DEVELOPMENT OF RECOMBINANT *SACCHAROMYCES CEREVISIAE* FOR IMPROVED D-XYLOSE UTILISATION

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis 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.

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Date:

SUMMARY

Plant biomass is potentially an inexhaustible source of bioenergy. To be more useful in an industrialised context, conversion to liquid biofuel is necessary, which could provide the motor vehicle market with energy. To enable fermentation of both hexose and pentose sugars present in plant biomass, many researchers have introduced eukaryotic D-xylose utilisation metabolic pathways into *S. cerevisiae* as these yeasts cannot utilise D-xylose. The aim of this study was to increase D-xylose utilisation and lower the xylitol production found with the eukaryotic pathway, thus redirecting carbon to the increased production of ethanol.

In order to reduce xylitol yield a two-fold approach was followed. Firstly *S. cerevisiae* transformed with eukaryotic XR and XDH genes were subjected to random mutagenesis and selection for improved D-xylose utilisation. Unfortunately no mutant superior to the parental strain with respect to D-xylose utilisation, lowered xylitol production and improved ethanol production was obtained.

Subsequently a bacterial xylose isomerase (XI) gene was introduced into *S. cerevisiae*. Bacterial xylose isomerase converts D-xylose to xylulose in a single step, while eukaryotic pathways produce the intermediate xylitol. The chosen gene encodes for a putative xylose isomerase gene (*xylA*) from the bacterium *Bacteroides thetaiotaomicron*, which has not previously been transformed into yeast. When the native *xylA* was expressed in *E. coli* and *S. cerevisiae* no XI activity was found, nor growth on D-xylose sustained. Lack of activity was surmised to be due to an amino acid modification, or possibly due to a vastly different codon bias in yeast compared to the *Bacteroides* strain. Northern analysis revealed that no D-xylose transcript was formed. A synthetic D-xylose isomerase gene (SXI) based on the *B. thetaiotaomicron* XI amino acid sequence, but optimised for *S. cerevisiae* codon bias, was designed and manufactured. *S. cerevisiae* transformed with the synthetic gene showed sustained, non-pseudohyphal growth on D-xylose as sole carbon source, both on solid and liquid medium. This ability to utilise D-xylose represents a significant step for recombinant *S. cerevisiae* to potentially ferment D-xylose for bioethanol.

OPSOMMING

Plant biomassa is potensieel 'n onuitputlike bron van bio-energie. Om in die huidige industriële konteks van groter nut te wees, en die motor-industrie met energie te voorsien, is omskakeling na 'n vloeistof-energievorm nodig. Om die fermentasie van beide heksoses en pentoses teenwoordig in plantbiomassa te bewerkstellig, het verskillende navorsingspanne eukariotiese D-xilose-afbraak metabolise weë na *S. cerevisiae* oorgedra om dié gis die vermoë te gee om D-xilose af te breek. Die doel van hierdie studie was om D-xilose-verbruik in geneties gemodifiseerde *S. cerevisiae* te verhoog en die hoeveelheid xilitol wat met die eukariotiese sisteem verkry word, te verminder om 'n hoë etanol opbrengs te handhaaf.

Twee moontlikhede is ondersoek om die xilitol opbrengs te verminder. Eerstens is 'n rekombinante *S. cerevisiae* met die xilose reduktase (XR) en xilitol dehidrogenase (XDH) gene aan nie-spesifieke mutagenese onderwerp en vir verbeterde D-xilose verbruik geselekteer. Ongelukkig kon geen mutante wat beter as die oorspronklike ras D-xilose kon gebruik, en etanol produseer met relatief min xilitol opbrengs, gevind word nie.

Daarna is 'n bakteriese D-xilose-afbraak geen na *S. cerevisiae* oorgedra. Bakteriese xilose isomerases skakel D-xilose om na xilulose in 'n enkele stap, terwyl die eukariotiese paaie die tussenganger xilitol produseer. Die gekose *xylA* geen wat vir xilose isomerase (XI) van die bakterium *Bacteriodes thetaotaomicron* kodeer, is vir die eerste keer in gis getransformeer. Toe die natuurlike *xylA* geen In *E. coli* en *S. cerevisiae* uitgedruk is, is geen XI-aktiwiteit of volhoubare groei op D-xilose waargeneem nie. Die tekort aan aktiwiteit is aan 'n aminosuurverandering, of aan die groot verskil tussen kodonkeuse ("codon bias") in gis teenoor die *Bacteroides* ras toegeskryf. Noordkladanaliese het bepaal dat geen mRNA spesifiek tot die XI-geen geproduseer is nie. Die xilose isomerase geen van *B. thetaiomicron* is toe sinteties ontwerp, met die DNA-volgorde vir die *S. cerevisiae* kodonkeuse geoptimiseer. *S. cerevisiae* wat met die sintetiese geen (SXI) getransformeer is, het aanhoudende, nie-pseudohife groei op D-xilose as enigste koolstofbron op beide soliede en in vloeibare medium getoon. Die vermoë om D-xilose te verbruik verteenwoordig 'n betekenisvolle stap tot die fermentasie van D-xilose na etanol met geneties gemodifiseerde *S. cerevisiae*.

DEDICATION

This thesis is dedicated to my parents. To my father François de Villiers, with many thanks for all his love and support, and to my mother Gai de Villiers, who is not alive to see it.

ACKNOWLEDGEMENTS

PREFACE

This thesis is presented as a compilation of five chapters. Each chapter is introduced separately. The experimental chapters are not written in manuscript format for journals.

Chapter 1 General introduction and aims of the study: "Introduction and project aims"

Chapter 2 Literature Overview: "D-Xylose utilising microorganisms and development of *Saccharomyces cerevisiae* metabolism for efficient bioethanol production from xylose"

Chapter 3 Experimental Work: "Mutation and selection of *Saccharomyces cerevisiae* with the recombinant D-xylose utilising *Pichia stipitis* pathway for enhanced xylose utilisation"

Chapter 4 Experimental Work: "Cloning, characterisation and expression of the unique *Bacteroides thetaiotaomicron* xylose isomerase gene (*xylA*) in *Saccharomyces cerevisiae*"

Chapter 5: General Discussion and Conclusions

CHAPTER 3: EXPERIMENTAL WORK:

MUTATION AND SELECTION OF *SACCHAROMYCES CEREVISIAE* WITH

THE RECOMBINANT D-XYLOSE UTILISING *PICHIA STIPITIS* PATHWAY FOR

ENHANCED XYLOSE UTILISATION

CHAPTER 4: EXPERIMENTAL WORK:
CLONING, CHARACTERISATION AND EXPRESSION OF THE UNIQUE
BACTEROIDES THETAIOTAOMICRON D-XYLOSE ISOMERASE GENE (*XYLA*)
 IN *SACCHAROMYCES CEREVISIAE*

CHAPTER 1

INTRODUCTION AND PROJECT AIMS

1   INTRODUCTION

Internationally energy needs are on the increase, which increases the pressure to find sustainable energy sources (Zaldivar et al., 2001). Plant biomass produced worldwide is one such renewable resource. Yearly the agricultural sector produces many tons of biomass waste products, which can be used as a substrate for microorganisms to convert to useful products. Currently there are significant efforts to make renewable carbohydrates economically feasible as a major source of fuel (Aristidou and Penttilä, 2000). Ethanol alcohol is a major fermentation commodity product, which has already been used successfully in automotive vehicles (Zaldivar et al., 2001).

To make the production of ethanol production from biomass economical, processes to release the component fermentable sugars from biomass must be inexpensive and the biological conversions must be effective; thus utilising all the sugars, both pentoses and hexoses (Zaldivar et al., 2002). D-Xylose sugar is found at high levels in lignocellulose and is the second-most common fermentable sugar in nature (Aristidou and Penttilä, 2000). Many organisms are capable of fermenting D-xylose to ethanol. Yeasts including *Pichia stipitis* use a xylose reductase enzyme (XR) to convert D-xylose to xylitol. The xylitol is then converted into xylulose with xylitol dehydrogenase (XDH) (Hahn-Hägerdal et al., 2001). In bacterial pathways the enzyme used is xylose isomerase (XI), which directly converts D-xylose to xylulose (Wovcha et al., 1983). Unfortunately none of the natural D-xylose fermenters are capable of producing high enough levels of ethanol for industrial implementation to be feasible. The organism best at fermenting hexose sugars to ethanol, the yeast *Saccharomyces cerevisiae*, cannot naturally ferment pentose sugars such as D-xylose, which by weight makes up some 17-31% of plant biomass sources (Jeffries and Shi, 1999). This yeast can, however, ferment xylulose to ethanol.

Previous work with recombinant *S. cerevisiae* for D-xylose fermentation has shown that low levels of ethanol tend to be produced when using the *P. stipitis* enzymes XR and XDH (Zaldivar et al., 2002; Ostergaard et al., 2000). This is due to a variety of factors including yeast metabolism channelling approximately 30% of the D-xylose sugar into its sugar-alcohol intermediate xylitol, because of cofactor imbalance and

unfavourable thermodynamics. For cost-effective fermentation of D-xylose to ethanol the yeast strain should efficiently consume D-xylose and ferment it to ethanol, while producing little xylitol. With random mutagenesis and selection over several generations on D-xylose, recombinant *S. cerevisiae* has been shown to improve the ability to convert D-xylose to ethanol, and reduce xylitol production (Wahlbom et al., 2003). Rationally determining which mutations were responsible for this improvement is difficult, as the strain used was an industrial polyploid mutant (Sonderegger et al., 2004).

Expression of xylose isomerases in *S. cerevisiae* has been disappointing (Gárdonyi and Hahn-Hägerdal, 2003). Recently Harhangi et al. (2003) found a xylose isomerase in a rumen filamentous fungus, *Piromyces* sp. strain E2. This is unusual because fungi normally possess XR and XDH, rather than XI. The group successfully cloned and expressed the gene in *S. cerevisiae*, which led to efficient conversion of D-xylose to xylulose. The protein sequence of the *Piromyces* sp. strain E2 XI enzyme is very similar to that of the bacterium *Bacteroides thetaiotaomicron*.

1.1   AIMS OF THIS STUDY

A goal for the renewable energy industry is for *S. cerevisiae* to metabolise D-xylose with ethanol as the major product under anaerobic conditions. This study addressed the problem of recombinant D-xylose utilisation in *S. cerevisiae* from two angles. Firstly *S. cerevisiae* expressing *P. stipitis* D-xylose utilisation genes were investigated. The project aimed to produce a haploid *S. cerevisiae* strain capable of more efficient conversion of D-xylose to ethanol, compared to the recombinant parent strain. This could be divided into the following objectives:

• To use random mutagenesis and selection to develop *S. cerevisiae* strains for D-xylose fermentation.

• To quantify the difference if any between these strains and the parent with techniques such as growth curves and enzyme assays to determine differences in:

o   aerobic and anaerobic growth on D-xylose as sole carbon source

o   D-xylose consumption of the recombinant strain

Secondly the expression of a prokaryotic xylose isomerase gene was investigated in *S. cerevisiae*. This investigation took advantage of the high homology between the *Piromyces* sp. strain E2 XI and *B. thetaiotaomicron* putative XI. The aims included:

- The use of PCR to obtain the xylose isomerase gene (*xylA*) from *B. thetaiotaomicron* genomic DNA

- The cloning of the *xylA* gene into plasmid pGEMT-Easy™ (Promega) and transformation of *E. coli* HB101 (Lönn et al., 2002)

- Subclone the *xylA* gene into a yeast expression vector and transform *S. cerevisiae*

- Test the expression and activity levels of XI in *S. cerevisiae*

- Determine growth of the *S. cerevisiae* on D-xylose

- To quantify whether the transformed *S. cerevisiae* strain and the parent differed in aerobic growth on D-xylose as sole carbon source with:

o growth on solid and liquid media

o enzyme assays

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CHAPTER 2

LITERATURE OVERVIEW

D-XYLOSE UTILISING MICROORGANISMS AND DEVELOPMENT OF
SACCHAROMYCES CEREVISIAE METABOLISM FOR EFFICIENT BIOETHANOL
PRODUCTION FROM D-XYLOSE

2.1 INTRODUCTION

The twentieth century has been the hydrocarbon century. Petroleum and diesel have been used to fuel motor vehicles. Solvents have been produced cheaply from crude oil and nylon and other now ubiquitous plastics have the same origin. Fossil fuels including crude oil were created over millions of years (Aristidou and Penttilä, 2000). Although advances in technology can both reveal new deposits and improve the economics of production from known sites, existing fossil fuels will not be able to sustain predicted future demands (Hahn-Hägerdal et al., 2001)

Energy alternatives are necessary and due to their potential sustainability, renewable fuels with plant biomass as the raw material are being considered. Ethanol from biomass fermentations is one option as this liquid fuel is already used in Brazil for motor vehicles, and in many countries as octane booster (de Carvalho Lima et al., 2003). In addition, ethanol produces far lower pollution levels than do current petrol and diesel fuels (Sheehan and Himmel, 1999).

Plant biomass essentially consists of cellulose, hemicellulose and lignin, together known as lignocellulose (Jeffries and Jin, 2000). Cellulose and hemicellulose are polymers hydrolysable to sugars which can be biologically fermented to ethanol (Aristidou and Penttilä, 2000). A current drawback to the process is that the sugars include pentoses, but no microorganism is currently available which both efficiently uses pentoses and produces ethanol. The yeast *Saccharomyces cerevisiae* is the most efficient ethanol producing organism, but does not naturally use pentose sugars. The pentose sugar D-xylose is the second-most common sugar after glucose and abundantly available in hemicellulose (Geng et al., 2003). Given that D-xylose can make up between 5% and 20% of plant biomass of plant biomass, an *S. cerevisiae* which could ferment it would substantially improve the yields of ethanol from lignocellulose hydrolysate (Aristidou and Penttilä, 2000). This would in turn reduce costs by up to 25% and enhance economic competitiveness with petrol. The extent of the engineering and economic challenges associated with lignocellulose fermentations in general are discussed in the following section. In addition this work gives an overview of D-xylose metabolism by microorganisms and the genetic modification of *S. cerevisiae* with both eukaryotic and bacterial enzymes for D-xylose

utilisation. The focus is on the development and improvement of *S. cerevisiae* D-xylose metabolism.

2.2 ETHANOL FROM LIGNOCELLULOSE

2.2.1 Bioethanol: a renewable, low pollution petrol alternative

Petrol for use in motor vehicles is a non-renewable product derived from fossil oil. Since the known supply of crude oil and coal is limited, and fossil fuels were created over a period of millions of years, available fossil fuels cannot sustain current rates of demand (Aristidou and Penttilä, 2000).

Several alternatives as fuels for motor vehicles are being investigated. Many of these: such as electric cars, fuel-cell cars or solar-powered vehicles involve extensive redesign, both of motor vehicles and of infrastructure to distribute the relevant fuel. One biologically natural and renewable fuel, which is being thoroughly researched and has already been used with success in Brazil, is ethanol.

A major advantage of bioethanol is its flexibility and fuel blends of up to 15% ethanol require no modifications made to current petrol-driven motor vehicles, while many current vehicles have been developed that can use 85% ethanol concentrations or E85 (Mielenz, 2001). At the height of bioethanol production in Brazil 90% of new cars bought in that country were solely powered by hydrous alcohol, which contains no petrol (de Carvalho Lima et al., 2002). Ethanol technically speaking has only two-thirds of the calorific value of petrol, but as the octane rating is higher, in practice ethanol gives an additional 15% w/w energy (Wheals et al., 1999).

This higher octane means ethanol is already used as an oxygenate to help control carbon-monoxide and other exhaust emissions (Zaldivar et al., 2002). Nitrous oxides and hydrocarbons in petrol can produce ozone, but with the use of bioethanol this can be reduced by between 20 and 30%. When ethanol is used instead of petrol net carbon-dioxide emissions can be reduced by between 60% and 90% (Zaldivar et al., 2001). Sheehan and Himmel (1999) cite the 1990 USA Clean Air Act Amendments, which showed that 95% ethanol blends (E95) reduce sulphur oxide emissions by up to 80%. Ethanol combustion essentially results in carbon dioxide and water, producing fewer greenhouse gases and other toxic compounds overall than does petrol, although acetaldehyde levels are higher (Wheals et al., 1999).

While oil prices were low around 1999, the use of ethanol as a fuel additive to reduce carbon monoxide, nitrogen oxide and hydrocarbon emissions was what drove ethanol production in America (Wheals et al., 1999). In 2004 3.41 billion gallons of ethanol was produced in America as biofuel and octane booster additive (Dineen, 2005). In South Africa too, this octane boosting property could be of increasing value, as lead and heavy metal octane boosters are being phased out (Lynd et al., 2003). In the United States there is significant research interest and currently a tax incentive for ethanol. It was hoped that by 2007, when the incentive falls away that bioethanol will be competitive (Zaldivar et al., 2001). This year the energy bill conference report H.R 6 became law (Shaw, 2005). It allows American taxpayers a rebate on installation of clean fuel pumps, and blends with 85% ethanol or higher qualify as clean fuels. In addition a renewable fuels standard (RFS), was introduced which requires a minimum of 4 billion gallons of renewable fuels in 2006, increasing to 7.5 billion in 2012. Ethanol from lignocellulosic or waste gains 2.5 RFS gallon credit for every gallon, this is particularly good news for the industry.

Recently a bio-ethanol powered car competed in the Le Mans 24-hour endurance race and had a top-speed of 318km/h, the second-fastest of 49 competitors, showing that speed not be sacrificed (Cohen, 2004). Even if future cars use hydrogen cells, bioethanol can be used as their fuel source (Aristidou and Penttilä, 2000).

2.2.2 Bioethanol: a sustainable, locally produced fuel

Advantages of ethanol as fuel source include its potential sustainability, while oil sources are limited (Aristidou and Penttilä, 2000). In addition most countries do not possess sufficient oil, coal or gas sources to be self-sufficient. This reliance on foreign fuel can be a disadvantage at times of economic downturn or political or international instability. Currently bioethanol is more expensive than petrol, however in 1999 the indirect costs per barrel of oil were estimated to be US$45 with respect to pollution and up to US$9 for intervention in Middle Eastern oil supplies, substantially increasing the direct cost of US$13 per barrel (Wheals et al., 1999). The ethanol produced locally by fermentations will not be subject to the political and economic vulnerability associated with petrol from crude oil produced by only a limited number of countries.

2.2.3 Bioethanol could utilise available biomass

Ethanol can be produced from fermentations with microorganisms – for example the yeast *S. cerevisiae* – grown on widely available agricultural products. There are several agricultural processes which generate vast volumes of biological waste. This is often underutilised from an energetic point of view, as it can usually not be used industrially, although it is biodegradable. As feedstock is one of the major costs in bioethanol production, these very inexpensive substrates could substantially reduce commercial production costs (Zaldivar et al., 2002).

According to at least one estimate the United States could convert biomass to 270 billion gallons of ethanol yearly, thus producing twice as much ethanol as the currently required petrol (Aristidou and Penttilä, 2000). In South Africa advantages of bioethanol would include the use of agricultural residues and forestry waste products currently generated in South Africa at a rate of 17.3 Mega tonnes (Mt) per year and the removal of invasive plant species could generate an additional 8.7 Mt (Lynd et al., 2003). If this alone were converted with 50% efficiency to liquid fuels this would provide 38% of South Africa's current energy use in the transport sector. Additional biomass could be generated if crops were to be planted in non-crop, non-wilderness, non-forest area specifically for these energy goals. It should be borne in mind that some of the residues are already used, although not necessarily with greatest efficiency, for example the sugar industry produces steam and power for internal consumption from bagasse.

2.2.4 Chemical composition of plant biomass sources

Plant matter from wood and agricultural residues consisting of cellulose, hemicellulose and lignin is generically known as lignocellulose (Jeffries and Jin, 2000). The composition of lignocellulosic components from biomass is complex and varies greatly between plant species (Jeffries and Jin, 2000; Aristidou and Penttilä, 2000). Plant waste in the case of wheat straw, consists of 17.1% lignin, 38.8% celluloses and 39.5% hemicelluloses (Sun and Tomkinson, 2003). Typically plants have a hemicellulose fraction of 10-35% (Aristidou and Penttilä, 2000). The five major sugars in lignocellulose are the hexoses D-glucose, D-mannose and

D-galactose and the pentoses D-xylose and D-arabinose (Jeffries and Jin, 2000). Cellulose can be degraded by a variety of microorganisms to produce the fermentable hexose sugar glucose (Aristidou and Penttilä, 2000). Figure 1 shows sugar and lignin content in typical hardwood, softwood and sugarcane bagasse plants.

Hemicelluloses vary widely between plant species and this lack of uniformity increases the challenges for making this substrate amenable to fermentation (Geng et al., 2003). Hemicelluloses fall into various categories of which xylans are the most abundant. Xylan is a polymer, which typically consists of a backbone of D-xylose, joined together by β-1,4 bonds, and has a variety of side chains including acetyl groups and furanoses. This polymer may include other sugars such as arabinose, rhamnose, galactose and glucose and groups of 4-*O*-methyl-D-glucuronic acid units and phenolic acids including *p*-coumaric acid and ferulic acid, which further increases the complexity of its degradation (Sun and Tomkinson, 2003; Larsson et al., 2001). Xylans vary between plant species, a major difference between hardwood and softwood xylans is that in addition to 4-*O*-methyl-D-glucuronic acid substitutions, softwood xylan also has α-arabinofuranose units, as illustrated in Figure 2. D-xylose can make up between 5 and 20% of the biomass (Aristidou and Penttilä, 2000).

For the process of biological ethanol production through fermentation to be cost-effective and competitive in price with petrol, it is necessary that all the sugars in the available substrate be used (Zaldivar et al., 2002). If all pentoses as well as hexoses were used, bioethanol costs could be reduced by 25% (Aristidou and Penttilä, 2000).

2.2.5 Complete biomass utilisation presents a unique challenge to fermenting microorganisms

Clearly because lignocellulosic components are not homogenous, the fermentation will not be as simple as a glucose or beer wort fermentation. For industrial fermentations of ethanol from lignocellulose a specialised fermentative organism is required (Zaldivar et al., 2001). It must be resistant to toxins in the lignocellulosic hydrolysate, be able to utilise and ferment both pentoses and hexoses, without catabolite repression, produce a high yield of ethanol to carbon utilised and do so at

a rate making the process industrially feasible. Unfortunately there is not currently a single microorganism that conforms to the required specifications.

2.2.6 The bioethanol production process

The process producing ethanol from lignocellulose has many steps (Aristidou and Penttilä, 2000). Firstly, in pretreatment the lignin must be separated to free cellulose and hemicellulose from the complex. A variety of saccharification processes are used on lignocelluloses to make the sugars available to the fermenting microorganisms and their enzymes (Zaldivar et al., 2001). Some of the best known include low temperature concentrated acid hydrolysis, high temperature dilute acid
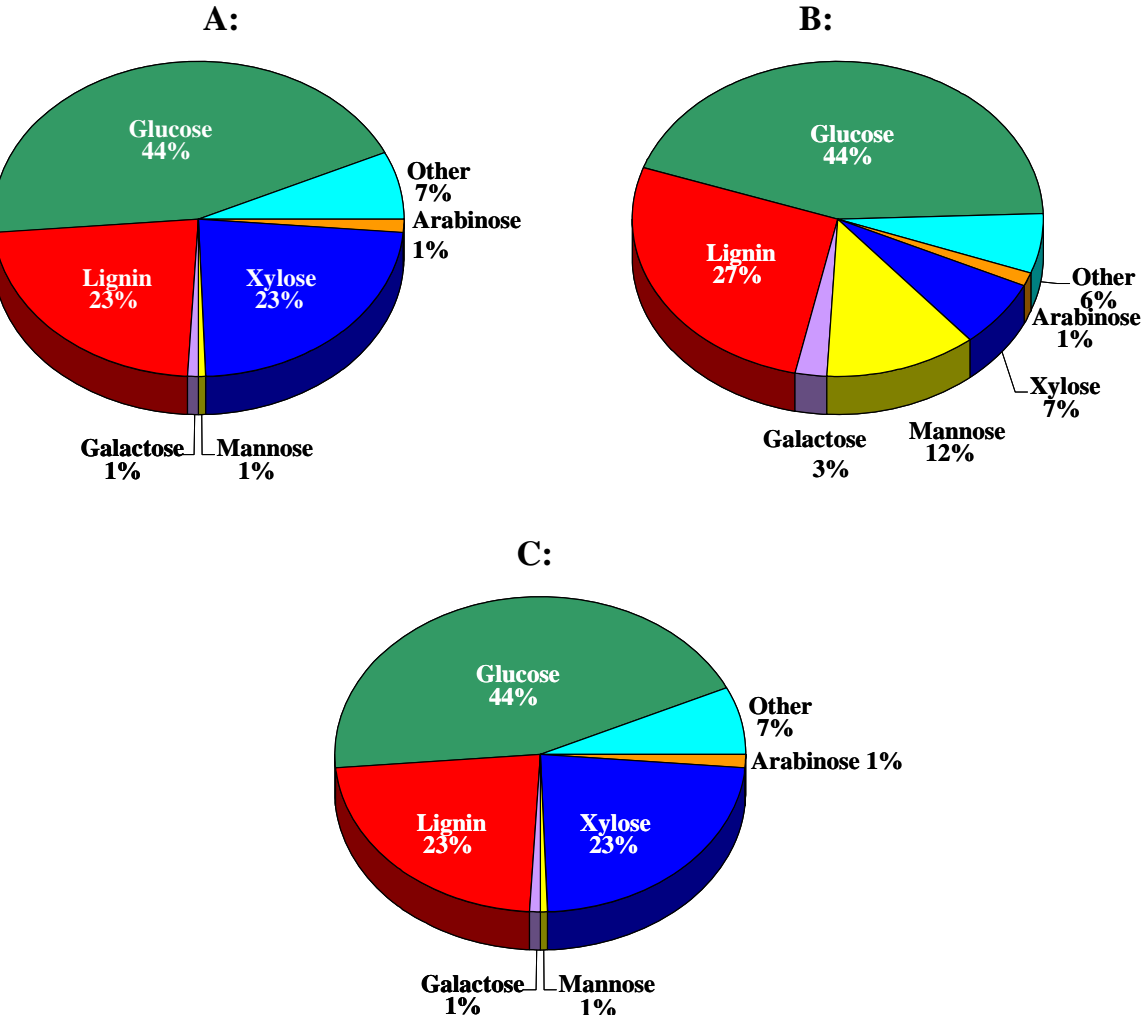
Figure 1: A schematic representation of the dry weight composition of (A) hardwood, (B) softwood and (C) bagasse. Amongst different plant species the composition may vary (Puls and Shuseil, 1993).

hydrolysis and enzymatic hydrolysis. These processes hydrolyse the cellulosic materials: cellulose to release glucose and hemicellulose to release hexose and pentose monomers. A disadvantage of acid hydrolysis is the production of inhibitory substances such as aromatic compounds, furaldehydes and aliphatic acids (Larsson et al., 2001). This often necessitates steps to detoxify the mixture before sensitive microorganisms can ferment it, although some microorganisms are resistant to phenolic compounds and other lignocellulose hydrolysis products. Figure 3 compares the processes for acid hydrolysis fermentations with simultaneous enzyme saccharification and fermentation. In some cases the further optional step of addition of nutrients is used to stimulate the fermenting microorganisms.

A:

B:
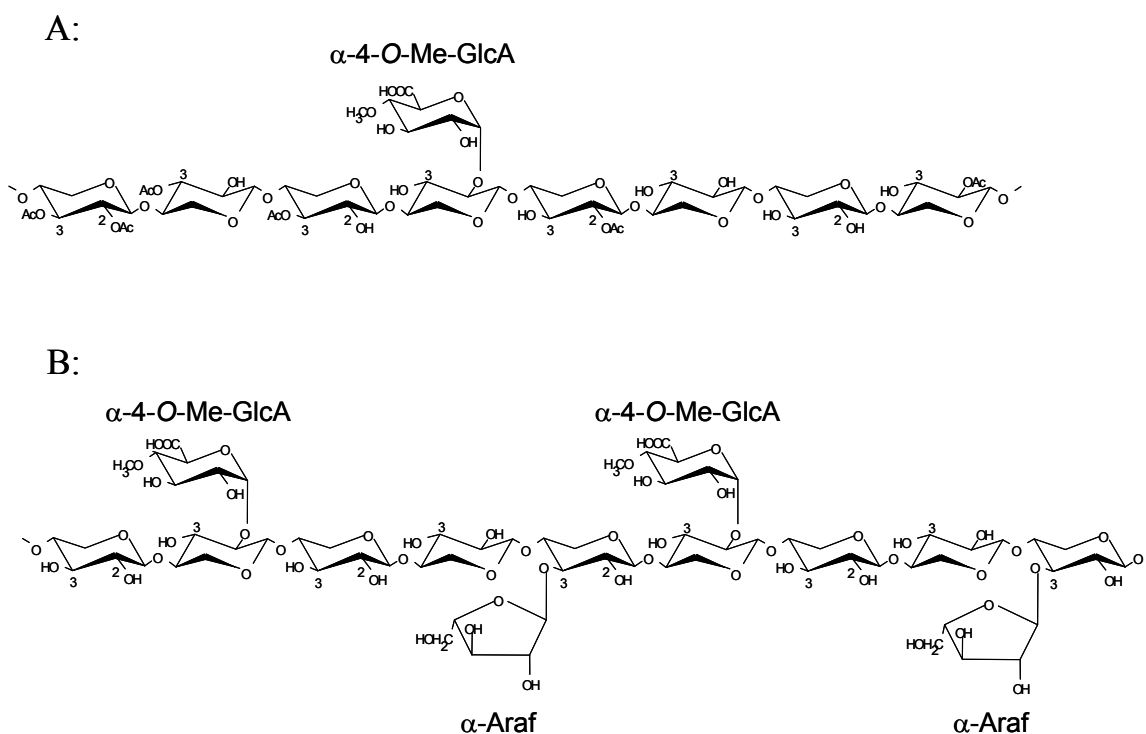
Figure 2: The differences in xylan structure between (A) hardwood xylan and (B) softwood xylan (Sunna and Antranikian, 1997). The carbon atoms at which substitutions take place are numbered. Ac: acetyl group, α-4-*O*-Me-GlcA: α-4-*O*-methylglucuronic acid, α-Araf: α-arabinofuranose

2.2.7 The economics of bioethanol

Every additional processing step costs money and time, reducing the efficiency and profitability of the fermentation. To this end research to reduce steps in the processing and fermentation of lignocellulosic materials is being undertaken (Zaldivar et al., 2002). Ideally cellulose and hemicellulose should be saccharified and fermented together, rather than first being separated. When cellulases are added from external sources and not produced by the fermenting microorganism then the system is known as simultaneous saccharification and fermentation (SSF) (Zaldivar et al., 2001). If the microorganism could produce its own cellulases then the process is direct microbial conversion (DMC). If all the sugars in the substrate could be utilised by one microorganism the process could be substantially streamlined and made more economical and therefore better able to compete financially with petrol.

Wooley et al. (1999) presented a model to estimate the costs of bioethanol technology. Currently both petrol refineries and current wet mill maize ethanol plants, which are commonly used in the USA, are more cost effective than attempts to produce ethanol from lignocellulose. The main additional cost of lignocellulose fermentation is due to the addition of enzymes and the pre-treatment processes. Final costs per litre of ethanol will also largely be determined by the cost of the biomass serving as substrate, which some studies show to be the largest expense (Wooley et al., 1999; Hahn-Hägerdal et al., 2001). The industrial plant design needs to include facilities to convert carbohydrate biopolymers to sugars, if this processremains separate from the fermentation. Microorganisms will ferment both hexose and pentose sugars to ethanol. Plant design includes considerations for recovery of the produced ethanol and the disposal of residual waste residues. Savings in waste disposal and electricity can be made if waste residues are burnt to provide electricity for the plant. Improvements in cost-effectiveness are also made when by-products having a higher value than the ethanol are sold. Half of the profit from wet mill maize ethanol plants producing ethanol from starch is from co-products (Wheals et al., 1999). Glycerol is sold, the yeast is incorporated into distiller's grains as a protein supplement for animal feed, and the aqueous residue known as stillage left after distillation is used as fertilizer.
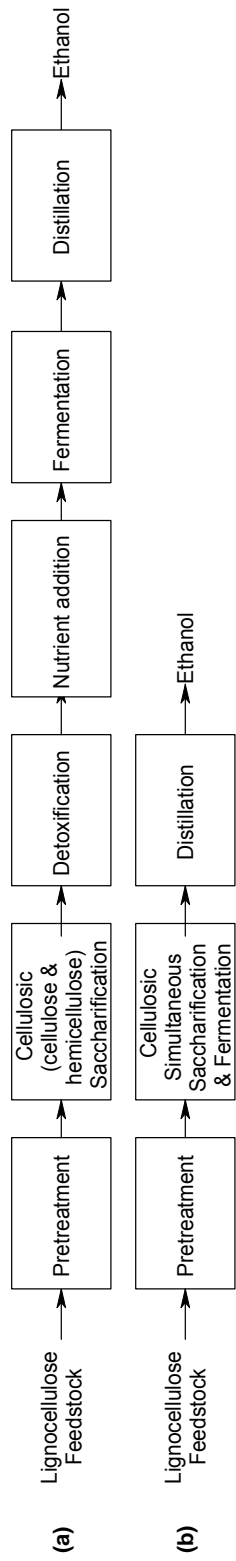
Figure 3: Comparison of (a) a typical acid hydrolysis fermentation and (b) simultaneous enzyme saccharification and fermentation (Mielenz, 2001)

2.2.8 Bioethanol: a practical alternative

Ethanol from lignocellulose has great potential to be an economically viable, sustainable energy resource. Ethanol from fermentations can use readily available and under-utilised resources to produce a cleaner product than fossil fuels. Current and past utilisation of fossil fuels has encouraged unrealistic expectations with regard to energy. These sources are non-renewable, but humanity appears to have no expectations of reducing energy requirements, as shown by the increasing worldwide consumption of transportation fuel (Hahn-Hägerdal et al., 2001). In developed countries a single human consumes the energy equivalent of a 30 tonne primate (Moses and Brown, 2003). Energy expenditures are not being reduced and the underdeveloped countries have every expectation of developing similar environmentally disastrous energy economies. Non-renewable fossil fuels cannot in the long- or even medium-term sustain these expectations.

Currently the increasing global energy needs make the expansion of oil fields and drilling into otherwise pristine areas, such as the Arctic National Wildlife Refuge and the National Petroleum Reserve in Alaska, very likely (Drew, 2005). Serious exploration of alternative energy sources shows that the environment is perceived as having a great value. In the process of completely using up the global supply of fossil fuels, vast volumes of carbon dioxide will be released (Hahn-Hägerdal et al., 2001). Already the atmospheric CO_2 concentration has increased globally by 30% in the past hundred years (Shaw et al., 2002). Amongst other effects, this can exacerbate global warming and lead to changes of plant community composition, in addition elevated CO_2 suppresses root allocation and net plant biomass production. Yet by eliminating wilderness areas and changing the global climate, the earth may not remain a suitable habitat for humans.

Reduced wastefulness in agriculture, industry and the home could lower global energy consumption substantially. In addition money will have to be spent on environmentally sustainable energy sources. This not only includes government subsidies for such alternatives but that customers have the choice to spend more to invest in a sustainable future, as some already believe they do by purchasing 'organic' products.

Not only does ethanol from biomass have great potential to be a sustainable energy source, but with advances in engineering and biotechnology, given time, this environmentally friendly characteristic will not make it unaffordable (Zaldivar et al., 2002).

2.3　D-XYLOSE UTILISING YEAST AND EUKARYOTIC PATHWAYS IN RECOMBINANT D-XYLOSE UTILISING *SACCHAROMYCES CEREVISIAE*

2.3.1　Natural D-xylose fermenting yeasts

A variety of bacteria and yeast can naturally utilise D-xylose (Jeffries, 1983). Currently these organisms are being evaluated on their ability to utilise lignocellulose and effectively ferment it to ethanol. Almost every potential lignocellulose-fermenting organism has some disadvantages ranging from being sensitive to compounds in the hydrolysate, not having established genetic systems for their manipulation, not naturally fermenting pentoses, having special oxygen requirements or even being overly sensitive to the major metabolite, ethanol (Aristidou and Penttilä, 2000). An advantage that yeasts have over bacteria is that they tend to have higher ethanol tolerance (Jeffries and Jin, 2000).

Many types of yeast can grow on D-xylose but only a few catalyse conversion to ethanol anaerobically (Jeffries, 1985; Kuyper et al., 2004). The three most studied yeasts, which produce significant ethanol from D-xylose, are *Candida shehatae*, *Pachysolen tannophilus* and *Pichia stipitis*. All yield most ethanol under micro aerobic conditions (Jeffries and Jin, 2000). The organisms *P. stipitis* and *C. shehatae* have the best aerobic D-xylose fermentation rates yet described (Jeppsson et al., 1999). Similarities between *C. shehatae* and *P. stipitis* led some researchers to believe they were the asexual and sexually perfect stages of the same organism, but this is not the case (Jeffries, 1985). *P. stipitis* is capable of fermenting all the sugars found in wood, including D-xylose for which it uses a native D-xylose utilising pathway. *P. stipitis* D-xylose utilisation enzymes are repressed by glucose and require D-xylose for induction (Ho et al., 1998). It produces 0.45 g ethanol.g^{-1} D-xylose and up to 2.23% ethanol from D-xylose substrates before ethanol toxicity stops fermentation (Jeffries and Jin, 2000). This is substantially lower than the 10-15% ethanol that *S. cerevisiae* regularly yields on D-glucose before toxicity is too great for further fermentation (Hettenhaus, 1998). *S. cerevisiae* ferments D-glucose between 6 and 35 times faster than native D-xylose utilising yeast can ferment D-xylose (Jeffries, 1985).

2.3.2 *S. cerevisiae* as potential D-xylose fermenting yeast

P. stipitis requires very carefully controlled low levels of oxygen, but cannot ferment under strictly anaerobic conditions (Jeppsson et al., 1999). On the other hand, *S. cerevisiae* can grow and produce ethanol under a range of conditions from completely aerobic to anaerobic and vast amounts of ethanol are produced under standard glucose anaerobic fermentations, making it the microorganism which produces the best yield of ethanol from fermentable substrate in the shortest time (Jeppsson et al., 1996). Absolute anaerobic conditions are also not required. The ability of *S. cerevisiae* to survive under high ethanol conditions also reduces the number of precautions needed to ensure sterility, as the high ethanol concentration it produces is toxic for most other organisms. More importantly, methods of harvesting high concentrations of ethanol are more efficient and less costly than for low concentrations of ethanol, as processes are viable from 5% w/w and higher, thereby making an economic difference to the viability of bioethanol processes (Jeffries and Jin, 2000).

S. cerevisiae is also resistant to many of the toxic compounds found in lignocellulosic hydrolysates, while other organisms are more sensitive (Zaldivar et al., 2002). This makes *S. cerevisiae* the preferred organism for fermentations on lignocellulosic hydrolysates as it performs better than any other yeast or bacterium on non-detoxified lignocellulosic hydrolysates (Lindén and Hahn-Hägerdal, 1989). The major drawback of *S. cerevisiae* is that it cannot naturally grow on or ferment D-xylose.

S. cerevisiae has the further advantage of being a preferred organism with which to work because of its food-grade status from centuries of use in the bread and alcohol fermentation industries. This means that a variety of breeding and modification processes have already been developed to improve or modify strains for specific processes. At the same time, *S. cerevisiae* is a model organism with its whole genome sequenced and available (Anonymous, 1996). The metabolism of this yeast is better understood than most others, since substantial research has been done on its biochemistry, genetic modification and other selection techniques are therefore reasonably easy to apply.

2.3.3 *S. cerevisiae* native D-xylose utilising enzymes

Although *S. cerevisiae* cannot convert the pentose sugar D-xylose to ethanol (Hahn-Hägerdal et al., 2001), *S. cerevisiae* can convert xylulose to ethanol under oxygen-limited conditions (Senac and Hahn-Hägerdal, 1990; Yu et al., 1995). Therefore *S. cerevisiae* naturally possesses the genes needed for xylulose metabolism (Toivari et al., 2004). This suggests that only the upper part of the D-xylose utilisation pathway is inactive in *S. cerevisiae* (Jeffries, 1983).

For *S. cerevisiae* to utilise D-xylose it is necessary for D-xylose to be converted to its isomer xylulose. Yeasts which naturally use D-xylose, convert D-xylose to xylitol with xylose reductase (XR), and xylitol to xylulose using xylitol dehydrogenase (XDH) (Amore et al., 1991). This metabolism of D-xylose and the *P. stipitis* enzymes needed for D-xylose utilisation by *S. cerevisiae* are shown in Figure 4(a). Phosphorylation of xylulose by xylulose kinase (XK) before entering the pentose phosphate pathway (PPP) also seems to be critical in D-xylose metabolism (Hahn-Hägerdal et al., 2001). Therefore a recombinant *S. cerevisiae* engineered to have a complete D-xylose utilisation system should at least express the *XYL1* and *XYL2* genes and overexpress the native xylulose kinase (*XKS1*) gene.

Recently Toivari et al. (2004) were able to produce a *S. cerevisiae* with D-xylose utilisation activity by overexpressing native genes. This pathway is shown in Figure 4(b), allowing comparison with the corresponding *P. stipitis* genes in Figure 4(a). The *S. cerevisiae* gene *GRE3* (*YHR104w*) encodes a non-specific aldose reductase (AR), and is functionally similar to the *P. stipitis XYL1* gene coding for xylose reductase (Träff et al., 2001). While the *P. stipitis* enzyme can use either NADH or NADPH as a cofactor, the *S. cerevisiae GRE3* gene can only use NADPH.

<div align="center">(a)</div> <div align="center">(b)</div>
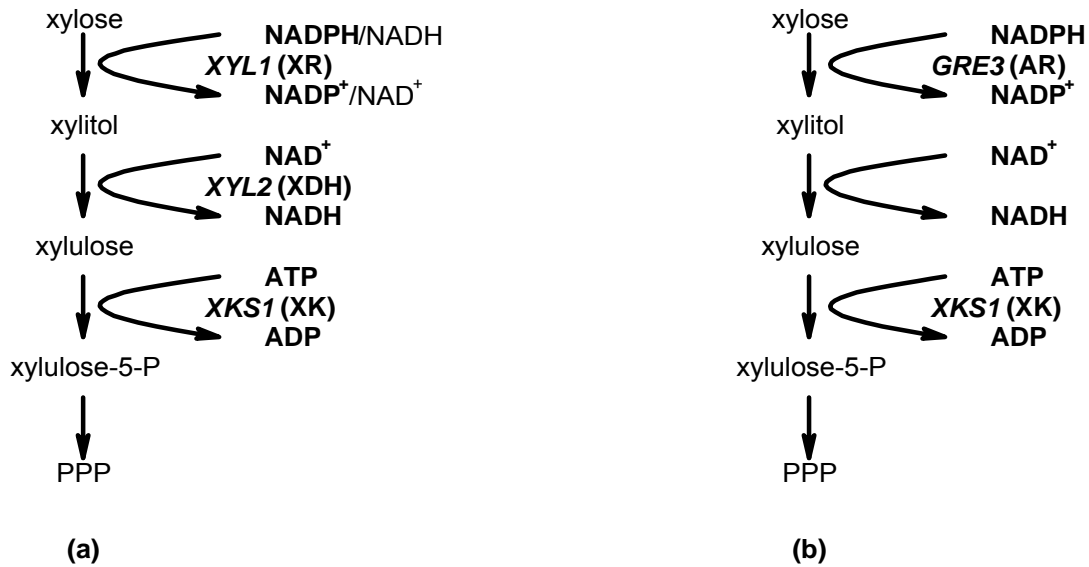
Figure 4: (a) *P. stipitis* D-xylose utilising enzymes introduced into the *S. cerevisiae* metabolic pathway. (b) Putative D-xylose utilising enzymes native to the *S. cerevisiae* metabolic pathway.

In addition a *S. cerevisiae XYL2* gene (*YLR070c*) encoding an XDH was also overexpressed (Richard et al., 1999; Toivari et al., 2004). This enzyme, like *P. stipitis* XDH requires NAD$^+$ as cofactor. *S. cerevisiae* sorbitol dehydrogenases (SDH) are also capable of performing the function of XDH (Toivari et al., 2004).

When the *S. cerevisiae GRE3* and *XYL2* genes were overexpressed in *S. cerevisiae*, it produced higher levels of xylitol than did strains transformed with *P. stipitis* genes for XR and XDH (Toivari et al., 2004). *P. stipitis* enzymes expressed in *S. cerevisiae* led to higher biomass and greater ethanol yield on D-xylose than did overexpression of the native enzymes in *S. cerevisiae*.

2.3.4 *S. cerevisiae* and strain background

Literature on *S. cerevisiae* and D-xylose utilisation contains some vastly different statements on the use of xylulose by this organism. Kuyper et al. (2004) and Jeffries (1983) stated that non-recombinant *S. cerevisiae* could not grow fermentatively on either D-xylose or xylulose. This is supported by Walfridsson et al. (1996) who reported that *S. cerevisiae* apparently does not grow on xylulose in minimal medium, and does not increase in dry weight by growth on D-xylose. In contrast, Jeffries

(1981) stated that only the upper part of the D-xylose utilising pathway is missing in *S. cerevisiae*, since it can grow on xylulose as sole carbon source. Richard et al. (2000) corroborate this by saying the very slow growth on D-xylulose as sole carbon source is enhanced by *XKS1* overexpression, while other researchers found overexpression to be lethal (Rodriguez-Peña et al., 1998). In part the contradictory findings can be explained by different culture conditions including medium and oxygenation, yet large differences remain between recombinant *S. cerevisiae* strains.

Most research in the past few years on D-xylose utilisation in recombinant *S. cerevisiae* has been done in various strains of *S. cerevisiae* transformed with the *P. stipitis* enzymes xylose reductase (XR) and xylitol dehydrogenase (XDH). Different research groups have obtained different results. Among the best performing strains were a strain based on *S. cerevisiae* strain 1400 [pLNH32] (expressing *P. stipitis* XR and XDH and overexpressing native XK enzymes) producing 0.45 g ethanol.g^{-1} D-xylose with 0.1 xylitol.g^{-1} D-xylose as byproduct and capable of simultaneous fermentation of xylose and glucose anaerobically (Ho et al., 1998). A more recent strain TMB3255 of CEN.PK background produced 0.41g ethanol.g^{-1} D-xylose and 0.05 g xylitol.g^{-1} D-xylose. This yeast, in addition to expressing XR, XDH and XK, had deletions in parts of the oxidative pentose phosphate pathway (Jeppsson et al., 2002).

Since the same enzymes have been used in many strains, and no research appears to better the 0.45 g ethanol.g^{-1} D-xylose threshold, this indicates that the particular starting host *S. cerevisiae* strain is one of the most influential factors in D-xylose utilisation. Toivari et al. (2004) compared a *S. cerevisiae* CEN.PK2-based strain and S150-2B-based strain transformed with the same D-xylose utilising enzymes. The CEN.PK2-based strain had substantially higher growth rate, specific D-xylose consumption and specific xylitol production rate, showing the significant effect of different parental strains. More successful *S. cerevisiae* strains may have genetic or physiological variations which improve D-xylose transport, produce fewer non-specific aldoses, upregulate carbon flux through the pentose phosphate pathway or in some other way improve their potential for D-xylose metabolism. This may well also explain differing conclusions with respect to the xylulose utilisation by *S. cerevisiae*.

2.3.5 Heterologous gene expression systems in industrial settings

There is now substantial research experience to draw upon in D-xylose utilising *S. cerevisiae*. Research project considerations include the appropriate *S. cerevisae* host strain, D-xylose utilisation genes, transformation techniques and markers available, whether further manipulation will take place and the experimental selection and culture conditions for the yeast.

Strain background also has general importance for commercial fermentations and genetic manipulation apart from natural D-xylose utilisation ability. Industrial *S. cerevisiae* strains usually have the advantage over laboratory strains for commercial fermentations due to tolerance to high cell density and pressures encountered within factory settings (Zaldivar et al., 2002). Disadvantages include that they can be difficult to transform due to a thick cell wall and that producing auxotrophic strains is not easy since they are often polyploid. Likewise it is more difficult to analyse industrial strains with sporulation or other methods to determine whether essential genes have been deleted, or where mutations have occurred. Laboratory strains are generally less tolerant towards industrial conditions. The desirable characteristics of laboratory yeasts are that transformation methods have been optimised for them and they are generally better for research as, being haploid or diploid, the effect of gene changes are comparatively simple to determine. Therefore the choice of strain is important not only for manipulation and research, but for utility for future industrial scale-up.

In the development of a gene-expression system for eventual industrial use there are many practical considerations. Since the genes used need to be retained at stable levels one consideration is the plasmid type used to transform yeast strains. This will influence both the metabolic strain on the organism and financial outlay on medium components, both of which should be minimised. Since hemicelluloses and other undefined media are likely to be used in eventual industrial applications, selection for genetic markers on plasmids is unlikely to be strong enough to make auxotrophic strains industrially competitive. Even when auxotrophic strains have been complemented with wild-type genes, they may have metabolic differences to their

wild-type counterparts (Pronk, 2002). Auxotrophic strains require nutritional supplementation and may grow at different rates to prototrophic strains, as absorbing the essential compound from the medium may differ metabolically from manufacturing it. In addition strains which carry the complementing gene on a multicopy plasmid, may show differences in biomass density or product formation to the prototrophic strain (Beretta et al., 1991). At the same time the public backlash against foreign genes combined with the expense of adding antibiotics to lignocellulosic hydrolysates and media for inocula makes toxic dominant selectable markers unattractive.

To prevent plasmid loss, without expensive defined medium or antibiotics, integrated plasmids present a good option. However some researchers have successfully used high-copy number plasmids in medium with D-xylose as sole carbon source and suggest that the need to metabolise D-xylose is sufficient selection in *S. cerevisiae* to prevent plasmid loss, maintaining the transformants (Ho et al., 1998). The copy-number of the plasmid can affect the fitness of a cell and the amount of enzyme produced. While high-copy number plasmids may lead to high enzyme expression, this may in some circumstances put undue stress on the cell. With integrated plasmids usually only a single copy of the gene for an enzyme is integrated, so expression is often lower but the 'metabolic burden' is also lighter.

Another important factor for expression is the chosen promoter. For good D-xylose metabolism constitutive promoters could be chosen, or promoters used that are activated by pentoses in the media. The successful strain *S. cerevisiae* 1400 [pLNH32] used constitutive glycolytic promoters (Ho et al., 1998). Non-constitutive promoters will clearly be inactive if the activating compound is not present.

2.3.6 D-xylose transport in *S. cerevisiae*

Theoretically both the carbon sources D-glucose and D-xylose should result in a maximum yield of 0.51 g ethanol.g^{-1} sugar (Ostergaard et al., 2000). In practice the yields differ substantially, presumably because the metabolism of D-xylose by recombinant *S. cerevisiae* is significantly different from glucose. Glucose fermentation produces few by-products, whereas the unwanted by-product xylitol is

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produced in D-xylose fermentations at levels close to those of the desired product ethanol.

One limitation on D-xylose metabolism may be in transporting D-xylose into the yeast cell (Eliasson et al., 2000a). Research by Kotyk (1966) confirmed competition between D-glucose and D-xylose for sugar transporters in *S. cerevisiae*. Work on recombinant xylose utilising *S. cerevisiae* on mixtures of D-xylose and D-glucose showed the rate of D-xylose uptake increased with decreasing D-glucose concentrations (Eliasson et al., 2000a). Very high levels of D-xylose block D-glucose utilisation and this activity is membrane-associated (Kotyk, 1966). Also the activation energy for D-xylose suggests that movement of the sugar into the cell may be limiting at temperatures of 15ºC to 30ºC.

Hamacher et al. (2002) stated that transport of D-xylose into recombinant *S. cerevisiae* cells is not mediated by specific pentose transporters. In *S. cerevisiae* hexose sugars are transported by the hexose transporter subfamily comprising 18 proteins, namely Hxt1-17 and Gal2 (Wieczorke et al., 1999). Cells where all 18 hexose transporters have been deleted, are unable to import or grow on D-xylose (Hamacher et al., 2002). It was shown that at 2% D-xylose concentrations the transporters with moderately low affinity for D-glucose, namely Hxt2, Hxt4 and Hxt5, and the high affinity hexose transporters Hxt6, Hxt7, and Gal2 support growth on D-xylose (Hamacher et al., 2002). Of these, the high-affinity D-glucose transporter, Hxt 7 appeared to confer the highest D-xylose activity. These transporters mediate transport of the substrates down a concentration gradient by facilitated diffusion. The affinity that these transporters have for D-glucose is 200 times greater than the affinity for D-xylose (Eliasson et al., 2000a). However ethanol production from D-xylose was not increased when the monosaccharide transporters were overexpressed (Hamacher et al., 2002). Gárdonyi et al. (2003) supported this, showing in *S cerevisiae* TMB3001 that D-xylose transport was not the rate limiting step in D-xylose utilisation, and in TMB3260, which could utilise D-xylose 70% faster in oxygen-limited batch fermentation, the transport only influenced the fermentation rate where extracellular D-xylose concentrations were lower than 1 g.L^{-1}. The two research groups thus concluded, in direct contradiction to the suggestion by Eliasson

et al. (2000a), that D-xylose uptake does not appear to determine D-xylose flux and, although uptake can be improved, it is not the limiting factor in the metabolism of D-xylose (Hamacher et al., 2002; Gárdonyi et al., 2003).

2.3.7 Glucose catabolite repression in *S. cerevisiae*

S. cerevisiae experiences catabolite repression, namely it prefers to utilise glucose above any other carbon source (Van Zyl et al., 1999). *S. cerevisiae* ATCC 23860, without heterologous xylose utilising genes, tested on various mixtures of D-xylose, D-glucose, D-xylulose and xylitol showed no utilisation of D-xylose or D-xylitol. In glucose and xylulose mixtures consumption of D-xylulose began only once D-glucose had completely been depleted (Hsiao et al., 1982). If *S. cerevisiae* is to eventually ferment lignocellulosic hydrolysates, the organisms must be exposed to D-glucose and D-xylose simultaneously. In *E. coli* the phosphotransferase system transports and phosphorylates glucose, thereby causing catabolite repression of other substrates (Nichols et al., 2001). *E. coli* with deleted *ptSG* no longer exhibit this catabolite repression and successfully utilise glucose simultaneously with other sugars (Nichols et al., 2001). Similarly recombinant *S. cerevisiae* strains, which are not subject to catabolite repression, should be significantly more successful at simultaneous utilisation of D-glucose and D-xylose alleviating the negative effect of glucose repression on xylose metabolism.

However, current recombinant *S. cerevisiae* ferment D-xylose anaerobically very slowly if at all. Many researchers grow or ferment their yeast on a combination of D-glucose and D-xylose (Ho et al., 1998). *S. cerevisiae* always prefers to metabolise D-glucose so that in a mixture all the D-glucose will be used before all the D-xylose is metabolised, giving a diauxic growth pattern. Work on *hxt1-7 S. cerevisiae* strains showed mutants with reduced glucose transport had defects in glucose repression and to trigger repression of other hexose sugars, glucose must be taken up by the cell (Reifenberger et al., 1997). This may well also be true of glucose repression of D-xylose. Therefore the cell is unlikely to evolve into a more efficient, faster, D-xylose fermenter if glucose is an available substrate, although glucose metabolism may help produce certain essential metabolic intermediates while D-xylose is metabolised.

Research by Wu et al. (2004) on a diploid *S. cerevisiae* FE1679/ΔHO grown in a chemostat under aerobic conditions examined the effects of glucose starvation. Amongst the upregulated genes were those encoding high-affinity glucose transporters, glucose kinases, PPP-enzymes and tricarboxylic acid (TCA) cycle enzymes and glutamate-dehydrogenase enzymes, suggesting that regulation of carbon and nitrogen metabolism are not completely independent. The genes encoding oxidative PPP and TCA enzymes were activated, providing intermediates and ATP for cellular growth. Conditions with D-xylose as sole carbon source may mirror glucose starvation. However where D-xylose is the only carbon source, carbon is fluxed through the non-oxidative PPP, and oxygen levels in fermentations are low. In this case the PPP and TCA cycles cannot provide energy and intermediates with the same efficiency as seen by Wu et al. (2004). Tsuboi et al. (2003) studied *S. cerevisiae* mutants where *JEN1*, which encodes a pyruvate transporter, was not repressed by glucose. The mutants showed a phenotype corresponding to insensitivity to MIG1 protein located in the nucleus, which repressed transcription of certain genes when glucose is present. MIG1 protein is dephosphorylated and moved to the cytoplasm when there is no longer glucose present. Although it is unclear whether the results are true for other strains, a strain, which does not exhibit catabolite repression for the non-fermentable carbon source pyruvate, may also allow simultaneous metabolism of D-xylose with glucose. Whether such changes in glucose regulation would modify metabolism to reduce the efficiency of glucose-utilisation, or reduce the fitness of recombinant *S. cerevisiae* on lignocellulosic hydrolysates would need to be experimentally determined.

Kuyper et al. (2005) selected for an improved, recombinant D-xylose utilising *S. cerevisiae* on xylose/glucose mixtures with prolonged anaerobic cultivation on xylose/glucose mixtures in automated sequencing batch reactors. Selection was induced by the addition of extra D-xylose after all the glucose and D-xylose had been sequentially utilised, which followed a diauxic pattern. The strain continued to consume D-xylose preferentially to glucose although the K_m for D-xylose decreased and the transport capacity for D-xylose improved to the point where it was nearly four times higher than for glucose. Thus a selection strategy, while not eliminating glucose catabolite repression, was able to increase the rate of D-xylose utilisation.

S. cerevisiae is also much more efficient at transporting glucose into the cell and fermenting it than for D-xylose. At high glucose concentrations the cell is induced into vacuole protein degradation of the high-affinity hexose transporters Hxt 6 and Hxt 7 (Krampe et al., 1998). One can only speculate that since these transporters are so essential to importing D-xylose into the cell, that their degradation will be detrimental to the speed of D-xylose transport and metabolism, in the presence of glucose (Hamacher et al., 2002).

One alternative to D-xylose as sole carbon source is to use a non-repressive carbon source such as raffinose as co-metabolite or extremely low concentrations of glucose, as levels as low as 0.05 mM can compete with D-xylose uptake (Jeffries and Jin, 2000). Van Zyl et al. (1999) compared glucose and raffinose as co-metabolites with D-xylose in recombinant D-xylose utilising *S. cerevisiae*. The D-xylose consumption rate increased when glucose or raffinose was used as cosubstrates. Raffinose led to higher D-xylose consumption than glucose. Glucose led to stress as evidenced by moderate levels of glycerol and acetic acid and high initial ethanol concentrations, while raffinose resulted in more gradual ethanol formation, less acetic acid and no glycerol formation. Thus, use of glucose-grown precultures and cells can be counter-productive. Therefore it would make sense that an organism developed and evolved on D-xylose alone, or with other sugars than glucose will more likely be able to metabolise D-xylose at similar rates to glucose and without catabolite repression.

2.3.8 Cofactor imbalance in recombinant D-xylose utilising *S. cerevisiae*

Most eukaryotic pathways for D-xylose degradation begin with xylose reductase (XR) and xyltitol dehydrogenase (XDH) enzymes. For many XR enzymes both NADH and NADPH can act as cofactors for conversion of D-xylose to xylitol, but NADPH is favoured, as is the case in the *P. stipitis* pathway (Hahn-Hägerdal et al., 2001). To convert xylitol to xylulose, NAD^+-mediated XDH is used (Richard et al., 1999). This means that the cofactors are not replenished. (See Figure 4(a)). Since *S. cerevisiae* does not have an effective transhydrogenase enzyme to interconvert NADPH and NADH, once the cell's pool of cofactors has been used, they are effectively taken out

of circulation (Kuyper et al., 2004). Due to this cofactor imbalance, metabolism of D-xylose under anaerobic conditions tends to be halted, resulting in a large amount of the unwanted by-product xylitol, but very little ethanol.

Most current work on D-xylose utilising pathways in *S. cerevisiae* remains focused on improving strains which have been transformed with *P. stipitis* enzymes. Yet despite possessing these genes, up to now no recombinant *S. cerevisiae* has been shown to both grow and ferment D-xylose as sole carbon source under completely anaerobic conditions. Furthermore, even aerobic growth rates on D-xylose fall far short of the yields theoretically possible, which are obtained in practice on glucose. Ho et al., (1998) produced an *S. cerevisiae* strain capable of anaerobic fermentation on D-xylose as sole carbon source, but it could not grow anaerobically. The effects of oxygen are shown by a recombinant *S. cerevisiae* with *P. stipitis* XR and XDH, and overexpressed native XK, cultivated on mixtures of 15 g.L^{-1} D-xylose and 5 g.L^{-1} D-glucose (Hahn-Hägerdal et al., 2001). The ethanol level remained approximately 0.34 g.g^{-1} carbohydrate as the oxygen level rose, although glycerol and xylitol levels were lower and the biomass was increased.

The fermentation problems may partly be due to the equilibrium constants for reduction of D-xylose to xylitol (Hahn-Hägerdal et al., 2001) and for oxidation of xylitol to xylulose (Rizzi et al., 1988), both favouring xylitol production. In addition, up to the same mass of xylitol as ethanol is produced showing redox constraints in recombinant *S. cerevisiae*, this is wasteful since the process is aimed at producing large volumes of ethanol (Wahlbom et al., 2001). In *S. cerevisiae* overexpressing native enzymes, even more xylitol is produced possibly because the cofactor for AR (NADPH) is not renewed by the dehydrogenase (Toivari et al., 2004).

Many scientists have attempted to solve the redox imbalance. One option would be a xylose reductase with greater specificity for NADH, or alternatively a xylitol dehydrogenase with NADP$^+$-specificity, however attempts to generate such an enzyme through mutation, as reviewed by Hahn-Hägerdal et al. (2001), have succeeded only in decreasing specific activity or affinity for D-xylose. Since redox imbalance is reduced in the presence of other carbon sources and oxygen, it has

been thought that an enzyme to regenerate cofactors might alleviate the cofactor mismatch. D-xylose passes through the pentose phosphate pathway so certain glycolysis metabolites are no longer produced. If D-xylose is not the sole carbon source then some carbon can cycle through glycolysis, the various intermediates thus produced may have a positive effect on the redox imbalance.

It is speculated that the D-xylose fermenting yeast *P. stipitis* does not suffer this problem due to its possessing two redox sinks unknown in *S. cerevisiae*. An alternative cyanide-insensitive oxidase may efficiently regenerate NAD^+ for polyol oxidation, and D-arabinitol dehydrogenase (ArDH) may, by reducing D-ribulose to D-arabinitol, pull xylulose-5-P further in the pathway (Walfridsson et al., 1995). These two mechanisms enhance *P. stipitis* capability to balance the cell's redox requirements during D-xylose metabolism. Since *P. stipitis* also ferments under conditions of low oxygen but never completely anaerobically, the available oxygen may be used to balance cofactor metabolism, while using the enzymes XR, XDH and its own XK.

The rates of D-xylose fermentation are also very slow. Part of the challenge for D-xylose metabolism is increasing the fermentation rate of D-xylose to ethanol. As can be seen in the following section, overexpression of the native *S. cerevisiae* XK has already partially alleviated the problem as xylitol is fluxed further along the pathway enhancing ethanol production.

2.3.9 Optimising XR, XDH, XK enzyme ratios for D-xylose utilisation

Since D-xylose utilisation requires carbon flux through the recombinant pathway, the amount of each enzyme must be produced in the correct ratio for optimal D-xylose utilisation. At one point XR:XDH:XK enzyme activity ratios were extensively investigated in the hope that the optimal ratio would alleviate the effect of unfavourable equilibrium constants. Most researchers conclude that high levels of XDH compared to XR substantially reduce the amount of xylitol produced (Johansson et al., 2001). Strains with different XR:XDH activity ratios were cultivated under O_2 limitation and the strain with the highest XR:XDH ratio produced 0.82 g xylitol.g D-xylose^{-1}, but the strain with the lowest ratio produced no xylitol

(Walfridsson et al., 1997). *S. cerevisiae* has its own xylulokinase genes, only the longer 1803 bp *XKS1* gene codes for active enzyme (Johansson et al., 2001). When this gene was overexpressed in recombinant *S. cerevisiae* the ethanol yield doubled, xylitol was reduced by 70-100% and acetate was also reduced. However, D-xylose utilisation was reduced by at least half. Specific D-xylose consumption has also been shown to be enhanced by XK overexpression (Hahn-Hägerdal et al., 2001). Ethanol production from D-xylose was higher from a recombinant *S. cerevisiae* strain with chromosomally integrated XK (Wahlbom et al., 2001) than a strain where the gene is present in multiple copies (Johansson et al., 2001). This supports models suggesting that XK should not be too highly expressed, as it requires ATP. The rest of the pathway cannot regenerate ATP fast enough, leading to depletion when XK is highly expressed (Rodriguez-Peña et al., 1998; Hahn-Hägerdal, 2001). This pathway has the characteristics of one with so-called 'turbo design' (Tuesink et al., 1998). In such cases a reaction early on in a pathway requires energy from ATP, in this case the xylulose phosphorylation reaction using XK, and ATP is regenerated downstream in the pathway. *S. cerevisiae* with overexpressed XK on xylulose as sole carbon source may show substrate-accelerated cell death as a large volume of xylulose is phosphorylated at a faster rate than the rest of the pathway can produce ATP, leading to runaway accumulation of xylulose-5-phosphate and therefore eventual cell death. A kinetic data model of the D-xylose utilisation metabolic pathway in *S. cerevisiae* now exists and experiments support the finding that to reduce xylitol formation the optimal XR:XDH:XK ratio is 1:10:4 (Hahn-Hägerdal et al., 2001).

2.3.10 The role of the pentose phosphate pathway and glycolysis in pentose fermentation by *S. cerevisiae*

Work on recombinant D-xylose utilising *S. cerevisiae* has shown that improvement of D-xylose transport and optimisation of XR, XDH and XK ratios do not improve D-xylose utilisation sufficiently for effective fermentation to ethanol. Assimilation of xylulose-5-phosphate thus also requires attention. D-xylose is converted to xylulose and brought into yeast metabolism via conversion to xylulose-5-phosphate by XK in an ATP-dependent step. Xylulose-5-phosphate then enters the PPP. In the oxidative part of the PPP, hexose phosphates are converted to pentose phosphates, while in the non-oxidative PPP the opposite occurs (Jeffries, 1983). The PPP is an

important link in yeast metabolism as its metabolites can exit into the Embden-Meyerhof pathway (EMP), enter nucleotide synthesis or amino acid synthesis, the cofactor NADPH is produced when CO_2 is released in the oxidative PPP (Jeffries, 1983). Xylulose-5-phosphate enters the non-oxidative PPP, which can lead only to the EMP or biosynthetic pathways.

S. cerevisiae metabolically channels D-xylose, like other five-carbon sugars, through the pentose phosphate pathway. Strains have been developed with improved capacity for pentose metabolism by making a variety of changes to the pentose phosphate pathway, suggesting that significant modification of natural *S cerevisiae* biochemical pathways is necessary for good D-xylose utilisation (Walfridsson et al., 1995). As *S. cerevisiae*'s glucose metabolism draws carbon through glycolysis, some D-xylose fermentation problems may be due to pentose sugars being channelled through the PPP. The non-oxidative part of the PPP has insufficient capacity, as shown by accumulation of sedoheptulose-7-phosphate rather than conversion to fructose-6-phosphate and erythrose-6-phosphate, as illustrated in Figure 5 (Aristidou and Penttilä, 2000).

Under aerobic conditions the oxidative part of the PPP provides NADPH required for D-xylose reduction, and reducing power is exchanged between the NADPH and NADH pool, regenerating cofactors (Jeffries, 1985). Pentose sugars are utilised only under aerobic conditions, whereas *S. cerevisiae* can use D-glucose completely anaerobically. The native D-xylose utilising *P. stipitis* also requires a low level of oxygen for D-xylose fermentations, although its xylose reductase can use both NADPH and NADH as cofactors (Lindén and Hahn-Hägerdal, 1989). Unless conditions are aerobic or an additional carbon source is simultaneously metabolised with D-xylose the lack of intermediates from glycolysis prevents D-xylose flux. Some of the glycolysis reactions may regenerate the cofactors NADPH and NAD^+ alleviating the cofactor imbalance and subsequent xylitol formation, in a similar way to oxygenation.

One problem with D-xylose fermentation could be that the PPP does not activate ethanologenic enzymes, because signalling metabolites do not reach high enough

concentrations (Walfridsson et al., 1995). Xylulose fermenting *S. cerevisiae* has signalling metabolite levels almost ten times lower than on glucose (Senac and Hahn-Hägerdal, 1990). Phosphoglucose isomerase (PGI1) interconverts glucose-6-phosphate and fructose-6-phosphate in glycolysis, shown in Figure 5. *S. cerevisiae pgi1* deletion mutants are incapable of growth on glucose as sole carbon source. Strains with deletions to trehalose synthesis, or reduced PGI1 activity showed substantial increase of ethanol from xylulose (Eliasson et al., 2000b). A recombinant *S. cerevisiae* expressing *P. stipitis XYL1* and *XYL2*, showed enhanced ethanol production to a third of theoretical yield when respiration was inhibited by antimycin A (Kötter and Ciriacy, 1993). This confirms that the ethanol from D-xylose pathway requires the stimulation of ethanologenic enzyme pyruvate decarboxylase (PDC1, PDC5), which converts pyruvate to acetaldehyde (Figure 5). In strains with lower PGI1 activity the signalling intermediates fructose-6-phosphate and fructose 1,6-bisphosphate accumulated intracellularly to levels that activated ethanologenic enzymes (Eliasson et al., 2000b). Thus inadequate inactivation of respiratory enzymes and stimulation of ethanol enzymes is a factor in the suboptimal D-xylose utilisation in recombinant *S. cerevisiae*.

When Eliasson et al. (2000b) deleted the *GND1* gene encoding gluconate 6-phosphate dehydrogenase (6PGDH), the ethanol yield from xylulose improved by almost a third. 6PGDH converts 6-phosphogluconate to ribulose-5-phosphate and releases CO_2 (See Figure 5) (Jeppsson et al., 2002). This improvement may be because carbon-dioxide formation is reduced, keeping more carbon for ethanol (Hahn-Hägerdal et al., 2001). The *ZWF1* gene encodes glucose-6-phosphate dehydrogenase (G6PDH), which catalyses the oxidation of glucose-6-phosphate to 6-phosphoglucono-δ-lactone, as shown in Figure 5 (Miosga and Zimmermann, 1996). This enzyme has significant control over the regulation of the oxidative PPP (Jeffries, 1983). Deletion of the *GND1* or *ZWF1* genes lowers the activity of the PPP, as does decreasing PGI1 activity (Jeppsson et al., 2002). Decreased flux in the PPP lowers the NADPH levels, which causes D-xylose to be preferentially reduced with NADH. Thus, conversion of D-xylose to xylulose is balanced as XR consumes NADH and XDH produces this cofactor. Since the activity of the oxidative PPP was lowered this reduced both xylitol production and the consumption of D-xylose.
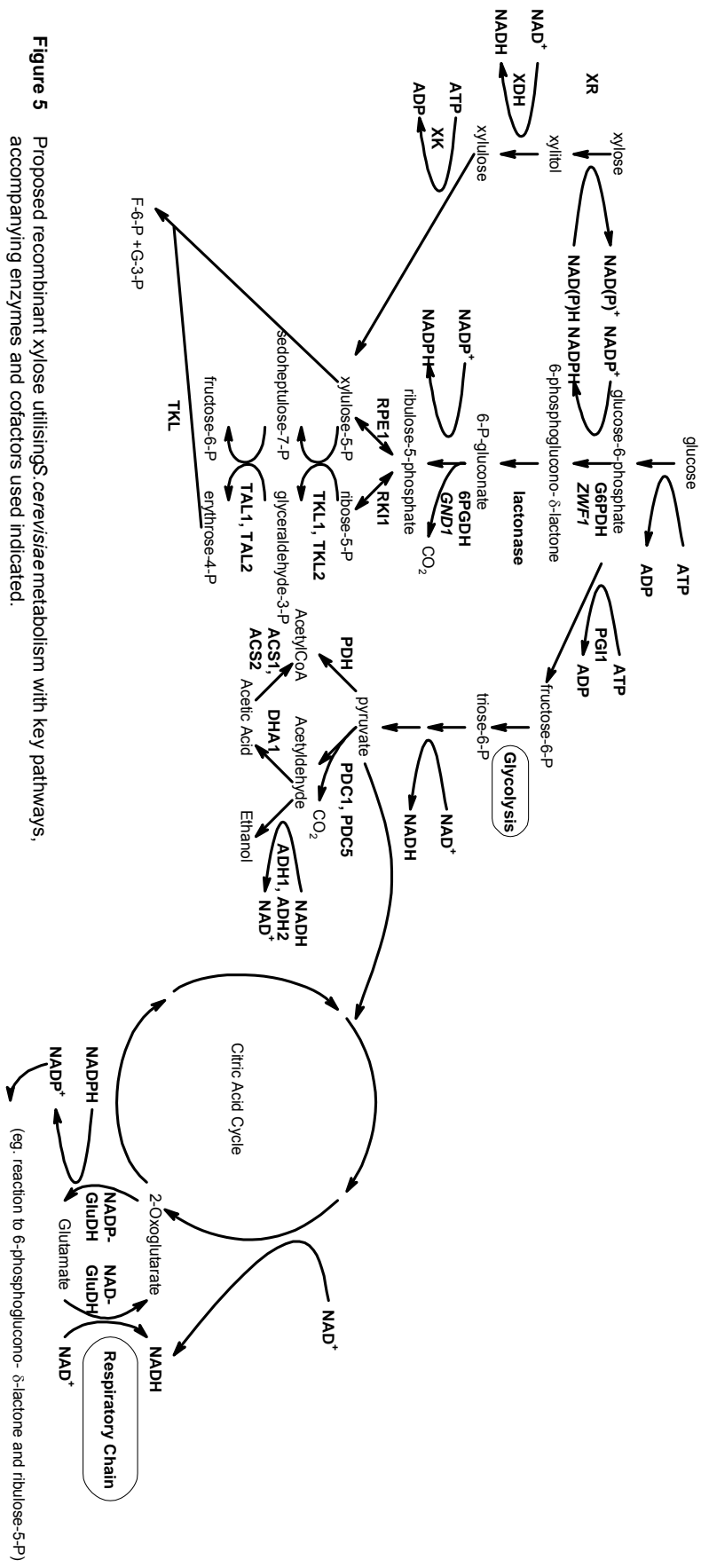
Figure 5 Proposed recombinant xylose utilising *S. cerevisiae* metabolism with key pathways, accompanying enzymes and cofactors used indicated.

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Figure 5 shows the RPE1 and RKI1 enzymes which begin the non-oxidative part of the PPP (Miosga and Zimmermann, 1996). The *RPE1* gene encodes the D-ribulose-5-phosphate 3-epimerase enzyme which reversibly catalyses conversion of D-xylulose-5-phosphate to D-ribulose-5-phosphate. Eliasson et al. (2000b) found that deletion of the *RPE1* gene was lethal when *S. cerevisiae* mutants were grown on xylulose as sole carbon source, although flux through that reaction is generally low (Wahlbom et al., 2001). Mutants show normal growth on glucose, but cannot grow on a xylose/xylulose mixture. This can be understood by noting on Figure 5 that xylulose-5-phosphate must be converted to ribulose-5-phosphate by the RPE1 enzyme, and to ribose-5-phosphate by the RKI1 enzyme to utilise D-xylose. The *RKI1* gene encodes D-ribose-5-phosphate ketol-isomerase, which reversibly converts ribulose-5-phosphate to ribose-5-phosphate (Miosga and Zimmermann, 1996). Deletion mutants were not even viable on glucose, leading the researchers to suggest that the primary function of RKI1 is in an essential part of metabolism, as the loss of function in the non-oxidative pentose phosphate pathway should not cause lethality.

Walfridsson et al. (1995) suggested the enzymes transaldolase (TAL1, TAL2) and transketolase (TKL1, TKL2) control PPP flux and cause a bottleneck for D-xylose utilisation in recombinant *S. cerevisiae*. The group overexpressed both the *TAL1* and *TKL1* genes in a recombinant *S. cerevisiae* (expressing *P. stipitis XYL1* and *XYL2* genes). Overexpression of *TKL1* did not enhance growth but the native transaldolase levels were shown to be insufficient for pentose phosphate metabolism. Under the experimental conditions D-xylose provided carbon for growth and xylitol but ethanol production was unchanged (Walfridsson et al., 1995). This showed that TAL enzymes do limit the flux in *S. cerevisiae*, but overexpression of TAL1 protein did not eliminate xylitol formation, and therefore does not alleviate the cofactor imbalance.

Boles et al. (1993) corrected glucose growth defects in *S. cerevisiae pgi1* deletion mutants by overexpressing the NAD-dependent glutamate dehydrogenase (NAD-GluDH) encoded by the *GDH2* gene. An artificial transhydrogenase cycle was

produced as dehydrogenase enzymes with different cofactor specificities were expressed simultaneously, as seen in Figure 5. The NADPH-dependent glutamate dehydrogenase (NADP-GluDH), encoded by *GDH1*, which uses NADPH converts 2-oxoglutarate to glutamate and produces NADH. These two cytosolic enzymes allowed for efficient cycling of cofactors and relieved the *pgi1* mutation. Similarly the redox imbalance of the xylose reduction and oxidation pathway was reportedly alleviated by just such a system (Aristidou and Penttilä, 2000).

As described above, substantial work was done on the metabolism around the PPP. Almost all of the genes in the pathway have been studied and appear to influence D-xylose flux through the pathway. The effect of overexpression or deletion of any single gene has not, however, enhanced D-xylose fermentation to levels comparable to D-glucose fermentation. In recombinant *S. cerevisiae* glucose flux is 2.2 times higher than D-xylose flux (Eliasson et al., 2000b). According to Hahn-Hägerdal et al. (2001) this low rate is the limitation to anaerobic growth on D-xylose rather than a low ATP yield from pentoses, which theoretically should be the same as from hexoses.

Control analysis allows systems to be described in terms of individual reaction steps, which in the case of metabolism are the separate enzyme reactions, and the influence or control each step has over the entire system (Hofmeyer and Westerhoff, 2001). The theory of metabolic control analysis suggests that engineering single genes will not make a significant difference to flux unless the control coefficient for the enzyme encoded by that gene is larger than 0.6 (Johansson and Hahn-Hägerdal 2002). In practice the problem with D-xylose flux in *S. cerevisiae* seems to be that control of the pathway is shared throughout the metabolic steps. Thus the D-xylose fermentation problems cannot be solved with directed genetic engineering given current understanding of D-xylose metabolism in yeast.

2.3.11 Random mutation and selection could be a useful adjunct to genetic engineering

Yields from D-xylose utilisation in recombinant *S. cerevisiae* are not as high as they theoretically should be. *S. cerevisiae* naturally possesses most of the genes needed for D-xylose metabolism (Toivari et al., 2004). Yet D-xylose fermentations are slow,

and in general not completely anaerobic. Researchers have suggested a variety of places in biochemical pathways where the problem could reside. These, which have each been briefly discussed, include a transport limitation, catabolite repression by glucose, metabolism through the PPP with limitations caused by lack of intermediates from glycolysis and cofactor imbalance (Eliasson et al., 2000a; Van Zyl et al., 1999; Walfridsson et al., 1995; Hahn-Hägerdal et al., 2001). Single gene transformations in *S. cerevisiae* have shown that XR and XDH are essential and overexpression of XK substantially assists with flux. Deleting parts of the oxidative PPP has made further changes. Suggested improvements include introducing a better D-xylose transporter to improve D-xylose transport and a transhydrogenase to enable yeast to replace cofactors.

The flux problem may well not be solved by selectively cloning more genes, as each step in the xylose pathway has so little control over flux (Johansson and Hahn-Hägerdal, 2002). As shown by very different results on different strain backgrounds, the underlying metabolism, though incompletely understood, is crucial. For this reason various top-down strategies for breeding and selection, like protoplast fusions or production of hybrids through mating, have promise and have been more extensively reviewed elsewhere (Hahn-Hägerdal et al., 2001). In these systems greater genetic variation is obtained and selection methods are used to determine which strains have desired traits. In addition, some good results have been obtained by a combination of random mutation and selective evolution over time. Ferea et al. (1999) cultivated diploid *S. cerevisiae* over more than 250 generations in glucose limited conditions and showed an increased fitness of the evolved strains with respect to glucose limitation, this shows in principle the success of selection over time. The D-xylose metabolising enzymes XR, XDH and XK are all produced intracellularly. A selective evolution strategy could be particularly effective, as less well-adapted strains do not have access to sugars and metabolites produced by the improved cells. Selective evolution strategies are specially powerful as they harness the organism's own survival capacity, and while the research can subsequently enhance knowledge of the relevant biochemistry, this understanding is not necessary to begin the study.

For this reason random mutation of recombinant *S. cerevisiae* combined with aerobic growth on mixtures of D-glucose and D-xylose with gradual increase in D-xylose concentration and lowering of oxygen has been explored. Wahlbom et al. (2003) did such a study on the industrial polyploid *S. cerevisiae* strain TMB3400 which derived from a genetically variable population of which some clones had greater fitness on D-xylose than the parent strain. The improved *S. cerevisiae* strain TMB3400 may be the result of physiological conditioning, changes in expression of the three core D-xylose metabolism genes or their actual protein structure, or improvements in expression of other genes regulating D-xylose flux. It is unlikely that the D-xylose utilisation genes were directly modified, although testing this was not within the scope of the study. A similar study using random mutagenesis and selection to produce a haploid strain would be particularly valuable, if it also shows better ethanol production from D-xylose, as any specific genetic changes would be easier to analyse.

Thus a vast body of knowledge of recombinant *S. cerevisiae* with the *P. stipitis* xylose utilisation pathway is available. In addition improvement strategies such as mating and selective evolution have not been exhausted. Yeasts and fungi are however not the only microorganism capable of ethanol production from D-xylose, and as bacteria have differences in their metabolic xylose utilisation pathway, they require consideration of their own.

2.4 D-XYLOSE ISOMERASE IN RECOMBINANT D-XYLOSE UTILISING *SACCHAROMYCES CEREVISIAE*

2.4.1 Natural D-xylose utilising bacteria

Not only yeasts and fungi, but many strains of both native and genetically engineered bacteria can ferment D-xylose to ethanol. Bacteria tend to have low ethanol tolerance and produce mixtures of metabolic products (Jeffries and Jin, 2000). This reduces the efficiency of bioethanol production and harvest. Among the vast number of bacterial strains utilising D-xylose are *Clostridium* sp., native and recombinant *Escherichia coli*, *Klebsiella oxytoca*, *Lactobacillus* sp. and recombinant *Zygomonas mobilis* (Jeffries and Jin, 2000). As an example, the recombinant D-xylose-adapted bacterium *E. coli* KO11 (de Carvalho Lima et al., 2002) can metabolise pentoses and hexoses from corn-cob hydrolysate producing up to 0.47 g ethanol.g^{-1} D-xylose, close to the theoretical maximum of 0.51 g.g^{-1} D-xylose. The final ethanol concentration of 4% produced remains lower than the yield needed for economical harvest. Thus other strategies than simple bacterial fermentations are needed for economical ethanol fermentations from lignocellulose feedstocks.

2.4.2 D-xylose isomerase groups

Xylose isomerase (XI), which is also known as glucose isomerase (GI), is capable of isomerising various aldose sugars, and is generally an intracellular enzyme (Bhosale et al., 1996). Xylose isomerase enzymes have great commercial value in industry as they convert D-glucose to D-fructose, which is used for the production of high-fructose corn syrup. The other reaction catalysed by these enzymes is reversible isomerisation of D-xylose to xylulose, which is of interest for lignocellulose fermentations to ethanol. When D-xylose is isomerised the reaction reaches equilibrium when the product, xylulose, reaches 15% (Kersters-Hilderson et al., 1987)

D-xylose catabolism is encoded by bacterial operons which contain genes for D-xylose isomerase (*xylA*), D-xylulokinase (*xylB*), regulatory protein (*xylR*) and sometimes a D-xylose permease transporter (*xylT*) (Lokman et al., 1991). Affinity for D-xylose varies between the XI from different organisms (Kuyper et al., 2003). For example, while XIs from *Streptomyces* have K_ms between 0.08 mM to 33 mM, *Bacillus* species XIs vary from 1 mM to 10 mM. The temperature optima tend to lie

between 60ºC and 80ºC and the optimal pH range between 7 and 9, depending on the source (Bhosale et al., 1996). XIs include divalent metal cations which improve activity. XIs tend to be best activated by Mg^{2+}, but Co^{2+} helps to hold the quaternary structure of the enzyme and offers superior enzyme stabilisation. On the other hand, a variety of inhibitors are known for these enzymes, these include some metal ions as well as the molecules xylitol, arabitol, sorbitol, mannitol and lyxose (Bhosale et al., 1996).
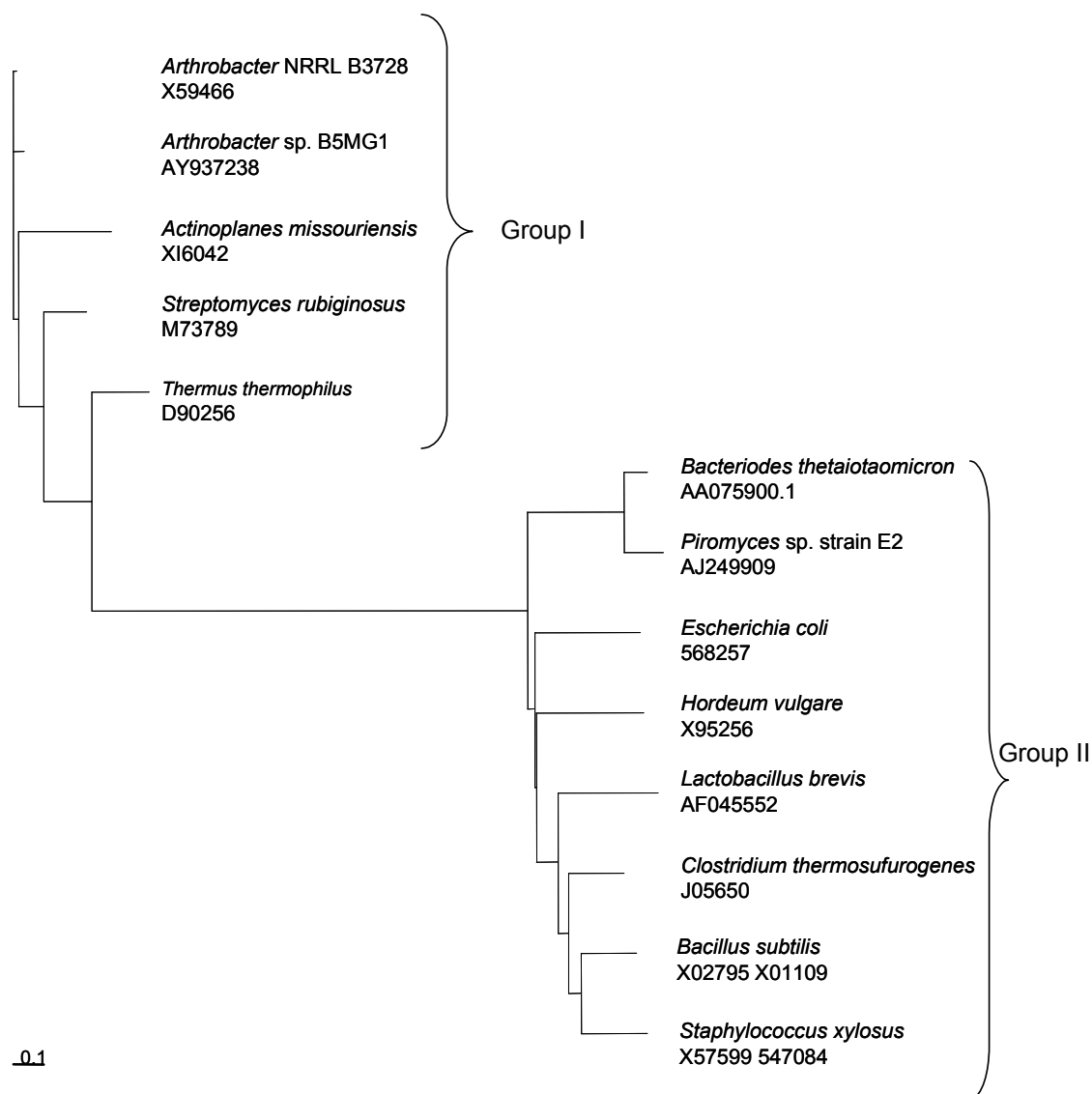
Figure 6: Phylogenetic tree of amino acid sequences of XIs clearly differentiating between the Group I and Group II, and indicating homology between the *Piromyces* and *Bacteroides* XIs. Horizontal lines show the degree of homology.

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XIs have been classified into two main groups (Bhosale et al., 1996). The Group I includes XIs from the high G+C Gram positive bacteria, such as from *Actinoplanes, Streptomyces* and *Arthrobacter species.* XIs from the thermophilic *Thermus* bacterial species are also found in this group, having an alkaline pH optimum and a molecular weight of approximately 45 kDa.

Group II includes all the other XIs. Examples include those from *E. coli*, *Bacillus*, *Clostridium* (also known as *Thermoanaerobacter*), and *Thermotoga* species. This group also includes the eukaryotic XI documented from barley, *Hordeum vulgare*. These XIs have a neutral pH optimum and an extended N-terminal region increasing the molecular weight to approximately 50 kDa. Figure 6 uses a phylogenetic tree to compare the Group I and Group II XIs.

2.4.3 D-xylose isomerase mechanism

XI has substantial commercial importance but the mechanism and even structure are poorly understood (Bhosale et al., 1996). Some XIs have been shown to function in dimeric form, such as *E. coli* XI, while others form tetramers like the *S. rubiginosus* XI, which functions as connected active dimers. The tetrameric form of the enzyme can be seen in Figure 7. XI monomers are formed by a β-barrel surrounded by eight helices and link with neighbouring monomers by an extended C-terminal tail (Whitlow et al., 1991). The active site consists of glutamate, aspartate, histidine residues, and two divalent metal ions in the deep pocket made near the C-terminus of the barrel (Whitlow et al., 1991; Asbóth and Náray-Szabó, 2000). XIs require divalent metal ions, kinetic studies have shown Mg^{2+}, Co^{2+}, Mn^{2+} and Fe^{2+} help enzyme activity and stability (Asbóth and Náray-Szabó, 2000).

Initially XI was thought to have a cis-enediol mechanism, like that for triose-phosphate isomerase (Whitaker et al., 1995). X-ray diffraction studies on the crystalline protein of *S. rubiginosus* D-xylose isomerase suggested histidine might be the base to abstract a proton from the sugar (Carrell et al., 1989). Similarly mutation studies done on *E. coli* XI showed histidines His-101 and His-271 were required for activity (Batt et al., 1990). While His-271 bound a metal ion in the active site, His-101 was suggested as the base mediating the reaction. Site directed mutagenesis

showed that for *Clostridium thermosulfurogenes* XI only His-101 is required (Lee et al., 1990). It was suggested that the substrate is in an open chain conformation and produces a cis-enediol intermediate (Carrell et al., 1989; Collyer and Blow 1990a). Carrell et al. (1989) speculated that proton transfer occurs through a water molecule bound to the substrate.
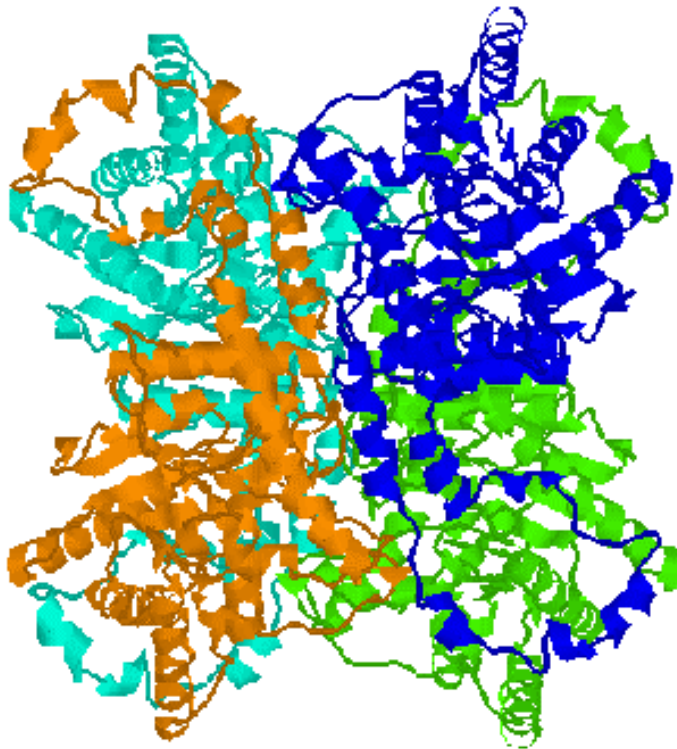
Figure 7: Rasmol visualisation of the tetrameric form of *Arthrobacter* strain B3728 xylose isomerase built from identical subunits (pdb accession number: 1xlg).

XI differs from triose-phosphate isomerase in that it requires metal cofactors, transfers hydrogen without a solvent and has a k_{cat}/K_m several orders of magnitude lower (Farber et al., 1989). Lee et al. (1990) suggested that the essential histidine residue stabilises the transition state and enabled a hydride shift catalysed by a metal ion to occur. This was supported by Collyer and Blow (1990b) as no bases suitable for a cis-enediol intermediate could be determined from crystal structures. Asbóth and Náray-Szabó (2000) also concluded that the mechanism should be a base-catalysed hydride shift as this is more energetically favourable than a cis-enediol

mechanism in the presence of Mg^{2+}, while acid-catalysed hydride shift is unfavourable whether or not Mg^{2+} is present.

In conclusion, unlike the enzyme triose phosphate isomerase, xylose isomerases use a general base catalysed enediol mechanism (Lee et al., 1990). Whitaker (1995) suggested that the sugar in the form of a closed α-D-xylopyranose ring binds the enzyme, after which D-xylose is bound into an open conformation by two divalent cations (Whitlow et al., 1991). The hydride shift occurs by hydrogen transfer from carbon 2 to carbon 1 of the molecule and, as can be seen in Figure 8, is not by movement of solvent (Meng et al., 1993). The process is completed by closure of the substrate to form an alpha-D-xylulose ring.

aldose   cationic form   ketose
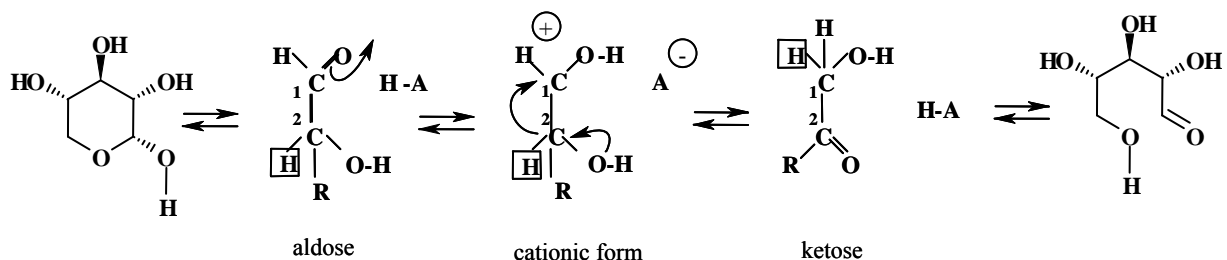
Figure 8: Hydride shift mechanism of xylose isomerase

2.4.4 Bacterial xylose isomerase expression in *S. cerevisiae*

Unfortunately, native *S. cerevisiae* cannot efficiently utilise pentose sugars such as D-xylose but can metabolise its isomerised monomer xylulose (Hamacher et al., 2002). D-xylose sugar must be transported into the cell, where it is converted to xylulose. Most bacteria possess an XI, which converts D-xylose directly to xylulose without xylitol as intermediate (Aristidou and Penttilä, 2000). The advantage above XR and XDH is that no redox cofactors are necessary and no redox imbalance will be generated by the production of xylulose from D-xylose.

Numerous attempts to express bacterial XIs in *S. cerevisiae* failed. Group I XI from the Gram positive bacterium *Actinoplanes missouriensis* expressed in *S. cerevisiae* produced specific mRNA under the yeast *GAL1* promoter, but no activity presumably due to a missing initiation codon in the transcripts (Amore et al., 1989). Similarly Moes et al. (1996) attempted expression of *C. thermosulfurogenes* XI, in

S. cerevisiae and although specific mRNA was produced, no activity was detected. This Group II XI has 64% similarity to *E. coli xylA* and had activity in *E. coli*. The researchers speculate that the problem may be in forming active XI tetramers from monomers in the yeast cytosol.

Ho et al. (1983) expressed the *E. coli xylA*, a member of the Gram negative Group II XIs, in *S. cerevisiae* under the yeast *TRP5* promoter but activity was almost undetectable. The *E. coli* initiation of translation was used but it was suggested that other genetic elements are needed to stabilise XI in yeast as experiments showed post-translational modifications did not cause the problem. Sarthy et al. (1987) also attempted expression of the *E. coli xylA* gene under the yeast *ADH1* promoter. Yeast transformants were incapable of growth on D-xylose as carbon source, the enzyme was 10^3 times less active than native *E. coli* XI and very few of the XI molecules were soluble.

Expression of the *Bacillus subtilis xylA* gene in *S. cerevisiae* (Amore et al., 1989) was also unsuccessful because the protein that resulted was insoluble and catalytically inactive. Sarthy et al. (1987) suggested the lack of XI activity in *S. cerevisiae* was due to improper disulfide bridge formation or improper protein folding, problems with post-translational modifications or yeast internal pH. Hahn-Hägerdal et al. (2001) conclude the problem was not due to post-translational modifications, and since yeast internal pH is close to neutral it should not have caused problems with expression. The *E. coli* and *B. subtilis* XIs may be inactive in yeast for the same reason as they are both group II enzymes and share more than 50% homology (Amore et al., 1989).

Hallborn (1995) chose to express a *Lactobacillus pentosus* XI because it has a low pH optimum and belongs to a different subgroup than the *E. coli* and *B. subtilis* XIs which were unsuccessful cloned into *S. cerevisiae*. The XI from *L. brevis* has a high affinity for D-xylose and therefore it was expected that the *L. pentosus* XI, which has high homology to the *L. brevis* enzyme, would also have high affinity for D-xylose. The amino acid sequence also predicted that the enzyme should be stable in yeast. *S. cerevisiae* transformed with the *L. pentosus* xylose isomerase gene were unable

to grow on D-xylose and had no xylose isomerase activity. Other DNA alignments of *L. pentosus* xylose isomerase have shown it belongs with *B. subtilis* and *E. coli* XIs in the same similarity group (Lokman et al., 1991). It was suggested that co-expression of a chaperone or fusion to a secretion sequence might be necessary for XI activity in *S. cerevisiae* (Hallborn, 1995).

Walfridsson et al. (1996) appear to have been the most successful with the cloning of the Group I *Thermus thermophilus xylA* bacterial xylose isomerase in *S. cerevisiae*. The recombinant XylA monomer was similar in size to the native enzyme. The subunits of this enzyme are 387 residues in size, and form tetramers (Lönn et al., 2002). The recombinant *S. cerevisiae* grown on D-xylose used 3 times more D-xylose than the control strain and yielded 0.125 g ethanol.g^{-1} D-xylose (Walfridsson et al., 1996). The XI activity of 1 U.mg^{-1}.min^{-1} was greatest at the pH optimum of 7. Unfortunately the temperature optimum of 85°C made the gene and expression system incompatible, as *S. cerevisiae* grows well at 30ºC. In addition xylitol, which is known to inhibit XI, was produced by the strain due to non-specific NADPH-linked aldose reductase, probably from the *GRE3* gene.

Streptomyces rubiginosus XI has 60% identity at DNA level and 80% protein level similarity to the *T. thermophilus* XI, but has significant activity at room temperature (Gárdonyi and Hahn-Hägerdal, 2003). When the *S. rubiginosus* xylose isomerase was expressed in *S. cerevisiae*, it formed insoluble protein without activity, despite the similarities to the *T. thermophilus* XI.

The xylose isomerase genes from a number of bacteria have been cloned into *S. cerevisiae*. Unfortunately, although the reasons for this are little understood, only the strain transformed with the *T. thermophilus xylA* was capable of significant D-xylose utilisation. Reasons for the lower activity by most XIs cannot consistently be ascribed to difficulties with transcription, translation or differences in codon usage patterns between the bacterial strains and yeast. It is possible that the *T. thermophilus* XI folds correctly as the protein structure is stabilised by many ion-bridges (Gárdonyi and Hahn-Hägerdal, 2003).

2.4.5 Improvement of D-xylose utilisation in *S. cerevisiae* expressing *xylA*

Xylose isomerases are known to be competitively inhibited by xylitol (Hahn-Hägerdal et al., 2001). For this reason, and to preserve carbon for ethanol production, it was thought that eliminating xylitol production in *S. cerevisiae* transformed with the *T. thermophilus xylA* would improve ethanol yield. Träff et al. (2001) deleted the *GRE3* gene encoding a non-specific AR, and expressed the *T. thermophilus* XI and overexpressed the native *XKS1* gene in a CEN.PK21-C *S. cerevisiae* grown at 30ºC. No AR activity was detected in the modified strain, and xylitol production decreased by half. Xylose isomerase activity appeared unchanged whether AR was present or not. Ethanol was only produced by the Δ*gre3* strain, with XI. The strain overproducing XK produced no ethanol, suggesting that too high an XK level is as much of a problem as too low. None of the recombinant strains grew on D-xylose in contrast to the Walfridsson et al. (1996) study which used *S. cerevisiae* SH158, which produces a higher yield of ethanol from D-xylose than CEN.PK21-C. In addition the activity of XI is doubled at the fermentation temperature of 38ºC used by Walfridsson et al. (1996). Therefore, deletion of *GRE3* and overexpression of XK unexpectedly did not enhance ethanol production from a recombinant D-xylose utilising *S. cerevisiae* expressing *T. thermophilus xylA* (Träff et al., 2001).

Lönn et al. (2003) expressed mutated *T. thermophilus xylA* in *S. cerevisiae* strains CEN.PK113-7A and CEN.PK2-1C with *GRE3* deletion and *XKS1* overexpression. As opposed to Träff et al. (2001), they found that strains with deleted *GRE3* produced no xylitol and yielded twice as much ethanol (Lönn et al., 2003).

2.4.6 Mutations in D-xylose isomerase and improvements to xylose isomerases

The *xylA* gene encoding XI from *T. thermophilus* has successfully been expressed in *S. cerevisiae* (Walfridsson et al., 1996). However, the activity at the growth temperature for *S. cerevisiae* is very low, as the temperature optimum is 85ºC. Lönn et al. (2002) used random PCR mutagenesis to produce *T. thermophilus* XIs with increased function at lower temperatures. Mutant enzymes had one or two mutations on the surface, far from the catalytic site. Enzymes were tested in *E. coli* and mutants had increased activity compared to the wild-type at 30ºC, but activity

remained low in this range and more extensive testing occurred at 60ºC, where the mutants had a catalytic rate constant (k_{cat}) of almost an order of magnitude higher. The increased relative activity was due to the increased k_{cat}. In addition, mutants had higher K_i for xylitol, suggesting lower inhibition by xylitol than for the wild-type. The changes may have improved the k_{cat} by increasing the enzyme flexibility and thereby lowering the energy needed to change conformation. At the same time, this change in rigidity reduced the thermal stability of the mutants.

In a follow-up study, the modified XIs were tested in *S. cerevisiae* to see whether they would improve D-xylose utilisation, given their moderately improved activity at 30ºC (Lönn et al., 2003). The study produced very low activity for yeast grown at 30ºC and 45.5 mU.mg^{-1} protein at 40ºC. This low activity compared to total protein suggested misfolding in yeast. Ethanol was only measured when XI had activity greater than 30 mU.mg^{-1}. The improved catalytic rate constants of the mutant enzymes, did not have any effect on ethanol levels or D-xylose consumption. D-xylose consumption correlated with K_m for D-xylose, and the wild-type enzyme performed best. D-xylose consumption in *S. cerevisiae* expressing the mutant XIs was lower than for strains which express the enzymes XR, XDH and XK. As Walfridsson et al. (1996) and Träff et al. (2001) obtained higher expression with different plasmid systems, Lönn et al. (2003) suggested that high XI activity levels are critical for D-xylose consumption by recombinant *S. cerevisiae*.

2.4.7 Eukaryotic xylose isomerases

Kristo et al. (1996) published information on a eukaryotic XI from the barley plant *H. vulgare*. This is a group II XI with confirmed activity. Other plant XIs have been identified from DNA sequences but activity has not been confirmed (Harhangi et al., 2003). XIs have been reported in the yeast *Candida utilis* grown on D-xylose, and in *Rhodotorula gracilis* (Jeffries, 1983). Bhosale et al. (1996) also reported on a glucose isomerase in *Candida boidinii*. Among the XIs found in mycelial fungi are those of a *Malbranchea* sp. and *Neurospora crassa* (Hahn-Hägerdal et al., 2001). In addition *Aspergillus oryzae* has been documented as producing GI (Bhosale et al., 1996). Except for *R. gracilis*, for these and other fungi and yeasts the putative XI

activity is very low so D-xylose metabolism probably occurs through XR and XDH (Jeffries, 1983; Gárdonyi and Hahn-Hägerdal, 2003).

Thus various aldose isomerases have sporadically been reported from eukaryotic sources. These could be a potential source of XIs compatible with *S. cerevisiae* expression systems, once the genes have been identified.

2.4.8 Eukaryotic xylose isomerase expression in *S. cerevisiae*

As previously mentioned, several groups have tried to express prokaryotic XI genes in S. *cerevisiae* with little success. According to Harhangi et al. (2003) the route of D-xylose metabolism is not known in anaerobic fungi. Recently this group showed that *Piromyces* sp. strain E2 uses the enzymes XI and XK to metabolise D-xylose, and has no detectable XR or XDH.

The XI gene of *Piromyces* sp strain E2 cloned into *S. cerevisiae* strain BJ1991 under the *GAL1* promoter is the first eukaryotic XI documented to be transformed in *S. cerevisiae*. When expressed in *S. cerevisiae* XI activity was observed, although low: 25 ± 13 nmol.min^{-1}mg^{-1} protein. In a follow-up study Kuyper et al. (2003) grew *Piromyces* anaerobically on minimal medium and D-xylose at 39°C. XI specific activity was 0.08 U.mg^{-1} protein while XK activity was 0.2 U.mg^{-1} protein and was also present during growth on fructose.

When *S. cerevisiae* CEN.PK 113-5D was transformed with the *Piromyces* XI, under control of the *TPI1* promoter, the activity was significantly greater than in the native organism: 1.1 U.mg^{-1} protein (Kuyper et al., 2003). The heterologous enzyme had a $K_m=20\pm1$ mM for D-xylose while *Streptomyces* XI vary from $K_m=0.08$-33 mM, so the heterologous XI does not have a particularly high affinity for D-xylose. The enzyme is Mg^{2+} dependent and Co^{2+} can also restore activity. Recombinant *S. cerevisiae* with *Piromyces* XI could grow very slowly on 2% D-xylose as sole carbon source, and showed the highest XI activity under aerobic conditions. While the *S. cerevisiae* reference strain showed negligible use of D-xylose when D-xylose and D-glucose were cosubstrates, the recombinant strain used between 20-50% of the D-xylose. Since the recombinant strain used less than 10% of the D-xylose in a xylose/glucose

mixture under anaerobic conditions, obtaining anaerobic growth of recombinant *S. cerevisiae* on D-xylose as sole carbon source is still a significant challenge. The authors speculated that the enzyme may have better activity in *S. cerevisiae* than bacterial XIs because *Piromyces* and yeast cytosol have more similar conditions for protein folding than found in the cytosol of bacteria. This suggests genetic engineering of the XI metabolic pathway in *S. cerevisiae* may yet be successful.

2.4.9 *Piromyces* xylose isomerase is homologous to the unique, putative, *Bacteroides* xylose isomerase

The expression of the unusual *Piromyces* fungal xylose isomerase in *S. cerevisiae* suggested that there may be unique xylose isomerases which could be compatible with *S. cerevisiae* as expression system. This would have the significant advantage that xylitol would not be a by-product as is the case with the xylose reductase and xylitol dehydrogenase enzymes.

Homology searches on the Genbank database revealed that the *Piromyces* XI sequence (with accession number AJ249909) has high homology of 83.1% at amino acid level with a putative xylose isomerase from the genome sequence of gram negative *Bacteroides thetaiotaomicron* (accession number AA075900.1). This is shown in Figure 9. At DNA level the sequences are closer to 70% similar. To our knowledge the putative xylose isomerase of *B. thetaiotaomicron* has not been isolated and characterised and the gene has not been cloned or expressed. The significant homology with the *Piromyces* XI, which has been successfully cloned into *S. cerevisiae,* justified attempting expression in this host, as a highly similar protein might also be functional.

2.4.10 *Bacteroides* family and taxonomy

The genus *Bacteroides* is a member of the Bacteroidetes/Chlorobi superphylum, which diverged from other bacteria before Gram positive and gram negative organisms differentiated. *Bacteroides* thus has similarities to both gram positive and gram negative organisms (Comstock and Coyne, 2003). It is part of the Cytophaga-Flavobacteria-Bacteriodes (CFB) phylum, also known as the Bacteriodetes phylum (Nelson et al., 2003; Kuwahara et al., 2004). These bacteria then fall under class

Bacteroides, order Bacteriodales and family Bacteriodaceae (Xu and Gordon, 2003). *Bacteroides* species are obligate anaerobes and ferment carbohydrates for carbon and energy (Xu and Gordon, 2003; Guthrie et al., 1985). *B. thetaiotaomicron* is related to *B. fragilis*, another member of the mammalian gut, with overall DNA homology of 45% (Cooper et al., 1997). *B. thetaiotaomicron* is also part of the phylogenetic group which includes *Porphyromonas* species (Gupta et al., 2003).

```
Upper line: Bacterium B. thetaiotaomicron putative XI        from 1 to 438
Lower line: Fungus Piromyces sp. E2 XI AA sequence           from 1 to 437

identity= 83.1%(364/438) gap=0.00%(0/438)
1      MATKEFFPGIEKIKFEGKDSKNPMAFRYYDAEKVINGKKMKDWLRFAMAWWHTLCAEGGDQF
       ||  ||  ||  | |||||||||||| || |||||||    ||||||||||||||||| || |||
1      MA KEYFPQIQKIKFEGKDSKNPLAFHYYDAEKEVMGKKMKDWLRFAMAWWHTLCAEGADQF

63     GGGTKQFPWNGNADAIQAAKDKMDAGFEFMQKMGIEYYCFHDVDLVSEGASVEEYEANLK
       |||||| ||||    |||   || | ||||| ||| || ||||||||||||| | |||| |||
62     GGGTKSFPWNEGTDAIEIAKQKVDAGFEIMQKLGIPYYCFHDVDLVSEGNSIEEYESNLK

124    IVAYAKQKQAETGIKLLWGTANVFGHARYMNGAATNPDFDVVARAAVQIKNAIDATIELG
       |||  | || ||||||||| ||||||| ||||| |||||||||||| |||||||||| ||||
123    VVAYLKEKQKETGIKLLWSTANVFGHKRYMNGASTNPDFDVVARAIVQIKNAIDAGIELG

184    GENYVFWGGREGYMSLLNTDQKREKEHLAQMLTIARDYARARGFKGTFLIEPKPMEPTKH
       ||||||||||||||||||||||||||||| | ||| |||||| |||||||||||||||||||
183    AENYVFWGGREGYMSLLNTDQKREKEHMATMLTMARDYARSKGFKGTFLIEPKPMEPTKH

244    QYDVDTETVIGFLKAHGLDKDFKVNIEVNHATLAGHTFEHELAVAVDNGMLGSIDANRGD
       ||||||||| |||||||| |||||||||||||||||||||||||| ||| ||||||||||||
243    QYDVDTETAIGFLKAHNLDKDFKVNIEVNHATLAGHTFEHELACAVDAGMLGSIDANRGD

304    YQNGWDTDQFPIDNYELTQAMMQIIRNGGLGTGGTNFDAKTRRNSTDLEDIFIAHIAGMD
       |||||||||||||| ||| || | ||| ||  ||||||||||||||||||||||| ||| |||
303    YQNGWDTDQFPIDQYELVQAWMEIIRGGGFVTGGTNFDAKTRRNSTDLEDIIIAHVSGMD

364    AMARALESAAAALLDESPYKKMLADRYASFDGGKGKEFEDGKLTLEDVVAYAKTKGEPKQT
       |||||||| || || |||| || ||||||| | || |||||||||| |  | |  ||||||
363    AMARALENAAKLLQESPYTKMKKERYASFDSGIGKDFEDGKLTLEQVYEYGKKNGEPKQT

424    SGKQELYEAILNMYC
       |||||||||||  ||
423    SGKQELYEAIVAMYQ
```
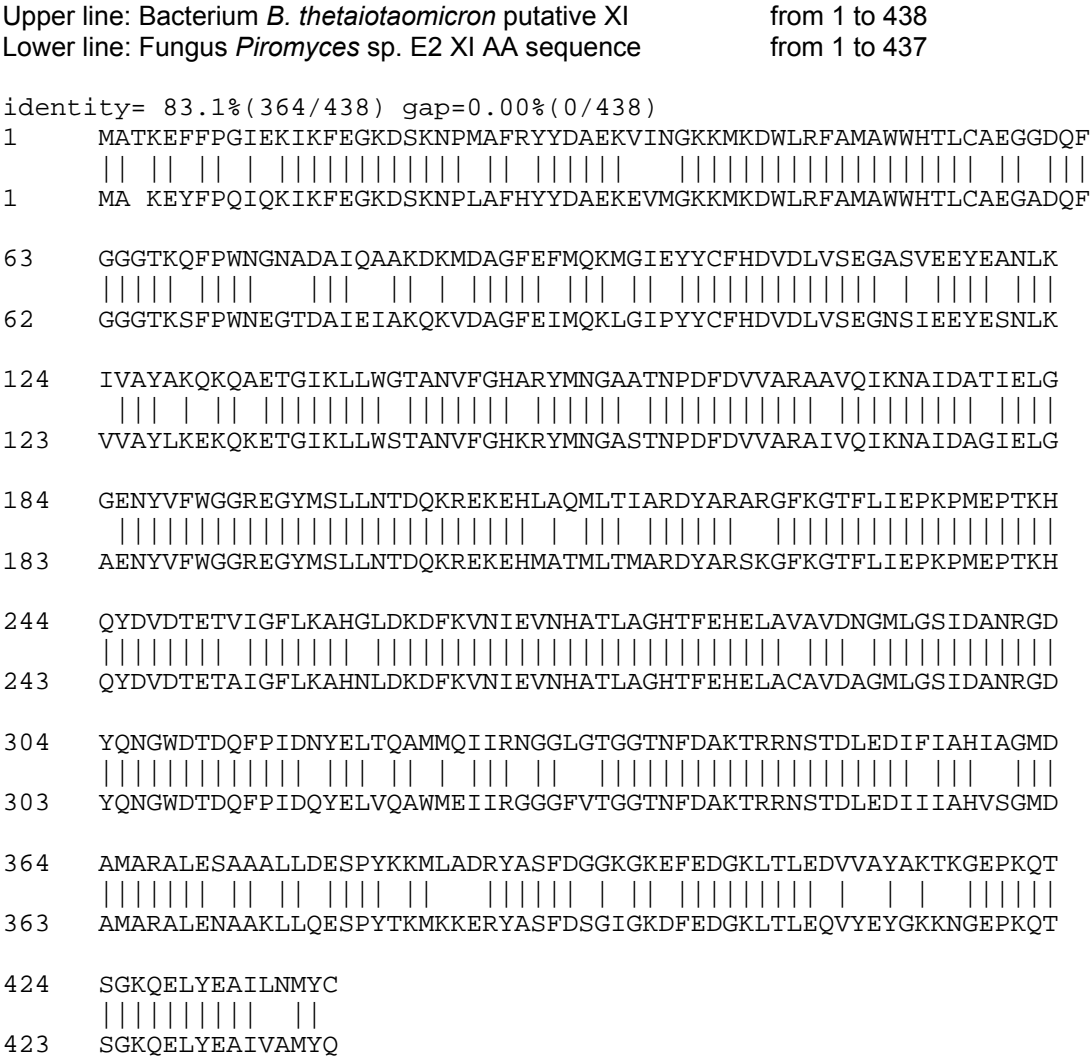
Figure 9:   Amino acid alignment of the bacterium *B. thetaiotaomicron* putative xylose isomerase with the fungus *Piromyces* sp. E2 xylose isomerase.

B. thetaiotaomicron occurs in large numbers in the intestinal microbiota of both humans and other mammals (Comstock and Coyne, 2003). According to Kuwahara et al. (2004) *Bacteroides* sp. make up 30% of the bacteria in the human gut. In

humans *B. thetaiotaomicron* provides the ability to degrade plant polysaccharides in the colon (Xu and Gordon, 2003). Approximately 10% of the energy obtained through digestion is from plant polysaccharides, indigestible to humans, being converted to short chain fatty acids by bacteria in the intestine. *B. thetaiotaomicron* has a plethora of polysaccharide-degrading enzymes in its glycobiome with many substrate specificities compared to other sequenced bacteria of the same niche (Kuwahara et al., 2004). Recent analysis of the *B. thetaiotaomicron* genome has shown it has 172 glycosylhydrolases, more than in any other bacterium sequenced (Comstock and Coyne, 2003). These genes include a potential 11 α-xylosidases, 3 β-xylanases and 8 β-xylosidases, suggesting that this organism can degrade hemicelluloses, and therefore it is likely to possess a functional xylose isomerase (Xu and Gordon, 2003).

2.4.11 Horizontal transfer and *Bacteroides* gene expression in other organisms

Facultative gram negative bacteria such as *E. coli* share the characteristic of a gram negative cell wall with *Bacteroides* sp., although they are in a separate phylogenetic group (Shoemaker et al., 1996b; Guthrie et al., 1985). In general neither can the IncP plasmids which have a broad host range across *E. coli* and similar genera, replicate in *Bacteroides* sp., nor do promoters from the one bacterium work in the other (Shoemaker et al., 1996b). *Bacteroides* sp. did not express ampicillin, sulfonamide, tetracycline or trimethoprim *E. coli* drug resistance genes and *E. coli* did not gain resistance from a *Bacteroides* clindamycin and erythromycin gene (Guthrie et al., 1985). However a gene *bexA* from *B. thetaiotaomicron*, encoding a multi-drug efflux transporter, provided a susceptible *E. coli* host with resistance to norfloxacin, ciprofloxacin and ethidium bromide (Miyamae et al., 2001).

Non-replicating *Bacteroides* units (NBUs) are non-selftransmissible 10 to 12 kb elements, which require self-transmissible conjugative plasmids (CTs) to be mobilised from *Bacteroides* donors to *Bacteroides* recipients (Shoemaker et al., 1996a). It was found that NBU1 from *Bacteroides* could be mobilised into *E. coli* (Shoemaker et al., 1996b). The integrations into the *E. coli* chromosome occurred at very low frequencies and were not site-specific, unlike integrations into *Bacteroides*

sp. This shows that DNA from *Bacteroides* is capable of transfer into *E. coli* and the encoded *mob* proteins functioned to integrate the NBU into the chromosome.

A DNA library from *B. thetaiotaomicron* in *E. coli* was analysed for chondroitin lyase activity (Guthrie et al., 1985). Chondroitin lyases can degrade chondroitin 6-sulfate, chondroitin 4-sulfate, and desulfated chondroitin sulfate (Guthrie and Salyers, 1987). The clones with activity were identified and the 7.8 kb fragment, including the original promoter regions, was subcloned into a multi-copy plasmid, but it was not possible to detect a protein of the correct size from recombinant *E. coli* or an *in vitro* transcription-translation system (Guthrie et al., 1985). The chondroitin lyase gene in *E. coli* had very low specific activity and was expressed weakly. Some differences were found between the protein in *E. coli* and in *B thetaiotaomicron*. It was suggested that the *E. coli* host began the gene's translation from a different initial site or that this protein undergoes post-translational modification in *B. thetaiotaomicron*, which did not occur in *E. coli*.

The above case studies suggest that although in some cases *E. coli* can recognise *B. thetaiotaomicron* promoters and produce functional protein from its genes, the expression and activity in such cases is very weak. Similarly, the putative *B. thetaiotaomicron* xylose isomerase expressed in *E. coli*, even under an *E. coli* promoter, may be incorrectly modified after translation and show only weak or no activity. The *B. thetaiotaomicron* genome analysis showed many mobile genetic elements including a 33 kb plasmid, several homologs of self-transmitting conjugation transposons and many transposase-encoding genes (Comstock and Coyne, 2003). This suggested that gene transfer is a reality in this organism.

Garcia-Vallvé et al. (2000) used a variety of methods to compare the glycosyl hydrolase (GH) DNA sequences of rumen fungi with bacterial sequences to determine their origin. Previously the mannanase gene *manA* in *Piromyces* sp. has been suggested to be of bacterial origin. Codon usage analysis clusters showed that the rumen fungi GH genes cluster more closely to related orthologous genes, than to genes from taxonomically similar organisms. Almost all sequences in the GH group have greater similarity to bacterial than fungal sequences. This is not true of the non-

GH group, where fungal genes cluster with fungal genes. Although XI is not a glycosyl hydrolase, the *Piromyces* XI also has greater similarity to bacterial sequences than to other eukaryotic sequences.

Rumen communities provide ideal conditions for gene transfer between the many different organisms there, and in vitro gene transfer between ruminal bacteria has been shown to occur (Gupta et al., 2003; Garcia-Vallvé et al., 2000). For *Orpinomyces* sp cyclophilin B GHs the codon bias is similar to other genes in the yeast, suggesting the genes have adjusted to the typical usage of the organism (Garcia-Vallvé et al., 2000). *Piromyces* has three mannanase sequences in a gene family which otherwise only has bacterial sequences, and with high homology to them.

This suggests that rumen fungi, including *Piromyces* sp have acquired the genes for plant polysaccharide degradation from bacterial members in the rumen community. Although the direction was not determined, strong evidence has been found for horizontal transfer occurring in the human colon between gram negative and gram positive bacteria (Garcia-Vallvé et al., 2000). It is possible even that horizontal transfer occurs as entire operons thus few recombination events are necessary for entire new substrates to be utilised. The *B. thetaiotaomicron* genome itself may contain foreign DNA obtained from other colon residents by horizontal gene transfer (Comstock and Coyne, 2003). Thus, it is possible that the XI in *Piromyces* was originally acquired through horizontal transfer from *Bacteroides thetaiotaomicron*. If so, then some changes have occurred as the sequences differ at DNA level although the amino acid sequence is very similar. In addition, differences in base sequence and codon bias between the two sequences can be explained by amelioration of the acquired gene to host genome characteristics.

2.4.12 Peptide folding and posttranslational modifications

Heterologous protein expression in organisms unrelated to the original host does not always lead to active product. Protein expression is a complex system controlled through transcription, mRNA stability and splicing, translation, post-translational modifications and protein degradation (Day and Tuite, 1998). Although many

problems with recombinant expression are due to mRNA or translational difficulties, even a correctly translated polypeptide may not lead to an active protein. XIs consist of two identical or four identical subunits (Bhosale et al., 1996). Correct enzyme structure therefore requires that the polypeptide correctly fold into subunits (tertiary structure) and the subunits correctly associate with one another and with the correct divalent cations (quaternary structure). These changes occur after a polypeptide has been produced by translation. In posttranslational modification a polypeptide may have amino acid changes, some portions removed or molecules such as polysaccharides added (Fairbanks and Andersen, 1999).

In the case of XI expressed in *S. cerevisiae,* the problem may also be one of protein trafficking. If XI polypeptides contain a signal on the N-terminal end sequence, which in the native organism directs it towards one of a variety of locations, for example, the endoplasmic reticulum for modification, or to be integrated into the plasma membrane or secreted. If *S. cerevisiae* fails to follow these directions this would lead to the protein not being modified in the ER, or being incorrectly directed within the cell. Since most XIs are intracellular proteins, the problem would not be the secretory part of this pathway.

Some of the XIs expressed in *S. cerevisiae* formed insoluble aggregates and had low activity (Amore et al., 1989; Sarthy et al., 1987). This suggested that since mRNA was formed, and a polypeptide was produced, the problem may be that in yeast the xylose isomerase does not attain the correct conformation through the mutual attraction of hydrophobic areas, and a natural tendency of the structure towards the lowest potential energy. For some proteins molecular chaperones are needed to assist them into the active conformation (Bu et al., 2005). Examples of such chaperones are the *E. coli* DnaK-DnaJ-GrpE and GroEL-GroES. It is possible that these or other species-specific chaperones might assist some recombinant XIs expressed in *S. cerevisiae* to attain the active conformation.

Bu et al., (2005) examined the effect of overexpression of the *E. coli* chaperone GroEL-GroES on the highly expressed xylulokinase of *Bacillus megaterium*, which formed insoluble protein when expressed in *E. coli* BL21(DE3)pLys5. GroEL-GroES

is thought to enhance correct folding of partially folded polypeptides by minimising aggregation. With GroEL-GroES overexpression a greater proportion of the produced xylulokinase was soluble and the activity increased concomitantly. This study increased solubility and activity of a recombinant protein by coexpression of a chaperone. Although in this case both the native strain and recombinant host were bacteria, it is possible that for D-xylose utilising enzymes including XI expressed in *S. cerevisiae* the solubility and low activity most often experienced, may be improved by a similar strategy. Whether GroEL-GroES would indeed be successfully expressed in yeast, or assist with XI folding in a eukaryotic host, remains to be seen.

In conclusion, XI expressed in yeast could potentially solve the cofactor imbalance problems seen when *S. cerevisiae* has been transformed with the *P. stipitis* pathway. However bacterial XIs have generally not been successfully expressed due to the enzyme forming insoluble aggregates or the monomers not combining as active dimers and tetramers. Eukaryotic XIs may exist which are better expressed in *S. cerevisiae*, or coexpression of chaperones may prevent the protein becoming insoluble and promote the formation of catalytically active dimers and tetramers.

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CHAPTER 3

EXPERIMENTAL WORK

MUTATION AND SELECTION OF *SACCHAROMYCES CEREVISIAE* WITH THE RECOMBINANT D-XYLOSE UTILISING *PICHIA STIPITIS* ENZYME PATHWAY FOR ENHANCED D-XYLOSE UTILISATION

3.1 INTRODUCTION

Plant matter includes up to 40% hemicellulose (Sun and Tomkinson, 2003). Hemicelluloses are high-energy polymers of which xylans are the most abundant (Geng et al., 2003). For fermentations to ethanol with biomass substrates to be economical, the D-xylose sugar must be utilised along with the hexose sugars. *S. cerevisiae* is a good fermenter of glucose, but does not naturally grow on D-xylose (Hahn-Hägerdal et al., 2001). D-xylose utilisation in recombinant *S. cerevisiae* appears to lead to severe metabolic problems due to cofactor imbalance, especially under anaerobic conditions where fewer mechanisms to regenerate cofactors are available (Hahn-Hägerdal et al., 2001).

The aim of this study was to improve the ability of a recombinant *S. cerevisiae* to utilise D-xylose under anaerobic conditions. The culture conditions used D-xylose as sole carbon source anaerobically, the combination of which is stressful to the yeast. Therefore stress was reduced in other ways. It is known that nitrogen limitations can induce pseudohyphal growth and prevent cell division amongst other undesirable metabolic results, and therefore it was decided to use rich medium for anaerobic fermentations and selection (Radcliffe et al., 1997). A haploid industrial *S. cerevisiae* strain Sigma α was transformed with the plasmid YIpLoxZEO (Jeppsson et al., 2002) to confer D-xylose utilisation ability on the strain. As the plasmid was integrative, selective pressure was unnecessary apart from for initial selection. The strain was subjected to random mutagenesis and selected for improved D-xylose utilisation ability under anaerobic conditions.

3.2 MATERIALS AND METHODS

3.2.1 Strains and transformation

The integrative plasmid YIpLoxZEO (Figure 1) (Jeppsson et al., 2002) carries the genes encoding xylose reductase (*XYL1*) and xylitol dehydrogenase (*XYL2*) from *Pichia stipitis* and xylulose kinase (*XKS1*) from *S. cerevisiae*. The *XYL1* gene was under the *ADH2* promoter and terminator and the *XYL2* and *XKS1* genes were under the *PGK1* promoter and terminator. The YIpLoxZEO plasmid was digested with the *NdeI* restriction enzyme so that the plasmid would integrate in the yeast chromosome at the *HIS3* gene. The auxotrophic haploid laboratory *Saccharomyces cerevisiae*

strain Sigma MAT α was transformed using the LiOAc/DMSO method (Hill et al., 1991). Details of strains and plasmids are shown in Table 1.
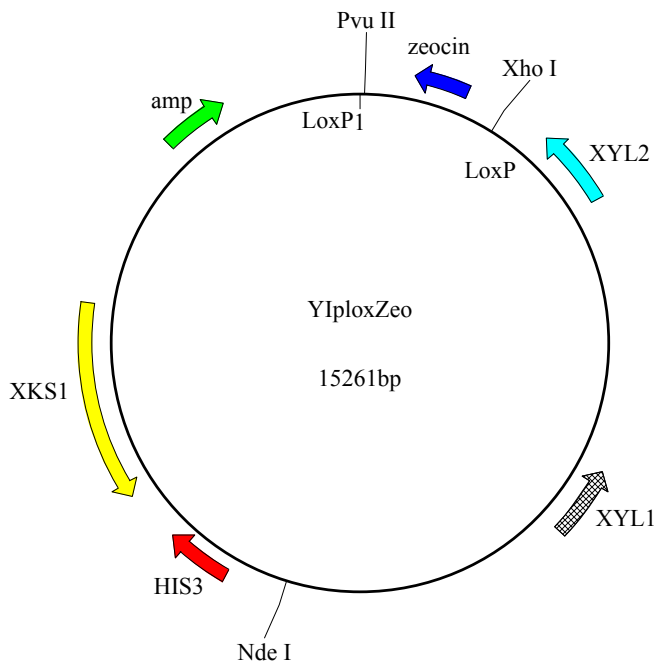
Figure 1:   Schematic representation of the plasmid YIpLoxZEO used in this study.

Table 1:   **Microbial strains and plasmids**

Strain or Plasmid	Genotype	Source/ Reference
Yeast strains:		
S. cerevisiae Sigma L5366h1 MAT α Strain was designated *S. cerevisiae* Sigma (reference strain)	*ura3-52*	Gagiano et al. (1999)
S. cerevisiae Sigma L5366h1 MAT α [YIpLoxZEO] Strain was designated *S. cerevisiae* Sigma [YIpLoxZEO]	*ura3-52, XYL1, XYL2, XKS1,*	This work
Plasmid:		
YIpLoxZEO	*XYL1, XYL2, XKS1, HIS3, ZEO^R*	Jeppsson et al. (2002)

3.2.2 Media and culture conditions

Yeast transformants were selected using rich medium (YP) [containing 10 g.L^{-1} yeast extract (Merck), 20 g.L^{-1} peptone (Merck) and 20 g.L^{-1} glucose (YPD) or 20 g.L^{-1} xylose (YPX) as carbon source] and supplemented with 100 μg.mL^{-1} zeocin. Standard cultivations were done using either selective synthetic (SC) minimal medium [containing 20 g.L^{-1} glucose or 20 g.L^{-1} D-xylose, 5 g.L^{-1} ammonium sulphate, 1.7 g.L^{-1} yeast nitrogen base without amino acids (Difco)(pH 6.0), 20 g.L^{-1} agar where necessary and supplemented with all growth factors except histidine], or rich medium as above, not necessarily with zeocin supplementation. Bengal Rose Agar medium, selective for yeast was prepared [containing 10 g.L^{-1} glucose, 5 g.L^{-1} tryptone, 1 g.L^{-1} KH$_2$PO$_4$, 0.5 g.L^{-1} MgSO$_4$, 0.05 g.L^{-1} Rose Bengal and 3 g.L^{-1} agar] this was supplemented with three antibiotics at the final concentration indicated: ampicillin (0.1 mg.mL^{-1}), kanamycin (0.03 mg.mL^{-1}) and chloramphenicol (0.09 mg.mL^{-1}).

3.2.3 Confirmation of yeast transformants

The presence of the integrated plasmid in yeast transformations was confirmed by polymerase chain reaction (PCR). Genomic DNA extracted from S. cerevisiae cultures (Hoffman and Winston, 1987) was used as template DNA. Primers were designed for a 870 bp region of the P. stipitis XYL1 gene from the sequence described by Amore et al. (1991).

(Pstixyl1-5')XYL1 5' primer sequence: 5'TTCTATTAAGTTGAACTCTGGTTAC3'

(Pstixyl1-3')XYL1 3' primer sequence: 5'TCAGCGAAATCTTGTTCG3'

Taq polymerase (Promega) was used as directed. Annealing and extension was done for 30 sec at 55ºC and 1 min at 72ºC, respectively to produce the 870bp product.

3.2.4 Mutagenesis and analysis of mutants

S. cerevisiae Sigma [YIpLoxZEO] was randomly mutated using ethyl methane sulfonate (EMS) (Lundblad, 1998). S. cerevisiae [YIpLoxZEO] was grown to OD$_{600}$=1.0 in YPD medium. Cells were washed in sterile dH$_2$O and then resuspended in 100mM sodium phosphate buffer (pH 7.0). Aliquots of cell suspension (100 μL) were treated with 0, 3, 4 and 6 μL EMS. After incubation at

30°C for 1 hour, 900 μL of 5% sodium thiosulphate ($Na_2S_2O_3$) was used to neutralise the EMS. Dilutions of cells to 10^{-3}, 10^{-4} and 10^{-5} were made and dilutions plated out on YP plates containing 20 g.L^{-1} D-xylose as sole carbon source, to select for surviving colonies with D-xylose utilisation ability.

Colonies showing significant aerobic growth on this medium were then inoculated into test tubes containing Durham tubes to determine gas production under anaerobic conditions, in YPD and YPX. Mutants showing promising gas production were to be subsequently placed under anaerobic fermentative conditions for further selection with D-xylose as sole carbon source. Alternatively after EMS mutation the cell aliquots treated with differing volumes of EMS were inoculated directly into anaerobic serum flasks after neutralisation with $Na_2S_2O_3$. The total volume of YP medium was 70 mL with 50 g.L^{-1} D-xylose.

3.2.5 Fermentations

Aerobic fermentations were done in shake flasks with YP medium to test the optimal concentration of D-xylose to support growth in a range between 20 g.L^{-1} and 100 g.L^{-1}. Anaerobic fermentations of *S. cerevisiae* [YIpLoxZEO] and the reference strain were done to provide samples for XR, XDH and XK enzyme activity assays. These took place in closed serum bottles with rich medium and 10 g.L^{-1} D-glucose together with 10 g.L^{-1} D-xylose as carbon source.

Subsequent anaerobic fermentations in closed serum bottles were done on YP medium with xylose as sole carbon source, supplemented with 0.42 g.L^{-1} and 0.01 g.L^{-1} ethanol-dissolved Tween 80 and ergosterol respectively (Sonderegger et al., 2004). A range of differing D-xylose concentrations were tested to optimise cell growth. Yeast growth was compared on D-xylose concentrations of 20, 50, 100, 200 and 300 g.L^{-1} in rich medium.

All subsequent fermentations were done in a total volume of 70 mL rich medium with 50 g.L^{-1} D-xylose as carbon source, supplemented with Tween 80 and ergosterol.

In all cases the medium was made up in the bottles at a lower volume to allow for the later addition of sterile D-xylose syrup and autoclaved with the rubber stoppers on. As D-xylose is unsuitable for autoclaving with nitrogenous compounds, a stock solution was made up and autoclaved separately.

D-xylose syrup (500 g.L^{-1} stock solution) and dH$_2$O were combined with the cell inoculum and administered aseptically via needle and syringe into the medium, to make up to the correct sugar concentration. Aerobic cultures of *S. cerevisiae* grown on rich medium with 10 g.L^{-1} glucose and 10 g.L^{-1} D-xylose were used as inoculum for anaerobic fermentations by inoculating to an absorbance at 600 nm of 0.4. At all times the flasks were kept anaerobic. Flasks were stirred on a magnetic stirrer pad to keep cells in suspension during incubation at 30ºC for up to 10 days. A syringe needle stuffed with cotton wool was left in the stopper to relieve build up of CO$_2$.

3.2.6 XR, XDH and XK enzyme activity assays

Cell growth: Enzyme assays were used to determine the activity of xylose reductase (XR), xylitol dehydrogenase (XDH) and xylulokinase (XK) enzymes, comparing these activities in Sigma [YIpLoxZEO] to that of the Sigma reference strain. Yeast cultures of the two strains were prepared in triplicate in 70 mL anaerobic serum flasks with 10 g.L^{-1} glucose and 10 g.L^{-1} D-xylose, as previously described.

Cell extraction: At 24 hr intervals, 7.5 mL medium samples with cells were drawn for enzyme assays using a sterile needle and syringe. The yeast cell samples were centrifuged at 5000 rpm (2.3*g*) for 5 min and resuspended in 500 μL 10 mM phosphate buffer (pH 7.2). Glass beads were added for vigorous vortexing six times for 15 seconds with 1 min intervals on ice. Samples were centrifuged at 5000 rpm for 5 min and the supernatant retained as cell extract used for assays (Kersters-Hilderson, 1982).

Enzyme Assays: Enzyme assays from each flask of yeast cells were done in triplicate. All of the assays were done in UV transparent 96 well plates with reaction volumes of 200 μL and values were read at absorbance 340 nm on a μ-Quant

spectrophometer. Assay units were in nkat.mL^{-1} where 1 nkat.mL^{-1} is 1 nmol of substrate converted in 1 second. Assays were incubated for 20 min.

Xylose Reductase (XR) Assay: Samples were not treated with β-mercaptoethanol as it decreases the observed activity (M Jeppsson, personal communication). Reactions contained reagents with the indicated final concentrations: 100 μL of triethanolamine buffer pH 7.0 [100 mM], 4 μL NADPH [0.2 mM], 76 μL of cell extract sample and at zero time 20 μL of D-xylose [350 mM], the starting reagent, was added. The decrease in absorbance at 340 nm, which indicates NADPH oxidation, was monitored - based on the principle of Smiley and Bolen (1982).
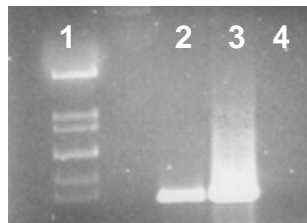
Xylitol Dehydrogenase (XDH) Assay: Samples were not treated with β-mercaptoethanol as it decreases the observed activity (M Jeppsson, personal communication). Reactions contained the following reagents at the indicated final concentrations: 100 μL of glycine buffer with MgCl$_2$ pH 9.0 [100 mM, 50 mM], 6 μL NAD$^+$ [3 mM], 74 μL cell extract sample and at zero time 20 μL xylitol [300 mM], as starting reagent, was added. Reduction of NAD is indicated by rapid increase in absorbance at 340 nm and shows active xylitol dehydrogenase - based on the principle of Rizzi et al. (1988).

Xylulose Kinase (XK) Assay: Reactions contained the following reagents at the indicated final concentrations: 100 μL Tris-HCl with MgCl$_2$ [50 mM, 2.0 mM], 16 μL phosphoenolpyruvate [0.2 mM], 4 μL NADH [0.2 mM], 1 μL pyruvate kinase [10 U], 1 μL lactate dehydrogenase, 60 μL cell extract sample, 10 μL xylulose [8.5 mM] (start reagent) and 8 μL ATP [2.0 mM]. A stable reading of absorbance at 340 nm is required before xylulose is added, after which absorbance readings at 340 nm are taken over time and used to produce a linear curve known as slope 1. Then ATP is added and the absorbance readings taken over time are used to produce a linear curve known as slope 2. The xylulose kinase reaction is calculated by subtracting the values of slope 1 from slope 2 - based on Shamanna and Sanderson (1979)

3.3 RESULTS

3.3.1 Confirmation of yeast transformants

S. cerevisiae Sigma [YIpLoxZEO] was successfully transformed with plasmid YIpLoxZEO as confirmed by PCR amplification of the 870 bp *XYL1* gene fragment present on the plasmid, as seen in Figure 2. Lane 1 is marker, all other lanes contain DNA which has undergone a PCR reaction to amplify for the *XYL1* fragment: lane 2 genomic DNA from transformed *S. cerevisiae* Sigma [YIpLoxZEO], lane 3 a positive control of [YIpLoxZEO] plasmid and lane 4 a negative control of genomic DNA from untransformed *S. cerevisiae* Sigma. The arrow indicates the 870 bp fragment. The transformed strain shows growth on rich medium agar plates with D-xylose as carbon source, whereas the parent strain *S. cerevisiae* Sigma shows very poor growth.

Figure 2: PCR to confirm integration of [YIpLoxZEO] plasmid into yeast

3.3.2 Mutagenesis and analysis of mutants

After exposure to EMS mutagenesis, *S. cerevisiae* Sigma [YIpLoxZEO] survival rates from plate counts were about 13%, within the recommended 10-50% range, for the EMS mutations. Following mutagenesis yeast aliquots were plated out on YPX. Individual yeast colonies isolated from YPX media were inoculated into test tubes containing Durham tubes. All of these samples showed an increase in cell density and bubble formation characteristic of the formation of CO_2 with glucose as carbon source. None of the samples showed CO_2 formation in Durham tubes with D-xylose medium.

The EMS treated *S. cerevisiae* Sigma [YIpLoxZEO] that was directly transferred to liquid medium with D-xylose as sole carbon source under anaerobic conditions, showed virtually no yeast growth, and contamination by bacteria.

3.3.3 Fermentations

Attempts to grow and ferment *S. cerevisiae* [YIpLoxZEO] anaerobically in minimal medium with D-xylose as carbon source led to virtually no biomass formation, the untransformed reference strain showed no growth. In aerobic shake flask

fermentations with D-xylose concentrations varying between 20 g.L^{-1} and 100 g.L^{-1}, the optimal D-xylose concentration appeared to be 50 g.L^{-1}, as this showed greatest turbidity and yielded the most yeast biomass when D-xylose was sole carbon source. *S. cerevisiae* [YIpLoxZEO] was grown anaerobically on rich medium with D-xylose as sole carbon source at differing concentrations from 20 g.L^{-1} to 300 g.L^{-1}. Although the highest biomass was apparent with a 50 g.L^{-1} D-xylose concentration, yeast biomass took a very long time to form and a bacterial contaminant overtook the yeast in growth in all samples. *S. cerevisiae* [YIpLoxZEO] grown anaerobically in serum flasks on YP medium with 10 g.L^{-1} D-glucose together with 10 g.L^{-1} D-xylose showed essentially the same amount of growth and growth rate as yeast grown anaerobically on glucose as sole carbon source. The growth was rapid, healthy and non-pseudohyphal. In Figure 3 this growth is shown, with *S. cerevisiae* [YIpLoxZEO] compared to the reference strain, the transformed strain produces more biomass than the reference strain.
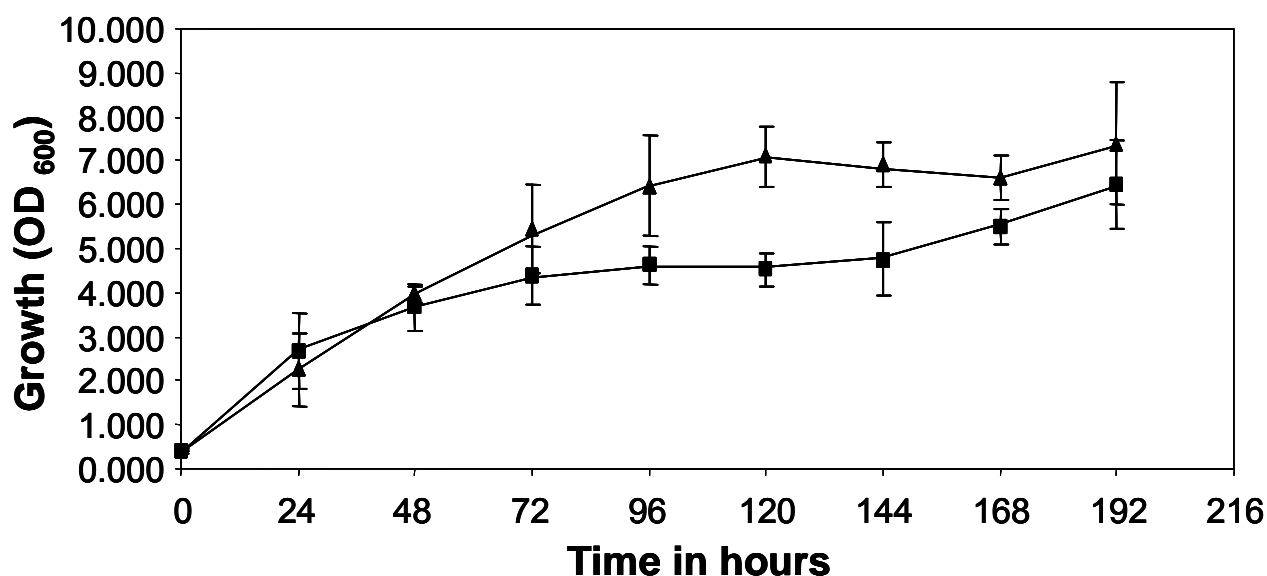

Figure 3: Anaerobic growth on rich medium with 10 g.L^{-1} D-glucose and 10 g.L^{-1} D-xylose in triplicate, was used to compare growth of *S. cerevisiae* Sigma reference strain (■) with *S. cerevisiae* [YIpLoxZEO] (▲). Error bars indicate deviations from the mean.

3.3.4 Assays

Anaerobic fermentations of *S. cerevisiae* Sigma reference strain and *S. cerevisiae* YIpLoxZEO were done on rich medium with 10 g.L^{-1} D-xylose and 10 g.L^{-1} D-glucose. Samples were taken and prepared for enzyme assays as previously described. The results of the XR, XDH and XK enzyme essays were inconsistent and showed no significant difference between the negative control of yeast without the xylose utilisation genes and the transformed yeast capable of growth on D-xylose. As an example Figure 4 shows the XR assay results of samples taken at daily intervals over the course of 4 days. *S. cerevisiae* Sigma reference strain was grown in triplicate in flasks 1, 2 and 3 while the transformed strain was grown in flasks 4, 5 and 6. Each point on the graph represents three readings taken from a single flask, with standard deviations from the mean indicated.

On a standard curve 10 mM NADPH levels registered as 33.3 nkat.mL^{-1}. As levels obtained in the assay were higher, it is clear that the total NADPH concentration in each reaction was not only due to added cofactor. Therefore the cell extracts contributed NADPH and the total NADPH concentrations were unknown, which renders calculations inaccurate. The error bars often overlap and therefore the differences between the reference and transformed strains are possibly not significant. Furthermore samples taken from replicate flasks of the same strain show great variability.

3.3.5 Purification of yeast strains

As bacterial contamination repeatedly plagued efforts to grow the recombinant yeast on D-xylose media, efforts were made to ensure strain purity. Repeated aseptic single colony transfer of yeast on Bengal Rose Agar supplemented with antibiotics led to very low bacterial load, but did not purify the culture. The contamination was not caused by improper aseptic technique, as only one bacterial contaminant was consistently found. This bacterium was designated Bacterium X and brought into pure culture. None of the pure cultures of Bacterium X grew in liquid medium when exposed to any of the antibiotics. Bacterium X was grown in TB medium with antibiotics ampicillin (0.1 mg.mL^{-1}), kanamycin (0.03 mg.mL^{-1}), chloramphenicol (0.09 mg.mL^{-1}) or streptomycin/penicillin mixture (as directed). In the *S. cerevisiae* Sigma reference strain and *S. cerevisiae* Sigma [YIpLoxZEO] yeast cultures exposed

to antibiotics the bacterial count fell, although they continued to show contamination by Bacterium X, even when all antibiotics were combined. Attempts to grow the yeast culture on YP medium with high glucose concentrations (90 g.L^{-1} and 150 g.L^{-1}), which should lead to high ethanol concentrations (approximately 60 g.L^{-1} and 100 g.L^{-1}), also did not eliminate the contaminant. Retransformation of the yeast with integrative plasmid YIpLoxZEO resulted in strains with the same bacterial contamination. Further investigation revealed that the original, untransformed Sigma culture was contaminated.

3.3.6 Identification of bacterial contaminant

Comparison of the 1kb sequence of 16S rRNA of Bacterium X, obtained by PCR with universal primers (Rawlings, 1995), with the Genbank database showed greatest similarity to 16S rRNA genes of various *Staphylococcus* species. This supports the biochemical profile results that Bacterium X is a *Staphylococcus*. The best alignment is shown below in Figure 5. The closest matches for Bacterium X was with *S. pasteuri*, which showed an average of 99.73% similarity for these two sequences. Thus Bacterium X could be identified with high confidence as *S. pasteuri*.

3.4 DISCUSSION

A haploid industrial *S. cerevisiae* strain Sigma α was transformed with the plasmid YIpLoxZEO to confer D-xylose utilisation ability on the strain. The transformed yeasts were then grown under conditions which should preferentially select for improved ability to use the carbon source D-xylose.

Attempts were made to grow the yeasts aerobically, and in a mixture of 10 g.L^{-1} D-xylose and 10 g.L^{-1} D-glucose, because the reference strain, *S. cerevisiae* Sigma α, could not tolerate xylose as sole carbon source. This however did not apply sufficient pressure for the *S. cerevisiae* Sigma [YIpLoxZEO] yeast to improve its ability to ferment D-xylose. To increase environmental pressure on the yeast to efficiently anaerobically ferment D-xylose to ethanol, it was kept under anaerobic conditions with D-xylose as the sole carbon source, rather than combined with D-glucose as by several other researchers (Ho et al., 1998). Medium was supplemented with Tween 80 and ergosterol to reduce anaerobic stress on the yeast,

as *S. cerevisiae* can only manufacture such sterols in the presence of molecular oxygen (Sonderegger et al., 2004). In all cases when samples were placed under
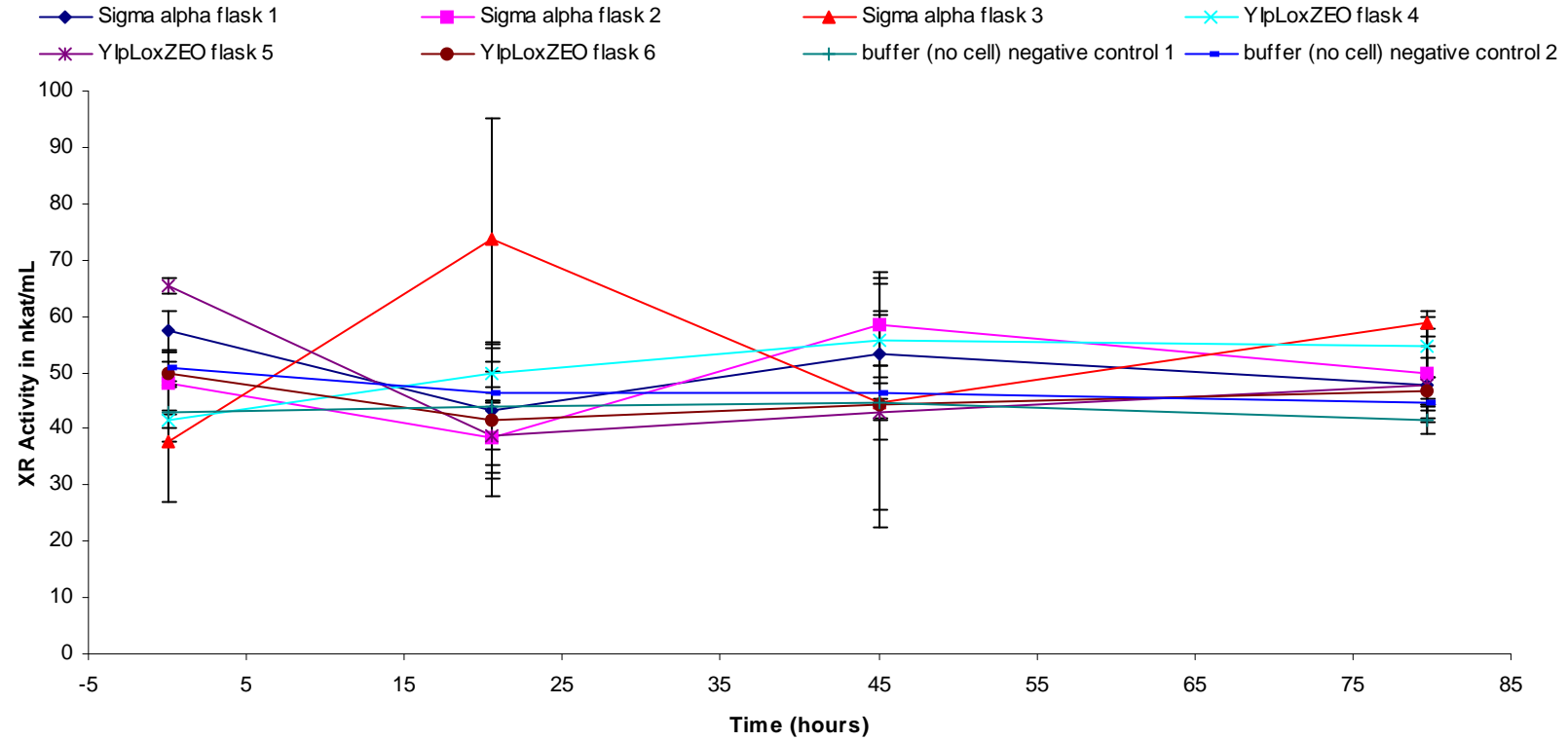
Figure 4: Graph of XR activity in *S. cerevisiae* YIpLoxZEO compared to the reference strain Sigma from anaerobic fermentation rich medium with 10g/L glucose and 10g/L xylose

Upper line: Bacterium X universal primer sequence   from 2 to 745

Lower line: *S. pasteuri* 16S rRNA sequence   from 530 to 1273

```
identity= 99.87%(743/744) gap=0.00%(0/744)
2      CGTAGGTGGCAAGCGTTNTCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTTTTA
       |||||||||||||||| |||||||||||||||||||||||||||||||||||||||||||
530    CGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTTTTA

62     AGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGAAAACTTGA
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
590    AGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGAAAACTTGA

122    GTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGA
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
650    GTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGA

182    ACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGATGTGCGAAAGCGTGGG
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
710    ACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGATGTGCGAAAGCGTGGG

242    GATCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTA
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
770    GATCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTA

302    GGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACG
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
830    GGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACG

362    ACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGG
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
890    ACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGG

422    TTTAATTCGAAGCAACGCGAAGAACCTTACCAAATCTTGACATCCTTTGACCGCTCTAGA
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
950    TTTAATTCGAAGCAACGCGAAGAACCTTACCAAATCTTGACATCCTTTGACCGCTCTAGA

482    GATAGAGTCTTCCCCTTCGGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCG
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1010   GATAGAGTCTTCCCCTTCGGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCG

542    TGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAAGCTTAGTTGCCATC
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1070   TGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAAGCTTAGTTGCCATC

602    ATTAAGTTGGGCACTCTAAGTTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACG
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1130   ATTAAGTTGGGCACTCTAAGTTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACG

662    TCAAATCATCATGCCCCTTATGATTTGGGCTACACACGTGCTACAATGGACAATACAAAG
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1190   TCAAATCATCATGCCCCTTATGATTTGGGCTACACACGTGCTACAATGGACAATACAAAG

722    GGCAGCTAAACCGCGAGGTCAAGC
       ||||||||||||||||||||||||
1250   GGCAGCTAAACCGCGAGGTCAAGC
```

Figure 5: Alignment of Bacterium X sequenced with 'right primer 16S reverse' universal primer (Rawlings, 1995) with *S. pasteuri* 16S rRNA sequence.

anaerobic conditions with xylose as sole carbon source, yeast growth was very slow with samples overrun by a bacterial contaminant. The yeast displayed extreme pseudohyphal growth showing the growth conditions are stressful (Radcliffe et al., 1997). In cases when the conditions were aerobic the same preculture produced higher yeast biomass with a more normal yeast morphology and the extremely low levels of bacterial contamination were more difficult to distinguish. Similarly the contamination was not discovered during growth on medium with 10 g.L^{-1} glucose and 10 g.L^{-1} D-xylose, as yeast growth was healthy. The result that 50 g.L^{-1} D-xylose is optimal for yeast growth may therefore be invalid, as at least part of the biomass yield was bacterial in origin.

The *S. cerevisiae* Sigma [YIpLoxZEO] was randomly mutated with EMS to accelerate formation of different yeast mutants. These were selected to determine those with better D-xylose utilisation and fermentative ability than the original transformed strain. The mutated yeast and control showed no CO_2 formation in Durham tubes with D-xylose. This strongly suggested that all of the mutant strains, and the untreated *S. cerevisiae* Sigma [YIpLoxZEO], continued to suffer redox imbalance leading to ineffective fermentation of the pentose sugar D-xylose.

An alternative mutation protocol based on the EMS method above was developed to allow the various yeast mutants to directly compete with one another in anaerobic fermentations. The conditions were more stressful as yeast was directly inoculated into anaerobic serum flasks rather than first being selected on D-xylose plates in aerobic conditions. Those mutants which could best utilise D-xylose anaerobically, should out-compete others and attain the highest cell numbers monitored with OD_{600}, within the anaerobic serum flasks. Unfortunately all samples cultivated under these conditions showed very slow yeast growth, and the presence of the coccus, Bacterium X. No yeast mutant was found to be superior to the parent *S. cerevisiae* Sigma [YIpLoxZEO] at D-xylose utilisation.

As seen in the results the XR assay did not consistently show greater enzyme activity in the transformed strain than the reference strain by XR activity. In addition the assay should show an immediate rise in absorbance at 340 nm, which then falls, and

this difference indicated the XR activity. Unfortunately a reliable reading for the maximum level was not attained. In addition NADPH levels were problematic, as the assay registered higher levels than were added. Since unpurified cell extract will contain NADPH, this is unsurprising, however as the assay depends on the molar relationship between NADPH and xylulose, unknown NADPH quantities affect the accuracy of the assay. For this reason the assay was dismissed as unreliable. Similarly data from the XDH and XK assays, which similarly rely on cofactors available in cell extract, also showed these assays could not be used (data not shown). *S. cerevisiae* native enzymes do not produce significant D-xylose utilising activity (Toivari et al., 2004). The bacterial contaminant very likely did not influence the assay result as bacteria use the enzyme xylose isomerase (XI), which is not cofactor linked. Some bacteria do however possess a xylulose kinase enzyme; for which xylulose is the substrate and ATP is necessary, so bacterial contamination may have influenced the XK assay.

The XR, XDH and XK assays did not reveal significant activity in the recombinant yeast strains. Perhaps the activity of the recombinant enzymes was very low in the recombinant yeast. Activity did exist as shown by assimilation of D-xylose over time under aerobic conditions. This activity however may not have been strong enough to show significant values in the assay, in which cell extracts were incubated for only twenty minutes.

After multiple generations of streaking out single colonies of yeast on Rose Bengal Agar supplemented with antibiotics, it was concluded that the yeast could not be completely purified of Bacterium X contamination using standard microbial methods. Due to the stress conditions under which *S. cerevisiae* must be placed for experiments on D-xylose, no level of contamination is acceptable, as the bacterium rapidly out-competes the yeast under anaerobic conditions with D-xylose as sole carbon source.

Several cultures were obtained which appeared as pure colonies on plates, and also under the microscope to be pure, but when cultivated anaerobically on D-xylose, were found overrun with bacteria. This showed the inoculum was not pure. The

contamination was not caused by improper aseptic technique, as that would have led to a variety of different contaminants. It is speculated that the bacteria attached to the yeast cell and this attachment, perhaps with extracellular polysaccharides forming a biofilm may have contributed to the antibiotic resistance in co-culture with *S. cerevisiae*.

The contaminant was classified in the hope of identifying and eliminating it. Microscope work identified Bacterium X as a coccus. Tests with an Api 20 strip (bio Mérieux) for *Staphylococcus* and *Micrococcus* used according to manufacturer's instructions suggested that it might be a *Staphylococcus*. This was supported by 16S rRNA analysis, which further showed the strain was in all probability *S. pasteuri*.

Literature shows that *S. pasteuri* could grow under the culture conditions in this study, as the bacterium can grow on protein sources alone, without a fermentable carbon source (Rantsiou et al., 2005). At least one *Staphylococcus* species, *S. xylosus*, has an operon for D-xylose utilisation (Sizemore et al., 1991). *S. xylosus* and *S. pasteuri* are not in the same species group, with the former a member of *S. saprophyticus* and the latter falling under *S. epidermis* (Takahashi et al., 1999). According to Freney et al., (1999) more than 90% of *S. pasteuri* species do not produce acid from D-xylose under aerobic conditions, while *S. xylosus* does.

Identification of the strain as *S. pasteuri* did not suggest any further methods for purification than those previously attempted. As it was impossible to obtain a yeast culture of *S. cerevisiae* Sigma YIpLoxZEO without *S. pasteuri* contamination, it was impractical to continue with this strain. Retransformation of the yeast was then undertaken. Unfortunately this, too, resulted in yeast with the same bacterial contamination. Further investigation revealed that the untransformed stock Sigma culture was contaminated and was also not purifiable given the above techniques. For the purposes of research, pure strains are necessary as interactions between species increase the complexity of studies.

In addition to the problems with the yeast strain, the enzyme assays had been found to be difficult to work with and to reveal results showing greater variation within

samples than between the samples and negative control. The only other reliable method to determine the difference between the initial strain and the selected strain is High Performance Liquid Chromatography to measure levels of sugars and metabolites including xylitol and ethanol after fermentation. Unfortunately given that HPLC is a screen, not a selection and that it is an end-point analysis, it was impractical to use. At the same time, aerobic selection for D-xylose utilisation did not assist anaerobic D-xylose utilisation. Anaerobic fermentations in serum flasks and in Durham tubes on D-xylose as sole carbon source placed too much stress on *S. cerevisiae* Sigma [YIpLoxZEO] for survival. Thus another selection method would have been necessary for successful evolution of anaerobic D-xylose utilisation ability in yeast.

A better D-xylose utilising recombinant *S. cerevisiae* strain was not developed via random mutagenesis and selection and therefore given these insurmountable difficulties it was decided to terminate this line of investigation and explore an alternative approach.

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CHAPTER 4

EXPERIMENTAL WORK

CLONING, CHARACTERISATION AND EXPRESSION OF THE UNIQUE
BACTEROIDES THETAIOTAOMICRON D-XYLOSE ISOMERASE GENE (*XYLA*) IN
SACCHAROMYCES CEREVISIAE

4.1 INTRODUCTION

Many researchers have introduced eukaryotic D-xylose utilisation pathways into *S. cerevisiae*, to make this yeast capable of D-xylose utilisation (Zaldivar et al., 2001). The aim of the study was to increase D-xylose utilisation and lower the xylitol production found with the eukaryotic pathway, to redirect this carbon to increased ethanol levels. Bacterial D-xylose isomerases convert D-xylose to xylulose in a single step, while eukaryotic pathways produce the intermediate xylitol (Hahn-Hägerdal et al., 2001).

Although numerous groups have expressed xylose isomerase genes in *S. cerevisiae*, to date only two genes have led to active protein production. One of these genes originated from the fungus *Piromyces* sp. E2 (Harhangi et al., 2003). The amino acid sequence of the *Piromyces* XI protein showed similarity to a putative xylose isomerase encoded in the genome sequence of the bacterium *Bacteroides thetaiotaomicron*.

In this chapter the unique *B. thetaiotaomicron* putative xylose isomerase gene, which has not previously been transformed into yeast, was introduced into *S. cerevisiae*. The gene was cloned into the yeast shuttle vector YEpENO and transformed into *S. cerevisiae* CEN.PK strain CEN.PK21-C (Hamacher et al., 2002). The well-characterised CEN.PK yeast strain was chosen for fermentation ability and hardiness, while its haploid laboratory strain status aided ease of molecular research (Johansson et al., 2001). The CEN.PK family has been chosen as reference strains and is frequently used in xylose utilisation work, easing comparison of these results with international publications (van Dijken et al., 2000).

4.2 MATERIALS AND METHODS

4.2.1 Isolation and subcloning for the D-xylose isomerase (XI) - encoding gene

For DNA manipulations, standard protocols were followed (Sambrook et al., 1989). Restriction endonucleases and T4 DNA ligase were purchased from Roche Molecular Biochemicals and used according to the manufacturers directions. DNA digested with restriction endonucleases was eluted from agarose gels with the method from Tautz and Renz (1983).

4.2.2 *Bacteroides* strain, genomic DNA isolation and PCR amplification of XI gene

Strains and plasmids used in this work are indicated in Table 1. The anaerobic rumen bacterium *B. thetaiotaomicron* was received as a preculture. Genomic DNA was isolated from *B. thetaiotaomicron* using a protocol for DNA extraction from gram-negative bacteria (Engebrecht, 1998) and used for polymerase chain reaction (PCR) amplification of the putative xylose isomerase gene, and for sequencing. Primers were designed for the 1317 bp *B. thetaiotaomicron* putative xylose isomerase gene from Genbank sequence (Accession number: AA075900.1) the 5' primer included an *NruI* site, and the 3' primer a *SalI* site for cloning (shown in bold). The ATG in the 5' primer is the start of the XI coding sequence.

5' primer sequence *XI*: 5'GGCC**TCGCGA**ATGGCAACAAAAGAATTTT3'

3' primer sequence *XI*: 5'GCGC**GTCGAC**TTAGCAATACATATTCAGAATTG3'

Taq polymerase was used as directed. Annealing and extension was done for 30s at 53ºC and 95s at 72ºC, respectively to produce the 1317bp product.
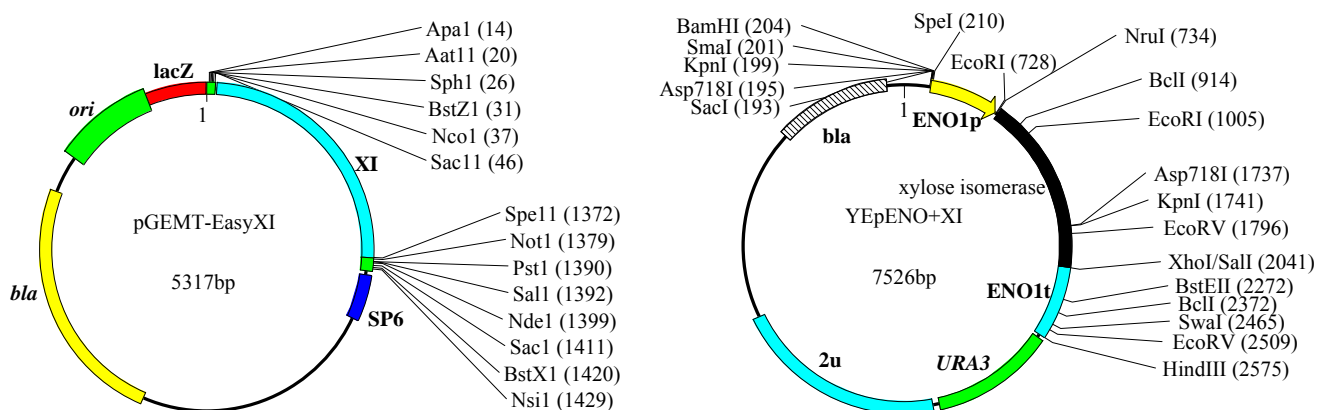

Figure 1: Plasmid maps of pGEMT-EasyXI and YEpENOXI

4.2.3 Plasmids, host strains and transformation method

The resultant *xylA* fragment was gel purified and ligated into commercial plasmid pGEMT-Easy™ (Promega), with the resultant plasmid designated pGEMT-EasyXI. Plasmid pGEMT-EasyXI was transformed into *E. coli* HB101, a Δ*xylA* deletion mutant, using the CaCl₂ method (Engebrecht, 1998), for initial enzyme work.

The putative *xylA* gene was subcloned from the pGEMT-EasyXI plasmid as a *NruI-SalI* fragment into the yeast shuttle plasmid YEpENO opened with *NruI* and *XhoI*, inserted between the *ENO1* promoter and terminator sequences, yielding plasmid YEpENOXI. Construction of the expression cassette with the *ENO1* promoter sequence and *ENO1* terminator sequence, was described by McBride (2005). The structure of bacterial plasmid pGEMT-EasyXI and yeast plasmid YEpENOXI are shown in Figure 1.

A haploid *Saccharomyces cerevisiae* CEN.PK 21-C (*ura3, his3, leu2, trp1*) was transformed with YEpENO and YEpENOXI using the bicine method (Klebe et al., 1983). Yeast was selected and routinely cultured using selective synthetic (SC) minimal medium [5 g.L^{-1} (NH$_4$)$_2$SO$_4$, 1.7 g.L^{-1} yeast nitrogen base without amino acids (Difco) (pH 6.0), supplemented with all growth factors except uracil (SC^{-URA}), containing 20 g.L^{-1} glucose or 20 g.L^{-1} D-xylose]

4.2.4 Selection of bacterial strains

Xylose isomerase activity in *E. coli* HB101 transformants was screened using McConkey Agar supplemented with 20 g.L^{-1} D-xylose as carbon source. The medium also contained 20 g.L^{-1} lactose but this should not affect the screen as the bacterial strains had a lactose permease mutation in *lacY* rendering them incapable of using lactose. Red colouring of colonies or surrounding agar indicated a fall in pH caused by use of the sugar, whereas yellow colouring indicated a rise in pH that was due to utilisation of the amino acids. Colonies showing red colouring were selected. *E. coli* HB101 and transformants were grown on LB (5 g yeast extract, 10 g tryptone, 10 g NaCl, 20 g Agar per litre) supplemented with ampicillin (100 mg.L^{-1}). Transformants were also grown in liquid TB medium (12 g tryptone, 24 g yeast extract, 4 mL glycerol per litre and buffered with potassium phosphate buffer), also supplemented with ampicillin (100 mg.L^{-1}) where necessary. Minimal M9 medium [containing 12.8 g.L^{-1} Na$_2$HPO$_4$7H$_2$O, 6 g.L^{-1} KH$_2$PO$_4$, 1 g.L^{-1} NaCl, 2 g.L^{-1} NH$_4$Cl, 1.7 g.L^{-1} yeast nitrogen base without amino acids (Difco), 5 g.L^{-1} (NH$_4$)$_2$SO$_4$, 20 g.L^{-1} agar and 20 g.L^{-1} D-xylose added after autoclaving] was also used to grow transformants.

Table 1: **Microbial strains and plasmids**

Strain or plasmid	Genotype	Source/ Reference
Plasmid:		
pGEMT-Easy™	commercial plasmid	Promega
pGEMT-EasyXI	with putative *B. thetaiotaomicron xylA* gene (XI)	This work
YEpENO	*URA3 ENO1$_p$ ENO1$_t$ bla*	McBride, 2005
YEpENOXI	*URA3 ENO1$_p$ ENO1$_t$ bla* with putative *B. thetaiotaomicron xylA* gene (XI)	This work
Strain:		
B. thetaiotaomicron	wild-type	Prof P. Weimer USDA Agricultural Research Service
E. coli HB101	*F⁻Δ(gpt-proA2)62 leuB6 glnV44 ara-14 galK2 lacY1 Δ(mcrC-mrr) rpsL20(Str^r) xyl-5 mtl-1 recA13*	ATCC
E. coli HB101[pGEMT-EasyXI]	*F⁻Δ(gpt-proA2)62 leuB6 glnV44 ara-14 galK2 lacY1 Δ(mcrC-mrr) rpsL20(Str^r) xyl-5 mtl-1 recA13/* pGEMT-EasyXI	This work
S. cerevisiae CEN.PK21-C	MATa *leu2-3,112 ura3-52 trp1-289 his3-Δ1 MAL2-8^c SUC2*	Hamacher et al., 2002
S. cerevisiae CEN.PK21-C [YEpENO]	MATa *leu2-3,112 ura3-52 trp1-289 his3-Δ1 MAL2-8^c SUC2/URA3 ENO1$_p$ ENO1$_t$ bla*	This work
S. cerevisiae CEN.PK21-C [YEpENOXI]	MATa *leu2-3,112 ura3-52 trp1-289 his3-Δ1 MAL2-8^c SUC2/URA3 ENO1$_p$ XI ENO1$_t$ bla*	This work

S. cerevisiae CEN.PK21-C [YEpENOSXI]	MATa *leu2-3,112 ura3-52 trp1-289 his3-Δ1 MAL2-8^c SUC2/URA3 ENO1$_p$ synthetic xylose isomerase (SXI) ENO1$_t$ bla*	This work

4.2.5 Confirmation of yeast strains

The presence of the plasmid YEpENOXI in *S. cerevisiae* [YEpENOXI] transformants was confirmed by PCR. Genomic DNA extracted from *S. cerevisiae* cultures (Hoffman and Winston, 1987), was used as template DNA. The same primers and conditions were used as for amplification of the XI gene from the *B. thetaiotaomicron* DNA. The CENPK 21-C strain transformed with empty YEpENO plasmid served as reference strain.

4.2.6 D-xylose isomerase activity determination

E. coli HB101 pGEMT-XI transformants were inoculated from –80$^°$C storage cultures into 5 mL TB supplemented with ampicillin and incubated on a wheel overnight at 37$^°$C. Spectophotometric readings were taken of cultures at 600 nm. Up to 3.9 mL of each culture was spun down, washed and resuspended in ice-cold potassium phosphate buffer (0.05 M, pH 7.8, 0.25 mM dithiothreitol) and broken with glass beads (Vongsuvanlert and Tani, 1988).

Similarly *S. cerevisiae* [YEpENO] and *S. cerevisiae* [YEpENOXI] cultures were inoculated from –80$^°$C storage cultures into 5 mL SC^{-URA} 20 g.L^{-1} glucose cultures and incubated overnight on a rotating wheel at 30$^°$C. Cells were spun down, resuspended in potassium phosphate buffer and broken with glass beads. Cells and extracts were kept on ice. 200µL of cell extract was used for Biorad DC protein determinations according to manufacturers instructions (Biorad Laboratories).

To determine xylose isomerase activity cysteine-carbazole assays were used (Nakamura, 1968). 200 µL of reagent mix (250 µL 0.01 M D-xylose, 3 750 µL 0.2 M potassium phosphate buffer pH 7.8, 750 µL 0.1 M MgSO$_4$7H$_2$O, 5 250 µL dH$_2$O) was combined with 100 µL cell extract and incubated at 50$^°$C for 10 min. Alternatively

0.1 M solutions of $CoSO_47H_2O$, $MnCl_2$, and $FeSO_47H_2O$ were used instead of $MgSO_47H_2O$. The reaction was heat-inactivated at $100^{\circ}C$ for 1 min. The following reagents were added on ice: 1.8 mL 75% H_2SO_4, 60 μL 24 g.L^{-1} L-cysteine-HCl, then 60 μL 1.2 g.L^{-1} carbazole (dissolved in ethanol). The reaction was mixed well and incubated at $50^{\circ}C$ for 10 min. Absorbance was measured at 537 nm to detect the pink colour compound hydroxymethylfurfurol. The assay was calibrated using xylulose to produce standard curves, and with an industrial enzyme (Maxazyme), showing that the assay does pick up xylose isomerase activity.

4.2.7 Sequencing of gene

Sequencing of genomic DNA from *B. thetaiotaomicron* was undertaken and confirmed the Genbank published sequence. Plasmid isolation of YEpENOXI was undertaken and sent for sequencing with the *XI* 5' and 3' primers, at the Central Analytical Facility DNA sequencer (University of Stellenbosch).

4.2.8 Northern blots

RNA was isolated from shake flask cultures (2 X SC^{-URA} containing 20 g.L^{-1} glucose) of *S. cerevisiae* CEN.PK 21-C [YEpENO] and *S. cerevisiae* CEN.PK 21-C [YEpENOXI] at 24 and 48 hours after incubation. RNA isolations and Northern hybridisations were carried out as described by Sambrook et al. (1989).

A 1% (wt/vol) formaldehyde gel was run with 30μg total RNA samples. Denatured 1 300bp putative *xylA* DNA fragment served as positive control and a High Range RNA ladder and loading buffer from Fermentas were used. RNA was transferred to a positively charged nylon membrane (Osmonics). All membrane hybridisations included shaking. Prehybridisation was done for 30 min in DIG Easy Hyb at 50°C, followed by hybridisation with probe in DIG Easy Hyb overnight at 50°C. Incubation in low stringency buffer (2 X SSC, 0.1% SDS) occurred for 2 X 5 min at room temperature. Incubation in high stringency buffer (0.1 X SSC, 0.1% SDS) occurred for 2 X 15 min at 50°C.

DNA probes were prepared by labelling a 1500 bp fragment from the 5S rDNA of *S. cerevisiae* (to bind to the rRNA transcripts), and the 1300 bp putative xylose

isomerase gene of *B. thetaiotaomicron* with dioxigenin hexanucleotide primers, using the Random Primed DIG Labelling Kit (Roche Molecular Biochemicals) according to the manufacturers instructions. The DIG-labelled 5S rDNA fragment was used to probe the rRNA levels as internal standard in the Northern blots.

4.2.9 Design of synthetic XI

The amino acid sequence of the *B. thetaiotaomicron* XI was used to design a genomic sequence comprising of codons more optimal for *S. cerevisiae*, as the original sequence has a codon bias index (CBI) of 0.07 in *S. cerevisiae*. The CBI of this sequence was designed to be 0.93 and designated SXI. The gene was manufactured by GenScript Corporation. The synthetic SXI gene was introduced into plasmid construction pUC57 and transformed into *E. coli* HB101.

4.2.10 Yeast plasmid construction

The SXI gene was excised from the pUC57 plasmid using restriction enzymes *EcoRI* and *XhoI* and cloned into the yeast plasmid YEpENO cut with the same enzymes. The new plasmid was designated YEpENOSXI. Plasmid YEpENOSXI was transformed into *S. cerevisiae* CENPK21-C as previously described.

4.2.11 Confirmation of recombinant *E. coli* strains

E. coli HB101 transformants were tested for the plasmid by small scale plasmid DNA isolations which when cut with the restriction enzymes *EcoRI* and *XhoI* showed fragments of the required size, namely 1 300 bp.

4.2.12 Confirmation of yeast strains

S. cerevisiae CEN.PK21-C transformants were tested for SXI DNA by genomic extraction (Hoffman and Winston, 1987) and PCR using the *ENO1* primers (McBride, 2005).

5' primer *ENO1*: 5'GGATCCACTAGTCTTCTAGGCGGGTTATC3'

5' primer *ENO1*: 5'AAGCTTGCGGCCGCAAAGAGGTTTAGACATTGG3'

Taq polymerase MO267L (New England Biolabs) was used as directed. Annealing and extension was done for 30s at 50ºC and 150s at 72ºC, respectively. In the case of the reference strain [YEpENO] the product length was 1 030 bp for the *ENO1*

promoter and terminator. For the yeast [YEpENOSXI] the product was 2 400 bp, including the 1 317 bp *SXI* gene.

4.2.13 SDS-PAGE analysis

Cell samples of *S. cerevisiae* strains CEN.PK21-C [YEpENO] and [YEpENOSXI] were taken over 4 days. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% gel (wt/vol) was prepared according to the method of Laemmli (1970). Cell extracts were prepared as for cysteine-carbazole assays. Protein fractions (10 µL) were boiled for 3 min in SDS gel loading buffer (0.1 M Tris Cl pH 6.8, 0.2 M dithiothreitol, 4% SDS, 0.2% bromophenol blue and 20% glycerol) and loaded, 5 µL of protein marker (Fermentas) was loaded. Proteins were visualized by Coomassie brilliant blue staining (Sambrook et al., 1989).

4.3   RESULTS

4.3.1 Selection of bacterial strains

Major selection of bacterial transformants for the plasmid was by selection on ampicillin medium. Although the pGEMT-Easy plasmid is primarily a cloning rather than expression tool, it has previously been used for expression of *T. thermophilus* XI in *E. coli* (Lönn et al., 2002). Transformants were grown on McConkey Agar as red zones would indicate utilisation of the xylose. Transformants which showed redder zones, and therefore possible D-xylose utilisation as well as the XI insert in the correct orientation to the *lac* promoter, were transferred to M9 minimal medium with D-xylose as sole carbon source. The reference strain *E. coli* HB101 did not yield colonies on minimal medium with D-xylose as sole carbon source. However, none of the *E. coli* HB101 pGEMT-EasyXI transformants grew on D-xylose, indicating that the *xylA* deletion was not complemented by the *B. thetaiotaomicron* putative xylose isomerase.

4.3.2 Confirmation of yeast strains

The newly transformed strain (designated *S. cerevisiae* [YEpENOXI]) showed gel bands characteristic of the size of the putative xylose isomerase gene (+-1 300 bp). This indicated that the plasmid YEpENOXI was present in the yeast. PCR was used to test for the native *Saccharomyces* yeast promoter *GPD1,* which is not on the

plasmid on genomic DNA from the yeast strains. This confirmed that the different strains were indeed *S. cerevisiae*.

S. cerevisiae [YEpENOXI] showed growth on SC^{-URA} with glucose as carbon source, as did reference strain *S. cerevisiae* [YEpENO], whereas the parent strain (not shown) did not, thus showing complementation of the uracil deletion mutation (Figure 2a). Unfortunately *S. cerevisiae* [YEpENOXI], like *S. cerevisiae* [YEpENO] and the parent strain did not grow on SC^{-URA} with D-xylose as sole carbon source (Figure 2b). This showed that the putative *B. thetaiotaomicron* XI gene did not confer the ability to utilise D-xylose on *S. cerevisiae*.
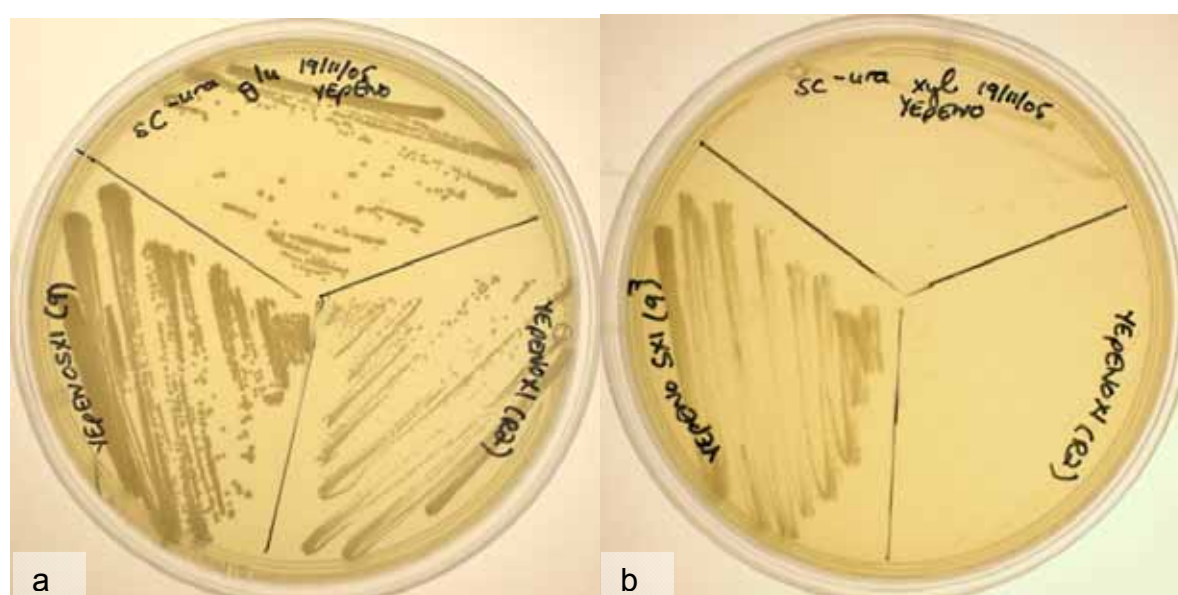

Figure 2: *S. cerevisiae* strains [YEpENO], [YEpENOXI] and [YEpENOSXI] on (a) SC^{-URA} 20 g.L^{-1} glucose and (b) SC^{-URA} 20 g.L^{-1} D-xylose

Table 2:   Xylose Isomerase Activity

	Activity 18 hrs (fkat.mL^{-1})	Activity 42 hrs (fkat.mL^{-1})
background	15±3	16±2
CEN.PK21-C [YEpENO]	15±5	17±5
CEN.PK21-C [YEpENOXI]	20±5	10±5

4.3.3 D-Xylose Isomerase Activity Determinations

D-xylose isomerase activity was measured with the cysteine-carbazole method using cell extracts from CEN.PK21-C [YEpENO], and CEN.PK21-C [YEpENOXI] after 18 hours and 42 hours of growth. Table 2 shows that all values, both for the reference strain and the transformed strain remain in the background range. The effect of different metal cofactors Mg^{2+}, Co^{2+}, Fe^{2+} and Mn^{2+} was investigated with cell extracts from CEN.PK21-C [YEpENO], and two of the CEN.PK21-C [YEpENOXI] transformed strains, grown overnight in triplicate. Once again the activity values were in the background range. None of the cofactors led to significantly better performance than did Mg^{2+}.

4.3.4 Sequencing of XI gene

Sequencing of the YEpENOXI plasmid revealed a mutation, which would change a lysine (K) to glutamine (E) in position 242. This was found also to be the case within the pGEMT-EasyXI plasmid. The protein most homologous to *B. thetaiotaomicron* xylose isomerase for which protein crystal structure data exists is *Thermotoga neapolitana* XI (pdb accession number: 1AOE). This protein shows a corresponding lysine at position 239 and as seen in the Rasmol visualisation in Figure 4 this mutation appears on the surface of the protein, not in the active site.

4.3.5 Northern blots

The 5S rDNA probe as an internal standard revealed RNA of experimental quality. Lane 1 contained marker and lanes 2 and 3 RNA from *S. cerevisiae* CEN.PK21-C [YEpENO] after 24 and 48 hrs of growth respectively. Similarly lanes 4 and 5 contain RNA from *S. cerevisiae* CEN.PK21-C after 24 and 48 hrs of growth. Lane 6 contains the positive control of denatured *B. thetaiotaomicron* XI DNA. Probing with the xylose isomerase probe showed no mRNA specific to the putative *xylA* gene. The 1 300 bp *xylA* DNA fragment used as positive control was detected.
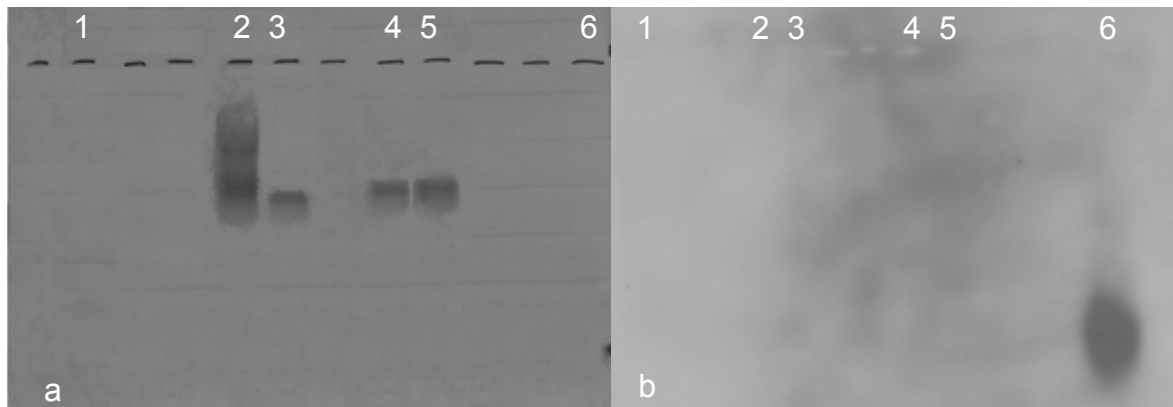
Figure 3: Northern blot (a) shows 5s rRNA probing (b) shows the XI probing

4.3.6 Yeast transformant with synthetic gene growth on D-xylose

S. cerevisiae strains CEN.PK21-C [YEpENO] and [YEpENOSXI] were grown on SC^{-URA} (glucose) as seen in Figure 2a. *S. cerevisiae* CEN.PK21-C [YEpENOSXI] and the control *S. cerevisiae* CEN.PK21-C [YEpENO] were grown on SC^{-URA} with D-xylose as sole carbon source, as seen in Figure 2b. *S. cerevisiae* CEN.PK21-C [YEpENOSXI] grew on D-xylose as sole carbon source showing that the inability to utilise D-xylose was complemented for.

In addition CEN.PK21-C [YEpENO] and [YEpENOSXI] were inoculated into SC^{-URA} medium with 20 g.L^{-1} D-xylose as sole carbon source, from SC^{-URA} 20 g.L^{-1} glucose plates, for growth curves. Figure 5 shows that the CEN.PK21-C [YEpENOSXI] strain grows significantly better and in a sustainable fashion on D-xylose over 200 hours, in contrast to the reference strain [YEpENO], that displayed stunted growth.

4.3.7 SXI activity

More than 50 *S. cerevisiae* CEN.PK21-C [YEpENOSXI] transformants were grown on SC^{-URA} (20 g.L^{-1}) glucose) and extracted as previously described. The enzyme activity in fkat.mL^{-1} was measured after overnight growth. Yeast CEN.PK21-C [YEpENO], was the yeast reference strain. Activity of both the reference strain and all [YEpENOSXI] transformants was lower than 40 fkat.mL^{-1}, which is in the background range. Another experiment tracked the activity over 4 days. Again all values were in the low fkat.mL^{-1} range, therefore although values dropped over time for both the reference strain and transformant this is likely an artifact of the assay. In
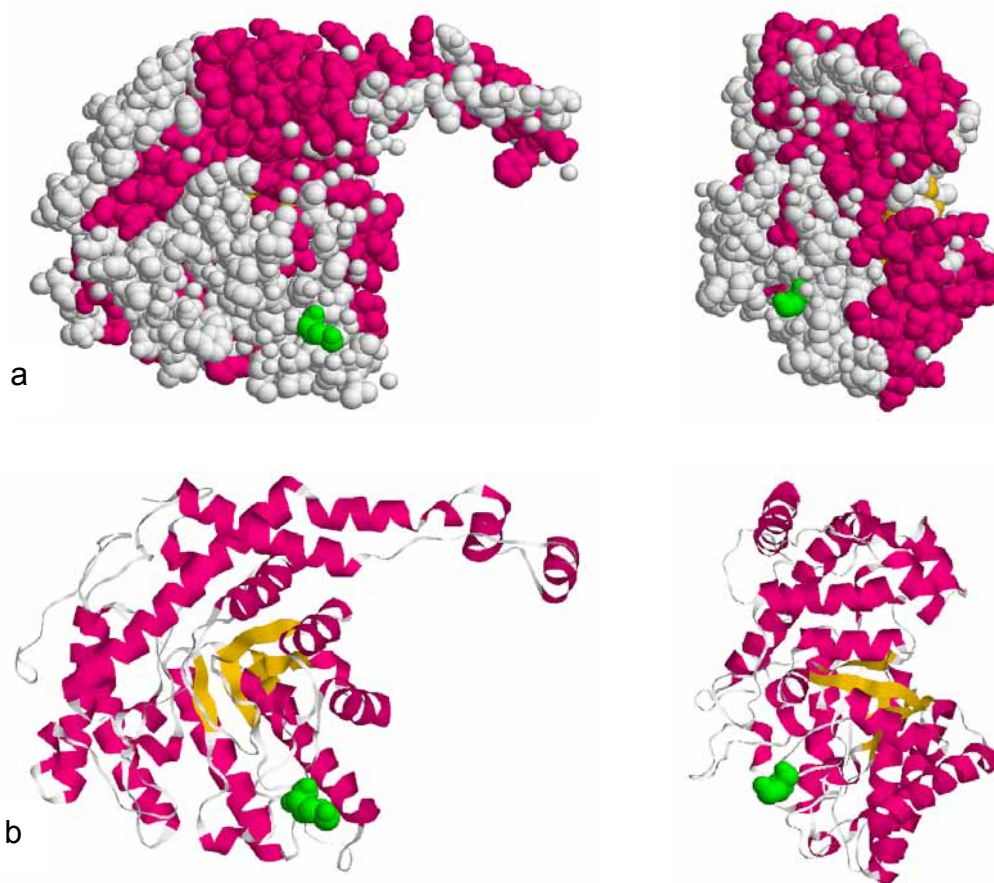
Figure 4: *B. thetaiotaomicron* xylose isomerase monomer, from *Thermotoga Neapolitana* xylose isomerase protein data showing the substituted amino acid on the enzyme surface in green. (a) space-filling model (b) α-helix and β-sheet model

addition the activity was measured using differing pH Tris-maleate buffers between pH 5.06 and pH 9.13. The assay showed no significant differences with pH, but the assay values are in the background range. As none of the assays gave values significantly different to the background values, these graphs are not shown.
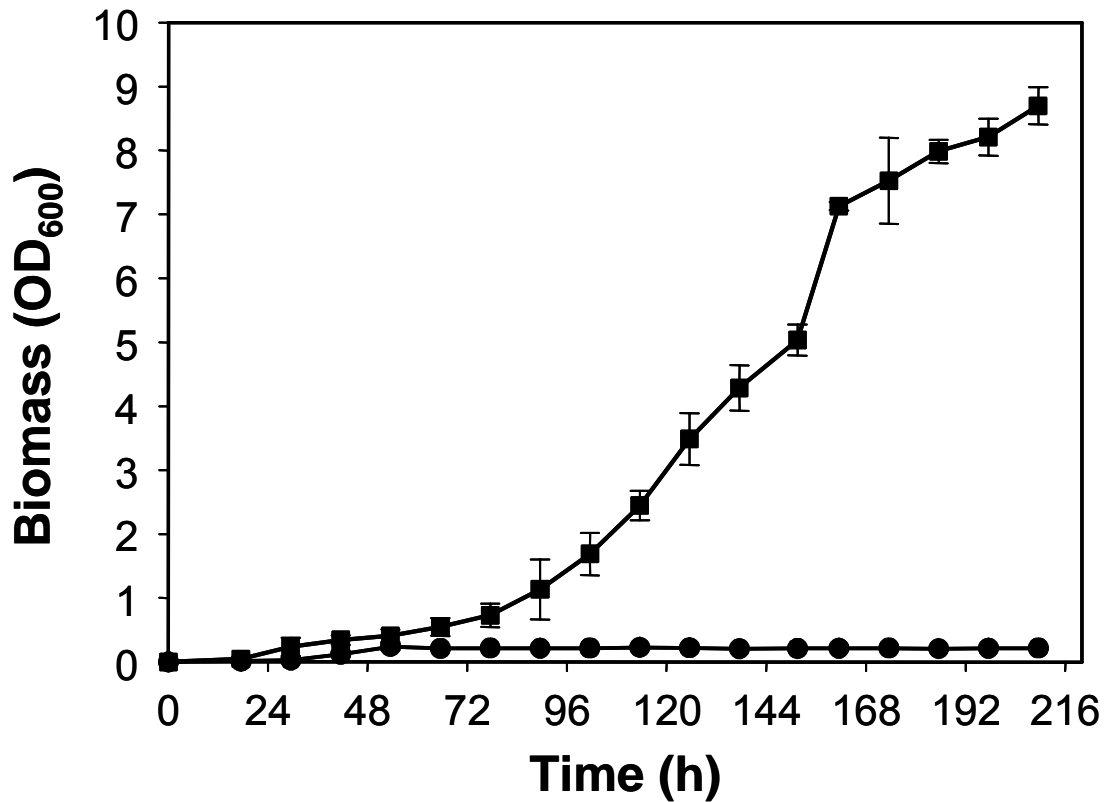
Figure 5: Aerobic growth of yeast reference strain (•) and [YEpENOSXI] (■) strain showing superior and sustained growth on D-xylose by the transformant.
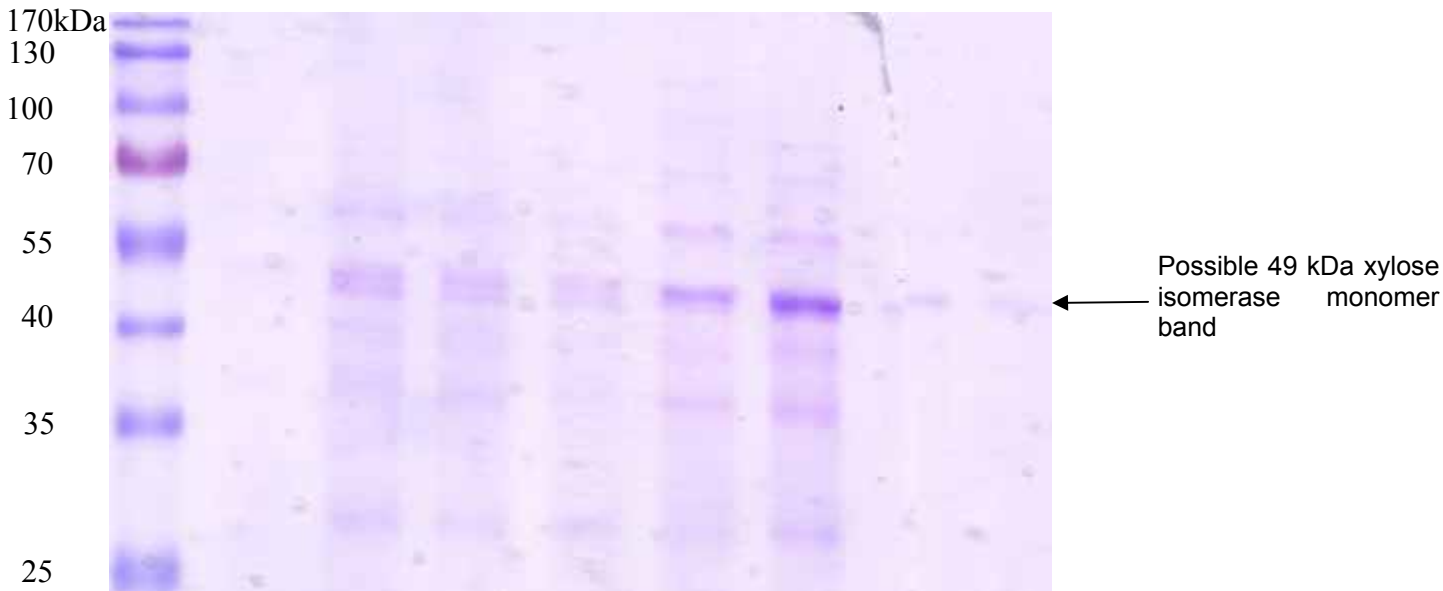

Figure 6: Protein gel showing darker bands in 49 kDa range for days 1 and 2 for the transformed than for the reference strain.

4.3.8 SDS-PAGE analysis

The *B. thetaiotaomicron* protein monomer should be approximately 49 kDa. The protein gel in Figure 6 shows amongst other proteins, a more prominent protein species between 55 and 40 kDa for the [YEpENOSXI] strain, especially on day 2, than the reference [YEpENO] strain. The position of the prominent protein species corresponded with the predicted molecular weight of the XI monomer. Proteins in other regions show similar intensities between the reference and transformed yeasts. Another PAGE analysing the growth medium over 4 days shows a slight band in the 49kDa position on days 3 and 4 for the [YEpENOSXI], and not for the reference strain (data not shown).

4.4 DISCUSSION

The *B. thetaiotaomicron* putative xylose isomerase (XI) gene was chosen due to the high homology on protein level that it shows to the *Piromyces* xylose isomerase (Harhangi et al., 2003). The reasons for the lack of activity of the native *B. thetaiotaomicron* putative XI in *S. cerevisiae* are unknown. Since the sequencing showed the amino acid at position 242, which was lysine in the *B. thetaiotaomicron* putative enzyme, was substituted by glutamine in the transformed strains, it may have influenced the activity. Crystallographic data of other XIs suggest that the mutation is not in the active site, as indicated in Figure 4. The substitution may however, lead indirectly to a change in binding or activity. Another surmise could be that the putative xylose isomerase is not an active enzyme even in *B. thetaiotaomicron*. A further possibility is that the codon bias is suboptimal in *S. cerevisiae*, or that the peptide does not fold appropriately during posttranslational modification.

The northern blot showed no mRNA specific for the XI gene transcript. This strongly suggested that the lack of activity is not solely due to the amino acid substitution, as mRNA would presumably be formed, but the protein would be inactive. This suggests that mRNA is not produced or is produced in a truncated and highly unstable form, which is degraded too quickly to analyse. Since the *xylA* gene is

under the constitutive *ENO1* promoter, it is more likely that an unstable mRNA is produced than that no mRNA is produced.

The xylose isomerase of *Piromyces* sp E2 codon usage was compared to the codon bias for *S. cerevisiae* as determined by Sharp and Cowe (1991). This showed a reasonable match with a CBI of 0.69 suggesting that expression of *Piromyces* xylose isomerase would not fail on the grounds of codon usage. The group Harhangi et al. (2003) showed low but detectable protein and activity levels.

On the other hand, when the *B. thetaiotamicron* xylose isomerase was calibrated for codon usage it differed significantly from the optimal patterns of *S. cerevisiae*, giving a low CBI of 0.07. Problems in expression and activity of the protein xylose isomerase in *S. cerevisiae* might therefore be traced to suboptimal codon bias. It is interesting to see that the xylose isomerases of *B. thetaiotamicron* and *Piromyces* differ so significantly with respect to codons (DNA level), when at the amino acid level they are 83.1% similar.

The xylose isomerase gene was manufactured synthetically to have a codon bias of 0.93. This gene also was designed to have the original amino acid sequence that *B. thetaiotaomicron* did, with no amino acid substitutions. Yeast transformants with the synthetic gene were found to grow on minimal medium with D-xylose as sole carbon source, where the reference strain did not. This was true for both solid and liquid media, and in liquid medium the transformed strain showed increasing growth for more than four days, while the reference strain attained far lower levels, which reached a plateau after two days.

Yeast samples with the SXI gene conferring growth on D-xylose did not show greater activity with the cysteine-carbazole assay than the reference strain, which could not grow on D-xylose. The enzyme assay itself was validated by standard curves with xylulose, and the use of an industrial xylose isomerase (Maxazyme). This suggests that xylose isomerase activity for this enzyme is not meaningfully measured in this assay. The assay may not be sufficiently sensitive, or given the comparatively short incubation time may not mimic the actual working conditions of the enzyme. For this

reason, it was not possible to determine the optimum pH, temperature or metal cofactor for the recombinant *B. thetaiotaomicron* xylose isomerase enzyme.

The growth activity on D-xylose in the yeast strain with the synthetic gene, and lack thereof with the native sequence, confirms the effect of codon bias on mRNA stability and protein expression. Although the mRNA levels were not specifically tested for the synthetic gene, growth activity suggests that mRNA was produced. Furthermore the SDS-PAGE analysis shows high expression of protein from the transformant, which corresponds to a protein species of the correct size of approximately 49 kDa. Although this species is less prominent after day 2, it is possible that xylose isomerase enzyme in the medium from lysed cells assists with the growth seen in Figure 5 after 2 days. Proteins in other regions show approximately the same intensity between the reference strain and transformed strain, strongly suggesting that the prominent band is the XI monomer. Without antibody detection methods, however, it is not possible to be absolutely certain of this. As the native *Bacteroides thetaiotaomicron* xylose isomerase protein has never been isolated, or heterologously expressed before, no antibodies have been raised against it, and it is not possible to do a western blot.

The growth of *S. cerevisiae* on D-xylose represents a significant step for bioethanol production from lignocellulose, as this yeast cannot naturally utilise D-xylose. Further work should explore whether ethanol is produced, and how the transformant reacts to growth in a mixture of glucose and D-xylose. Should the strain produce ethanol under these conditions it should be tested on hydrolysed plant biomass. Since the assay is not a reliable indicator of activity, but activity clearly exists, other outcomes should be used to assess the success of the yeast. Analysis of the metabolic products with HPLC would be a reliable method to determine how much D-xylose is utilised and ethanol produced, as for the ethanol fuel industry these parameters are more important than enzyme activities, which can vary greatly between the laboratory and industrial conditions.

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

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Globally energy demands are increasing, especially in much of the third world, and current fossil resources will be stretched further than is sustainable in the long term (Zaldivar et al., 2001). Research suggests that alternatives will be necessary in the near future to meet these demands. One resource, which is both underutilised and ubiquitous, is the energy in plant biomass (Aristidou and Penttilä, 2000). Approximately two-thirds of plant biomass is composed of cellulose and hemicellulose. Cellulose yields glucose and hemicellulose a variety of sugars including large amounts of D-xylose, upon hydrolysis. The glucose and other sugars can be fermented by microorganisms to ethanol, which can be used as an alternative to petroleum (Zaldivar et al., 2001).

Of all known microorganisms, *S. cerevisiae* has the highest yield and tolerance to ethanol when grown on hexose sugars. Given its excellent fermentative properties, and resistance to toxins in hydrolysed lignocellulose, it is a natural candidate for bioethanol fermentation (Zaldivar et al., 2002). Unfortunately this yeast is unable to utilise pentose sugars, including D-xylose (Hahn-Hägerdal et al., 2001). Various approaches have been taken to confer *S.cerevisiae* with the ability to ferment D-xylose, and improve its ability to use plant biomass resources to provide a long-term, sustainable alternative to petrol.

5.1 MUTATION AND SELECTION OF RECOMBINANT *SACCHAROMYCES CEREVISIAE* FOR ENHANCED D-XYLOSE UTILISATION

S. cerevisiae does not have sufficient native enzyme activity for growth on D-xylose (Senac and Hahn-Hägerdal, 1990). *S. cerevisiae* strains have successfully been transformed with the *P. stipitis* genes *XYL1* and *XYL2* for production of enzymes XR and XDH, which confer D-xylose utilisation ability (Hahn-Hägerdal et al., 2001). Unfortunately these yeast struggle to use D-xylose under anaerobic conditions, or when it is the sole available carbon source (Zaldivar et al., 2002). The main reason for this is thought to be because XR and XDH use different cofactors. As they do not renew one another's cofactors this leads to a cofactor imbalance. When cofactors are no longer available the reaction from D-xylose to xylulose can no longer take place, leading to high levels of xylitol being produced, rather than the production of

the desired metabolite ethanol (Toivari et al., 2004). In addition recombinant *S. cerevisiae* show strong catabolite repression, leading to preferential utilisation of glucose above D-xylose (Van Zyl et al., 1999). These factors are problematic for industrial use of *S. cerevisiae* on lignocellulosic hydrolysates for the production of ethanol.

Recombinant *S. cerevisiae* metabolism has been extensively analysed (Hahn-Hägerdal et al., 2001). Although a variety of strategies have been attempted, and additional genes cloned, the cofactor imbalance continues to lower ethanol yields. Since D-xylose utilisation has many steps, all of which share in the D-xylose flux, an evolutionary approach would allow the yeast to develop solutions which have not yet been predicted by rational metabolic design. Mutation and selection of an industrial strain resulted in some progeny with improved fitness on D-xylose than the initial strain (Wahlbom et al., 2003). For this reason a part of this study explored the mutation and selection of the recombinant *S. cerevisiae* strain Sigma [YIpLoxZEO] for enhanced D-xylose utilisation.

Growth of the strain and mutated strains, under anaerobic conditions with D-xylose as the sole carbon source, revealed that the conditions were highly stressful to the yeast as it grew very slowly and in pseudohyphal form. These selection conditions were apparently too harsh, while under conditions which included glucose the selective pressure was not great enough to select for better D-xylose utilising strains. Unfortunately an improved D-xylose utilising strain was not obtained. However, this work has not sufficiently explored the possibility of random mutagenesis and selection, or other evolutionary selective methods and therefore these approaches could still give insight into recombinant *S. cerevisiae* for improved D-xylose utilisation.

5.2 EXPRESSION OF THE *BACTEROIDES THETAIOTAOMICRON XYLA* GENE IN *SACCHAROMYCES CEREVISIAE*

Another approach to D-xylose utilisation in *S. cerevisiae* is that of transformation with a bacterial *xylA* gene for xylose isomerase (XI) (Moes et al., 1996). This enzyme directly converts D-xylose to xylulose, which the yeast can ferment. Expression of bacterial XIs in *S. cerevisiae* have not generally been successful as insoluble protein

or inactive monomers usually result (Hahn-Hägerdal, 2001). The *xylA* from *T. thermophilus* provided functional XI activity in yeast (Walfridsson et al., 1996). Recently an unusual fungal XI was found in *Piromyces* sp. strain E2, this too provided low activity in *S. cerevisiae* (Harhangi et al., 2003). The amino acid sequence for the *Piromyces* enzyme is very similar (83.1%) to that of a putative xylose isomerase from the bacterium *B. thetaiotaomicron,* for which the genome sequence is available.

Genomic DNA was isolated from *B. thetaiotaomicron*, the *xylA* sequence amplified, cloned to expression vectors and transformed into *E. coli* HB101 and *S. cerevisiae* CEN.PK21-C. No XI activity was found either in the bacterium, or in the yeast, and neither was capable of growing on D-xylose following transformation. Previously, XI genes have been expressed in yeast, but the lack of activity was due to post-translational problems. Northern analysis showed that no mRNA specific to the *B. thetaiotaomicron xylA* gene was produced, this showed that the lack of activity was probably not due to post-translational problems. Instead it must have originated at mRNA level, either through inadequate transcription, degradation of mRNA or from problems with translating the mRNA. One possibility could be that the codon bias of the *B. thetaiotaomicron* gene was incompatible with *S. cerevisiae* as expression system, which would cause problems with translation.

5.2.1 Codon usage bias and mRNA processing

Heterologous protein expression in organisms unrelated to the original host does not always lead to active product. Protein expression is a complex system controlled through transcription, mRNA stability and splicing, translation, post-translational modifications and protein degradation (Day and Tuite, 1998). Both regulation controlling transcription, and the various mechanisms for activation and deactivation of extant enzymes may well be better understood than post-transcriptional gene regulation (Pesole et al., 2001). Post-transcriptional control of protein synthesis is partly exerted by the rates of mRNA synthesis, translation and mRNA decay (Jacobson and Peltz, 1996). These are affected by transport of mRNA between the nucleus and cytoplasm, stability of mRNA, translation efficiency and distribution within the cell (Pesole et al., 2001).

The mRNA itself carries signals about its half-life. An mRNA requires a 3' poly-adenylated tail and also a guanosine cap on the 5' end for active translation (Howe, 2002). The cap is necessary for the ribosome and translation complexes to bind and find the initiation codon (Tourrière et al., 2002). When the poly-A tail is shortened or polypeptide translation terminates prematurely, an mRNA decay pathway is triggered (Day and Tuite, 1998). In one mRNA decay pathway the poly-A shortening is followed by decapping and then exonucleolytic 5'-3' mRNA degradation occurs. In so-called nonsense-mediated mRNA decay mRNAs containing premature termination codons have the cap removed without deadenylation of the poly-A tail (Tourrière et al., 2002). In yeast, formation of the 3' poly-A tail of mRNA requires a variety of polyadenylation signals. Since the poly-A tail is essential for translation, a heterologous gene without polyadenylation signals recognisable to yeast, could lead to mRNA degradation prior to translation (Zhao et al., 1999). In addition other signals are found in mRNA untranslated regions (UTRs) (Pesole et al., 2001). The cap, secondary structures, RNA-protein interactions, upstream open reading frames and ribosome entry sites may all be affected by 5'UTR mediated regulation, while 3'UTR mediated regulation further affects antisense RNA, RNA-protein and multiprotein complex interactions as well as affecting cytoplasmic polyadenylation elements and the size of the poly-A tail. Thus mRNAs intrinsically contain many signals essential to correct translation, and which affect mRNA life-time. Heterologous genes containing signals dissimilar to host signalling, may result in mRNA that is incorrectly spliced, has a short half-life or is directed towards decay pathways, which will lead to low levels, truncated or no translated protein.

Heterologous expression can take place because the genetic code is almost universal with nucleotide triplets coding for the same amino acids between almost all life forms. Since the early 1980s it has become apparent that for the mRNA transcripts of highly expressed genes, organisms tend to prefer the use of one of a set of synonymous triplets above the others for protein production, this tendency is referred to as the organism's codon usage bias (Comeron and Aguadé, 1998). Codon bias is thus one of the regulatory mechanisms for protein expression.

Codon bias in mRNAs may reflect selective evolutionary pressures. Highly expressed genes experience selective pressure to have stable mRNA transcripts for which translation is rapid. If a gene encodes triplets corresponding to rare tRNA isoacceptors then translation pauses until the correct tRNA appears at random. As the tRNA is rare, at this point an incorrect amino acid may be built in, or termination occur prematurely. The most frequently used triplets are considered to be optimal for protein production of a specific organism. To a large extent optimal codons therefore correspond to the most frequently occurring yeast isoacceptor tRNA anticodon for a specific amino acid. The protein levels of genes with many optimal codons also tend to be higher.

Bennetzen and Hall (1982) defined a measure of codon usage bias, namely the codon bias index (CBI) as a number between 0 and 1, where a value of 1 would indicate that every single triplet in the mRNA is a preferred one while a codon bias of 0 would suggest completely random triplets. If a gene has a score substantially below 0 then there are more non-preferred triplets in the sequence than would be expected by chance. The 'optimal' triplets for *S. cerevisiae* were originally chosen by analysing the codons used in 8 highly expressed genes (Bennetzen and Hall, 1982). Subsequently Sharp and Cowe (1991), analysing 30 high expression genes confirmed these optimal triplets. A recent study found that the CBI and similar indices remain relevant models when measured against the large datasets available now (Jansen et al., 2003). On the other hand, Friberg et al. (2004) showed that measures such as these more accurately predict mRNA levels than protein levels and Comeron and Agaudé (1998) show that depending on the study, different measures of codon bias may be appropriate.

When heterologous proteins from one organism are expressed in a closely related species, translation occurs with relative ease. One reason for this is the similar 'codon usage bias' between the two organisms. On the other hand, when the DNA for a heterologous protein has 'non-optimal' codons for the organism in which it is expressed this can dramatically lower the translation rates. For this reason it can be helpful to determine the pattern of codon usage for a particular organism. In the case of *B. thetaiotaomicron* XI expressed in *S. cerevisiae* the CBI is a very low 0.07. This

very poor codon bias suggested that translational pauses may well lead to premature termination in *S. cerevisiae* (Sharp and Cowe, 1991). Naturally, other factors such as the availability of chaperones and post-translational folding patterns also may have an effect.

5.2.2 Codon optimised *xylA* expression in *S. cerevisiae*

A synthetic *xylA* gene (called SXI) was designed to have a CBI of 0.93 and, after cloning into the same plasmid as used before, was transformed into *S. cerevisiae* CEN.PK21-C. In this case the synthetic *xylA* gene conferred on *S. cerevisiae* transformants the ability to grow on solid media with D-xylose as sole carbon source. In addition, while the reference strain showed only residual growth, the transformant grew in liquid medium with D-xylose as sole carbon source in a sustained fashion for longer than a week. Although the growth on D-xylose clearly showed the production of active xylose isomerase, the assays used were not able to detect the activity. In addition, SDS-PAGE showed a prominent protein species at the size corresponding to the 49 kDa predicted molecular weight of the SXI monomer.

Kinetic analysis of the SXI enzyme would be valuable, as the native enzyme has not been studied and very little is known, however, it is clear that the recombinant enzyme is active under growth conditions in the yeast cytosol. Thus anaerobic fermentations to assess anaerobic growth of the *S. cerevisiae* [YEpENOSXI] transformant on D-xylose, with particular emphasis on whether ethanol is produced, and at what levels would be more valuable to the fuel industry. In addition metabolism of *S. cerevisiae* [YEpENO] with D-xylose and glucose as cosubstrates could determine the effect of catabolite repression, the metabolic products should be analysed by HPLC. Ultimately, if ethanol is produced, aerobic and anaerobic growth of the strain on lignocellulosic hydrolysate and the ethanol levels produced should be determined. In addition, the recombinant *S. cerevisiae* with the synthetic XI may also be improved by an evolutionary selection approach, if current ethanol levels are uneconomical. Kuyper et al. (2005) used selection to improve the use of D-xylose in the presence of glucose and thereby reduce the diauxic growth pattern, a similar approach may be useful to improve *S. cerevisiae* [YEpENOSXI] for ethanol production on lignocellulosics.

5.3   CONCLUSIONS

From the data presented the following can be concluded:

1.   Under the experimental conditions in this study recombinant *S. cerevisiae* with *P. stipitis XYL1* and *XYL2* genes showed very poor anaerobic growth on D-xylose as sole carbon source. It is possible that use of a cosubstrate may reduce stress, but for a mutation and selection study the cosubstrate should not reduce the selectivity of the conditions, and the effects of catabolite repression must be taken into account (Van Zyl et al., 1999).

2.   *B. thetaiotaomicron* native *xylA* cloned in this work did not give D-xylose utilisation ability to *E. coli* or *S. cerevisiae*. No XI activity could be detected in either microorganism with cysteine-carbazole assays.

3.   A synthetic *B. thetaiotaomicron* XI was manufactured with codon bias optimised for *S. cerevisiae*.

4.   The recombinant enzyme synthetic *xylA* probably forms the predicted 49 kDa monomer as seen on SDS-PAGE, activity was not, however, measurable with cysteine-carbazole assays.

5.   The synthetic gene conferred on *S. cerevisiae* transformants the ability to grow on D-xylose as sole carbon source on solid medium. In liquid medium, growth by transformants was sustained and productive for more than a week, while the reference strain was unable to sustain growth on D-xylose as sole carbon source.

In this work a heterologous *S.cerevisiae* capable aerobic D-xylose utilisation has been created. *S. cerevisiae* is unable to utilise D-xylose, but is in most other respects a good candidate for ethanol production from lignocellulose.   The growth of *S. cerevisiae* [YEpENOSXI] on D-xylose therefore represents a significant step for economical bioethanol production from plant biomass.

5.4 REFERENCES

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