strain of *S. cerevisiae* on xylose utilisation and fermentation and show that constitutive repression improves xylose consumption even in the presence of high glucose concentration.

3.3 MATERIALS AND METHOD

3.3.1 Strains. *Saccharomyces cerevisiae* CEN.PK 2-1D was transformed with *XYL1*, *XYL2* genes from *Pichia stipitis*. This resulted in the strain YUSM 1001d (hereafter referred to as YUSM), which is the control strain used in this study. All strains derived from YUSM for this study are given in Table 3.1. Plasmids were cloned and amplified in the bacterial host *Escherichia coli* DH5α.

3.3.2 Recombinant DNA methods, plasmid construction and transformation. Standard procedures for isolation and manipulation of DNA were used throughout this study (Ausubel *et al*., 1995). Restriction enzymes (from Roche, Mannheim, Germany or Fermentas, Vilnius, Lithuania), T4 DNA-ligase and Expand Hi-Fidelity DNA polymerase (Roche) were used according to the specifications of the supplier. *Escherichia coli* was transformed as described by Inoue *et al*. (1990), while the lithium acetate method (Gietz *et al*., 1992) was used for yeast transformations.

The *MIG1* gene from the YUSM strain was isolated by the polymerase chain reaction (PCR) technique [using primers MIG1-F (CCCAAGCTTATGCAAAGCCCATATCC) and MIG1-R (GGAAGATCTTCAGTCCAT GTGTGGG)] as a 1533-bp fragment with flanking *Hin*dIII and *Bgl*II sites (underlined above) and cloned into the pGEM-T Easy® vector (Promega, Madison, WI, USA). Removal of a 337-bp fragment between the *Cla*I and *Nhe*I sites resulted in plasmid pGEM-MIG1Δ5. Similarly, removal of a 347-bp fragment between the *Swa*I and *Nhe*I sites resulted in plasmid pGEM-MIG1Δ17 (Östling *et al*., 1996). *Hin*dIII/*Bgl*II digests were used to excise the *MIG1* (1533 bp), *MIG1*Δ5 (1200 bp) and *MIG1*Δ17 (1186 bp) fragments from these respective pGEM constructs. The *MIG1 Hin*dIII/*Bgl*II fragments were blunt-ended by using the Klenow component of polymerase I (Ausubel *et al*., 1995) and inserted into the blunt-ended *Eco*RI site of plasmid pSTAH (Table 3.1). This resulted in plasmids pHMIG1, pHMIGΔ5 and pHMIGΔ17, respectively, with *MIG1* and its disruptants being under control of the yeast phosphoglycerate kinase I gene (*PGK1*) promoter ($PGK1_p$) and terminator ($PGK1_t$) sequences.

TABLE 3.1 Yeast strains and plasmids used in this study

Name	Description
Strain	
YUSM	CEN.PK 2-1D *XYL1-2::URA3*
YDM1	YUSM *Δmig1*::*KanMX4*
YCR5	YUSM *Δmig1* pHMIGΔ5::*HIS3*
YCR17	YUSM *Δmig1* pHMIGΔ17::*HIS3*
YOM	YUSM *Δmig1* pHMIG1::*HIS3*
Plasmid	
pSTAH	YIp5-*Δura3*::*HIS3*:*PGK1*p-*PGK1*t
pHMIG1	YIp5- *Δura3*::*HIS3*:*PGK1*p-*MIG1*-*PGK1*t
pHMIG1Δ5	YIp5- *Δura3*::*HIS3*:*PGK1*p-*MIG1*Δ5-*PGK1*t
pHMIG1Δ17	YIp5- *Δura3*::*HIS3*:*PGK1*p-*MIG1*Δ17-*PGK1*t

The *MIG1* deletion cassette from the *S*. *cerevisiae* deletion library strain (BY4742 Δ*mig1*) was obtained using PCR with MIG1-F and MIG1-R primers. This Δ*mig1* cassette was used to delete *MIG1* in YUSM by homologous recombination, thus creating strain YDM1. In order to develop the constitutive catabolite repression strains, *Nsi*I-digested plasmids pHMIG1, pHMIGΔ5 and pHMIGΔ17 were integrated at the *HIS3* locus of YDM1. The resultant strains were named YOM, YCR5 and YCR17, respectively. All gene integrations and deletions were verified by Southern blots (data not shown).

3.3.3 Media and growth conditions. Bacteria were grown in Luria-Bertani (LB) medium (Ausubel *et al*., 1995) at 37 °C. Ampicillin-resistant (Ap^R) *E*. *coli* transformants were selected on LB medium containing 100 mg/l ampicillin. Yeast strains were cultivated at 30 °C in either a rich medium, YPD (containing in w/v: 1% yeast extract, 2% peptone and 2% glucose), or a synthetic

complete medium, SC [containing 2% w/v glucose, 0.67% w/v yeast nitrogen base without amino acids (Difco, Detroit, MI, USA), supplemented with essential amino acids (Sherman *et al*., 1983)]. Deletion strains, in which the *MIG1* gene was disrupted, were grown in YPD medium containing 200 mg/l geneticin (G418; Sigma-Aldrich, St. Louis, MO, USA). Solid media contained 20 g/l agar. Yeast strains were stored in rich medium as 15% (v/v) glycerol stocks at –80 °C, while bacterial cultures were stored in LB medium as 40% (v/v) glycerol stocks.

For batch fermentation, a defined mineral medium (Verduyn *et al*., 1992), containing 20 g/l glucose and 50 g/l xylose, was used. Amino acids were added as required for strain auxotrophy (40 mg/l histidine, 40 mg/l tryptophan and 240 mg/l leucine), while ergosterol and Tween 80 were dissolved in boiling 96% (v/v) ethanol to final concentrations of 0.01 and 0.42 g/l, respectively and added to anaerobic cultures. For N-limitation, 5 g/l ammonium sulphate (Verduyn *et al*., 1992) was reduced to 0.6 g/l (Klein *et al*., 1998). For aerobic growth, a single colony was inoculated in appropriate auxotrophic selective media and incubated at 30 °C in a rotary wheel. From exponential-phase cultures, about 10^5 cells/ml were inoculated into 50 ml SC medium and incubated at 30 °C and 200 rpm. Growth was followed as optical density measurements at 600 nm (OD_{600}) at regular intervals, using a UV-Vis spectrophotometer (UV-1601, Shimadzu, Japan).

3.3.4 Fermentation. Inocula for batch fermentation were prepared by inoculating single colonies into a 1-litre baffled shake-flask containing 100 ml defined mineral medium, at 30 °C for 15 h, in an orbital shaker at 150 rpm. Cells were harvested by centrifugation (5000 *x g*, 4 °C, 5 min) and re-suspended in sterile water before inoculating into the fermentation medium to an OD_{600} of 0.25. Fermentation was performed in a BIOFLO III (New Brunswick, USA) bioreactor with a working volume of 1 litre, at 30 °C and 200 rpm. Nitrogen gas containing < 5 ppm O_2 (ADR class 2 1A; AGA, Sweden) was passed through the column at a flow rate of 0.2 litre/min, as measured by a gas-flow regulator (Bronkhorst, The Netherlands). The pH of the medium was automatically maintained at 5.5 ± 0.2 by the addition of 3 M potassium hydroxide. Antifoam (Dow Corning, Midland, MI, USA) was added at 0.5% v/v. Batch fermentation was carried out with 20 g/l glucose and 50 g/l xylose in defined mineral medium. Samples taken at regular time intervals were immediately filtered through a 0.22-μm filter (Advantec MFS, Irvine, CA, USA) and stored at –20 °C until further analysis. N-limited chemostat cultivation was started in batch mode, with 20 g/l glucose as the sole carbon source. When glucose was close to depletion, as observed by OD_{600} and CO_2 measurements, fresh N-limited defined mineral medium was fed into the fermenter at a dilution rate (D) of 0.1 h^{-1}. The volume was kept constant at 1 litre by continuous removal of medium from the fermenter using norprene tubing and peristaltic pumps (Masterflex, Cole-Parmer Instruments, Chicago, USA). For each strain three different feed conditions were employed: (*i*) G20X10 – containing 20 g/l glucose and 10 g/l xylose; (*ii*) G10X10 – containing 10 g/l glucose and 10 g/l xylose; and (*iii*) G02X10 – containing 2 g/l glucose and 10 g/l xylose. Steady-state conditions were achieved when the biomass and CO_2 levels remained stable for at least three consecutive volume changes (differing by < 2%). Chemostat cultivation was performed in duplicate for all strains. Samples taken during steady state conditions were

immediately filtered through a 0.22-µm filter (Advantec MFS, Irvine, CA, USA) and stored at –20 °C until further analysis.

3.3.5 Analyses of sugars and fermentation products. Substrates consumed and metabolites formed were analysed using a Waters HPLC system (Waters Corporation, Milford, MA, USA) equipped with an Aminex HPX-87H column (Bio-Rad laboratories, Richmond, VA, USA), connected to a refractive index detector (RID-6A, Shimadzu) and an UV detector (Waters-2487; Waters Corporation) in series. A mobile phase of 5 mM H_2SO_4 at a flow rate of 0.6 ml/min and a column temperature of 45 °C was used to analyse glucose, xylose, xylitol, glycerol, acetate, and ethanol. The measured ethanol values were then corrected by using redox balance equations (Roels, 1983). Off-gas analysis during fermentation was continuously monitored by a CO_2 and O_2 monitor type 1308 (Brüel & Kjær, Nærum, Denmark), using photo acoustic and magneto acoustic detection for CO_2 and O_2 respectively (Christensen *et al*., 1995). Cell dry weight was determined by filtering a known volume of culture broth through a pre-dried (350 W for 4 min in a microwave oven), pre-weighed Supor membrane of 0.45-µm (Gelman Sciences, Ann Arbor, MI, USA). The filter was weighed again after being washed with three volumes of distilled water and dried in a microwave oven at 350 W for 8 min. The difference in the filter weight was used to calculate the dry weight in accordance with the absorbency measured. Dry weights were determined in duplicates. Trehalose and glycogen were assayed as described by Parrou and François (1997), using trehalase and amyloglucosidase (Sigma-Aldrich), respectively.

3.4 RESULTS AND DISCUSSION

3.4.1 Development and evaluation of MIG1 mutants

Improving xylose utilisation is of importance in mixed-sugar fermentation where the presence of easily fermentable sugars could cause catabolite repression. Since glucose (the most common sugar present in fermentation) and xylose share the same transporters in *S*. *cerevisiae* (Hamacher *et al*., 2002), an attempt was made to mimic and prolong a glucose-like environment in a xylose background. In the YUSM recombinant *S*. *cerevisiae* strain overexpressing the *P*. *stipitis* xylose reductase and xylitol dehydrogenase genes (Table 3.1) constitutive catabolite repressed *MIG1* disruptants MIG1Δ5, MIG1Δ17 were created according to Östling *et al*. (1996). The strains YCR17 and YCR5, when evaluated for growth in a 20 g/l maltose-containing medium as a means of verifying constitutive catabolite repression, grew comparably slower than YDM1. When the medium contained 10 g/l glucose and 10 g/l maltose, however, YCR17 initially grew more slowly than the other strains but later followed a pattern similar to that of YUSM (data not shown). When the strains were grown in a medium containing 20 g/l xylose as the sole carbon source, YCR17 showed the highest maximum growth rate (0.005 h^{-1}) compared to the control YUSM (0.001 h^{-1}), closely followed by YCR5 and YOM (*PGK1*$_P$*-MIG1- PGK1*$_t$)(Fig. 3.1). YDM1 (growth rate 0.002 h^{-1}) grew two-fold better than the control. Based on growth pattern in xylose medium, three groups of strains emerged – those that were similar to that of (*i*) YCR17,

(*ii*) YDM1 and (*iii*) YUSM. Therefore, the xylose fermentation performance of YCR17 and YDM1 compared to YUSM was further investigated under anaerobic batch fermentation.

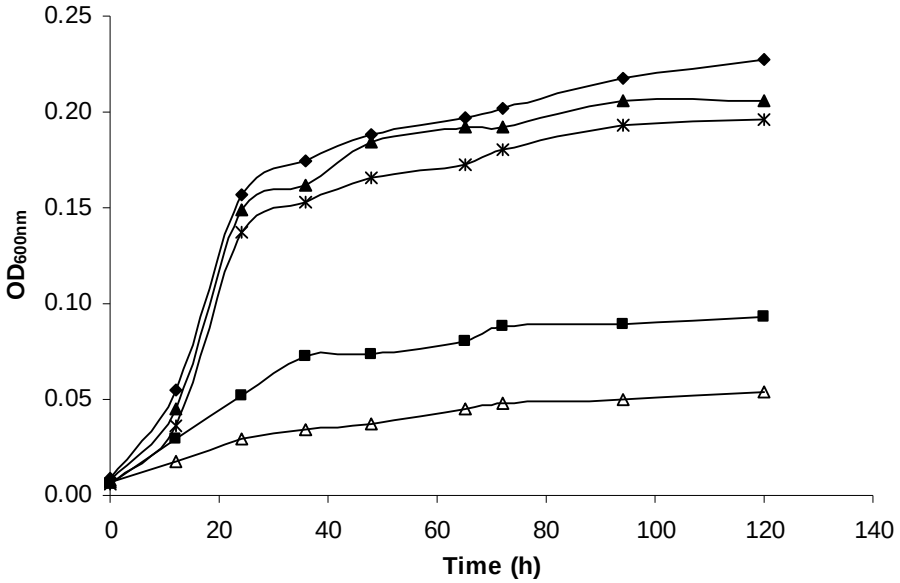

FIG. 3.1 Aerobic growth profile of various strains in SC medium with 20 g/l xylose as the sole carbon source, in shake-flask cultures at 30 ºC at 200 rpm. Strains profiled are: YUSM (△), YDM1 (*Δmig1*) (■), YCR5 (▲), YCR17 (◆), YOM (✳). From these, YUSM, YDM1 and YCR17 were selected for fermentation.

Previous reports have shown that xylose uptake and utilisation is enhanced by/in the presence of low/optimal amounts of glucose, although the exact amounts are difficult to determine (Kötter and Ciriacy, 1993; Eliasson *et al*., 2000). Meinander and Hahn-Hägerdal (1997) have shown that high concentrations of glucose in the fermentation medium possibly saturate the transport machinery, resulting in very little xylose transport, so that efficient utilisation of xylose occurs only when low glucose levels are present in the medium. Under aerobic conditions a constitutive catabolite repression mutant may maintain the hexose transporters in the active state due to the 'glucose/repression signal', leading to more sugar – in this case, xylose – being transported and consumed by the cell. This was reflected by the fourfold increase in biomass production observed for YCR17 compared to the control strain YUSM.

3.4.2 Batch cultivation

Anaerobic batch cultivation with 20 g/l glucose and 50 g/l xylose was compared for YUSM, YDM1 and YCR17, to evaluate their xylose fermentation capacities. All three strains fermented glucose within 24 h, while only about one-fifth of the xylose was consumed after 100 h, which could be attributed to the expression of mainly low-affinity transporters during batch cultivations (Roca *et al*., 2004). Xylose is predominantly transported into the cells *via* the high-affinity

transporters (Hamacher *et al*., 2002; Gardonyi *et al*., 2003). Roca *et al*. (2004) used a high amount of xylose to overcome the poor affinity and uptake rate of xylose in *S*. *cerevisiae* (Kötter and Ciriacy, 1993), but xylose consumption by our strains was still comparably low. Xylose consumption was faster in the presence of low glucose in the medium, which is in agreement with the findings of previous studies (Meinander and Hahn-Hägerdal, 1997; Lee *et al*., 2002).

TABLE 3.2 Xylose concentrations and metabolite yields measured from anaerobic batch fermentation in mineral medium with 20 g/l glucose and 50 g/l xylose, for the three strains tested (values represented are averages of two individual fermentation)

Strain	Xylose levels (g/l)		Yield from total sugars (g/g)					
	Initial	Consumed	$Y_{S,X}$*	$Y_{S,Xol}$ †	$Y_{S,Gly}$ ‡	$Y_{S,Ace}$ §	$Y_{S,E}$**	$Y_{X,Xol}$ ††
YUSM	48.6 ± 0.3	12.6 ± 0.3	0.09 ± 0.003	0.37 ± 0.001	0.06 ± 0.003	0.05 ± 0.005	0.19 ± 0.002	0.95 ± 0.01
YDM1	48.3 ± 0.3	9.2 ± 2.0	0.10 ± 0.012	0.35 ± 0.05	0.07 ± 0.02	0.05 ± 0.002	0.17 ± 0.02	0.99 ± 0.03
YCR17	48.0 ± 0.7	13.5 ± 2.0	0.08 ± 0.0	0.38 ± 0.01	0.05 ± 0.004	0.05 ± 0.001	0.19 ± 0.009	0.96 ± 0.07

*Biomass yield, † xylitol yield, ‡ glycerol yield, § acetate yield, ** ethanol yield †† xylitol yield from only xylose

Glucose was totally consumed within 24 h from the start of fermentation for all the three strains. Fermentation was carried out for at least 100 h.

Once glucose was depleted, very little xylose was consumed by all three strains. At the end of batch fermentation YCR17 had consumed 8% more xylose (13.5 g/l) than the control strain YUSM (12.6 g/l) and 45% more than the mig1–deleted strain YDM1 (9.2 g/l). Xylose was mainly converted to xylitol, with the xylose to xylitol conversion being more than 90% (Table 3.2). Glycerol and acetate yields ranged from 0.05 to 0.07 g/g. Ethanol production increased rapidly during the initial stages of fermentation (up to 24 h). Once glucose was consumed, no increase in ethanol production was observed. In terms of biomass yields, the YDM1 strain had a slightly higher yield than YCR17 and YUSM (see Table 3.2). Although the three strains performed similarly under batch fermentation, there were slight differences in sugar consumption, so that the glucose consumption rate was slower for strain YDM1. But no major differences could be substantiated in batch fermentation.

3.4.3 Nitrogen-limited chemostat cultivation

N-limited chemostat cultivation, in which the growth rate of a culture is limited only by the amount of nitrogen source in the medium, was employed under anaerobic conditions to determine the effect of constitutive catabolite repression on xylose fermentation. Previously Roca *et al*. (2004) had used C-limited chemostat cultivation to study the effect of *mig1* and *mig1mig2* mutants on anaerobic xylose fermentation. In our case, N-limited chemostat cultivation was used as it provided the advantage of possibility to keep the residual concentrations of two sugars at distinct levels (Klein *et al*., 1998), which can help in substantiating the effect, constitutive catabolite repression, has, during anaerobic xylose fermentation. The glucose concentration, varied from 20 g/l (feed G20X10) and 10 g/l (feed G10X10) to 2 g/l (feed G2X10), with a constant xylose concentration of 10 g/l. The residual sugar concentration in all feed conditions was more than 5 g/l, most of which was xylose. In fact,

all three strains consumed glucose to completion under feed condition G2X10. For all strains the rate of glucose consumption decreased with decreasing glucose concentration in the feed (Fig. 3.2). YCR17 exhibited its highest xylose consumption rate, 0.21 g/g/h, with the G20X10 feed, but no difference with the other two feeds (Fig. 3.2).

TABLE 3.3 Sugar consumption and yield values from N-limited anaerobic chemostat cultivations performed with the three strains (values are averages of duplicate experiments with SD ≤ 10% A Students *t*-test was performed to determine the statistical significance at 95% confidence level)

Feed	Strains	Feed Sugar (g/l)		Xylose consumed (g/l)	Yield on total sugars (g/g)						$Y_{X, Xol}$ †
		Glucose	Xylose		Xylitol	Glycerol	Acetate	Biomass	CO_2	Ethanol	
G20X10*	YUSM	20.4	10.5	3.1	0.08	0.01	0.01	0.08	0.45	0.45	0.61
	YDM1	19.8	9.9	2.3	0.09	0.08	0.01	0.09	0.42	0.40	0.80
	YCR17	20.7	9.9	4.3	0.13	0.04	0.01	0.08	0.42	0.41	0.76
G10X10*	YUSM	10.2	9.9	1.7	0.10	0.02	0.01	0.12	0.46	0.44	0.74
	YDM1	9.9	10.2	2.3	0.16	0.04	0.01	0.12	0.43	0.40	0.84
	YCR17	10.5	10.5	2.7	0.16	0.03	0.02	0.17	0.42	0.40	0.79
G2X10*	YUSM	1.8	9.3	0.6	0.11	0.05	0.02	0.16	0.44	0.43	0.48
	YDM1	2.1	9.6	0.9	0.11	0.03	0.04	0.22	0.48	0.39	0.38
	YCR17	2.3	10.2	0.6	0.13	0.04	0.01	0.15	0.53	0.41	0.71

* G20X10 – feed with 20 g/l glucose; G10X10 – feed with 10 g/l glucose; G2X10 – feed with 2 g/l glucose; 10 g/l xylose added to all the three feeds.

† Xylitol yield on xylose only (g/g).

Similarly, the control strain displayed its highest xylose consumption rate 0.16 g/g/h in the G20X10 feed. YDM1, consumed xylose with the same rate (0.13-0.14 g/g/h) in all the three feed conditions. The amount of xylose consumed decreased with decreasing amount of glucose in the feed. With feed G20X10, YCR17 consumed more than 40% of the xylose in the medium (Table 3.3). This was two times more than that consumed by YDM1 and about 10% more than that consumed by YUSM. However, with feed G10X10, the pattern changed; YCR17 still consumed most xylose followed by YDM1 and YUSM, in decreasing order. With feed G2X10, YDM1 was more effective in xylose utilisation even though only 10% xylose was consumed in total. Thus, YCR17 showed increased xylose consumption in presence of high glucose, while xylose consumption was similar to that of the control strain YUSM when the glucose level was lower than the xylose level. Strain YDM1 showed increased xylose consumption when there was less glucose in the medium, as has also been observed by Roca *et al*. (2004).

As was observed in batch fermentation more than 75% of xylose was converted to xylitol (Table 3.3). Glycerol and acetate yields ranged from 0.01 to 0.08 g/g. Ethanol yields on consumed sugars varied between 0.39 and 0.45 g/g (Table 3.3). Biomass levels varied with feed conditions (Fig. 3.3). For the control strain biomass decreased with decreasing glucose concentration in the feed, a trend followed by the mutant strains. However, YCR17 displayed

higher biomass levels when glucose was high/equal in feeds G20X10 and G10X10 than the other two strains. In fact, strain YCR17 produced most biomass when there were equal amounts of glucose and xylose in the feed, which was slightly more than produced by the control strain. At the lowest glucose level, feed G2X10, YDM1 produced most biomass, while that of the other two strains was similar.

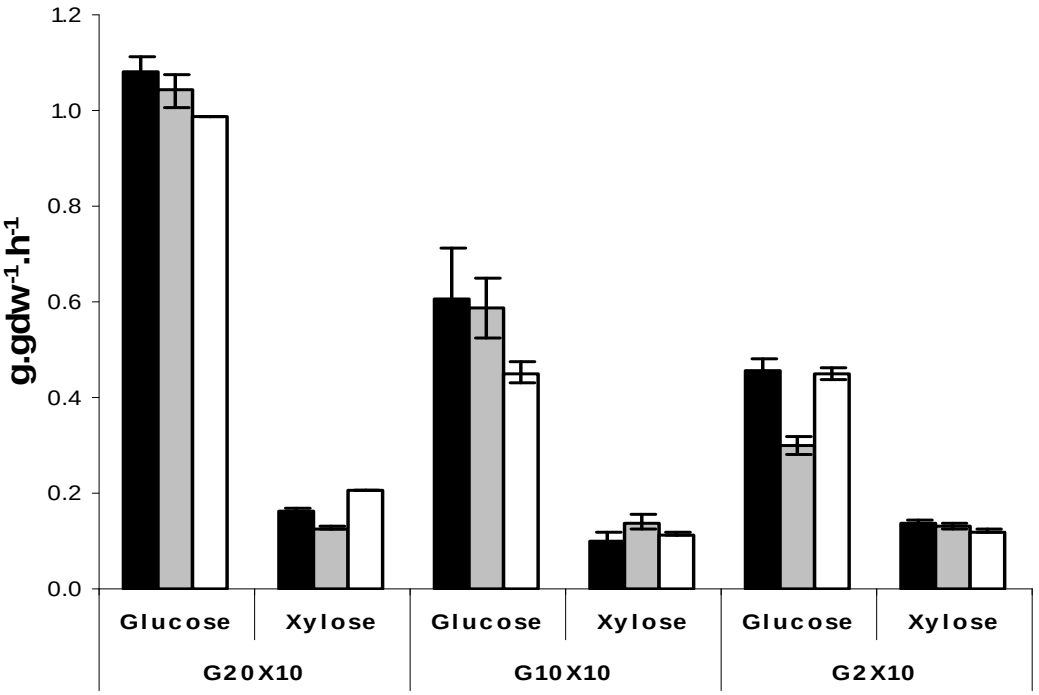

FIG. 3.2 Sugar consumption rates of the strains under N-limited chemostat conditions in mineral medium with 10 g/l xylose and 20 g/l glucose (G20X10), 10 g/l glucose (G10X10), and 2 g/l glucose (G2X10). Values, expressed as g/g.dw/h, are averages of two individual trials (the standard deviations are indicated by error bars). YUSM (black bars), YDM1 (grey bars) and YCR17 (white bars).

Our results emphasise the glucose-dependence of xylose consumption and metabolism under anaerobic conditions (Meinander *et al*., 1999; Eliasson *et al*., 2000). Although xylose transport is less efficient than that of glucose in *S. cerevisiae* (Kotyk, 1967; Busturia and Lagunas, 1986), there is evidence that it is not the main limitation for xylose utilisation under anaerobic conditions (Kötter and Ciriacy, 1993). The major problem is rather the metabolic flux through the pentose phosphate pathway (Fiaux *et al*., 2003; Johansson and Hahn-Hägerdal, 2002; Wahlbom *et al*., 2001; Karhumaa et *al*., 2005) as well as the regeneration of reduced co-factors under anaerobic conditions (Bruinenberg *et al*., 1983), which result in xylitol rather than ethanol production. In the YUSM strain background, about 70-90% of xylose consumed in both batch fermentation and chemostat was converted into xylitol. This could also suggest a deficiency in the xylulokinase (XK) activity in this strain (Ho *et al*., 1998; Richard *et al*., 2000; Johansson *et al*., 2001; Toivari *et al*., 2001; Jin *et al*., 2003). The xylulokinase gene *XKS1* was

not over-expressed in our strains, and it would be of interest to study the effect of constitutive catabolite repression in such a strain (Eliasson *et al*., 2000; Karhumaa *et al*., 2006).

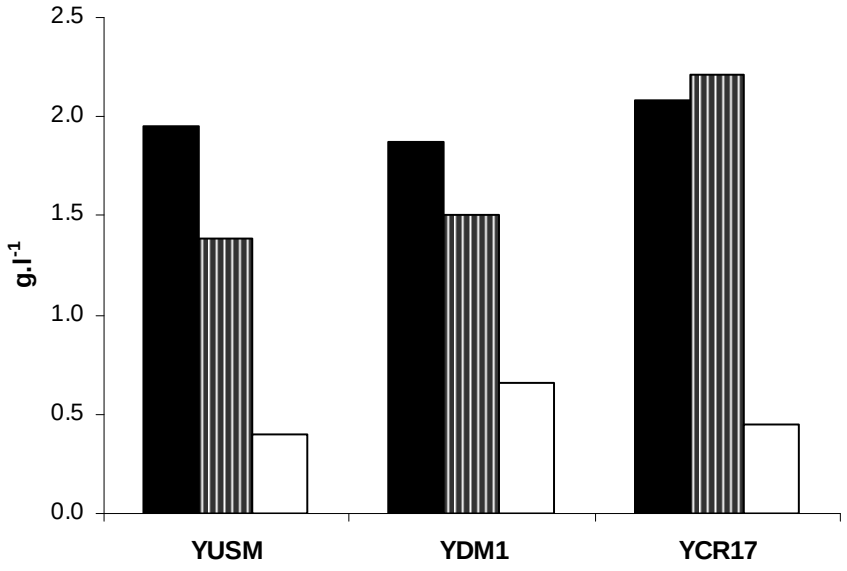

FIG. 3.3 Biomass produced during anaerobic N-limited chemostat cultivations with three different feeds – G20X10 (black bars), G10X10 (striped bars), G2X10 (white bars). Values represented are average of duplicate trials (SD ≤ 5%)

Biomass production varied between strains and feed conditions in N-limited chemostat. The fact that YCR17 had the highest biomass levels in feeds G20X10 and G10X10 shows that continued repression increases the co-utilisation of glucose and xylose. Nevertheless, the YDM1 strain produced more biomass at low glucose concentration. *MIG1* regulates the high-affinity glucose sensor *SNF3*, which detects low glucose conditions (Kaniak *et al*., 2004). Therefore the increased biomass formation by a *mig1* mutant could suggest the action of *SNF3* at low glucose concentration, which would increase the glucose uptake. Since xylose does not support anaerobic growth (Eliasson *et al*., 2000) there was a significant decrease in biomass production for all three strains with feed G2X10 (low glucose). Energy deficiency and/or ATP depletion has been observed at low dilution rates in N-limited chemostat cultures (Lidèn *et al*., 1995). With low glucose concentration (2 g/l) and xylose being close to a non-fermentable sugar unable to account for anabolic cellular activities, an overall cellular starvation response may result (Thomsson *et al*., 2005). Under such conditions yeast cultures may produce storage carbohydrates such as trehalose and glycogen (Lillie and Pringle, 1980; Panek, 1991; Rose and Vijayalakshmi, 1993). However, neither storage carbohydrate accumulated in the strains investigated (data not shown).

In conclusion the constitutive catabolite repression approach indicated that xylose consumption and metabolism could be considerably increased. This approach will further be evaluated in improved xylose-fermenting strains (Eliasson *et al*., 2000; Wahlbom *et al*., 2003a; Sonderegger *et al*., 2003; Karhumaa *et al*., 2006). Such strains produce more ethanol at a

faster rate and a fine-tuned constitutive catabolite repression could lead to faster xylose consumption in the presence of other sugars.

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Chapter 4

RESEARCH RESULTS II

Molecular cloning and functional expression of a novel *Neurospora crassa* xylose reductase in *Saccharomyces cerevisiae* in the development of a xylose fermenting strain

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Molecular cloning and functional expression of a novel *Neurospora crassa* xylose reductase in *Saccharomyces cerevisiae* in the development of a xylose fermenting strain

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4.1 ABSTRACT

The development of a xylose-fermenting *Saccharomyces cerevisiae* yeast would be of great benefit to the bioethanol industry. The conversion of xylose to ethanol involves a cascade of enzymatic reactions and processes. Xylose (aldose) reductases catalyse the conversion of xylose to xylitol. The aim of this study was to clone, characterise and express a cDNA copy of a novel aldose reductase (*NCAR-X*) from the filamentous fungus *Neurospora crassa* in *S. cerevisiae*. *NCAR-X* harbours an open reading frame (ORF) of 900 nucleotides. This ORF encodes a protein (NCAR-X, assigned NCBI protein accession ID: XP_956921) consisting of 300 amino acids, with a predicted molecular weight of 34 kDa. The *NCAR-X*-encoded aldose reductase showed significant homology to the xylose reductases of *Candida tenuis* and *Pichia stipitis*. When *NCAR-X* was expressed under the control of phosphoglycerate kinase I gene (*PGK1*) regulatory sequences in *S. cerevisiae*, its expression resulted in the production of biologically active xylose reductase. Small-scale oxygen-limited xylose fermentation using recombinant strains harbouring *NCAR-X* gene resulted in the production of less xylitol and at least 15% more ethanol, different to that of *PsXYL1-2*-containing strains. The *NCAR-X*-encoded enzyme produced by *S*. *cerevisiae* was NADPH-dependent and no activity was observed in the presence of NADH. The co-expression of the *NCAR-X* gene with the *P*. *stipitis* xylose reductase gene (*PsXYL1*) in *S. cerevisiae* constituted an important part of an extensive research program aimed at the development of xylolytic yeast strains capable of producing ethanol from plant biomass.

Key words: *Neurospora crassa*, *Saccharomyces cerevisiae*, xylose fermentation, xylose reductases.

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4.2 INTRODUCTION

D-Xylose reductase is a member of the well-characterised family of aldose reductases (aldo-keto reductases; EC 1.1.1.21). These enzymes catalyse the reversible reduction of aldehydes and ketones to their corresponding alcohols and are NAD(P)H dependent. These enzymes are ubiquitous among eukaryotes and have been reported in mammals, birds, amphibians, reptiles, fish, insects, yeasts and filamentous fungi. Research on aldose reductases has been focused mainly on two distinct, sometimes overlapping, levels: (i) mammalian aldose reductases, which have been implicated in many diabetic complications; and (ii) yeast and fungal aldose reductases, which have been essential in the conversion of agricultural waste to ethanol in the renewable fuel industry. In industries using fermentation processes, the yeast and fungal xylose (aldose) reductases have applications in the production of xylitol, a dental-caries preventing agent and low-calorie sweetener that can act as an insulin-independent sugar substitute for diabetics (Emodi, 1978).

There is huge potential for the conversion of lignocellulose waste to ethanol for use as a renewable energy source, but this has been limited by the inability of known microorganisms to utilise the pentose fraction of hemicellulose. This has led to studies that have focussed on finding novel xylose (aldose) reductases (Ditzelmuller *et al*., 1984; Ho *et al*., 1990; Billard *et al*., 1995; Bolen *et al*., 1996; Neuhauser *et al*., 1997; Panagiotou and Christakopoulos 2004), and expressing them in well-characterised yeast hosts such as *Saccharomyces cerevisiae* and *Pichia pastoris* (Amore *et al*., 1991; Takuma *et al*., 1991; Walfridsson *et al*., 1997; Handumrongkul *et al*., 1998; Hacker *et al*., 1999; Kang *et al*., 2003). Conversion of lignocellulose, which contains between 16% and 30% xylose, to ethanol involves a cascade of processes. Firstly D-xylose reductase (XR) converts xylose into xylitol. This is oxidised to xylulose by xylitol dehydrogenase (XDH), which is phosphorylated to xylulose-5-phosphate by a xylulokinase (XK). The xylulose-5-phosphate enters the pentose phosphate pathway leading to the production of secondary metabolites such as ethanol and glycerol. Most of the yeast and fungal aldose reductases (ARs) that have been identified and expressed up to now are predominantly dependent on NADPH (Rizzi *et al*., 1988; Amore *et al*., 1991; Billard *et al*., 1995; Yokoyama *et al*., 1995a; Panagiotou and Christakopoulos 2004). However, some enzymes that are more specific to NADH than NADPH, such as that of *C*. *parapsilosis* have been reported (Lee *et al*., 2003).

The filamentous fungi play a pivotal role in the decomposition of complex organic matter in nature. *Neurospora crassa* is one such fungus reported to grow on cellulose and hemicellulosic biomass and produce ethanol (Rao *et al*., 1983; Deshpande *et al*., 1986), although it cannot grow under complete anaerobic conditions. D-Xylose metabolising enzymes have been reported to be present in this organism (Rawat *et al*., 1993; Rawat and Rao, 1996), although no xylose reductase gene had been characterised until recently (Woodyer *et al*., 2005). With the availability of the *N*. *crassa*

genome database, Woodyer *et al*. (2005) identified and isolated a xylose reductase gene (Entrez Protein Accession number: AAW66609) using a homology search with other known xylose reductases.

Filamentous fungi have been shown to possess several isozymes in one species (Yokoyama *et al*., 1995b; Mayr *et al*., 2000; Nidetzky *et al*., 2003). Knowledge of as many isozymes as possible would help in understanding their function and could lead to novel characterisations.

This study forms an integral part of a comprehensive research program aimed at the development of an efficient xylose-fermenting *S. cerevisiae* strain that could potentially be used in the conversion of plant biomass to bioethanol. The specific aim of this study was to clone and characterise a second aldose reductase gene (*NCAR-X*) from *N*. *crassa*, and to express it on its own in a laboratory strain of *S. cerevisiae*, as well as in recombinant *S. cerevisiae* strains containing the xylose reductase (*XYL1*) and xylose dehydrogenase (*XYL2*) genes of *P. stipitis*. These *S. cerevisiae* transformants were compared under aerobic and oxygen-limited conditions.

4.3 MATERIALS AND METHOD

4.3.1 Strains and plasmids. All strains and plasmids used in this study are listed in Table 4.1. The aldose reductase genes were cloned in *S. cerevisiae* CEN.PK 2-1D (Entian and Kötter, 1998; van Dijken *et al*., 2000). All yeast strains were grown either in rich YPD medium (10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose) or selective minimal media (SC) [20 g/l glucose, 6.7 g/l yeast nitrogen base without amino acids (Difco, Detroit, MI, USA) supplemented with essential amino acids for auxotrophic selection (40 mg/l histidine, 40 mg/l tryptophan and 240 mg/l leucine)] (Sherman *et al*., 1983). For screening *N*. *crassa* cDNA library transformants, SCX medium (the same as SC, but glucose replaced with 20 g/l xylose) was used. For plasmid DNA amplification and cloning, *Escherichia coli* DH5α was used as the host. Bacterial transformants were grown in Luria-Bertani (LB) medium containing 100 mg/l of ampicillin (Ap) to maintain selection pressure in the transformants (Ausubel *et al*., 1995). Solid media contained 20 g/l agar. The yeast and bacterial strains were stored as glycerol stocks at -80℃, and grown at 30℃ and 37℃, respectively

4.3.2 Screening of the *Neurospora crassa* cDNA library. A fast-growing, xyloseutilising recombinant *S*. *cerevisiae* strain Y-X (Table 4.1; Institute for Wine Biotechnology, Stellenbosch University, Stellenbosch, South Africa) was transformed with a *N*. *crassa* cDNA yeast expression library (Swiegers *et al*., 2002). Screening was done on SCX plates and three colonies growing faster than the parent were isolated (named Y-XNc). In liquid SCX medium the Y-XNc strains exhibited a growth rate of 0.06 h^{-1} compared to the 0.04 h^{-1} growth rate of Y-X. Restriction digest analysis, using *Eco*RI and *Xho*I, of the plasmids from the screened strains revealed the presence of a 1-kb DNA fragment in each of the transformed strains. This DNA fragment was gel-isolated, cloned in pGEM-T Easy® vector (Promega, Madison, WI, USA) and sequenced. Cycle

sequencing was done using the BigDye™ Terminator v3.1 sequencing kit (Applied Biosystems, Foster City, CA, USA) and reactions were analysed in an ABI3100 (Applied Biosystems) automated sequencer. Analysis of the sequence revealed the presence of an ORF, which coded for a hypothetical protein (Entrez-protein accession code: XP_956921) in the *N. crassa* genome database. A web-based BLAST (http://www.ncbi.nih.nlm.gov/blast/) was performed with other known xylose reductases.

TABLE 4.1 Strains and plasmids used in this study

Strains	Description	Reference
CEN.PK 2-1D	*MATα*; *ura3*-52; *trp1*-289; *leu2*-3_112; *his3Δ*1; *MAL2-8*^{C}; *SUC2*	Entian and Kötter (1998)
CNAR	CEN.PK 2-1D transformed with pNcAR into *HIS3* locus	This work
CNARX2	CNAR transformed with pUSXYL2 into *URA3* locus	This work
YUSM	CEN.PK 2-1D transformed with pUSM1001 into *URA3* locus	This work
YUSMNAR	YUSM, transformed with pNcAR into *HIS3* locus	This work
Y-X	YUSM with undetermined mutation(s); faster xylose growth	Manuscript in preparation
Plasmids		
pUSM1001	YEp24 Δ2μ *ADH1*$_p$-*PsXYL1- ADH1*$_t$: *PGK1*$_p$-*PsXYL2- PGK1*$_t$ *URA3*	This work
pUSXYL2	pUSM1001 *PGK1*$_p$-*XYL2- PGK1*$_t$ *URA3*	This work
pSTAH	YIp5 *PGK1*$_p$-*PGK1*$_t$ *HIS3*	Thanvanthri Gururajan *et al*. (2007)
pNcAR	pSTAH *PGK1*$_p$-*NCARX-PGK1*$_t$ *HIS3*	This work

4.3.3 Plasmid constructions. Standard procedures for isolation and manipulation of DNA were carried out according to Ausubel *et al*. (1995). Restriction enzymes were obtained either from Roche (Mannheim, Germany) or Fermentas (Vilnius, Lithuania). T4 DNA ligase was obtained from Promega (Madison, WI, USA). The polymerase chain reaction (PCR) method was used for DNA amplification. *Ex-Taq* polymerase (TaKaRa Bio Inc., Shiga, Japan) was used in the PCR reactions according to the protocol of the manufacturer. Plasmid pUSM1001, created by removing the 2μm region of the pY7 vector (Walfridsson *et al*., 1997), contained the *P. stipitis XYL1* and *XYL2* genes (encoding xylose reductase and xylitol dehydrogenase, respectively) under the *S. cerevisiae* alcohol dehydrogenase (*ADH1*) and phosphoglycerate kinase (*PGK1*) promoters, respectively. The plasmid was linearised with *Apa*I and integrated into the *URA3* locus of the CEN.PK 2-1D strain. The *XYL1* cassette was removed from plasmid pUSM1001 by *Bam*HI and the resultant vector was ligated using T4 DNA ligase generating plasmid pUSXYL2. The *N. crassa* aldose reductase gene (*NCAR-X*) was obtained from the *S. cerevisiae* Y-XNc transformant by PCR using the plasmid DNA of this transformant as template and primers NcARF (GGAATTCATGGCTCCTCTCAGCA) and NcARR (CCTTAAGTTAGTCGCTGTCGCCA) (*Eco*RI sites underlined). The resultant 900 bp product was cloned into the YIp5-based shuttle vector pSTAH (Table 4.1) to produce plasmid pNcAR. This plasmid contained the *NCAR-X* gene under the control of the *S. cerevisiae* phosphoglycerate kinase I (*PGK1*) promoter (*PGK1*$_p$) and terminator (*PGK1*$_t$), and the histidine auxotrophic marker gene (*HIS3*) for selection.

4.3.4 Transformation. Bacterial transformation was carried out using the method of Inoue *et al*. (1990) and yeast transformation was carried out using the lithium acetate method (Gietz *et al*., 1992). *S*. *cerevisiae* CEN.PK 2-1D was transformed with plasmid pUSM1001 to yield the strain YUSM, which contained *PsXYL1* and *PsXYL2*. When strain CEN.PK 2-1D was transformed with plasmid pNCAR, transformant CNAR (containing the *NCAR-X* gene) was obtained. The same plasmid, pNCAR, when transformed into YUSM, yielded strain YUSMNAR, which thus contained the genes *PsXYL1, PsXYL2* and *NCAR-X*. Strain CNAR was transformed with plasmid pUSXYL2 to produce strain CNARX2, which contained the *NCAR-X* and *PsXYL2* genes. The presence of these genes in the *S. cerevisiae* transformants was verified by PCR and/or Southern blot hybridisations.

4.3.5 Growth in shake flasks. The five yeast strains used in this study were evaluated for growth in a defined mineral medium (Verduyn *et al*., 1992) containing 20 g/l glucose and 50 g/l xylose in a mix, or 50 g/l xylose alone, as carbon sources. Erlenmeyer flasks (250 ml) each containing 50 ml of medium were inoculated (in triplicate) with 10^5 cells/ml and incubated at 30℃ in an orbital shaker at 200 rpm for over a period of 160 h. Samples were withdrawn at regular intervals to measure growth as optical density measurements at 600 nm (OD_{600}), using a UV-Vis spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan).

4.3.6 Fermentation. Small-scale fermentations were carried out in stoppered, sealed bottles containing magnetic stirrers to facilitate stirring on a stirrer plate at 30℃. Each bottle (total capacity - 210ml) contained 200 ml of mineral medium with 20 g/l glucose, 50 g/l xylose and Ergosterol+Tween80 for anaerobic growth. Pre-grown cells were washed in sterile water and inoculated to an initial OD_{600} of 0.2. Proper sealing of the bottles with parafilm ensured oxygen-limited conditions, together with the constant stirring and tiny head space. Samples were withdrawn for analysis at regular intervals and under aseptic conditions using a sterile needle and syringe. Samples were filtered through a 0.22-µm acetate filter and stored at -20℃ until further analysis.

4.3.7 Analyses. Substrates consumed and metabolites formed were analysed using a Waters HPLC system equipped with an Aminex HPX-87H column (BioRad, USA), connected to a refractive index detector (RID-6A, Shimadzu, Japan). A mobile phase of 5 mM H_2SO_4 at a flow rate of 0.6 ml/min and a column temperature of 45℃ were used to determine the concentrations of glucose, xylose, xylitol, glycerol, acetate and ethanol. The ethanol values measured were then corrected by using redox balance equations (Roels, 1983). The cell dry weight of the cultures was determined by filtering a known volume of culture broth through a pre-dried (350 W for 4 min in a microwave oven), pre-weighed Supor membrane of 0.45-µm (Gelman Sciences, Ann Arbor, MI, USA). The filter was weighed again after being washed with three volumes of distilled water and dried in a microwave oven at 350 W for 8 min. The differences in the filter weights were

used to calculate the dry weights in accordance with the absorbency measured. Dry weights were determined in duplicates.

4.3.8 Enzymatic assay. Cells grown at 30 ℃ in mineral medium containing 50 g/l xylose, both aerobically and under oxygen-limited conditions as explained above, were collected through centrifugation (3000 ×*g*, 4℃) and a crude extract was obtained for xylose reductase assay using the protocol described in Eliasson *et al*. (2000). The assay was adapted to a microtitre plate reader (Powerwave-X, Bio-tek Instruments, Winooski, VT, USA) so that the final mix contained 1 M triethanolamine (Sigma-Aldrich, St. Louis, MO, USA) buffer (pH 7.0), 10 mM NAD(P)H (Roche, Mannheim, Germany), crude extract and 3.5 M xylose, in a total volume of 0.2 ml. The oxidation of NAD(P)H at 340 nm was traced over a period of time. One unit of enzyme activity was defined as the amount of enzyme needed to consume 1 µmol of NAD(P)H per minute under the specified conditions. Activities are represented as units per milligram (U/mg) of protein and are averages of triplicates. Protein quantification was done using the BCA-Pierce kit (Pierce, Rockford, IL, USA).

4.4 RESULTS AND DISCUSSION

N. *crassa* is capable of growth on xylose, showing xylose reductase activity under aerobic conditions (Chiang and Knight, 1960). In the search for an efficient xylose-metabolising yeast, a recombinant *S*. *cerevisiae* xylose mutant (Y-X) transformed with the *N*. *crassa* cDNA library yielded transformants showing faster growth. These transformants contained plasmids with a 1-kb insert that had a 900-bp ORF. This in turn coded for a protein of 300 amino acids. In order to physiologically evaluate its efficiency in xylose utilisation, it was decided to express the *N*. *crassa NCAR-X* aldose reductase gene in different genetic backgrounds of the *S*. *cerevisiae* CEN.PK2-1D strain, in combination with the *P*. *stipitis XYL1* and *XYL2* genes. The aim was to evaluate strains expressing the *N*. *crassa* gene together with the *P*. *stipitis* xylose pathway genes and also to determine their efficiency to complement the *PsXYL1*-deficient strain. Accordingly, four different strains were developed; these strains are listed in Table 4.1.

4.4.1 Sequence analysis of the *N. crassa NCAR-X* gene and encoded aldose reductase

The 900-bp ORF was sequenced and the sequence was compared to the NCBI database which showed 100% homology to a hypothetical protein from the *N*. *crassa* genome database (ID: NCU04510.3). BLASTp (http://www.ncbi.nlm.nih.gov/BLAST/) analysis showed the protein to be 67% identical to the glycerol dehydrogenase (*GCY1*) of *Aspergillus fumigatus* and 40-50% identical to aldo-keto reductases from varied eukaryotes such as *Hypocrea jacorina*, *Digitalis purpurea*, *Aspergillus nidulans*, *Mus musculus*, plants such as *Oryza sativa*, and the bacterium *Lactobacillus plantarum*.

The nucleotide sequence of the 900-bp *NCAR-X* ORF, together with its 300 amino acids encoded pro NCBI protein, are given in Fig. 4.1. Conserved domain analysis

showed this protein as belonging to the family of aldose reductases. The presence of conserved sequences at the N-terminal region (LXXGXXXPXXGXG), and at the active site region (GXXXXDXAXXY, containing the active aspartic acid and tyrosine residues, and LXXXXXXXXDXXXXH, containing the active histidine residue), has been shown to be characteristic of xylose reductase from other yeasts (Lee *et al*., 2003) and these regions are conserved in this enzyme and also in NcrassXR, the enzyme previously reported by Woodyer *et al*. (2005) (Fig. 4.2). The N-terminal region of many xylose reductases contain a highly conserved LNXG, with X being mostly either a serine residue or in some cases, aspartic acid. However, in the *N. crassa* aldose reductase, NCAR-X, a threonine residue is present instead of either of the serine (which is the case in NcrassXR) and aspartic acid residues. This is also the case with some human aldose reductases (Lee, 1998).

```
ATG GCT CCT CTC AGC AAG ACT CAC AAG CTC AAC ACG GGC GAC GAG ATC CCC GCC GTT GGC
 M   A   P   L   S   K   T   H   K   L   N   T   G   D   E   I   P   A   V   G

CTC GGA ACC TGG CAG TCC AAG CCT GGT CAG GTC GAG AAG GCC GTC GAG GCC GCC CTA CGT
 L   G   T   W   Q   S   K   P   G   Q   V   E   K   A   V   E   A   A   L   R

GCC GGC TAT ACT CAC ATC GAC ACC GCG TAT GCC TAC GGC AAC GAG AAG GAG GTT GGC CAA
 A   G   Y   T   H   I   D   T   A   Y   A   Y   G   N   E   K   E   V   G   Q

GGC ATC AAG GCC TCC GGC GTC CCA CGC GAG AAG ATC TGG CTC ACC ACC AAG CTG GAC AAC
 G   I   K   A   S   G   V   P   R   E   K   I   W   L   T   T   K   L   D   N

GAC TGG CAC AAG CAT GTC GCC GAG GCT ATC GAT ACG TCG CTC AAG AAC CTG GAC ACC CCC
 D   W   H   K   H   V   A   E   A   I   D   T   S   L   K   N   L   D   T   P

TAC GTT GAC CTC TAC CTG ATG CAC TGG CCC GCC AGT CTT GTC AAG GGG AAC ACC AAG GAG
 Y   V   D   L   Y   L   M   H   W   P   A   S   L   V   K   G   N   T   K   E

GTG TAC AAC GAC TGG GAC TTT GTG GAC ACC TGG CGC GAG ATG CAG AAG CTG GTT GAC ACC
 V   Y   N   D   W   D   F   V   D   T   W   R   E   M   Q   K   L   V   D   T

GGC AAG GTC AAG AAC ATT GGT GTC AGC AAC TTT GGC GTC AAG AAC CTG GAG AAG CTT TTG
 G   K   V   K   N   I   G   V   S   N   F   G   V   K   N   L   E   K   L   L

AGC GCC GAG AGC ACC AAG ATT GTT CCC GCC GTT AAC CAG ATC GAA CTT CAT CCT GGT AAC
 S   A   E   S   T   K   I   V   P   A   V   N   Q   I   E   L   H   P   G   N

CCG TCC CCC CAC CTT GTC GAG TAT CTG CGC TCG AAG GGC ATT CAT GCT TCG GCC TAT TCG
 P   S   P   H   L   V   E   Y   L   R   S   K   G   I   H   A   S   A   Y   S

CCC CTC GGT AGC AGC GAC TCG CCG CTT TAC AAG CTT AAC AGC CTC ACC AAG CTC GCC GAG
 P   L   G   S   S   D   S   P   L   Y   K   L   N   S   L   T   K   L   A   E

TCC AAG GGC AAG ACT GTG CAG CAG GTT CTC CTT AGA TGG GGT GTC CAG AAG GGC TGG AGT
 S   K   G   K   T   V   Q   Q   V   L   L   R   W   G   V   Q   K   G   W   S

GTG CTG CCT AAG AGC GTC ACT GAG GAG CGT ATC AAG GCC AAC ATT GAT CTC GAG GGC TGG
 V   L   P   K   S   V   T   E   E   R   I   K   A   N   I   D   L   E   G   W

AGC TTG ACC GAT GAG GAG ATT GCT CAG ATT GAT GAG GTT CAC AAG GAG AAC AGC TTC AAG
 S   L   T   D   E   E   I   A   Q   I   D   E   V   H   K   E   N   S   F   K

GTC TGC GGC GAT GAC TGG TTG CCT GTC AAG ATC TTC TTC GGT GCT GGC GAC AGC GAC TAA
 V   C   G   D   D   W   L   P   V   K   I   F   F   G   A   G   D   S   D   -
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FIG. 4.1 Nucleotide and deduced amino acid sequence of the *Neurospora crassa* aldose reductase gene (Entrez protein accession ID: XP_956921).

The aldo-keto reductase (AKR) motif, IPKSXXXXR, is present in both NcrassXR and NCAR-X, even though the isoleucine residue is replaced by a leucine residue (LPKS) in the latter enzyme. Catalytic features, found in both aldo-keto reductases and short-chain dehydrogenases/reductases (SDR), implicated to participate directly in catalysis

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CtenuiXR    (1) MSASIPDIKLSSGHLMPSIGFGCWKLANATAGEQVYQAIKAGYRLFDGAEDYGNEKEVGD
PstipiXR    (1) ----MPSIKLNSGYDMPAVGFGCWKVDVDTCSEQIYRAIKTGYRLFDGAEDYANEKLVGA
  NCAR-X    (1) MAPLSKTHKLNTGDEIPAVGLGTWQSKPGQVEKAVEAALRAGYTHIDTAYAYGNEKEVGQ
NcrassXR    (1) ---MVPAIKLNSGFDMPQVGFGLWKVDGSIASDVVYNAIKAGYRLFDGACDYGNEVECGQ

CtenuiXR   (61) GVKRAIDEGLVKREEIFLTSKLWNNYHDPKNVETALNKTLADLKVDYVDLFLIHFPIAFK
PstipiXR   (57) GVKKAIDEGIVKREDLFLTSKLWNNYHHPDNVEKALNRTLSDLQVDYVDLFLIHFPVTFK
  NCAR-X   (61) GIKAS-G--VP-REKIWLTTKLDNDWH--KHVAEAIDTSLKNLDTPYVDLYLMHWPASLV
NcrassXR   (58) GVARAIKEGIVKREELFIVSKLWNTFHDGDRVEPIVRKQLADWGLEYFDLYLIHFPVALE

CtenuiXR  (121) FVPIEEKYPPGFYCGDGNNFVYEDVPILETWKALEKLVAAGKIKSIGVSNFPGALLLDLL
PstipiXR  (117) FVPLEEKYPPGFYCGKGDNFDYEDVPILETWKALEKLVKAGKIRSIGVSNFPGALLLDLL
  NCAR-X  (115) KGNTKEVYN--------------DWDFVDTWREMQKLVDTGKVKNIGVSNFGVKNLEKLL
NcrassXR  (118) YVDPSVRYPPGWHFDGKSEIRPSKATIQETWTAMESLVEKGLSKSIGVSNFQAQLLYDLL

CtenuiXR  (181) --RGATIKPAVLQVEHHPYLQQPKLIEFAQKAGVTITAYSSFGPQSFVEMNQGRALNTPT
PstipiXR  (177) --RGATIKPSVLQVEHHPYLQQPRLIEFAQSRGIAVTAYSSFGPQSFVELNQGRALNTSP
  NCAR-X  (161) SAESTKIVPAVNQIELHPGNPSPHLVEYLRSKGIHASAYSPLG------------SSDSP
NcrassXR  (178) --RYAKVRPATLQIEHHPYLVQQNLLNLAKAEGIAVTAYSSFGPASFREFNMEHAQKLQP

CtenuiXR  (239) LFAHDTIKAIAAKYNKTPAEVLLRWAAQRGIAVIPKSNLPERLVQNRSFNTFDLTKEDFE
PstipiXR  (235) LFENETIKAIAAKHGKSPAQVLLRWSSQRGIAIIPKSNTVPRLLENKDVNSFDLDEQDFA
  NCAR-X  (209) LYKLNSLTKLAESKGKTVQQVLLRWGVQKGWSVLPKSVTEERIKANIDLEGWSLTDEEIA
NcrassXR  (236) LLEDPTIKAIGDKYNKDPAQVLLRWATQRGLAIIPKSSREATMKSNLNSLDFDLSEEDIK

CtenuiXR  (299) EIAKLDIGLRFNDP-WDWDNIPIFV------
PstipiXR  (295) DIAKLDINLRFNDP-WDWDKIPIFV------
  NCAR-X  (269) QIDEVHKENSFKVCGDDWLPVKIFFGAGDSD
NcrassXR  (296) TISGFDRGIRFNQP-TNYFSAENLWIFG---
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FIG. 4.2 Sequence alignment between xylose reductases from *Candida tenuis* (CtenuiXR), *Pichia stipitis* (PstipiXR), *Neurospora crassa* (NcrassXR) as described by Woodyer *et al*. (2005) and the second aldose reductase from *N. crassa* (NCAR-X) (this work). All conserved amino acid residues are shaded grey, while those in the catalytic domain are displayed in **bold**.

or cofactor binding, viz. aspartic acid 43 (stabilises lysine 77 through a salt bridge), tyrosine 49 (a proton donor during aldehyde reaction), lysine 77 (hydrogen bonds to and stabilises tyrosine 49), histidine 110 (directs the orientation of the substrate in the active site pocket), lysine 270 (binds to the 2'-phosphate of NADPH) and arginine 276 (contributes to tighter coenzyme binding) are conserved in NCAR-X (Bohren *et al*., 1994; Kubiseski *et al*., 1995; Kostrzynska *et al*., 1998; Lee, 1998) (Fig. 4.2). However, in the SDR family of enzymes, the critical lysine is only four residues downstream of essential tyrosine in the primary sequence, thereby forming an invariant YXXXK motif (Jörnvall *et al*., 1995). This motif is present in the NCAR-X studied, but not in the NcrassXR gene already reported (Fig. 4.2). While this YXXXK motif is present twice in yeast xylose reductases [*Pichia stipitis*, *Candida tropicalis*, *Pachysolen tannophilus* (Lee *et al*., 1998)], it is present only once in this NCAR-X. The other SDR motif (or the Wierenga co-enzyme binding motif of dehydrogenases) represented by a glycine motif (GXXXGXG region), which is present in most of the yeast xylose reductases is absent in NCAR-X. However, a recent study on the role of the glycine motif (SDR motif) in the *S*. *cerevisiae* xylose reductase has shown that it is not directly essential for coenzyme

binding (Chu and Lee, 2006). These findings place this aldose reductase in a unique position among the reductases isolated so far. It has some properties similar to mammalian aldose reductases and others similar to yeast xylose reductases. The predicted molecular weight of this enzyme is ca. 34 kDa. Phylogenetic analysis showed NCAR-X to be closely related to the other xylose reductase of *N. crassa* already reported (Entrez Protein Accession number: AAW66609) by Woodyer *et al*. (2005). Similar to the XR reported by Woodyer *et al*. (2005), NCAR-X also lacks cysteine 23 residue in the NADPH binding pocket but, in contrast, has a threonine 23 residue instead of leucine 20 reported by the authors.

4.4.2 Growth rates and fermentation performance of *S. cerevisiae* strains

The four recombinant yeast strains together with the control strain CEN.PK 2-1D were cultured in mineral medium with xylose as the sole carbon source. Cell growth was monitored as OD_{600} measurements at regular intervals (Fig. 4.3). The YUSMNAR strain, harbouring the cassettes expressing *PsXYL1-2* and *NCAR-X,* was the strain that grew best in xylose-containing media with a growth rate of 0.022 h^{-1}. It was followed by CNARX2 (containing *NCAR-X* and *PsXYL2*; growth rate 0.01 h^{-1}) and YUSM (containing *PsXYL1-2*; growth rate 0.005 h^{-1}). The CNAR strain (containing only *NCAR-X*) was comparatively slower to grow than the others (growth rate 0.003 h^{-1}), while

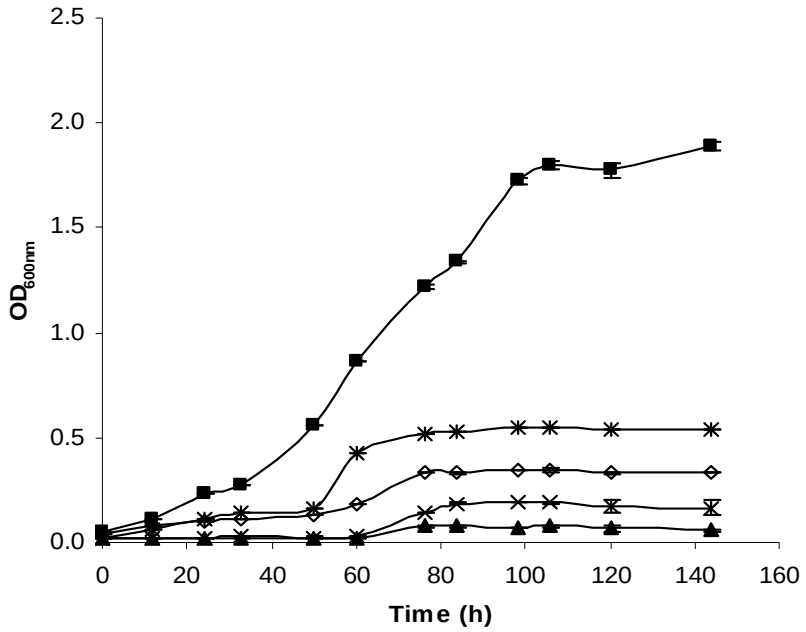

FIG. 4.3 Growth of CENPK (▲), CNAR (×), CNARX2 (∗), YUSM (◇) and YUSMNAR (■) in 50 g/l xylose containing mineral medium in shake-flasks at 30℃ and 200 rpm in horizontal orbital shaker under aerobic conditions. Represented values are averages of duplicate experiments and SD represented as error bars.

CEN.PK 2-1D strain showed negligible growth (rate 0.001 h^{-1}) in xylose. When the strains were grown in mineral medium containing 20 g/l glucose and 50 g/l xylose, no

significant difference was observed between the growth rates of the strains (data not shown).

Under oxygen-limited/anoxic conditions, with both glucose and xylose in mineral media, xylose utilisation was concomitant and faster with low amounts of glucose (Meinander and Hahn-Hägerdal, 1997; Öhgren *et al*., 2006). When glucose was depleted, xylose utilisation became slower. Analysis of substrates and metabolites (Table 4.2) showed that the highest xylose consumption (approx. 40% w/v) was by the YUSMNAR strain, followed by the YUSM, CNARX2 and CNAR strains. The CEN.PK 2-1D strain consumed only about one-tenth of the total xylose present in the medium. Most of the consumed xylose was converted to xylitol by the two strains containing *PsXYL1*. Interestingly, the xylitol conversion from xylose was lower for the strains containing *NCAR-X* not associated with *PsXYL1* than the corresponding conversion rates for the strains containing *PsXYL1*. In terms of xylitol yield from xylose consumed, strains YUSM and YUSMNAR produced almost three times more xylitol than the CNARX2 strain and about 1.5-times that of CNAR (Table 4.2). The absence of xylitol dehydrogenase in the CNAR strain might have been a reason for its lower xylose consumption and higher xylitol yield. With no apparent difference between strains YUSM and YUSMNAR for the xylitol yield from xylose, it is possible that there was no addition to the *P. stipitis* XR activity by the NCAR-X. It could be suggested that the *P. stipitis* XR was competitively more active than the NCAR-X, when they were both expressed together. When NCAR-X was expressed solely in the CNAR strain, there was an increase in xylitol yield. However, when it was expressed together with a xylitol dehydrogenase gene (CNARX2), the xylitol yield was decreased. This meant that the xylose was being channelled downstream of the metabolic pathway. Among the other metabolites produced, ethanol values were more or less similar between strains, except

TABLE 4.2 Xylose consumption and yields of different metabolites during small-scale oxygen-limited fermentation using mineral medium containing 20 g/l glucose and 50 g/l xylose. Physiological conditions are given in the Materials and Method section.

Strain	Xylose consumed (g/l)	Yield from total sugars (g/g)					Xylitol/xylose (g/g)
		Ethanol	Glycerol	Xylitol	Biomass	Acetate	
CENPK	5.97	0.41	0.067	0.01	0.07	0.013	*0.05*
CNAR	8.71	0.41	0.044	0.05	0.07	0.006	*0.16*
CNARX2	13.81	0.44	0.036	0.03	0.06	0.002	*0.07*
YUSM	16.94	0.39	0.026	0.12	0.05	0.011	*0.26*
YUSMNAR	20.16	0.39	0.024	0.13	0.05	0.010	*0.26*

Glucose was consumed to completion by all the strains. Values represented are averages of duplicate experiments (SD≤5%)

for strains CEN.PK 2-1D and CNAR (Table 4.2). With the lack of xylitol dehydrogenase downstream to process the xylose being utilised, it was plausible that the CNAR strains had low ethanol formation. However, it was interesting to note that strain CNARX2 produced almost the same amount of ethanol as that produced by strain YUSM. The

strains expressing the *NCAR-X* gene produced comparatively higher amounts of glycerol than the strains expressing the *PsXYL1* gene. CEN.PK 2-1D produced the highest amount of glycerol while having the lowest xylose consumption. Such a shift in metabolite production by the *NCAR-X* gene could help to improve the bio-transformation of sugar into alcohol, rather than the formation of pentitol, as seen in many other xylose reductases (Nidetzky *et al*., 1998; Oh *et al*., 1998).

TABLE 4.3 Specific xylose reductase activity of strains under aerobic growth in shake-flasks and oxygen-limited bottle fermentation. Mineral medium with 50 g/l xylose was used and cells were incubated at 30℃ and 200 rpm.

Strains	Spec. activity (U/mg)	
	Aerobic flasks	Fermentation bottles
CENPK	0.021 ± 0.002	0.003 ± 0.001
CNAR	0.032 ± 0.003	0.015 ± 0.001
CNARX2	0.037 ± 0.012	0.022 ± 0.002
YUSM	0.052 ± 0.002	0.027 ± 0.004
YUSMNAR	0.067 ± 0.005	0.037 ± 0.008

For oxygen-limited fermentation, 20 g/l glucose was added to facilitate cell growth. Enzyme activities were measured for crude extracts in triplicates and SD values are represented after the ± sign

4.4.3 Xylose reductase activities of the *S. cerevisiae* transformants

Xylose reductase activity of the strains grown aerobically and under fermentative conditions was determined. All strains showed (Table 4.3) higher specific activity during aerobic growth than under fermentation, using NADPH as cofactor. Little or no activity was observed with NADH as cofactor under both conditions. The highest xylose reductase activity was seen from strain YUSMNAR, which contained two heterologous aldose reductases. Additionally, both yeast strains that expressed *PsXYL1* had almost twice the activity of the strains that expressed *NCAR-X* alone. CNARX2, which contained *PsXYL2* together with *NCAR-X,* had a marginally higher XR activity than CNAR although it was lower than that of YUSM. The presence of XR-specific activity for the strains containing *NCAR-X* together with the fact that the presence of xylitol dehydrogenase increased xylose reductase activity, led to the consideration that the aldose reductase from *N*. *crassa* was a xylose reductase. Strain CEN.PK 2-1D showed xylose reductase activity under aerobic conditions even though it did not contain any heterologous xylose reductase, but *S*. *cerevisiae* has been shown to possess some endogenous, lowly expressed xylose reductases (Kuhn *et al*., 1995; Träff *et al*., 2002; Toivari *et al*. 2004; Träff-Bjerre *et al*., 2004). However, the activity was negligible for fermentation samples. The xylose reductase activity was considerably lower for all strains during fermentation, although a pattern similar to that of aerobic growth was seen with the YUSMNAR strain having the most activity followed by strains YUSM, CNARX2, CNAR and CEN.PK 2-1D in decreasing order. The increased *in vitro* xylose reductase activity of strain YUSMNAR in comparison to that of strains YUSM and CNARX2 in comparison to CNAR could be attributed to a possible add-on effect from

the *N*. *crassa* xylose reductase. However, this did not translate into increase in xylitol yields obtained between the two strains, which were the same. When comparing strains YUSM and CNARX2, similar in background except for the heterologous xylose reductase expressed, it was interesting to note that the ratio between *in vitro* aerobic and oxygen-limited activities for CNARX2 was lower than that for strain YUSM. Nonetheless, this showed that *NCAR-X* was active under oxygen-limited conditions in a recombinant *S*. *cerevisiae* host.

This is the first report, to our knowledge, pertaining to the expression of a xylose reductase from *N*. *crassa* in *S*. *cerevisiae*. The strain with the *NCAR-X* gene and the *XYL2* gene from *P*. *stipitis* was able to ferment xylose to levels that were very close to those produced by the strain with the *XYL1-2* genes of *P*. *stipitis*, showing at least a 15% increase in ethanol yield. However, when coexpressed with both reductases, the resultant recombinant strain showed only a slight increase in fermentation properties even though the *in vitro* enzyme activities were much higher. Thus, it is possible that *in vivo*, the *N*. *crassa* xylose reductase might be differentially regulated or might be inhibited by the xylose reductase from *P*. *stipitis*. However, the product profile of strain CNARX2 was in stark contrast to that of strain YUSM. It contained less xylitol and more glycerol and ethanol, even though no NADH-specific activity was observed. It would be of interest in the future to compare and contrast this enzyme with that of the one reported by Woodyer *et al*. (2005) and possibly express both in *S*. *cerevisiae*.

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Chapter 5

RESEARCH RESULTS III

Development and characterisation of a *Saccharomyces cerevisiae* recombinant strain with enhanced xylose fermentation properties

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Development and characterisation of a recombinant *Saccharomyces cerevisiae* mutant strain with enhanced xylose fermentation properties

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5.1 ABSTRACT

The purpose of this study was to help lay the foundation for further development of xylose-fermenting *Saccharomyces cerevisiae* yeast strains through an approach that combined metabolic engineering and random mutagenesis in a recombinant haploid strain that overexpressed only two genes of the xylose pathway. Previously, *S. cerevisiae* strains, overexpressing heterologous genes encoding xylose reductase, xylitol dehydrogenase and the endogenous *XKS1* xylulokinase gene, were randomly mutagenised to develop improved xylose-fermenting strains. In this study, two gene cassettes ($ADH1_P$-*PsXYL1*-$ADH1_T$ and $PGK1_P$-*PsXYL2*-$PGK1_T$) containing the xylose reductase (*PsXYL1*) and xylitol dehydrogenase (*PsXYL2*) genes from the xylose-fermenting yeast, *Pichia stipitis*, were integrated into the genome of a haploid *S. cerevisiae* strain (CEN.PK 2-1D). The resulting recombinant strain (YUSM 1001) overexpressing the *P. stipitis XYL1* and *XYL2* genes (but not the endogenous *XKS1* gene) was subjected to ethyl methane sulfonate (EMS) mutagenesis. The resulting mutants were screened for faster growth rates on an agar medium containing xylose as the sole carbon source. A mutant strain (designated Y-X) that showed 20-fold faster growth in xylose medium in shake-flask cultures was isolated and characterised. In anaerobic batch fermentation, the Y-X mutant strain consumed 2.5-times more xylose than the YUSM 1001 parental strain and also produced more ethanol and glycerol. The xylitol yield from the mutant strain was lower than that from the parental strain, which did not produce glycerol and ethanol from xylose. The mutant also showed a 50% reduction in glucose consumption rate. Transcript levels of *XYL1*, *XYL2* and *XKS1* and the *GPD2* glycerol 3-phosphate dehydrogenase gene from the two strains were compared with real-time reverse transcription polymerase chain reaction (RT-PCR) analysis. The mutant showed 10-40 times higher relative expression of these four genes, which corresponded with either the higher activities of their encoded enzymes or by-product formation during fermentation. Furthermore, no mutations were observed in

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the mutant's promoter sequences or the open reading frames of some of its key genes involved in carbon catabolite repression, glycerol production and redox balancing. The data suggest that the enhancement of the xylose fermentation properties of the Y-X mutant was made possible by increased expression of the xylose pathway genes, especially the *XKS1* xylulokinase gene.

Key words: mutagenesis, xylose fermentation, xylulokinase, xylitol dehydrogenase, xylose reductase, *XKS1*, *XYL1*, *XYL2*, *Saccharomyces cerevisiae*, yeast.

5.2 INTRODUCTION

Efficient conversion of xylose in lignocellulose-based raw materials by recombinant *Saccharomyces cerevisiae* is important for the production of fuel ethanol. It forms an integral part of a world-wide, long-term research effort aimed at the development of an economically viable and sustainable process for the efficient bioconversion of lignocellulose into biofuels, preferably by a single organism in a one-step process known as *consolidated bioprocessing* (Lynd 1996; Lynd *et al*., 2002).

Naturally occurring *S. cerevisiae* strains cannot ferment xylose. However, they are capable of utilising and fermenting an isomer of xylose, xylulose, at a rate 10-times slower than that of glucose consumption (Wang and Schneider, 1980; Chiang *et al*., 1981; Senac and Hahn-Hägerdal, 1990; Jeppsson *et al*., 1996). In this process, xylulose is metabolised through the pentose phosphate pathway, which feeds into glycolysis and the formation of ethanol. Hence, genes encoding the two-step reduction/oxidation of xylose to xylulose from the xylose-fermenting yeast *Pichia stipitis* have been expressed in *S. cerevisiae* (Kötter and Ciriacy, 1993; Tantirungkij *et al*., 1993). A recombinant *S. cerevisiae* strain, expressing the xylose reductase (XR) gene (*PsXYL1*) and xylitol dehydrogenase (XDH) gene (*PsXYL2*), was able to grow on xylose, albeit very slowly (Kötter *et al*., 1990; Hallborn *et al*., 1991).

The *XYL1* and *XYL2* encoded enzymes have different cofactor preferences. The *P. stipitis* XR enzyme can use both NADPH and NADH, with preference for NADPH (Verduyn *et al*., 1985; Rizzi *et al*., 1988; Kostrzynska *et al*., 1998), while its XDH enzyme is exclusively dependent on NAD^+ (Rizzi *et al*., 1989; Kötter *et al*., 1990). Normally, ethanol formation from glucose in *S. cerevisiae* is redox neutral, but when transformed with *XYL1* and *XYL2*, the XR and XDH enzymes' differing cofactor specificities led to a lack of NAD^+, especially under anaerobic conditions (Bruinenberg *et al*., 1984). It was found that *S. cerevisiae* also requires a carefully up-regulated *XKS1*-encoded xylulokinase activity (XK) to metabolise xylose to ethanol (Ho *et al*., 1998; Eliasson *et al*., 2000; Johansson *et al*., 2001).

In addition to using targeted metabolic engineering approaches (Bailey 1991) to generate xylose-fermenting strains of *S. cerevisiae*, strains overexpressing XR, XDH and XK have been further improved by random evolutionary engineering strategies (Sauer, 2001; Sonderegger and Sauer, 2003; Wahlbom *et al*., 2003a). The potential of evolutionary engineering was recently demonstrated with a xylose-utilising mutant from

a non-recombinant *S. cerevisiae* strain (Attfield and Bell, 2006). This strain had increased xylose reductase and xylitol dehydrogenase activities with no apparent increase in xylulokinase activity, although the anaerobic xylose fermentation properties of this strain have not been reported.

In light of the results reported to date regarding the development of xylose-fermenting *S. cerevisiae* strains, we believe that it is reasonable to explore ways of further improvement of these strains by subjecting them to a combination of genetic engineering, metabolic engineering, mutagenesis and/or directed evolutionary engineering strategies. The purpose of the present study was to overexpress the *P. stipitis XYL1* xylose reductase gene and *XYL2* xylitol dehydrogenase gene in a haploid laboratory strain of *S. cerevisiae* (CEN.PK 2-1D) without overexpressing a cloned version of the endogenous *S. cerevisiae XKS1* xylulokinase gene, and then to mutate the resulting recombinant strain. Therefore, there are two fundamental aspects that set this study apart from previously published or patented work on the development of xylose-fermenting *S. cerevisiae* strains. First, the rationale for conducting this investigation in a *haploid* laboratory strain as opposed to a more robust industrial strain with greater commercial application was to provide more scientific flexibility and opportunity for in-depth genetic analyses and research, which could later be translated and extended to industrial strains. Second, the reason why a cloned version of the endogenous *XKS1* gene was not overexpressed together with the heterologous *XYL1* and *XYL2* genes was to demonstrate that it is possible to generate a xylose-utilising strain by combining a metabolic engineering approach with classical random mutagenesis. Following this strategy, we succeeded in developing a recombinant *S. cerevisiae* mutant strain with a 20-fold increase in aerobic growth on xylose. The strain fermented xylose into ethanol and glycerol, rather than xylitol.

5.3 MATERIALS AND METHODS

5.3.1. Recombinant DNA methods, transformation. Standard procedures for isolation and manipulation of DNA were used throughout this study (Ausubel *et al*., 1995). Restriction enzymes (Roche, Mannheim, Germany), T4 DNA-ligase (Promega, Madison, WI, USA) and Takara *Ex-Taq* DNA polymerase (TakaRa Bio, Shiga, Japan) were used according to the specifications of the supplier. Bacteria were transformed as described by Ausubel *et al*. (1995), while the lithium acetate method (Gietz *et al*., 1992) was used for yeast transformations.

5.3.2. Microbial strains, plasmids and media. The sources and relevant genotypes of bacterial and yeast strains, together with the plasmids used in this study, are listed in Table 5.1. *Escherichia coli* DH5α was used for the amplification of the yeast integrating plasmid pUSM 1001 (Thanvanthri Gururajan *et al*. 2007). This plasmid carries two gene cassettes, containing the *P. stipitis* xylose reductase (*ADH1*$_P$-*PsXYL1*-*ADH1*$_T$) and xylitol dehydrogenase (*PGK1*$_P$-*PsXYL2*-*PGK1*$_T$) genes. The *XYL1* and *XYL2* genes are placed under the control of the *S. cerevisiae* alcohol dehydrogenase I (*ADH1*) and

phosphoglycerate kinase I (*PGK1*) regulatory sequences, respectively. Plasmid pUSM 1001 was stably integrated into the genome of the haploid *S. cerevisiae* strain CEN.PK 2-1D to generate recombinant strain YUSM 1001 (hereafter referred to as YUSM). Mutant strain Y-X was derived from the recombinant YUSM strain after it was mutagenised by ethyl methane sulfonate (EMS).

TABLE 5.1 Yeast strains and plasmids used in this study

Strains	Description	Source/Reference
CEN.PK 2-1D	*MATα*; *ura3*-52; *trp1*-289; *leu2*-3_112; *his3Δ*1; *MAL2-8*^{C}; *SUC2*	Entian and Kötter (1998)
CEN.PK 113-7A	*MATa; URA3*; *LEU2*;*TRP1*; *his3 Δ*1; *MAL2-8*^{C}; *SUC2*	IWBT, Stellenbosch
YUSM	CEN.PK 2-1D: pUSM 1001	Thanvanthri Gururajan *et al*. (2007)
YUSM 1001d	*MATa; URA3*; *LEU2*;*TRP1*; *his3 Δ*1; *MAL2-8*^{C}; *SUC2*: pUSM 1001	This work
CENPK 113-7A/Y-X	Diploid strain created by mating CEN.PK 113-7A with mutant Y-X	This work
CENPK 2-1D/YUSMd	Diploid strain created by mating CEN.PK 2-1D and YUSM 1001d	This work
YUSM d/Y-X	Diploid strain created by mating YUSM 1001d with mutant Y-X	This work
pUSM 1001	YIp5 ; *ADH1p-PsXYL1-ADH1t; PGK1p-PsXYL2-PGK1t*	Thanvanthri Gururajan *et al*. (2007)

Yeast strains were grown in rich YP (10 g/l yeast extract, 20 g/l peptone) media containing either 20 g/l glucose (YPD), xylose (YPX), sucrose (YPS), raffinose (YPR), maltose (YPM) or glycerol (YPG), and stored in glycerol stocks at -80℃. Yeast transformants were grown in a synthetic complete (SC) medium [6.7 g/l yeast nitrogen base without amino acids (Difco Laboratories, MI, USA), 20 g/l glucose (SCD) or 20 g/l xylose (SCX)] supplemented with amino acids essential for selection (50 mg/l tryptophan, 240 mg/l leucine, 50 mg/l histidine, and/or 40 mg/l uracil). Yeast strains were also grown in defined mineral medium (Verduyn *et al*. 1992). White, ampicillin-resistant (Ap^R) *E. coli* transformants were grown and selected in Luria-Bertani (LB) medium containing 100 mg/l ampicillin and 30 mg/l 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (Ausubel *et al*., 1995). Solid media contained 20 g/l agar (Difco). Yeast cultures were grown at 30℃ and bact eria at 37℃.

5.3.3. Development and characterisation of mutant Y-X. Recombinant *S. cerevisiae* strain YUSM was subjected to mutagenesis with ethyl methane sulfonate (EMS; Sigma-Aldrich, St. Louis, MO, USA). Yeast cells were incubated with EMS for 80, 100, 120 and 140 min to obtain survival rates of between 10% and 60%. Samples withdrawn at these time points were pooled together and plated on YPX plates at a concentration of 10^6 cells per plate. The plates were incubated at 30℃ for 5 days. At the end of incubation,

xylose-utilising colonies appeared at a rate of 1-5/10^8 cells. The colonies were replicaplated onto YPX plates and incubated at 30℃ for 2 days, at the end of which the fastest growing mutant culture was isolated and named *S. cerevisiae* Y-X.

To characterise mutant Y-X, it was crossed with the control strains CEN.PK 113-7A and YUSM 1001d (Table 5.1) by a replicaplating method (Sherman *et al*., 1991) on selective SCD medium. The diploid strains obtained were then plated onto rich agar medium with different carbon sources, viz. glucose (YPD), xylose (YPX), sucrose (YPS), raffinose (YPR), maltose (YPM) and glycerol (YPG) and incubated at 30℃ under aerobic and anaerobic conditions. For anaerobic conditions, anaerobic jars were used together with the AnaeroGen™ anaerobic system (Oxoid, Hampshire, England). After 40 h incubation for aerobic plates and 72 h for anaerobic plates, the growth of the cultures was evaluated visually. The fastest growing mutant, Y-X, was selected for further study.

5.3.4. Growth and fermentation. To compare the aerobic growth profile of YUSM and Y-X, the cultures were initially grown until mid-exponential phase in tubes containing SCD medium at 30℃ in a rotary wheel. The cells were then inoculated into Erlenmeyer flasks (250 ml) containing 50 ml of defined mineral medium (Verduyn *et al*. 1992), with 50 g/l xylose as the sole carbon source, to an initial optical density (measured at 600 nm; OD_{600}) of 0.01. Each culture was grown in triplicate. The flasks were incubated at 30℃ and 200 rpm in an orbital shaker. Growth was tracked by sampling at regular intervals and measuring the OD_{600} values using a UV-Vis spectrophotometer (UV-1601, Shimadzu, Japan). For anaerobic fermentation, cells of Y-X and YUSM were pre-grown until late exponential phase in defined mineral medium with 20 g/l glucose in shake flasks at 30℃ and 200 rpm. The cells were then collected by centrifugation (3000 ×g, 4℃), washed with sterile water and inoculated at a concentration of 0.05 g/l into the bioreactor. Fermentation was carried out in a BioFlo III fermentor (New Brunswick Scientific, New Brunswick, USA) containing defined mineral medium with 20 g/l glucose and 50 g/l xylose in a total working volume of 1 litre. The medium was supplemented with essential amino acids (according to the auxotrophic requirements of the strains), and ergosterol and Tween 80 for anaerobic growth. Antifoam (Dow Corning, Midland, MI, USA) was added at a concentration of 0.5 ml/l. Fermentation was carried out at 30℃ and 200 rpm, with a pH of 5.5 ±0.2 maintained by automatic addition of 3 M potassium hydroxide. Anaerobic conditions were maintained by the continuous sparging of nitrogen gas (less than 5 ppm oxygen, ADR class 2 1A; AGA, Malmö, Sweden) at a flow rate of 0.2 ml/min, controlled by a mass flow meter (Bronkhorst High-Tech, Ruurlo, The Netherlands). Carbon dioxide and oxygen levels in the bioreactor were analysed by an acoustic off-gas analyser (Type 1308, Brüel & Kjær, Nærum, Denmark) (Christensen *et al*., 1995). Samples were withdrawn at regular intervals and filtered through a 0.22 µm filter (Advantec MFS, CA, USA) before being stored at -20℃ until further analysis.

5.3.5. Chemical and sequence analyses. Acetate, ethanol, glucose, glycerol, xylitol and xylose were analysed using high-performance liquid chromatography (HPLC) (Waters Corporation, Milford, MA, USA) with an Aminex P87-H ion-exchange column (Bio-Rad laboratories, Hercules, CA, USA) connected in series to a refractive index detector (RID-6A, Shimadzu, Kyoto, Japan). The column temperature was 45℃, while the mobile phase was 5 mM sulfuric acid at a flow rate of 0.6 ml/min. Ethanol concentrations were corrected for evaporation by using the degree of reduction balance. No other products were detected by HPLC. Cell dry weight was determined by filtering a known volume of culture broth through a pre-dried (350 W for 4 min in a microwave oven), pre-weighed Supor membrane of 0.45-μm (Gelman Sciences, Ann Arbor, MI, USA). The filter was weighed again after being washed with three volumes of distilled water and dried in a microwave oven at 350 W for 8 min. The difference in the filter weights was used to calculate the dry weight. Dry weights were determined in duplicates.

For analysis of gene sequences, primers were designed to amplify both the promoter region and the open reading frame (ORF) of the gene by the polymerase chain reaction (PCR) technique. The PCR products were cloned into the sequencing vector pGEM-T Easy® (Promega) and transformed into *E. coli* DH5α. Transformants were grown on LB agar plates containing ampicillin and X-gal for selection of white (indicating the presence of an inserted DNA fragment in the pGEM-T Easy vector) Ap^R colonies. Plasmids were purified with Qiaprep® Spin Miniprep Kit (Qiagen GmbH, Hilden, Germany) and sequenced. Cycle sequencing was performed using the BigDye™ Terminator v3.1 sequencing kit (Applied Biosystems, Foster City, CA, USA) and reactions were analysed in either an ABI3100 (Applied Biosystems, Foster City, CA, USA) automated sequencer or in a Genetic analysis system SCE2410 (SpectruMedix LLC, State College, PA, USA).

5.3.6. Enzyme assay. Crude cell extracts were prepared from fermenting cells (Eliasson *et al*. 2000). Protein concentrations were determined by using the BCA Protein Assay Kit® (Pierce, Rockford, IL, USA) with bicinchoninic acid (BCA) as standard. Xylose reductase and xylitol dehydrogenase activities were determined by adapting previously described assay procedures (Eliasson *et al*. 2000) for use in a microtitre plate reader (Powerwave-X, Bio-tek Instruments, Winooski, VT, USA) whereby the assay mix had a final volume of 0.2 ml. Assays were performed in triplicate. One unit of enzyme activity was defined as the amount of enzyme needed to either reduce or oxidise 1 μmol of NAD(P)H per min under the specified conditions.

5.3.7. RNA extraction and real time analysis. Total RNA was obtained from selected fermentation samples of YUSM and Y-X by using the FastRNA® Pro Red Kit and FastPrep® Instrument (Qbiogene, Morgan Irvine, CA, USA), according to the manufacturer's protocol. RNA concentrations and purity were determined by using the NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and by agarose gel electrophoresis. To eliminate contaminating DNA in samples,

DNAaseI (Roche) treatment was performed when necessary. Complementary DNA (cDNA) from samples of total RNA was synthesised by using the reverse transcriptase from Superscript III RT-PCR kit (Invitrogen, Carlsbad, CA, USA), following the instructions in the manual. cDNA concentrations were measured using the NanoDrop® ND-1000 spectrophotometer and ≈ 70 ng of cDNA was used as a template for real-time PCR experiments, carried out in a LightCycler® (Roche) instrument. Transcripts of *XYL1*, *XYL2*, *XKS1* and *GPD2* (encoding glycerol 3-phosphate dehydrogenase) were analysed from samples obtained during fermentation. *ACT1*, encoding β-actin, was used as the housekeeping gene to normalise expression values (Giuletti *et al*., 2001; Bleve *et al*., 2003; Divol *et al*., 2006). Primers used for the synthesis of probes for each of these genes were designed using the software available at the primer3© website (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (Rozen and Skaletsky, 2000) and are listed in Table 5.2. The *ACT1* primers used were the same as those reported by Divol *et al*. (2006). Reactions were carried out in capillaries containing the template cDNA, primers and SYBR-green mix from Quantitect® SYBR-green PCR mix (Qiagen, Hilden, Germany), according to manufacturer's instructions. The reaction steps included the following: initial denaturation at 95℃ for 15 min, followed by 50 cycles at each of the following temperatures and times – 95℃ for 15 sec; 56℃ for 10 sec; 72℃ for 10 sec.

TABLE 5.2 Primers used for synthesis of probes for real-time PCR analysis

Name	Sequence
*Ps*XYL1-F646(Q)	5' – TTC GGT CCT CAA TCT TTC GT – 3'
*Ps*XYL1-R758(Q)	5' – GCT GGA GAC TTA CCG TGC TT – 3'
*Ps*XYL2-F441(Q)	5' – AGA CCA CGT CAG CTT GGA AC – 3'
*Ps*XYL2-R567(Q)	5' – AAG ACC AAC AGG ACC AGC AC – 3'
XKS1-F1395(Q)	5' – TTC AAA CGC AAG CTC ACA AC – 3'
XKS1-R1528(Q)	5' – CGT TTT TAG AAG CCC CAC CT – 3'
GPD2-F951(Q)	5' – TCA AAG GCT GGG TTT AGG TG – 3'
GPD2-R1123(Q)	5' – AGG CTG ACT TAC CGG TCT TG – 3'

After the completion of the QPCR reaction, melting curve analysis was performed. Samples were heated to 95℃, cooled to 56℃ for 15 sec before being ramped back to 95℃ in 0.1℃/sec increments. The melting temperatu res of the samples (T_m) were determined using the LightCycler Data Analysis® software. No primer-dimers were observed. Data obtained from the real-time PCR runs were analysed using the data analysis software provided by the manufacturer. The relative expression levels of the genes of interest in YUSM and Y-X were determined using the mathematical model proposed by Pfaffl (2001) and the assumption-free method proposed by Ramakers *et al*. (2003).

5.4 RESULTS

5.4.1. Development of a xylose-fermenting recombinant *S. cerevisiae* mutant

S. cerevisiae strain type CEN.PK 2-1D was transformed with the integrative plasmid pUSM1001, which contained the *P. stipitis XYL1* and *XYL2* genes, to create the reference strain YUSM. For developing the mutant, EMS was used as the mutagen with growth on xylose plates as the screening procedure. After 5 days of incubation at 30℃, colonies appeared at a rate of 1-5/10^8 cells. From a subsequent second screening on xylose medium, the fastest growing strain was isolated and named Y-X.

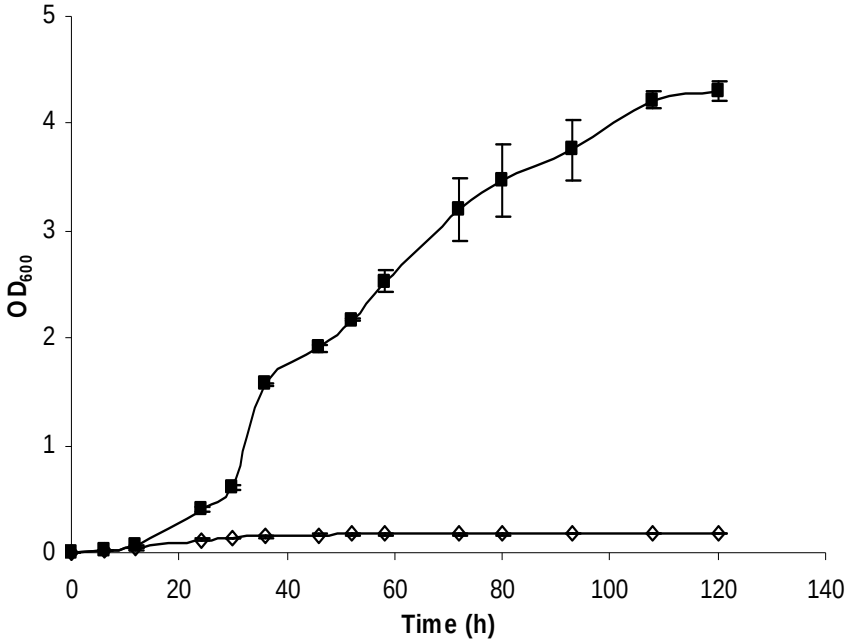

FIG. 5.1 Growth profile of the two recombinant *Saccharomyces cerevisiae* strains – parent strain YUSM (◇) and the mutant strain Y-X (■) in SCX medium at 30℃ and 200 rpm in orbital shaker. Values represented are average of triplicates and the standard deviation (SD) is indicated by error bars.

5.4.2. Growth analysis

Growth of strains Y-X and YUSM was assessed in defined mineral medium with xylose (20 g/l) as the sole carbon source (Fig. 5.1) at 30℃ and 200 rpm. The mutant strain Y-X had a growth rate of 0.042 /h on xylose, which represents a more than 20-fold increase compared to the parental strain YUSM.

5.4.3. Genetic analysis

The mutated strain was crossed with mating strains CEN.PK 113-7A and YUSM 1001d to obtain a heterozygous diploid CEN.PK113-7A/Y-X and a homozygous diploid YUSMd/Y-X, respectively. To evaluate the dominant/recessive characteristics of the

xylose phenotype, cell growth was observed in rich media with different carbon sources, as shown in Fig. 5.2. In all cases, growth differences were visually observed for the homozygous diploid and the heterozygous diploid. Similar results were obtained when diploids isolated independently from identical crosses were tested. This suggests that the xylose phenotype was dominant. Growth of the various strains on solid media also indicated that the mutant (and its derivative diploids) did not show any difference in utilising carbon sources other than xylose.

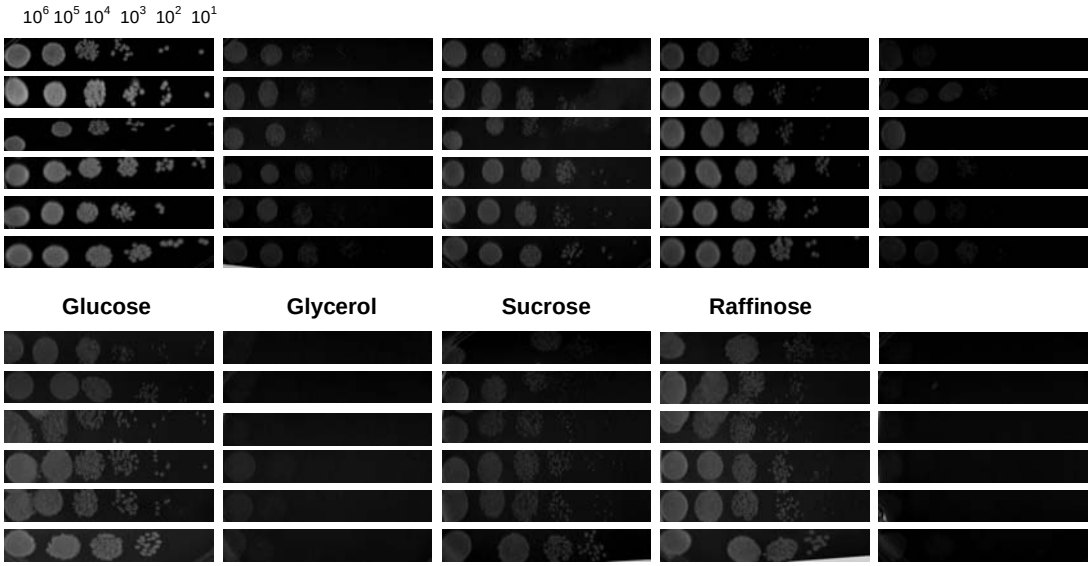

FIG. 5.2 Growth profile of the different haploid and diploid *Saccharomyces cerevisiae* strains developed, on rich solid media with different carbon sources. The top six rows represent strains grown aerobically, while the bottom six represent anaerobic growth. In both sets, the strains represented are: (from top to bottom) YUSM, Y-X, CEN.PK, CEN.PK/Y-X, CEN.PK/YUSM and YUSM/Y-X respectively. Each strain was serially diluted from 10^6 cells and spotted sequentially from left to right. Aerobic growth observed after 48 h and anaerobic growth observed after 72 h.

5.4.4. Fermentation

Anaerobic batch cultivation with the X-Y mutant and its parental strain YUSM was performed in controlled bioreactors using defined mineral medium containing 20 g/l glucose and 50 g/l xylose, supplemented with ergosterol and Tween 80 necessary for anaerobic growth of *S. cerevisiae* and the requisite amino acids as specified in the Materials and Methods section. The X-Y mutant consumed xylose twice as fast as its parental strain (Fig. 5.3). On the other hand complete glucose consumption took twice as long for the mutant strain as for the parent strain. Once glucose was depleted xylose consumption became negligible for the parental strain YUSM. It converted xylose mainly to xylitol, while ethanol was primarily formed from glucose (Figs. 5.3 and 5.4). Glycerol formation was in the range of 2 g/l. On the contrary, the mutant strain Y-X continued xylose consumption even after glucose depletion. It utilised more xylose and converted

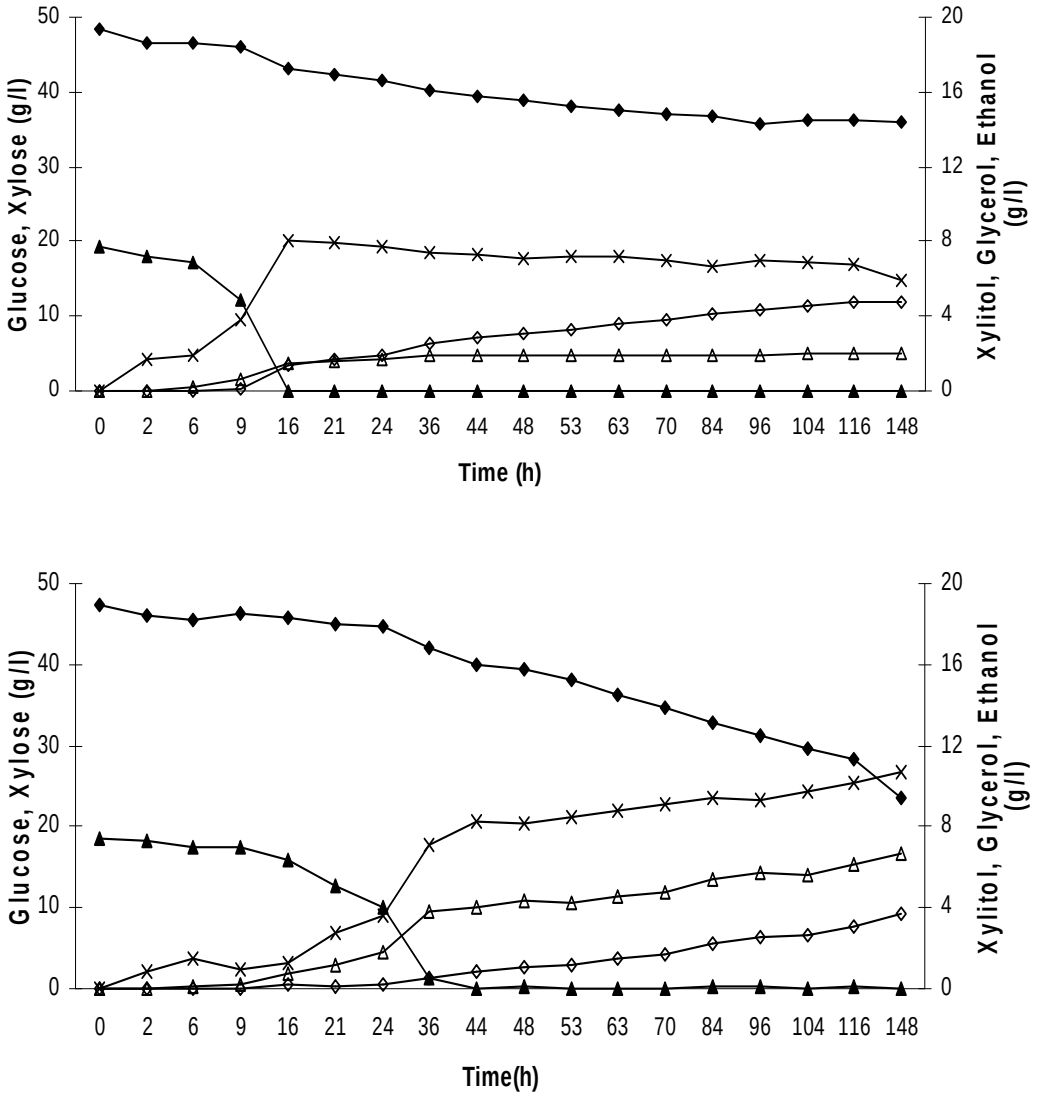

FIG. 5.3 Sugars utilised and metabolites formed during batch fermentation in minimal medium with 20 g/l glucose and 50 g/l xylose under complete anaerobic conditions at 30℃ and 200 rpm with parent strain YUSM (top chart) and mutant strain Y-X (bottom chart). Glucose -▲; Xylose -♦; Xylitol - ◊; Glycerol - Δ; ethanol – ×. Values represented are average of duplicate trials (SD <5%).

the sugar to ethanol and glycerol at the expense of xylitol formation (Fig. 5.4). While YUSM had a xylitol yield of 0.37 g/g sugar consumed, the corresponding figure for Y-X was only 0.20 g/g consumed sugar. For both the strains, acetate and biomass production were negligible under the chosen conditions.

5.4.5. Enzyme activity

The XR and XDH activities for the two strains are summarised in Table 5.3. The XR activity was determined with the two cofactors, NADPH and NADH. Both NADPH- and

NADH-dependent XR activities for Y-X were at least twice as high as those for YUSM. Furthermore, the XDH activity for Y-X was 5-fold increased. The XR/XDH ratio for Y-X was between 0.04 (NADH) and 0.07(NADPH), while for YUSM it was between 0.06 (NADH) and 0.16 (NADPH).

TABLE 5.3 Specific enzyme activities of control strain and mutant during fermentation with glucose and xylose at 30℃

Strains	XR		XDH
	NADPH	NADH	
YUSM	0.15 ± 0.01	0.056 ± 0.01	0.97 ± 0.002
Y-X	0.32 ± 0.02	0.167 ± 0.02	4.35 ± 0.01

Activities with the cofactors given are expressed as U/mg of protein from cell crude extract and are mean values of triplicates. SD values are represented after the ± sign.

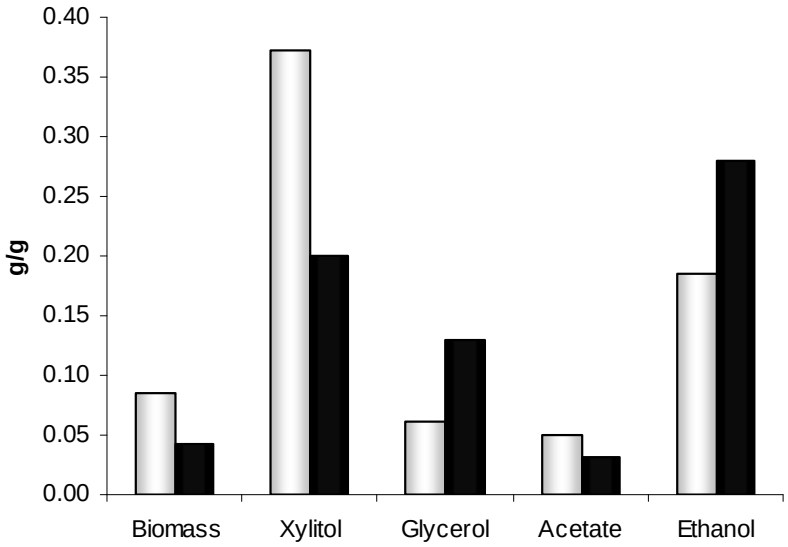

FIG. 5.4 Yields of various metabolites formed from total sugars consumed, expressed in g/g, during anaerobic fermentation by YUSM (white bars) and Y-X (black bars). Values represented are averages of two individual experiments (SD<5%).

5.4.6. Transcriptional expression of selected genes

Because the mutant showed increased xylose utilisation and glycerol production, the transcriptional expression of genes in the xylose metabolic pathway – XYL1, XYL2 and XKS1 – and the gene that is functional during anaerobic glycerol formation – GPD2 – (Ansell *et al.*, 1997; Björkqvist *et al.*, 1997; Valadi *et al*., 2004) were analysed by real-time RT-PCR. Selected samples from anaerobic fermentation representing the glucose phase (when both glucose and xylose were present in the bioreactor), and the xylose phase (when glucose was completely absent) were used to isolate total RNA.

Complementary DNA (cDNA) was reverse-transcribed from these samples. Real-time RT-PCR was performed for each of the four genes mentioned above, using gene-specific primers. The PCR efficiencies were usually ≥80%, determined by using the LinRegPCR© program (Ramakers *et al.*, 2003) in order to follow an assumption-free method of calculating the relative expression. Based on the calculations, the relative expression profiles of the four genes of the X-Y mutant to those of the parent strain YUSM are presented in Fig. 5.5. All four genes tested showed higher levels of expression in Y-X at all four time points. The increased levels of XYL1 remained consistent throughout the fermentation, while XYL2 transcript levels fluctuated between time points. XKS1 showed the highest increase in expression followed by GPD2 at 96 h. XKS1 levels were markedly higher when glucose was completely depleted.

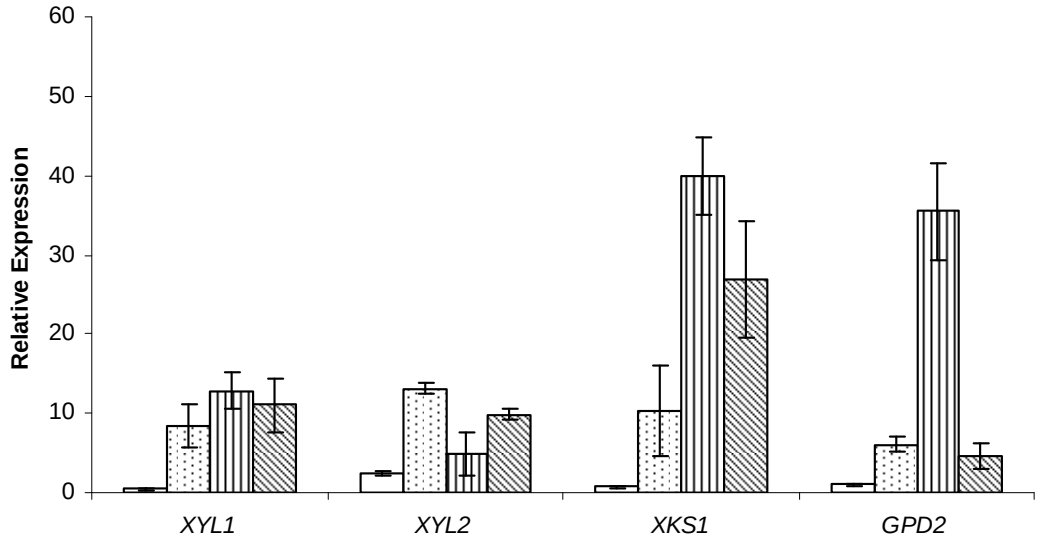

FIG. 5.5 Relative expression levels of four genes (given in the X-axis) of Y-X to that of YUSM during fermentation in minimal medium with 20 g/l glucose and 50 g/l xylose under anaerobic conditions, as determined by real-time PCR analysis of cDNA transcripts obtained during 2 h (white bars), 24 h (dotted bars), 96 h (vertical striped bars) and 144 h (diagonal striped bars) of fermentation. SD represented as error bars.

5.5 DISCUSSION

Most studies aimed at the development of xylose-fermenting *S. cerevisiae* strains report on strains overexpressing the first three genes involved in xylose conversion, *viz*. *XYL1, XYL2* and *XKS1*. Among the various strategies established for engineering yeast strains, random mutagenesis is an approach wherein the desired characteristic could be obtained without the knowledge of the exact factor(s) responsible for that desired change. Previously, Wahlbom *et al*. (2003a) randomly mutagenised a recombinant *S. cerevisiae* strain overexpressing all three genes of the xylose pathway and developed a mutant with improved properties. The current study was performed to create a random

mutant of a haploid recombinant *S*. *cerevisiae* strain overexpressing the *P*. *stipitis XYL1* and *XYL2* genes without overexpressing a cloned version of the endogenous *XKS1* gene and to evaluate its performance during anaerobic xylose fermentation. This could be seen in light of the recent development of a non-recombinant *S*. *cerevisiae* strain that showed increased XR and XDH (but not XK) activities and improved xylose growth by using a directed evolutionary engineering strategy (Attfield and Bell, 2006). Thus the parent strain YUSM was randomly mutagenised by EMS treatment. Since faster xylose utilisation was one of the desired goals, screening the mutants for growth on xylose plates constituted an effective selection procedure. The mutant strain, Y-X, showed a 20-fold increase in growth rate under aerobic conditions and more than twice the amount of xylose consumption during anaerobic fermentation. The mutant strain also showed slower glucose utilisation than the parent during fermentation, similar to that reported by Sonderegger *et al*. (2004). However, growth profiles of the mutant strain Y-X did not differ on other carbon sources tested.

During anaerobic batch fermentation the Y-X mutant was able to assimilate more xylose than the parent. This could be due to increased XR and XDH activities as reported by Eliasson *et al*. (2001), Jeppsson *et al*. (2003) and Karhumaa *et al*. (2007). When the enzyme activities for the mutant and the parent strains were compared, it was found that the mutant had at least 2-times more XR activity and 4-times more XDH activity than the parent. Real-time RT-PCR analysis showed a 10-fold increase of *XYL1* and *XYL2* expression for Y-X when compared to those of YUSM. The ratio of XR/XDH in the Y-X mutant was closer to 0.06 as described by Walfridsson *et al*. (1997) for efficient anaerobic xylose fermentation to ethanol. The reduction in xylitol levels, coupled with an increase in ethanol and glycerol suggested an altered cofactor affinity for the XR reaction, probably an increased preference for NADH. However, when XR activity was measured with the two cofactors, the increase was found to be proportional for both, meaning that the enzyme still preferred NADPH. Previously, Kuyper *et al*. (2004) proposed that at a NADPH:NADH ratio of one, the by-products for anaerobic xylose fermentation would be xylitol, glycerol and ethanol. However, Y-X showed 2-times more NADPH-dependent activity than NADH-dependent activity (Table 5.3). Sequence verification of the two ORFs and the respective promoter regions from the Y-X mutant did not show any mutation(s).

The altered by-product profile for the Y-X mutant suggested a shift in the metabolic flux towards redox balancing, as more glycerol was formed. For YUSM, which lacked XK overexpression, xylitol was the major by-product. It fits with the well-documented importance of xylulokinase overexpression for xylose fermentation to products other than xylitol (Ho *et al*. 1998; Eliasson *et al*. 2000; Richard *et al*. 2000; Johansson *et al*., 2001; Toivari *et al*., 2001; Jin *et al*., 2003; Karhumaa *et al*., 2005). Real-time RT-PCR analysis revealed a 40-fold increase in *XKS1* transcripts for Y-X, but subsequent sequence verification of the ORF together with the promoter region did not show any mutations. Thus, the increase in *XKS1* transcript levels can probably be ascribed to mutations in other regulatory genes or an increase in copy number. Increased glycerol

formation by Y-X suggested an increase in the glycerol flux and since *GPD2* is the gene involved in glycerol production under anaerobic conditions (Ansell *et al*., 1997; Björkqvist *et al*., 1997; Valadi *et al*., 2004), transcripts of this gene were compared between YUSM and Y-X. The results showed *GPD2* levels to be at least 5-times higher in Y-X (Fig. 5.5). Glycerol formation could also be due to redox-related changes during fermentation. A number of genes responsible for redox maintenance are present in the yeast (Bakker *et al*., 2001; Rigoulet *et al*., 2004). However, a few have been considered to be of importance during xylose fermentation. The coding sequences and promoters of various redox-related genes, mainly the endogenous aldose reductases (Träff *et al*., 2002; Träff-Bjerre *et al*., 2004) were verified but no mutation(s) could be determined.

The yield of fermentation by products by the mutant strain Y-X was comparable to that of strains overexpressing XR, XDH and XKS. Although a direct comparison was difficult due to the differences in fermentation procedures (Eliasson *et al*. 2000; Toivari *et al*. 2001), it was possible to compare the yield of xylitol and ethanol as well as the enzyme activities. Mutant strain Y-X showed lower xylitol and ethanol yields than strain TMB3001 (Eliasson *et al*. 2000) [0.20 g/g against 0.39 g/g xylitol; 0.29 g/g against 0.34 g/g ethanol] but higher glycerol yield [0.13 g/g against 0.078 g/g]. When compared with the XK overexpressing strain reported by Toivari *et al*. (2001), the mutant strain Y-X showed lower xylitol [0.2 g/g to 0.41 g/g], higher ethanol [0.29 g/g to 0.12 g/g] and glycerol yields [0.13 g/g to 0 g/g] under strict anaerobic conditions.

Although the presence of endogenous xylose pathway genes (Toivari *et al*. 2004 and references therein) had been established in *S*. *cerevisiae*, most studies reporting on improving xylose utilisation and fermentation by *S*. *cerevisiae* focussed on the expression of heterologous genes and the development of recombinant yeasts. Recently Attfield and Bell (2006) reported a non-recombinant *S*. *cerevisiae* strain evolutionarily engineered to utilise xylose. Compared with the parent strain this strain exhibited a significant increase in growth, which was coupled with a 4-fold and 80-fold increase in XR and XDH activities, respectively. However, there were no apparent differences in XK activity between the parent and the evolved strains. No data of xylose fermentation for the non-recombinant yeast has been reported so far. The parent strain used in the current study is similar to the aforementioned strain in that the XK was not overexpressed. However, due to a combination of metabolic engineering and random mutagenesis, the mutant strain Y-X displayed increased xylose utilisation and improved anaerobic xylose fermentation properties. Under anaerobic xylose fermentation conditions, the mutant showed increased levels of XR, XDH and XK. The development of this mutant has reiterated the requirement of increased xylulokinase activity for anaerobic xylose fermentation to obtain products other than xylitol. Based on the physiological characteristics exhibited by Y-X, attempts were made to establish the mutation(s) in Y-X, in vain. However, genome-wide characterisation analysis of previous random mutants (Wahlbom *et al*., 2003b) and evolutionary mutants (Sonderegger *et al*., 2004) have shown that the changes were not specific to a specific gene/pathway but were widespread at different metabolic and regulatory levels resulting in the improved

characteristic. As none of the structural genes investigated showed any mutations, it could be suggested that there might have been an increase in copy numbers. Proteome analysis might reveal the presence, if any, of post-translational modifications in the mutant; but the work previously reported (Salusjärvi *et al*. 2003) has shown that the changes were widespread at the protein level too.

5.6 Acknowledgements

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Chapter 6

GENERAL DISCUSSION AND CONCLUSIONS

6.1 GENERAL DISCUSSION AND CONCLUSIONS

Effective utilisation of xylose by *Saccharomyces cerevisiae* has been of particular interest in the quest for efficient bioethanol production during the past two decades. Xylose is the second most abundant carbon source present in plant biomass such as lignocellulose and if it can be converted to ethanol by fermentation, several problems faced by the renewable fuel industry could be alleviated. Using bioethanol as a fuel would help to reduce greenhouse gases in the atmosphere. However, no microorganisms have been found in nature capable of fermenting xylose to ethanol under anaerobic conditions, which is an important criterion in an industrial situation. Research has been focussed on developing a recombinant *S. cerevisiae* strain, containing xylose pathway genes from *Pichia stipitis*, capable of anaerobic xylose fermentation. Xylose consumption in this strain was found to be low due to possible cofactor differences between xylose reductase and xylitol dehydrogenase that resulted in a redox imbalance. The recombinant strain thus produced xylitol from xylose under anaerobic conditions rather than ethanol. Steps to overcome this imbalance through the addition of external electron acceptors helped to overcome the problem to a certain extent but these could not be applied industrially. Expression of heterologous transhydrogenases – enzymes that catalyse the conversion of NADPH to NADH and vice versa – in *S. cerevisiae* has been unsuccessful (Jeun *et al*. 2003). Expression of bacterial xylose isomerase – the enzyme that converts xylose to xylulose via a single isomerisation step instead of the two-step process in eukaryotes – has also met with limited success despite the efforts of a host of researchers. However, through systematic metabolic engineering and genetic manipulations, strains capable of anaerobic xylose fermentation have been developed although the xylose utilisation and fermentation abilities of these strains were affected by various shortcomings within the strains – the most predominant being the redox imbalance. The discovery of a fungal xylose isomerase and its successful expression in *S. cerevisiae* raised hopes of resolving the redox imbalance issue and developing a strain capable of yields closer to the theoretical maximum from xylose fermentation. Although this strain showed improved xylose utilisation, its fermentation was still not effective indicating a need for more genetic modification to improve its characteristics. It is safe to say that with recent advances, development of an ideal xylose-fermenting strain is closer than it was in the late 20th century.

In an industrial lignocellulose fermentation using a single organism such as yeast, it should be noted that the hydrolysate would be composed of a mixture of sugars. Presence of hexoses, such as glucose, would invariably affect the utilisation of other sugars due to the phenomenon of carbon catabolite repression. This will adversely affect the fermentation by prolonging the time and increasing the cost of the process. In the case of xylose, reports vary on the effect of glucose on this sugar. While there is unanimous acceptance that the hexose transporters are involved in xylose uptake, the

issues of glucose repression of xylose and the nature of this repression are debatable. Belinchon and Gancedo (2003) suggested that xylose itself could be a repressive carbon source. It is imperative for industrial applications to develop a yeast strain that can ferment both sugars almost simultaneously or at least in a shorter time. Thus, genetic manipulations of the recombinant *S. cerevisiae* strain, YUSM 1001, by deleting certain regulatory regions of the *MIG1* gene created a strain that was constitutively repressed by glucose. This strain, when subjected to nitrogen-limited conditions in chemostat cultivation, showed a 40% increase in xylose utilisation even in the presence of a high glucose concentration in the medium. This demonstrated that, with the use of such a strain, the cost and time of fermentation could be reduced because the yeast metabolises the two sugars in a shorter time. However, because of the lack of xylulokinase overexpression, almost all the xylose utilised was converted to xylitol and not ethanol. It was plausible to hypothesise that xylose utilisation could be enhanced with a strain overexpressing the *XKS1* gene. Additionally, by expressing the *mig1*-disruption cassette in a xylose-fermenting strain more suitable than the one used in this study (for instance, Eliasson *et al*. 2000; Sonderegger and Sauer 2003; Kuyper *et al*. 2004), the xylose utilisation capacity could be improved. The question of whether xylose is repressed or is repressive remains unanswered.

In Chapter 4, a cDNA library screening of *Neurospora crassa* for novel xylose-metabolising genes was described. *N. crassa* is a natural xylose-utilising fungus capable of converting cellulosic biomass, but is unable to grow anaerobically. Although the presence of xylose pathway genes has been reported and enzyme activity measured in this organism, no genes have been isolated nor sequences identified. With the availability of the whole genome sequence of *N. crassa* through the genome sequencing project, Woodyer *et al*. (2005) identified a xylose reductase (XR) gene from the fungus and characterised the enzyme. However, to the best of the author's knowledge, no *N. crassa* XR gene has been expressed in *S. cerevisiae*. During the screening for strains with improved xylose utilisation capacity, transformants that contained a 1000bp fragment from the cDNA library were isolated. Subsequent sequence analysis of the fragment showed a 900-bp open reading frame (ORF), which identified 100% with a previously unidentified protein in the *N. crassa* genome. BLAST analysis of this protein showed a similarity to several aldo-keto reductases. Thus, the gene was cloned in different recombinant *S. cerevisiae* backgrounds and evaluated for xylose growth, fermentation and enzyme activity to determine that it was in fact an XR-encoding gene. This would be the second XR-encoding gene of *N. crassa* to be reported. Many filamentous fungi are known to possess several isozymes. The screening and isolation of a XR gene led to the speculation that this XR could have different cofactor specificity from most of the NADPH-specific XRs isolated so far, but this *N. crassa* XR also had exclusive NADPH-specificity with little or no NADH-specific activity. When expressed in *S. cerevisiae*, the *N. crassa* XR-containing strain had a different product profile from the strain containing *P. stipitis* genes. This led to the idea that this enzyme might cause changes in the metabolic flux of the organism, which is an

aspect worth investigating in the future. Also, purification and complete characterisation of this enzyme would help in future studies. Apart from the application in the ethanol industry, aldose reductases also have applications in the medical field. The screening of more organisms for xylose-metabolising genes could shed more light on the understanding of xylose fermentation.

Strains with improved characteristics are usually developed by strenuous genetic manipulations of known metabolic pathways or by random mutagenesis with appropriate selection pressure. Conventional methods of introducing mutations include exposure to mutagens such as ultraviolet (UV) light and ethyl methane sulfonate (EMS). However, with the advent of molecular methodologies it has become possible to create site-specific mutations that can alter the characteristic of an enzyme thereby affecting the whole pathway. A basic requirement of mutagenesis is a strong screening procedure or selection pressure. With the advances made in methods and high-throughput techniques such as the use of microarrays, it has become possible to characterise mutations on a genome-wide scale. With this in mind, the reference strain, YUSM, was subjected to random mutagenesis and screened for improved growth on xylose medium. The fastest growing mutant strain, Y-X, was subjected to physiological characterisation. In liquid media under aerobic conditions, it showed a 10-fold increase in growth rate. Growth in other carbon sources showed no differences to the parent strain. Genetic analysis revealed the mutation to be dominant. Anaerobic batch fermentation showed a 70% xylose utilisation compared to a less than 30% xylose utilisation by the parent strain. The product profile was also different, with more glycerol production than ethanol and, more importantly, less xylitol formation. This could suggest that the mutation might be in one of the following: (i) one of the xylose pathway genes because the mutant showed higher growth and activity in xylose; (ii) one of the glycerol pathway genes as more glycerol was formed; (iii) one of the *MIG1*-related genes probably, due to reasons aforementioned; or (iv) one of the non-specific aldose reductases. To this end, the sequence of several genes together with their promoters representing the categories mentioned above, were verified but no mutations were observed. During the time of this study, several microarray and proteome analyses of xylose mutants were published (Wahlbom *et al*. 2003; Sonderegger *et al*. 2004; Jin *et al.* 2004; Salusjärvi *et al*. 2004; Kuyper *et al*. 2005). The underlying theme in most of the reports was that the improved characteristics were not due to changes in one particular gene or sets of genes, but rather in the whole central metabolic pathway. Thus it was difficult to point to a particular gene in those mutants as the cause of improved xylose utilisation. However, a real-time PCR analysis of the three xylose pathway genes together with *GPD2* was performed, which showed that the expression levels of these genes in the mutant were higher than those of the parent in xylose-containing media. While it would be tempting to perform a genome-wide transcriptional or translational analysis of the mutant, it would be more prudent and beneficial to try to alter the flux from glycerol towards ethanol production by using some strategies of Nissen *et al*.

(2000), Bro *et al.* (2006) and others to meet the objective of producing ethanol more efficiently.

Based on the different studies, it is once again emphasised that xylulokinase overexpression is critical for efficient xylose fermentation to ethanol. The redox-balancing steps in the cell are also important because they seem to play an important part in enhancing the xylose fermentation profile of the haploid random mutant. While many effective engineering approaches have resulted in yeasts capable of fermenting xylose, there might still be more surprises in nature that can alter the knowledge gained in this field so far.

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Appendix A

Evaluation of polygalacturonase activity in *Saccharomyces cerevisiae* wine strains

Appendix B

Engineering of an oenological *Saccharomyces cerevisiae* strain with pectinolytic activity and its effect on wine

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Evaluation of polygalacturonase activity in *Saccharomyces cerevisiae* wine strains

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Abstract

A total of 61 *S. cerevisiae* strains, 60 of them isolated from wine ecosystems, were evaluated for the presence of the gene encoding endopolygalacturonase (*PGU1*) and for polygalacturonase (PG) activity. Nine strains lack the gene *PGU1* and did not exhibit PG activity on plate assays. Of the 52 strains showing an amplified band corresponding to the size of *PGU1* gene, only 36 degraded polygalacturonic acid (PGA) and 17 did not degrade it at any of the pH values used. The coding region of the *PGU1* gene (ORF YJR153w) was not present in some PG activity negative strains. The *S. cerevisiae* UCLMS-39 strain was selected for its specific activity at different pHs, temperatures and oenological parameters. The temperature and pH optima were 50 °C and 3.5–5.5 respectively and it was only affected by ethanol. The *PGU1* gene was cloned and sequenced. The production of a biologically functional endoPG in *S. cerevisiae* UCLMS-39 brings us a step closer to improving the qualities of outstanding enological yeasts naturally lacking PG activity.

Keywords: *Saccharomyces cerevisiae*; Yeast; Endo-polygalacturonase; *PGU1* gene; Pectinase

1. Introduction

Pectic substances are structural heteropolysaccharides that occur mainly in the middle lamellae and primary cell walls of higher plants. They are primarily composed of galacturonic acid residues joined together by α(1-4)-glycosidic bonds. There are two types of pectins: homogalacturonic pectins, consisting solely of D-galacturonic acid, and rhamnogalacturonic pectins, in which the galacturonic acid chains are discontinued by rhamnose residues joined together by an α(1-2) bond, with possible side chains consisting of sugars such as galactose and arabinose [1]. The D-galacturonic acid residues of both types of pectins may be esterified with methanol, and pectins are further categorized as high-methoxyl or low-methoxyl, depending on their degree of methylation.

Pectinolytic enzymes work by hydrolysing the ester bond between galacturonic acid and methanol (pectin esterases) or by cleaving the glycosidic bonds of specific polymers (polygalacturonases (PG), pectin and pectate lyases), and are synthesized by plants [2] and microorganisms [3]. Fungal or bacterial genes encoding for these enzymes have been cloned and expressed in yeasts [4–6]. There are two types of PGs with widely differing techno-

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logical applications. Exopolygalacturonases (exo-PG) break down the distal groups of the pectin molecule, reducing chain length relatively slowly. Endopolygalacturonases (endo-PG) act randomly on all the links in the chain, reducing molecular dimensions and viscosity more rapidly [7]. Pectins may cause problems in the food industry by giving rise to turbidity and viscosity during the extraction, filtration, and clarification of fruit juices.

The fact that *S. cerevisiae* has been traditionally considered as devoid of PG activity underlines the importance of variability in this species [8]. Blanco et al. [9] showed that when wine is fermented using *S. cerevisiae* strains with PG activity, the clarification process is greatly facilitated and the filtration time is reduced by up to 50% in some cases. It has been reported [10–12] that some *Saccharomyces* strains isolated in wine making possess PG activity.

The *PGU1* gene encoding the endoPG of *S. cerevisiae* strains has been cloned and overexpressed [8,13,14], and the protein sequences of some of them show different amino acids from the protein sequence of the database SGD (http://genome-www.stanford.edu/Saccharomyces).

We therefore aimed to screen 60 *Saccharomyces* strains isolated from wine ecosystems, using the presence or absence of the *PGU1* gene associated with endoPG activity and the expression of such activity in several culture media to determine the relationship between the genetic background and the phenotype. Also, the *PGU1* gene from the strain that showed the best PG activity was cloned, sequenced, and its overexpression evaluated in a haploid *Saccharomyces cerevisiae* strain.

2. Materials and methods

2.1. Strains and plasmids

A total of 60 *S. cerevisiae* strains (UCLMS-1-4,-31, -33, -36-89) isolated from wine ecosystems [15,16] were used, and W3031b (*Mat*α, *ade*2-1*his*3-11, 15*leu2-3, 112 ura3-1 trp1-1 can1-100*) as laboratory strain. *Escherichia coli* DH5α [*end*A1 *hsd*R17 (rk^-mk^+) *sup* E44 *thi*λ^- *rec*A1 *gyr*A, *re*l A1 Δ (*lac*ZY A-*arg*F) U169 (ϕ 80*lacZ*Δ*M*15)] was used as host for cloning (Gibco-BRL). The *E. coli–S. cerevisiae* shuttle plasmid pYES2 (2 μm, *URA3*, *GAL1 P::CYC1 T*) was used in this study (Invitrogen).

2.2. Polygalacturonase activity

2.2.1. Qualitative analysis

PG enzyme production was assayed on agar plates containing 1% w/v polygalacturonic acid (PGA) (Sigma) and 1% w/v glucose at different pHs (8, 5.5 and 3.5) using a modified version of the methods described by McKay [17], Blanco et al. [18] and Charoenchai et al. [19]. The yeast strains were grown for 24 h on YPD. Five μl of media was inoculated onto PGA agar media, incubated at 30 °C for three and five days and developed following the recommendations of the authors cited above. All trials were performed in duplicate.

2.2.2. Quantitative analysis

Quantitative determinations of the activity were performed by a modification of the Nelson method [20] using PGA as a substrate. A standard curve was developed for the range of 0.04–0.1 $\mu mol\,ml^{-1}$ of galacturonic acid (GALA). The strains were grown in 0.67% w/v Yeast Nitrogen Base medium (YNB) supplemented with 1% w/v of glucose (without PGA) at 30 °C without shaking for three days [10], and 100 μl of culture supernatant was incubated at 37 °C in a reaction mix of 500 μl containing PGA (0.1%) and sodium acetate buffer pH 5.5 (50 mM) for 24 h. The reducing power was measured spectrophotometrically at 600 nm at 0 and 24 h, with the measure at 0 h considered as blank. The reaction was stopped with reactive A, described by Milner and Avignad [20], and the results were expressed as $\mu mol\,GALA\,ml^{-1}$ of supernatant ($\mu mol\,GALA\,ml^{-1}\,S$). A number of parameters that might affect enzyme stability during the winemaking process were assayed on UCLMS-39 supernatant. The effect of pH was tested over the range 3–7 (3, 3.5, 4, 5, 5.5, 6 and 7); and the temperature varied over the range 10–60 °C (10, 20, 30, 37, 40, 50 and 60). The concentrations of glucose assayed were 1%, 5%, 10%, 15% and 20% w/v. The influence of ethanol (4%, 8% and 12% v/v), acetic acid (0.1% v/v), glycerol (0.5% v/v) and SO_2 ($50\ \mu g\,ml^{-1}$) were also studied. All determinations were performed in duplicate from three independent cultures.

2.3. PGA degradation during yeast growth

Yeasts were grown in 0.67% YNB medium with glucose 1% w/v and PGA 0.5% w/v in 50 mM acetate buffer pH 5.5, and incubated at 30 °C without shaking for three days. Following incubation, all cultures were precipitated and 100 μl of supernatant was assayed directly for reducing power [20].

2.4. Screening for PGU1 gene using polymerase chain reaction

The coding region of the *PGU1* gene (ORF YJR153W from *S. cerevisiae* Genome Database) is 1086 bp long and could be amplified using genomic DNA obtained from *S. cerevisiae* strains as template [21] and two designed primers consisting of a *Bam*HI (underlined) restriction site linked to sequences flanking the ORF. CT-*PGU1* (5′-CTGCGGATCCTTAACAGCTTGCACCAGATCCAG-3′) and NT-*PGU1* (5′-GAGAGGATCCATGATTTCTGCTAATTCATTACTTATTT-3′).

The amplification reaction was performed in a Perkin–Elmer model 2400 Thermocycler under the following conditions: 50 μl reaction mixture was prepared with 1.25U *AmpliTaq* Gold DNA polymerase (Perkin–Elmer), 1 μM of each primer (Roche), 0.2 mM of each dNTP (Perkin–Elmer), 1x *AmpliTaq* Gold Buffer II (Perkin–Elmer), 2.5 mM $MgCl_2$ (Perkin–Elmer), and 5–30 ng yeast DNA. The mixture was subjected to an initial denaturing cycle of 10 min at 95 °C, followed by 30 cycles comprising 30 s at 95 °C, 30 s at 58 °C, and 1 min at 72 °C, with a final extension step of 10 min at 72 °C. Amplification products were analysed by electrophoresis on a 1.5% (w/v) agarose gel, in TBE 1x buffer stained with ethidium bromide (0.5 $\mu g\,ml^{-1}$).

2.5. Southern-blot analysis

Standard methods were used for yeast genomic DNA isolations [22]. The digoxigenin non-radioactive nucleic acid labelling and detection system was used for the Southern hybridization to confirm the presence or absence of *PGU1* gene in the *S. cerevisiae* strains. Southern hybridization was performed with the DIG Labeling Kit from Roche Biochemical Products (South Africa), using the method described in DIG's Application Manual. The genomic DNA of yeasts was cut once inside the respective PG gene, and once at both flanking genome sequences with the enzyme *Nco*I. The probe used was the 0.5 kb-long *EcoR*V fragment from *PGU1* gene.

2.6. Transformation protocols

E. coli strain was transformed following the method described by Inoue et al. [23], and the transformants were selected in LB medium complemented with ampicillin 100 $\mu g\,ml^{-1}$. *S. cerevisiae* transformation was achieved using the LiAc/SS-DNA/PEG protocol [24]. The selection of transformants was done on SC^{-Ura} medium. Standard methods for plasmid isolation, restriction and ligation reactions were used [22]. The *PGU1* gene amplified from *S. cerevisiae* 39 strain was cloned into the *Bam*HI site of yeast expression vector pYES, obtaining pGUa/b plasmids.

2.7. DNA sequencing

pGUa/b (*PGU1*b, pGU4a, pGU3b, pGU8b) were used as templates for sequencing *PGU1* gene previously extracted using QIAwell 8 Plasmid Kit (Qiagen). DNA sequences were determined by the dideoxynucleotide chain termination method [25] using oligonucleotides NT-*PGU*1, CT-*PGU*1, 2*PGU*1 (5′-GAGACGGTGATTCGGC-3′) and 3*PGU* 1 (5′-GAACCGGAATTCACAGC-3′) to obtain an adequate reading in both directions. The sequences of all constructs were verified by automated DNA sequencing (Servicio de Secuenciación, Universidad Autonoma de Madrid, Madrid, Spain).

2.8. Statistical analysis

Analysis of variance (one factor) was performed with the statistical programme SPSS (version 11.0) to check whether there were significant differences in the enzymatic activity in the various different study conditions.

3. Results and discussion

3.1. Screening and characterization of pectinolytic wine yeasts

Of the 61 *S. cerevisiae* strains, 25 were unable to hydrolyze PGA on agar plates at any of the pH assayed; the rest did so with different visual intensities depending on the pH.

Of the nine strains that degraded the PGA at the three pHs studied (UCLMS-39, -41, -46, -48, -65, -81, -82, -85, -89), four (UCLMS-39, -81, -85, -89) exhibited high activity.

The strains UCLMS-36, -44, -53, -60, -64, -66, -71, -78 presented activity at pH 3.5 and 5.5; UCLMS -54, -79 and -83 presented activity at 5.5 and 8. Some strains degraded PGA at only one pH: UCLMS-40 at pH 3.5, UCLMS-37, -38, -45, -50, -62, -68, -73, -75 and -86 at pH 5.5 and UCLMS-42, -52, -56, -69 and -70 at pH 8.

Evaluation of this activity on agar plates was most effective at pH 5.5.

3.2. Quantitative polygalacturonase assay

GALA released from PGA was quantified using the supernatants of the strains UCLMS-39, -41, -48, -53, -65, -81, -82, -85 and -89, with different hydrolytic capabilities. Some strains that did not show activity on agar plates were also assayed (UCLMS-1, -31, -33, -43, -63 and W3031b). The first three of these were selected for their good oenological properties [26].

Table 1 shows the relative PG activity, expressed as percentages of the maximum activity for the strains assayed on YNB-glucose (1) and YNB-glucose-PGA (2).

When strains were grown on YNB-glucose and the supernatant (S) was incubated in a reaction mix containing PGA at pH 5.5, 37 °C/24 h, the UCLMS-39 strain was the most active (100%), corresponding to 3.3 $\mu mol\,GALA\,ml\,S^{-1}$, followed by UCLMS-53 (70.3%) and UCLMS-81 (37.8%); the remaining strains presented very limited activity, about 80% less than in the UCLMS-39 strain.

With the inclusion of PGA in the medium of growth it was possible to estimate PG activity in terms of the

Table 1
Relative polygalacturonase activity (RA) for different *S. cerevisiae* strains grown in absence (1) or in presence (2) of PGA, expressed as percentages of the maximum activity

Strains	RA (%) (1)	RA (%) (2)
UCLMS-1	0.1	nd
UCLMS-31	0.3	nd
UCLMS-33	4.5	nd
UCLMS-39*	100	80.9
UCLMS-41	1.6	nd
UCLMS-43	1.7	nd
UCLMS-48*	17.6	83.1
UCLMS-53*	70.3	50.3
UCLMS-63	0.1	nd
UCLMS-65	1.2	nd
UCLMS-81*	37.8	100
UCLMS-82*	7.1	65.6
UCLMS-85*	14.0	89.7
UCLMS-89*	2.9	29.0
W3031b	2.0	nd

nd: Not detected.
* Significant differences ($p \leqslant 0.05$) in (1) and (2) media for the same strain.

appearance of reducing groups directly in the supernatant. This was possible because none of studied strains grow when GALA is the sole carbon source in YNB medium (data not shown), as previously described McKay [10]; although Gainvors and Belarbi [27] found that some strains use pectins as a carbon source only in the presence of other non-fermentable compounds like ethanol, glycerol and lactate.

The maximum value (100%), corresponding to 3.8 $\mu mol\,GALA\,ml\,S^{-1}$, was recorded for UCLMS-81, followed by UCLMS-85 (89.7%), UCLMS-48 (83.1%), UCLMS-39 (80.9%), UCLMS-82 (65.6%) and finally UCLMS-53 (50.0%). The UCLMS-89 strain was able to hydrolyze PGA on solid media with high intensity, even though on quantification assays showed weak activity.

ANOVA of the data obtained in both the studied media showed that the synthesis of this enzyme was significantly increased ($p \leqslant 0.05$) by the presence of PGA in the culture medium for UCLMS-48, -81, -82, -85 and -89. This suggests that for these strains, PG synthesis may be controlled by induction by galacturonates [28]. In contrast, PGA was not required to increase UCLMS-39 and -53 activity.

The strains that did not hydrolyze PGA on agar plates likewise did not hydrolyze PGA on liquid media.

3.3. PGU1 *gene screening using polymerase chain reaction*

PCR was carried out using specific primers to determine the presence or absence of whole *PGU1* gene of 61 yeast strains belonging to the genus *Saccharomyces*. S288C (*MATα*, *SUC2 mal mel gal2 CUP1*) was used as positive control.

In a total of nine strains of 61 studied strains (nearly 15%), the gene could not be amplified. Southern-blot hybridizations were performed to confirm the presence of *PGU*1 gene in the yeast genomes. The results showed that UCLMS-1, -3, -4, -31, -58, -59, -63, -67, -72 do not have the sequence encoding for the gene *PGU*1. Those strains did not exhibit PG activity on plate assays.

Of the 52 strains showing an amplified band corresponding to the size of *PGU1* gene, 36 degraded PGA, as shown by zones of different visual intensity around the colonies, and 17 did not degrade PGA at any of the pH values used. This third group of strains, showing a *PGU1* gene band but no ability to degrade PGA, may contain a promoterless PG gene or a non-functional transcription regulatory sequence for the gene, or they may express post-transcriptional modifications that result in a non-active peptide.

The results present here shows that such inconsistent occurrence of the *PGU1* gene in different strains of *Saccharomyces* suggests that this gene is not typical of *S. cerevisiae*, and hence may be used as an intraspecific marker for genetic and phenotypic characterisations. Our results disagree with those reported by Blanco et al. [13] and Naumov et al. [29], who suggested that the coding region of the *PGU1* gene (ORF YJR153w) was probably present and conserved in both PG positive and negative strains.

3.4. Properties of the UCLMS-39 polygalacturonase

Based on the results, the strain UCLMS-39 was selected for specific activity analysis. The UCLMS-1 strain was used as a negative control in all the assays. PG production in the strain UCLMS-39 is constitutive, because PGA is not required to induce the synthesis of this enzyme (Table 1). Additionally, the PG activity of the UCLMS-39 strain was primarily non-cell-associated.

The PG activity of *S. cerevisiae* UCLMS-39 strain using a modified version of the Nelson method [20] was measured at different pHs and temperatures (Fig. 1). The optimum pH for PGase activity was 3.5 (Fig. 1(a)); nevertheless, analysis of variance ($p \leqslant 0.05$) revealed no significant differences between 3.5 and 5.5.

The optimum temperature for PG was 50 °C (Fig. 1(b)), and there were significant differences at all the experimental temperatures. At temperatures below 50 °C the activity decreased gradually, whereas incubation at temperatures over 50 °C resulted in rapidly declining activity.

Yeast pectinases described by other authors [28] have also shown an optimum pH in the acidic region (3.5–5.5) and temperature optima between 40–50 °C, except for *S. cerevisiae* endoPG [30] and *Cryptococcus albidus* [31], for which the optimum temperatures were 25 and 37 °C respectively.

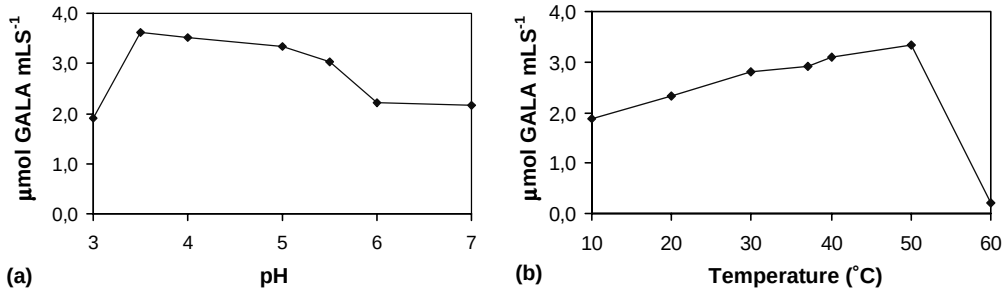

Fig. 1. Effect of different pH values at 37 °C (a) and temperatures at pH 5.5 (b) on the polygalacturonase activity in UCLMS-39 strain.

We further assessed the influence of various potential PG inhibitors that are present during wine making (Table 2). PG activity of the *S. cerevisiae* UCLMS-39 was indeed greatly inhibited by 4%, 8% and 12% v/v of ethanol (72%, 61% and 54% of the maximum value for each of these percentages respectively), however, glucose, acetic acid, glycerol and SO_2 had no significant inhibitory effect on UCLMS-39 PG activity.

PG activity of the *S. cerevisiae* UCLMS-39 was indeed greatly inhibited by ethanol, as described by Takayanagi et al. [32]. This may indicate that the enzyme could preferentially utilize water rather than alcohol as an acceptor for the GALA residues during catalysis of PGA, resulting in lowered reaction rates. However, glucose, acetic acid, glycerol and SO_2 had no significant inhibitory effect on UCLMS-39 PG activity; nevertheless, other authors have found that SO_2 [32] inhibited the enzyme activity.

3.5. Cloning and sequencing of PGU1 gene from S. cerevisiae *UCLMS-39*

In order to confirm that *PGU1* gene from UCLMS-39 encodes an active protein, it was cloned into pYES2 under the control of the inducible GAL promoter. Two types of plasmids were obtained: pGUa/b (pYES-*PGU1*) (a) in the same or (b) in the opposite orientation of GAL1 promoter. When *S. cerevisiae* W3031b (PG-), was transformed with pGUa and pGUb, all transformed strains were able to hydrolyse PGA on plates, indicating that *PGU1* gene was the one responsible for the PG phenotype.

The activity increased when galactose was used as the carbon source instead of glucose and when *PGU1* gene and *GAL*1 promoter were in the same orientation (pGUa).

The *PGU1* gene of strain UCLMS-39 showed more than 99% nucleotide identity to that of strain S288C (ORF YJR153W). One nucleotide is changed in the coding sequence (position 877). Protein differs by only one amino acid $Ser^{293} \rightarrow Gly$. The 361 amino acid sequence encoded by *PGU1* has a theoretical pI of 8.49 and a calculated molecular mass of 37.256 Da (http://us.expasy.org/tools/pi_tool.html). This amino acid change has also been described for *S. cerevisiae*, SCPP [8], now re-identified as *S. bayanus uvarum* [29] and *S. cerevisiae uvarum*, Scu [14]. Both sequences are functional.

It is demonstrated that *S. cerevisiae* UCLMS-39 strain synthesizes and excretes its own endoPG enzyme into the medium. This PG could be of potential use in enology thanks to its high tolerance of different compounds usually present in wine making. The production of a biologically functional endoPG in *S. cerevisiae* brings us a step closer to improving the qualities of outstanding enological yeasts naturally lacking PG activity.

Table 2
The effects of glucose, ethanol, acetic acid, glycerol and SO_2 upon *S. cerevisiae* UCLMS-39 polygalacturonase activity

Additions	RA (%)
[a]None (control)	100
[a]Glucose 20% w/v	102
[a]Glucose 15% w/v	104
[a]Glucose 10% w/v	103
[a]Glucose 5% w/v	99
[a]Glucose 1% w/v	102
[b]Ethanol 12% v/v	54
[b]Ethanol 8% v/v	61
[c]Ethanol 4% v/v	72
[a]Acetic acid 0.1% v/v	100
[a]Glycerol 0.5% v/v	97
[a]SO_2 50 mg ml^{-1}	107

RA (%). Indicates the experimental value in different conditions expressed as a percentage with respect to the control value.
[a,b,c] Different letters indicate significant differences ($p \leqslant 0.05$) between the parameters studied.

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Engineering of an oenological *Saccharomyces cerevisiae* strain with pectinolytic activity and its effect on wine

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Abstract

A pectinolytic industrial yeast strain of *Saccharomyces cerevisiae* was generated containing the *S. cerevisiae* endopolygalacturonase gene (*PGU*1) constitutively expressed under the control of the 3-phosphoglycerate kinase gene (*PGK*1) promoter. The new strain contains DNA derived exclusively from yeast and expresses a high polygalacturonic acid hydrolyzing activity. Yeast transformation was carried out by an integrative process targeting a dispensable upstream region of the acetolactate synthase locus (*ILV*2), which determines sulfometuron methyl resistance. Microvinification assays were performed on white and red musts with the transformed UCLMS-1M strain and with the same strain untransformed. It was found that the changes in the pectic polysaccharide contents did not directly affect the taste or flavor of the wine. From the data reported, it is deduced that the chief advantage of using the modified strain is that it improves the yield of must/wine extraction, while it also positively affects some variables relating to appearance.

Keywords: *Saccharomyces cerevisiae*; Polygalacturonase activity; *PGU*1; Wine

1. Introduction

Pectinases are enzymes able to degrade pectic substances by hydrolysing the ester bond between galacturonic acid and methanol (pectin esterases) or by cleaving the glycosidic bonds of specific polymers (polygalacturonases, pectin, and pectate lyases), and are synthesized by plants (Ali and Brady, 1982) and microorganisms (Pascualli et al., 1991). Pectins may cause problems in the food industry by giving rise to turbidity and viscosity during the extraction, filtration, and clarification of fruit juices.

Most commercial pectinase preparations used in the food industry are derived from *Aspergillus niger*, which in addition to producing large quantities of these

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enzymes is a GRAS (Generally Recognized as Safe) microorganism. However, this mould secretes other enzymes that are less desirable for the production of wine or fruit juices for instance arabinofuranosidase, which can cause turbidity (Whitaker, 1984).

Given the role played by yeasts, especially of the genus *Saccharomyces*, in fermented products, further research into their pectinolytic enzymes would be useful for two purposes: one, so that yeast can be used to synthesize then purify the enzymes for addition to fruit juices as clarification and extraction enhancers; and two, in the case of fermented products, so that the enzyme can be produced by the yeast as part of the process rather than having to be added to the medium.

Fungal or bacterial genes encoding for these enzymes have been cloned and expressed in yeasts (Laing and Pretorius, 1993; Lang and Looman, 1995; González-Candelas et al., 1995; Iguchi et al., 1997; Blanco et al., 1998; Gognies et al., 1999; Vilanova et al., 2000; Sieiro et al., 2003).

Probably the chief drawback to the use of yeast-derived pectinolytic enzymes in industrial processing is the low yield of fermentation activity. An alternative would therefore be to clone and overexpress the structural genes responsible for these enzymatic activities so as to obtain a wine yeast strain that facilitates must and wine clarification during fermentation, thus producing cost savings. Such strains could further enhance the color and the aroma of wines (Van Rensburg and Pretorius, 2000).

Gainvors et al. (1994a) found that the strain SCPP, recently identified as *Saccharomyces bayanus* (Naumov et al., 2001) produced three types of pectinolytic enzyme: pectin methyl esterase (PME), pectin lyase (PL), and polygalacturonase (PG). They also demonstrated (Gainvors et al., 1994b) that when the enzymatic extract from that strain was added to a fresh must, the effects on turbidity were the same as when a commercial enzymatic preparation (Endozyme) was added.

Blanco et al. (1997) reported that when PG+ strains of *Saccharomyces cerevisiae* were used in vinification, in some cases the filtration time was reduced by half without any appreciable changes in viscosity.

The *PGU*1 gene encoding the endoPG of *S. cerevisiae* strains has been characterized, cloned, and overexpressed (Blanco et al., 1998; Gognies et al., 1999, 2001; Jia and Wheals, 2000; Vilanova et al., 2000; Gainvors et al., 2000; Blanco et al., 2002, and Fernández-González et al., 2004), and the protein sequences of some of them show different amino acids from the protein sequence of the database SGD (http://genome-www.stanford.edu/Saccharomyces).

Blanco et al. (1998), Naumov et al. (2001), and Veiga-Crespo et al. (2004) suggested that the coding region of the *PGU*1 gene (ORF YJR153w) was probably present and conserved in both PG positive and negative strains, but Fernández-González et al. (2004) evaluated a total of 61 *S. cerevisiae* strains, 60 of them isolated from wine ecosystems for the presence of this gene and for PG activity, founded that 9 strains lacked the gene and of the 52 strains possessing *PGU*1 gene, only 36 showed PG activity. These results showed that such inconsistent occurrence of the *PGU*1 gene in different strains of *Saccharomyces* suggests that this gene is not typical of *S. cerevisiae*, and hence may be used as an intraspecific marker for genetic and phenotypic characterisations.

In this work the wine strain UCLMS-1, which has good enological qualities, was transformed with *PGU*1 gene from the strain UCLMS-39 (Fernández-González et al., 2004), transcriptionally bonded to the promoter of *PGK*1 gene, in order to enhance its expression during vinification. The *PGK*1 gene promoter presents high constitutive expression in the presence of glucose and is repressed by other non-fermentable carbon sources like glycerol (Kingsman et al., 1990). The data in the literature indicate that this promoter can increase the level of expression of different genes in *S. cerevisiae*, such as those for immunoglobulin (Wood et al., 1985), β-1,4-endoxylanase (Crous et al., 1995), α-amylase (Yánez et al., 1998), and alcohol acetyl transferase (Lilly et al., 2000).

Microvinification was assayed in white and red musts with the transformed UCLMS-1M strain, and with the same strain untransformed, and the results were compared.

2. Material and methods

2.1. Strains and media

Strains and plasmids used in this study are summarised in Table 1. The host used for maintenance

Table 1
Microbial strains and plasmids used in this study

Strains and plasmids	Relevant characteristics	References
Bacteria		
E. coli DH5 α	*end*A1 *hsd*R17 (r_k^- m_k^+) *sup* E44 *thi*1λ$^-$ *rec*A1 *gyr* A, *re*l A1 Δ (*lac*ZY A-*arg*F) U169 (φ80*lac*ZΔ M15)	Gibco-BRL
Yeasts		
S. cerevisiae W3031b	*Matα*, *ade2-1*, *his3-11,15 leu2–3,112 ura3-1 trp1-1 can1–100*	CBMSO, UAM-CSIC
S. cerevisiae WGUa/b	W303 1b pYES2-*PGU*1	Fernández-González et al. (2004)
S. cerevisiae WKU	W303 1b YCp50+PGK1p-*PGU*1	This work
S. cerevisiae UCLMS-39	Wine yeast strain isolated in our laboratory, pectinolytic activity	Fernández-González et al. (2004)
S. cerevisiae UCLMS-1	wine yeast strain isolated in our laboratory, pectinolytic activity-deficient control strain	Fernández-González et al. (2004)
S. cerevisiae UCLMS-1M	UCLMS-1 *SMR*1-410 *PGK1*p-*PGU*1	This work
Plasmids		
YCp50	*Hind*III – *Bam*HI – *Sph*I – URA3 – CEN4 – ARS1 – pMB1 ori – bla.	CBMSO, UAM-CSIC
YKU	YCp50-PGK1p-*PGU*1	This work
PWX509	bla SMR1-410	Casey et al. (1988)
PKUSa/b	pWX509-PGK1p-*PGU*1	This work

and propagation of the plasmids was *Escherichia coli* DH5α strain, which was grown in Luria–Bertani (LB) medium (bactotryptone 1%, yeast extract 0.5%, and NaCl 0.5%), supplemented with ampicillin (Ap; 100 μg/ml) or tetracyciline (TC; 25 μg/ml) as required. *S. cerevisiae* was cultivated at 30 °C in both media: SC (0.67% amino acid-free yeast nitrogen base, supplemented with the required aminoacids, and 2% glucose) and YPD (containing 1% yeast extract, 2% peptone, and 2% glucose). The haploid *S. cerevisiae* transformants were selected on synthetic complete (SC) medium without uracyl, and the wine yeast transformants were selected on SC plates complemented with sulfometuron methyl (SMM) 15 mg/l. Solid media contained 2% agar. Polygalacturonase (PG) activity evaluation of the yeast transformants, was carried out on plates complemented with polygalacturonic acid (PGA) as sustrate (Charoenchai et al., 1997), and the quantification was done using the method described by Milner and Avignad (1967), using PGA as substrate, and the results were expressed as μmol galacturonic acid (GALA) $\cdot$ ml^{-1} of supernatant (Fernández-González et al., 2004).

2.2. Enzymes and reagents

The restriction enzymes *Bam*HI, *Eco*RI, *Sal*I, *Xba*I, *Xho*I, and *Sma*I were supplied by Roche and *Sau*3A by Amersham Life Science. Other enzymes used in the handling of nucleic acids were: DNA ligase of bacteriophage T4, alkaline phosphatase (Roche), Klenow fragment of DNA polymerase I (*E. coli*; Promega), P*wo* DNA polymerase (Roche), Ampli*Taq* Gold DNA polymerase (Applied Biosystems), and RNAsa A (Sigma). The oligonucleotide primers were TIB molbiol (Roche). Other enzymes used were lysozyme and proteinase K, both from Sigma. All these enzymes were used in accordance with the protocols in the literature (Sambrook et al., 1989) or following the supplier's instructions.

The antibiotics ampicillin and tetracycline were supplied by Sigma and the herbicide sulfometuron methyl by Supergo.

The various organic and inorganic products were supplied by Merck, Panreac, Roche, Bio-Rad, and Sigma. The ingredients of the culture media were supplied by Difco, Pronadisa, Oxoid, and Panreac.

2.3. DNA manipulation, cloning, and transformation

The competent cells of *E. coli*, DH5α, were prepared according to the SEM (simple and efficient method; Inoue et al., 1990). The state of competence of cells was maintained for a prolonged period by freezing them with 7% DMSO at −70 °C. The

method described by Inoue et al. (1990) was used for *E. coli* transformations.

Small-scale DNA isolation from *E. coli* was obtained by alkaline lysis mini-preparations (Birnboim and Dolly, 1979). Alternatively, the plasmidic DNA of recombinant clones was purified with Promega's Wizard® Plus SV Minipreps (DNA Purification System) kit.

Yeast genomic DNA was obtained following the protocol "Isolation of genomic DNA for Southern Blot Analysis" (Rose et al., 1990), and recovery of plasmids for *E. coli* transformation was according to Hoffman and Winston (1987).

Yeasts were transformed following the protocol LiAc/SS-DNA/PEG described by Gietz and Shiestl (1995).

Standard procedures were used for restriction–ligation reactions (Sambrook et al., 1989).

2.4. Polymerase chain reaction (PCR)

The 583nt (−580 to +3) fragment containing the *S. cerevisiae PGK*1 gene promoter, was fused to the *PGU*1 gene coding region by PCR (Fig. 1) and the oligonucleotides used are in Table 2.

The oligonucleotide CT-*PGU*1 is reverse complementary (nt 1 to 33) to the nt sequence of ORF *PGU*1 (nt +1063 to +1086) and also contains the recognition sequence for the enzyme *Bam*HI (nt 5 to 10).

The oligonucleotide 5′-p*PGK*1 is homologous (nt 8 to 28) to the nt sequence in the region promoting the gene *PGK*1 (nt −580 to −560). This oligonucleotide further contains the recognition sequences for the restriction enzymes *Nco*I (nt 1 to 6) and *Bam*HI (nt 5 to 10) in its 5′ end (Yánez et al., 1998).

The oligonucleotide R-*PGU*1-p*PGK*1 is reverse complementary (nt 1 to 22) to the nt sequence of the ORF *PGU*1 (nt +1 to +22) and is also reverse complementary (nt 19 to 38) to the nt sequence of the chain promoting the gene *PGK*1 (nt −16 to +3).

The oligonucleotide F-p*PGK*1-*PGU*1 is homologous (nt 1 to 16) to the nt sequences of the region promoting the gene *PGK*1 (nt −13 to +3) and is also homologous (nt 13 to 30) to the nt sequence of the ORF *PGU*1 (nt +1 to +17).

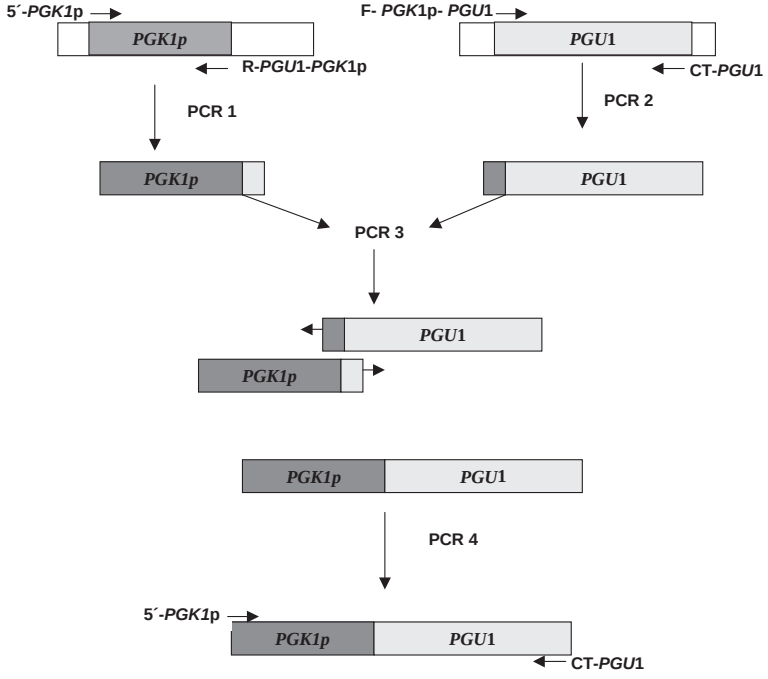

Fig. 1. Schematic representation of the fusion of the *S. cerevisiae* 3-phosphoglycerate kinase gene promoter (*PGK*1p) to the endopolygalacturonase gene (*PGU*1) coding region, using the PCR technique.

Table 2
Oligonucleotides used as primers to generate PCR products

Oligonucleotides	Sequences (5′ to 3′)	Nt
CT-*PGU*1	5′CTGCGGATCCTTAACAGCTTGCACCAGATCCAG 3′	33
5′-PGK1p	5′CCATGGAtcctccttcttgaattgatg 3′	28
R-*PGU1*-PGK1p	5′GTAATGAATTAGCAGAAATCATtgttttatatttgttg 3′	38
F-PGK1p-*PGU*1	5′ caaatataaaacaATGATTTCTGCTAATTC 3′	30

*Bam*HI recognition site (indicated by underlined letters) and *Nco*I (italics letters), 3-phosfoglicerate kinase gene promoter (*PGK1p*) sequence (small letters) and endopolygalcturonase gene (*PGU*1) coding sequence (capital letters). nt, nucleotide.

All amplification reactions were done in a Perkin-Elmer model 2400 Thermocycler apparatus. The volume of each reaction (PCR1, PCR2, and PCR4) was 50 μl, with a final concentration of: PCR buffer 1X; 0.2 mM of each dNTP; 1 μM of each of the oligonucleotides (5′-p*PGK*1 and R-*PGU*1-p*PGK*1 for PCR1; CT-*PGU*1 and F-p*PGK*1-*PGU*1 for PCR2; and 5′-p*PGK*1 and CT-*PGU*1 for PCR4); 1.25 U of P*wo* DNA polymerase; and 5–15 ng DNA. Amplification conditions were 94 °C/4 min followed by 30 cycles of 94 °C/30 s, 55 °C/30 s, and 72 °C/30 s (PCR1), 1 min (PCR2) and 2 min (PCR4), and finally 72 °C/5 min.

In the PCR3 reaction, the concentrations and components for a final volume of 50 μl were: PCR buffer 1X; 0.2 mM of each dNTP; 1.25 U of P*wo* DNA polymerase; and 100 ng of each of the reaction products of PCR1 and PCR2. Amplification conditions (hybridation) were 94 °C/4 min, followed by 30 cycles of 94 °C/30 s, 52 °C/1 min, and 72 °C/1.5 min, and finally 72 °C/10 min.

The fragment obtained by PCR3 was treated with *Bam*HI. T4 DNA ligase was used to bind it to plasmid YCp50, previously linearized with *Bam*HI, then *E. coli* DH5α was transformed with the plasmid, which formed (YKU). The constructions were checked by restriction and PCR4.

The plasmidic DNA extracted from the DH5α transformants with the target construction was used to transform *S. cerevisiae* W3031b, selecting by the Ura^+ phenotype and then by PG+.

2.5. Construction of a S. cerevisiae UCLMS-1M wine strain with pectinolytic activity

In order to obtain a wine yeast strain capable of hydrolysing pectins during vinification, the construction described in the previous section was used to transform the UCLMS-1 *S. cerevisiae* strain. The UCLMS-1. *S. cerevisiae* wine strain was isolated in wines from the Valdepeñas region (Spain) and selected as being of clear oenological interest to the wine-making of this region (Briones et al., 1995). Integration of this construction in the *ILV*2 locus (*SMR*1, *THI1*), ORF YMR108W, of chromosome XIII, which codifies for biosynthesis of acetolactate synthase (ALS), confering resistance to the herbicide sulphometuron methyl was the strategy followed (Falco et al., 1985) being used in the transformation of some industrial yeasts (Gasent-Ramirez et al., 1995; Marín et al., 2001). This was achieved with the integrative vector pWX509 (Casey et al., 1988) by insertion of the construction p*PGK1-PGU*1, obtained from the YUK plasmid using the *Bam*HI site. This fragment was treated with Klenow polymerase to obtain non-cohesive ends; then, after sub-cloning in the *Sna*BI site of the vector pWX509, *E. coli* was transformed by selecting Ap^R clones.

The plasmidic DNA (pKUSa/b) was treated with *Sal*I and the wine yeast was transformed with the resulting linear fragment, containing the *SMR*1 region, the p*PGK*1-*PGU*1 cassette and the 5′ and 3′ flanking sequences of locus *SMR*1, selecting for resistance to sulfometuron methyl (SMM^R).

Following transformation, it was found that the yeast (UCLMS-1M) expressed polygalacturonase activity in solid and liquid media (Fernández-González et al., 2004) and that it contained the target insert confirmed by PCR4.

2.6. Microvinification assays

Microvinification assays were performed in quintuplicate in white and red musts, following the traditional wine-making methods. The starter cultures

were *S. cerevisiae* UCLMS-1M and UCLMS-1 used as control.

White must from "Airen" grape variety and red must from "Cencibel" were stored at −20 °C until use. The musts with 30 ppm SO_2 added were used in the preparation of the inoculum and for microvinification trials. These assays were performed in 1-l Erlenmeyer flasks fitted with Müller valves. Samples were inoculated with 10^7 cells ml^{-1}. White musts were fermented at a controlled temperature of 18–20 °C until the sugars were exhausted. Red musts were fermented at 24–25 °C in contact with the skin for 72 h, and as much semi-fermented matter as possible was extracted by manual crushing. The yield of wine-must extraction was calculated by comparing the initial weight of the whole grapes and the final skin residue. Semi-fermentation after removal of the skin continued at the same temperature until the sugars were exhausted.

After fermentation, the wines were centrifuged to remove lies (2260×*g*, 4 °C/5 min) and then stored under refrigeration until analysis.

2.6.1. Viability of starter cultures

Samples were taken periodically to estimate cellular viability; these were plated on Wallenstein agar (Oxoid). The resulting colonies were counted after 48 h of incubation at 30 °C.

To ascertain the dominance of the inoculated strain, 10 isolates were selected at random and subjected to mitochondrial DNA restriction analysis using *Hin*fI (Querol et al., 1992). To confirm that the UCLMS-1M strain retained pectinolytic activity, these isolates were grown on PGA medium at pH 3.5 (Charoenchai et al., 1997).

2.6.2. Physicochemical parameters

The wines were analyzed for conventional parameters according to OIV recommendations. In addition, absorbances at 420 nm, 520 nm, and 620 nm were measured in red wines to determine the coloring intensity (IC=A420+A520+A620; Glories, 1984) and the tonality (*T*=A420/A520; Sudraud, 1958).

2.6.3. Filtration rate

Polygalacturonase activity of the *S. cerevisiae* UCLMS-1M strain on wine filtration behavior was determined by measuring the time required for 100 ml of final product to pass through a 0.45 μm filter using a tangential filtration system (Minitan, Millipore).

2.6.4. Measurement of viscosity

Viscosity was measured at 40 °C in 10 ml of filtered wine, using a Cannon-Fenske 5354/2 viscosimeter for transparent liquids (Comecta S.A). Kinematic viscosity (KV), expressed in centistockes (csk), was calculated by the formula KV=*Kt*, where $K_{40\ ^\circ C}$=0.0016709 and *t* is the time in seconds.

2.6.5. Major volatiles

Acetaldehyde, methanol, 1-propanol, 2-butanol, isobutanol, 2-methyl-1-butanol, 3-methyl-1-butanol, and ethyl acetate were analyzed using 3-pentanol as internal standard. A Perkin-Elmer gas chromatograph equipped with a VINICOL packed column was used (González and González Lara, 1994). The conditions were the following: detector temp.: 225 °C; Injector temp.: 200 °C; Carrier gas: N_2; Flow: 15 ml/min; injection volume: 1 μl. Oven program: initial temp. 40 °C/3 min; 6 °C/min up to 60 °C, isotherm 16 min; and 6 °C/min up to 126 °C, isotherm 12 min.

2.6.6. Sensory analysis

Sensory evaluations were performed to determine whether there were significant differences between wines made with the modified strain and with the control strain, in both white and red wines. All evaluations were carried out in a standardized tasting room (Spanish standard UNE 87004:1979; AENOR, 1997) using standard wine-tasting glasses (Spanish standard 87022: 1992 and ISO standard 3591:1977) (AENOR, 1997).

A triangular test (Spanish standard UNE 87006:1992 and ISO standard 4120:1983; AENOR, 1997) was designed to ascertain the effect of modified yeast strain on wine. The taste panel was composed of 25 tasters who were familiar with the product.

2.6.7. Statistical analysis

To determine whether there were significant differences between wines made with UCLMS-1 and UCLMS-1M strains, the Student's *t*-test was applied to data for related samples, using the SPSS statistical program (version 11).

3. Results and discussion

3.1. Modification of gene PGU1 expression

Once it was known that the PG+ activity of the strain UCLMS-39 was due to the gene *PGU*1 and that this was functional under its own promoter and the *GAL*1 promoter (Fernández-González et al., 2004), the *PGU*1 gene promoter was replaced by the strongly-expressing *S. cerevisiae PGK*1 gene promoter in order to raise the level of expression (Fig. 1).

In the first PCR (PCR1), a region containing the *PGK*1 gene promoter, nt −580 to +3, with *Nco*I and *Bam*HI recognition sequences at the 5′ end, and a short region "upstream" *PGU*1 gene, nt +1 to +22, at the 3′ end was amplified.

In another reaction mixture (PCR2), a region including a short portion "downstream" *PGK*1 gene promoter, nt –13 to +3, the 5′ end *PGU*1 gene, nt +1 to +1086, and a *Bam*HI recognition sequence at the 3′ end was amplified.

A third reaction (PCR3) produced a DNA fragment of 1665 bp containing the *PGK*1 gene promoter, nt −580 to +3, bonded to the *PGU*1 gene, nt +1 to +1086. This fragment was purified and bonded to the plasmid YCp50 before transformation of *E. coli* DH5α. The plasmidic DNA with the target construction (YKU-2, -15, and -16) was used to transform *S. cerevisiae* W3031b strain by selection in the appropriate media (see Material and methods).

3.2. Construction of a S. cerevisiae UCLMS-1M wine strain with pectinolytic activity

The *S. cerevisiae* UCLMS-1 strain lacking the *PGU*1 gene, as confirmed by PCR and Southern Blot (Fernández-González et al., 2004), was transformed with the p*PGK*1-*PGU*1 linear fragment. The pKUSa/b plasmids were treated with SalI, producing 30 transformed clones with the KUSa construction and 35 with the KUSb, SMM^R construction. More than half of these exhibited stable PG+ activity in a solid medium. *S. cerevisiae* UCLMS-1M strain is a genetically modified but not a transgenic organism since it contains no phage or bacterial sequences.

One transformed strain was randomly selected (UCLMS-1M), and the presence of the 1665 pb fragment corresponding to the construction p*PGK*1-*PGU*1 was confirmed by PCR4. This strain was also used for the quantification assays.

Table 3 shows the μmol GALA released by the transformed, the donor and the untransformed strains when grown without and with PGA.

The UCLMS-1M strain was much more active than the donor strain UCLMS-39 in all cases, and in only 1 h of contact with PGA it released large quantities of galacturonates, increasing progressively with time. Moreover, during growth it hydrolyzed PGA, and to a far lesser extent pectin, at both pH 3.5 and 5.5.

3.3. Microvinification assays

The kinetic behavior of all microvinification types was similar irrespective of the yeast strain used (UCLMS-1/-1M), and cell viability was adequate; counts were normal for this type of process.

As to implantation of the cultures, all the isolates analyzed presented the same mitochondrial DNA restriction profile as the starter cultures (patterns shown in Fig. 2). As can be observed in the plate with PGA, the isolates from microvinifications with the UCLMS-1M strain exhibited polygalacturonase activity.

Vilanova et al. (2000), used the recombinant strain M-20T with the pBJ16-*PGU*1 plasmid (Blanco et al., 1998), leaving its fermentation ability essentially unchanged. The results revealed that cells were gradually losing the plasmid. After fermentations, 70% of the cells still retained the plasmid, suggesting

Table 3
Polygalacturonase activity in supernatant and in broth-culture of donor (UCLMS-39), receptor (UCLMS-1), and modified (UCLMS-1M) yeast strains, expressed as μmol of galacturonic acid (GALA) released for mL of supernatant (μmol GALA mL S^{-1}), using polygalacturonic acid (PGA) as substrate

	(1) μmol GALA mL S^{-1}			(2) μmol GALA mL S^{-1}			
	1 h	5 h	24 h	PGA3.5	PGA5.5	PT3.5	PT5.5
UCLMS-39	1.01	2.05	3.26	3.06	2.94	0.38	0.30
UCLMS-1	0.00	0.01	0.02	0.01	0.05	0.00	0.02
UCLMS-1M	3.82	5.06	6.26	9.12	7.96	1.47	1.50

(1) GALA released from PGA, using the supernatant from 3 days of culture in YNB-glucose medium, incubated in PGA medium at 37 °C pH 5,5 at different times (1 h, 5 h, 24 h).
(2) Direct quantification of GALA released from PGA or pectin (PT) into the 3 days' culture medium at pH 3.5 or 5.5.

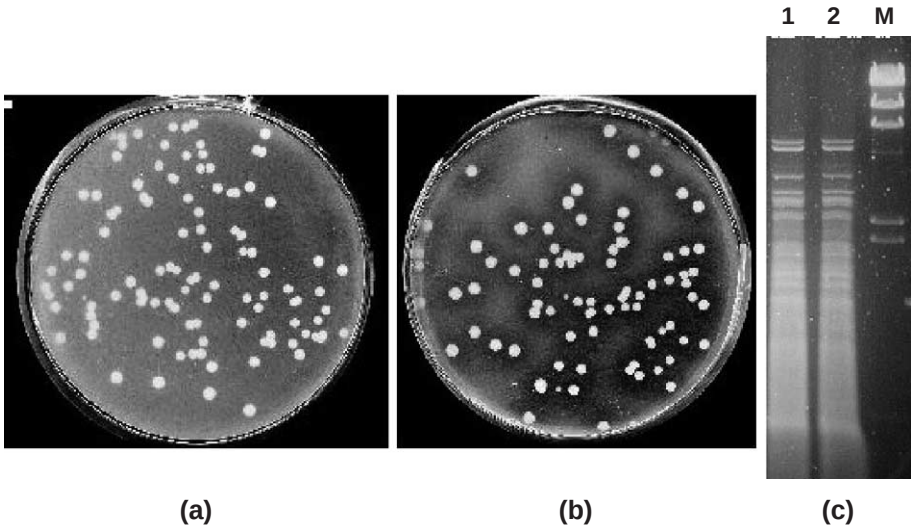

Fig. 2. Polygalacturonase activity on polygalacturonic acid medium at pH 3.5 after incubation at 30 °C/24 h. (a) UCLMS-1; (b) UCLMS-1M. (c) mtDNA patterns obtained using the restriction enzyme *Hin*fI, corresponding to UCLMS-1 and -1M strains (lines 1, 2). Line M. Marker II (Roche).

a high level of mitotic stability, but in this work, 100% of cells analyzed have PG activity because of chromosomal integration of the construction of interest (p*PGK*1-*PGU*1), and transformants were stable during vinification process.

The physicochemical parameters recorded for white and red wines are shown in Table 4. The Student's *t*-test for related samples showed no significant ($p \leq 0.05$) differences in any of the target physicochemical parameters in white wines. However, in red wines made with UCLMS-1M (RWM), the amount of reducing sugars was significantly ($p \leq 0.05$) higher than in the control (RWC). These findings were to be expected given that maceration with pectinolytic enzymes produces slow hydrolysis of the skins cell walls and hence a greater presence of enzyme-resistant

Table 4
Physicochemical parameters

	WWC		WWM		RWC		RWM	
	X	S.D.	*X*	S.D.	*X*	S.D.	*X*	S.D.
PH	3.84	0.02	3.84	0.03	4.15	0.05	4.14	0.04
Volatile acidity (g/L acetic acid)	0.10	0.01	0.11	0.00	0.10	0.02	0.10	0.01
Total acidity (g/L tartaric acid)	5.10	0.09	5.00	0.09	4.34	0.16	4.45	0.18
Residual sugars (g/L)	0.53	0.25	0.57	0.06	2.85[a]	0.31	4.67[b]	0.21
Alcohol degree (%v/v)	12.3	0.0	12.3	0.0	15.2	0.1	15.3	0.1
A420	0.118	0.001	0.131	0.007	0.384	0.039	0.455	0.021
A520					0.558[a]	0.062	0.703[b]	0.033
A620					0.154	0.022	0.189	0.007
IC=A420+A520+A620					10.96[a]	1.22	13.46[b]	0.61
T=A420/A520					0.69[a]	0.01	0.65[b]	0.01
Filtration time (s)	68.7	3.8	81.2	13.1	550.2	36.2	545.6	34.6
Kinematic viscosity (csK)	1.10	0.03	1.11	0.03	1.24	0.01	1.25	0.01
Yield of extraction (%)					75.3[a]	1.6	82.3[b]	0.5

WWC, white wine fermented with UCLMS-1; WWM, white wine fermented with UCLMS-1M; RWC, red wine fermented with UCLMS-1; RWM, red wine fermented with UCLMS-1M. *X*=mean value; S.D.=standard deviation. [a,b] Significant differences ($p \leq 0.05$).

pectic polysaccharides such as Rhamnogalacturonan II, which can increase the reducing power (Pellerin, 2001). We would note, however, that there were no significant differences in final alcohol concentration.

Also, the RWM sample exhibited more intense coloring accompanied by red tones (A520 nm), a feature very much appreciated in red wine fermentations, although the tone of the control was higher. This is consistent with the findings of González San José et al. (1998) in a study of the effect of treatment with commercial pectinolytic enzymes.

As regards the parameters identified with improvement of the process, the major technological improvement was an increased yield of extraction of semi-fermented from red grapes in wines inoculated with the UCLMS-1M strain, where the yield was 7% higher than in the control. This was because the secreted enzyme facilitated cleavage of the grape cell walls favoring also the extraction of anthocyans from anthocyanoplasts (Rogerson et al., 2000), which are responsible for increased colorant intensity. There were no significant differences in filtration time or kinematic viscosity between wines made with either strain.

Fig. 3 shows the GC analysis values of the major volatiles. The analyzed compounds did not differ significantly in the white wines; however, the methanol concentration increased in the red wines made with the UCLMS-1M strain, which is the logical outcome of degradation of the grape pectins. Nevertheless, these values were well below the maximum permitted by European regulations (500 mg/l).

3.3.1. Sensory analysis

A triangular test was conducted with white and red wines elaborated with UCLMS-1 and -1M strains. The statistical data revealed there were no significant differences (at the 95% level) between white wines elaborated with UCLMS-1 (WWC) and UCLMS-1 (WWM), and the same occurs for RWC and RWM; therefore, a more exhaustive sensory analysis was not possible because tasters were not able to difference the wines elaborated with UCLMS-1M and UCLM-1.

The results of the sensory analysis demonstrated that wines elaborated with the UCLMS-1M strain are of the same quality as those obtained with the UCLMS-1 strain and, moreover, they preserved the typical characteristic of wines elaborated with the Cencibel and Airen grape variety. Alterations to the polysaccharide content therefore have no direct effect on the taste or flavor of the wine.

Vilanova et al. (2002) obtained the same results with Albariño grape variety using the recombinant strain M-20T with the pBJ16-*PGU*1 plasmid (Blanco et al., 1998), leaving its fermentation ability essentially unchanged. Wines obtained with the recombinant strain and the untransformed counterpart did not differ in their physicochemical parameters or major sensory characteristics. With respect to wines obtained from must supplemented with commercial pectic enzymes, these wines display aromas that are less typical or not typical at all.

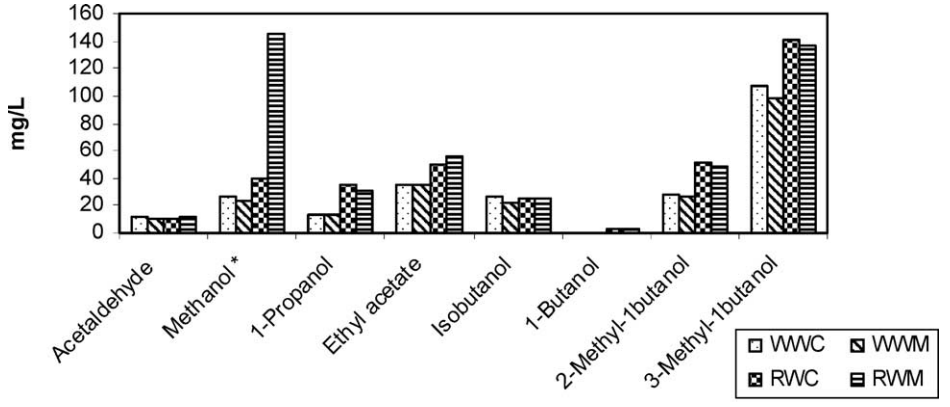

Fig. 3. Major volatile compounds in red and white wines, fermented with UCLMS-1 and UCLMS-1M. WWC, white wine fermented with UCLMS-1; WWM, white wine fermented with UCLMS-1M; RWC, red wine fermented with UCLMS-1; RWM, red wine fermented with UCLMS-1M. *Significant differences ($p<0.05$) in red wines.

4. Conclusions

The work reported is directed to the construction of a *S. cerevisiae* wine yeast strain, selected as being of clear oenological interest to the wine-making, containing *PGU*1 endopolygalacturonase gene constitutively expressed under the control of *PGK*1 gene promoter, used in trial fermentations in the search for an alternative to the use of commercial pectic enzymes of fungal origin.

Transformants obtained in this way are stable during vinification process, with solely nucleotide sequences of interest. The absence of bacterial DNA integrant structure, agree with the current GMO regulations in USA and Europe for the commercial application of recombinant DNA technology in food and beverage industry.

The major technological improvement was an increased yield of extraction of semi-fermented from red grapes in wines inoculated with the UCLMS-1M strain, where the yield was 7% higher than in the control, and increased colorant intensity.

When fermentation tanks are in short supply, the adventage of enzyme treatment is obvious, since the faster colour extraction will allow pomace to be pressed up earlier.

In white wines, any modification was observed, probably because of maceration techniques were not used, and Airen variety is a non-aromatic one.

However, additional work is required to determine the effectiveness of it in large-scale winemaking trials, in order to obtain information on the effect of the enzyme on the clarification and filtration of the wine.

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