Saccharomyces cerevisiae engineered for xylan utilisation

by

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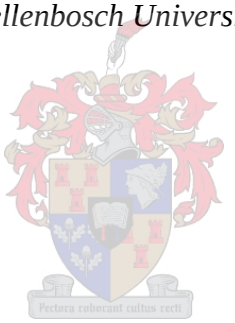

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March 2016

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Declaration by the candidate:

With regard to Chapters 1 to 5, the nature and scope of my contribution were as follows:

Nature of contribution	Extent of contribution
Planning and execution of experimental work, data analysis and preparation of draft manuscript	**90%**

The following co-authors have contributed to Chapters 2 to 4:

Name	e-mail address	Nature of contribution	Extent of contribution
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Date: ……..

Declaration by co-authors:

The undersigned hereby confirm that

1. the declaration above accurately reflects the nature and extent of the contributions of the candidate and the co-authors to Chapters 2 to 4,
2. no other authors contributed to Chapters 2 to 4, besides those specified above, and
3. potential conflicts of interest have been revealed to all interested parties and that the necessary arrangements have been made to use the material in Chapters 2 to 4 of this dissertation.

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SUMMARY

Alternative fuels or sources of energy need to be generated to complement or replace fossil based fuels since the growing global energy demand will soon exceed the fossil fuel supply. Brazil and the USA are currently the world's largest producers of bioethanol using sugar cane and corn starch as feedstock, respectively. However, financial feasibility of bioethanol technologies can only be attained when all of the fermentable carbon in plant biomass is converted to bioethanol. Cellulosic ethanol production has received much attention in the past decades due to the high cellulose composition of plant biomass. Yet xylan also represents a major component of lignocellulosic biomass that can be utilised for the cost-effective production of bioethanol.

In this study, a recombinant xylan-utilising *Saccharomyces cerevisiae* strain was engineered by co-expression of the xylanase (*xyn2*) of *Trichoderma reesei*, the xylosidase (*xlnD*) of *Aspergillus niger* and the codon-optimised *Bacteroides thetaiotaomicron* xylose isomerase (*xylA*) genes. The addition of the *Scheffersomyces stipitis* xylulose kinase (*xyl3*) gene and the disruption of the native aldose reductase (*GRE3*) gene increased the carbon flux through the pentose phosphate pathway and minimised carbon loss due to the production of xylitol, respectively. Cultivation on xylose as sole carbohydrate source under oxygen-limitation resulted in the recombinant strain producing ethanol from xylose at a maximum theoretical yield of ~90%, while displaying a complete respiratory mode under aerobic conditions. An increase in biomass was observed that coincided with an increase in enzyme activity. Furthermore, strain adaptation on xylose resulted in a strain with an improved xylose conversion rate.

A stable diploid *S. cerevisiae* strain overexpressing the *B. thetaiotaomicron* xylose isomerase encoding gene (*xylA*) and the *S. stipitis* xylulose kinase (*xyl3*) gene was constructed. The strain was used to compile metabolomics data at different time points when cultivated aerobically on xylose and glucose as respective sole carbohydrate sources. Cultivation on glucose resulted in a typical diauxic growth pattern on glucose and the production of ethanol due to the Crabtree effect. The UDP-D-glucose levels were approximately eight times higher with cultivation on xylose compared to glucose, indicating that the carbon is channeled towards biomass production. Glycerol was produced in response to ethanol and acetic acid toxicity and was substantially less with cultivation on xylose. Xylitol still accumulated despite the disruption of the *GRE3* gene, which suggests the presence of additional non-specific aldose reductases. The

concentration of phosphoenol pyruvate (PEP) was much lower with cultivation on xylose throughout the study, indicating that xylose does not induce the expression of pyruvate kinase (*PYK1*), which negatively affects the flux through the rest of glycolysis. The levels of fructose 1,6-bisphosphate (F1,6P) (an important modulator of the mitochondrial unspecific channel), was significantly lower with cultivation on xylose and contributed to the incomplete carbon catabolite response. Cultivation on xylose resulted in an increase in the pool size of the metabolites of the pentose phosphate pathway (PPP). The accumulation of sedoheptulose 7-phosphate suggests that the TAL1 enzyme is probably the rate-limiting enzyme activity of the PPP. This study is one of only a few that demonstrates xylose and xylan utilisation by a recombinant *S. cerevisiae* strain.

OPSOMMING

Alternatiewe brandstowwe of bronne van energie moet geskep word om fossielbrandstowwe te vervang, aangesien die groeiende globale energie-aanvraag binnekort die produksie van fossielbrandstowwe sal oorskry. Brasilië en die VSA is tans die wêreld se grootste bio-etanolprodusente en gebruik onderskeidelik suikerriet en mielie as voerstowwe. Die finansiële haalbaarheid van bio-etanoltegnologieë is slegs moontlik indien al die fermenteerbare koolstof in plantbiomassa na etanol omgeskakel word. Sellulolitiese etanolproduksie het in die afgelope dekades baie aandag ontvang as weens die hoë sellulose-inhoud van plantbiomassa. Tog verteenwoordig xilaan ook 'n belangrike komponent van lignosellulose-agtige biomassa wat vir die koste-effektiewe produksie van bio-etanol gebruik kan word.

In hierdie studie is 'n rekombinante xilaanbenuttende *Saccharomyces cerevisiae*-ras ontwerp deur gesamentlike uitdrukking van die *Trichoderma reesei* xilanase (*xyn2*), die *Aspergillus niger* xilosidase (*xlnD*) en die kodon-geoptimiseerde *Bacteroides thetaiotaomicron* xilose isomerase (*xylA*) gene. Die byvoeging van die *Scheffersomyces stipitis* xilulose kinase (*xyl3*)-geen en die ontwrigting van die natuurlike aldose reduktase (*GRE3*)-geen het onderskeidelik die koolstofvloei deur die pentosefosfaatweg verhoog en koolstofverlies weens die produksie van xylitol beperk. Die kweking op xilose as die enigste koolhidraatbron onder suurstofbeperkte toestande, het daartoe gelei dat die rekombinante ras etanol uit xilose teen 'n maksimum teoretiese opbrengs van ~90% vervaardig het, terwyl dit ‘n volledige respiratoriese metabolisme onder aërobiese toestande vertoon het. 'n Toename in biomassa is waargeneem wat met 'n toename in verder ensiemaktiwiteit ooreengestem het. Ras-aanpassing op xilose het verder tot 'n ras met 'n verbeterde xilosebenuttingstempo gelei.

‘n Stabiele diploïede *S. cerevisiae* ras is ontwerp waarin die *B. thetaiotaomicron* xilose isomerase (*xylA*)- en die *S. stipitis* xilulose kinase (*xyl3*)-gene oor-uitgedruk is. Die ras is vir die opstel van metaboloomdata op verskillende tydintervalle gebruik tydens die aërobiese kweking op onderskeidelik xilose en glukose as koolhidraatbronne. Kweking op glukose het tot 'n tipiese di-oukse groeipatroon en die produksie van etanol weens die Crabtree-effek gelei. Die UDF-D-glukosevlakke was ongeveer agt keer hoër met kweking op xilose in vergelyking met glucose, wat aandui dat die koolstof na biomassaproduksie gekanaliseer word. Gliserol was in reaksie op etanol- en asynsuurtoksisiteit geproduseer en aansienlik minder met kweking op xilose. Xilitol het steeds opgehoop ten spyte van die ontwrigting van die *GRE3*-geen, wat

dui op die teenwoordigheid van nog nie-spesifieke aldose reduktase-gene. Die konsentrasie van fosfoenolpirovaat (FEP) was deurgaans baie laer met kweking op xylose, wat daarop dui dat xilose nie die produksie van pirovaat kinase (PYK1) induseer nie, wat die vloei deur die res van glikolise negatiewe beinvloed. Die vlakke van fruktose 1,6-bisfosfaat (F1,6P) ('n belangrike moduleerder van die mitochondriale nie-spesifieke kanaal), was aansienlik laer met kweking op xilose en het tot die onvolledige koolstofkatabolietreaksie bygedra. Kweking op xilose het 'n toename in die poelgroottes van metaboliete van die pentosefosfaatweg (PPP) meegebring. Die opeenhoping van sedoheptulose 7-fosfaat dui daarop dat die TAL1-ensiem waarskynlik die tempobeperkende ensiemaktiwiteit van die PPP is. Hierdie studie is een van net 'n paar wat xilose- en xilaanbenutting deur 'n rekombinante *S. cerevisiae* ras demonstreer.

To my beloved sister, the late Juliet J Mert-Matolla, for being my inspiration to further my education

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PREFACE

The dissertation is presented as a compilation of separately introduced scientific manuscripts. Chapter 3 and 4 are written in the style of the journal to which the respective manuscript was or will be submitted. The Appendix has been published as a review article in a peer reviewed journal.

Chapter 3

Mert MJ, la Grange DC, Rose SH, van Zyl WH (2016) Engineering of *Saccharomyces cerevisiae* to utilize xylan as a sole carbohydrate source by co-expression of an endoxylanase, xylosidase and a bacterial xylose isomerase. J Ind Microbiol Biotechnol. DOI 10.1007/s10295-015-1727-1

Chapter 4

Mert MJ, la Grange DL, Rose SH, van Zyl WH (2016) Metabolic flux analysis of a xylose utilising *Saccharomyces cerevisiae* strain expressing the *Bacteroides thetaiotaomicron* xylose isomerase. Biotechnol Biofuels: *Submitted for publication*

Appendix

Den Haan R, Kroukamp H, Mert MJ, Bloom M, Görgens JF, van Zyl WH (2013) Engineering *Saccharomyces cerevisiae* for next generation ethanol production. *J Chem Technol Biotechnol* 88:983-991 (wileyonlinelibrary.com). DOI 10.1002/jctb.4068

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LIST OF ABBREVIATIONS

6PG	6-phosphogluconate
6PGDH	6-phosphogluconate dehydrogenase
6PGL	6-phosphogluconolactonase
Acetyl-CoA	acetyl coenzyme A
ADH	alcohol dehydrogenase
ADP	adenosine diphosphate
AFEX	Ammonia fibre explosion
ALDH	aldehyde dehydrogenase
AMP	adenosine monophosphate
AOX1	aldolase oxidase
ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
CAZy	carbohydrate-active enzymes database
CBP	consolidated bioprocessing
CCR	carbon catabolite repression
CO_2	carbon dioxide
CRP	cytoplasmic ribosomal proteins
DCW	dry cell weight
DHAP	dihydroxyacetone phosphate
DNA	deoxyribonucleic acid
EC	enzyme commission number
ENO	enolase
F1,6P	fructose 1,6-bisphosphate
F2,6P	fructose 2,6-bisphosphate
F6P	fructose 6-phosphate
FAD	flavin adenine dinucleotide

$FADH_2$	flavin adenine dinucleotide hydride
FBA	fructose-bisphosphate aldolase
G6P	glucose 6-phosphate
G6PDH/ZWF1	glucose 6-phosphate dehydrogenase / *Zwischenferment*
GAL	galactose permease
GAP	glyceraldehyde 3-phosphate
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GCN	general control non-depressible
gDNA	genomic deoxyribonucleic acid
GDP	glycerol 3-phosphate dehydrogenase
GH	glycoside hydrolase
GLK	glucokinase
GRE	aldose reductase
Gtons	gigaton
HMF	5-hydroxymethyl furfural
HPLC	high performance liquid chromatography
HXK	hexokinase
HXT	hexose transporter
IRA	inhibitor of RAS activity
LDH	lactate dehydrogenase
MIG	multicopy inhibitor of GAL gene expression
mRNA	messenger ribonucleic acid
MRP	mitochondrial ribosomal proteins
MUC	mitochondrial unspecific channel
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide hydride
NADP	nicotinamide adenine dinucleotide phosphate

NADPH	nicotinamide adenine dinucleotide phosphate hydride
OD	optical density
ORF	open reading frame
PCR	polymerase chain reaction
PDC	pyruvate decarboxylase
PDH	pyruvate dehydrogenase
PFK	phosphofructokinase
PGI	phosphoglucose isomerase
PGK	phosphoglycerate kinase
PGM	phosphoglycerate mutase
PHO	*p*-nitrophenyl phosphatase
PKA	protein kinase
PPP	pentose phosphate pathway
PYK	pyruvate kinase
R5P	ribose 5-phosphate
rDNA	ribosomal deoxyribonucleic acid
RGE	rapid growth element
RGT	restores glucose transport
RNA	ribonucleic acid
RPE	ribulose 5-phosphate epimerase
RPI	ribose 5-phosphate isomerase
rRNA	ribosomal ribonucleic acid
Ru5P	ribulose 5-phosphate
S1/S2/S3	sub layers 1/2/3
S7P	sedoheptulose 7-phosphate
SC	synthetic complete
SDS-PAGE	sodium dodecyl sulfate - polyacrylamide gel electrophoresis

SNF	sucrose non-fermenting
SOK	suppressor of kinase
TAL	transaldolase
ThPP	thiamine pyrophosphate
TKL	transketolase
TPI	triosephosphate isomerase
T6P	trehalose 6-phosphate
UDPase	uridylyl glucose pyrophosphorylase
UDP-D-glucose	uridine diphosphate-D-glucose
XDH	xylitol dehydrogenase
XI/XYLA	xylose isomerase
XK	xylulokinase
XLND	xylosidase
XR	xylose reductase
XYN	xylanase
YCK	yeast casein kinase
YNB	yeast nitrogen base
YPBX	yeast extract, peptone, Beachwood xylan
YPD	yeast extract, peptone, glucose
YPX	yeast extract, peptone, xylose

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CHAPTER 1

General introduction and project aims

1.1 GENERAL INTRODUCTION

Bioethanol is an attractive alternative to fossil fuels and needs to be produced to supplement the growing energy demand. Bioethanol production through microbial fermentation is an age-old technique used for the production of wine and beer. Using the same principles, the focus has shifted to energy security. The first ethanol plants primarily used sugar cane (Brazil) as feedstock. The USA currently uses starch-based resources, but these resources directly compete with human food and animal feed supply. The current emphasis is on sustainable liquid biofuel production from non-food plant biomass such as agricultural residues (and even dedicated energy crops), industrial and municipal solid waste, without adding to the pollution problem (Wyman 1999; Gray et al. 2006).

The conversion of plant cell walls (lignocellulose) to ethanol via microbial fermentation is one of the most attractive options for the production of environmentally friendly biofuels. Lignocellulose is composed largely of cellulose (insoluble fibres of β-1,4-glucan), hemicellulose (non-cellulosic polysaccharides including glucans, mannans and xylans) and lignin (a complex polyphenolic structure) (Thomson 1993; Aristidou and Penttilä 2000). The biological conversion of all the polysaccharides present in biomass requires the synergistic action of groups of hydrolytic enzymes such as cellulases, mannanases, xylanases and laccases. To date, cellulose conversion has received much interest due to it representing the main portion of the lignocellulose. Yet, the conversion of all the components to ethanol will increase the yield and the financial feasibility of the process.

Lignocellulose needs to be hydrolysed to fermentable sugars prior to fermentation. Unfortunately, pretreatment options and the addition of exogenous enzymes (cellulases and hemicellulases) surmount to one of the largest expenses of the bioethanol industry, resulting in an uneconomical process (Stephanopoulos 2007). A consolidated bioprocessing (CBP) approach, which combines enzymatic hydrolysis (substrate saccharification) and subsequent fermentation by the same host organism, would result in a more cost-effective process (van Zyl et al. 2007). A natural organism exhibiting all the desired qualities for saccharification and fermentation is yet to be identified. Whereas *Saccharomyces cerevisiae* is well suited for fermentation conditions, it is unable to hydrolyse the lignocellulosic components. Over the past three decades, much progress had been made in the genetic engineering of cellulolytic and hemicellulolytic *S. cerevisiae* strains by introducing genes that encode for cellulases and hemicellulases (la Grange et al. 2001; van Rooyen et al. 2005; den Haan et al. 2007; Ilmén

et al. 2011; Malherbe et al. 2014). Although *S. cerevisiae* cells can transport xylose by means of the hexose transporters, it cannot metabolise xylose. Although xylan-hydrolysing *S. cerevisiae* strains can be constructed with ease, the construction of xylan-utilising *S. cerevisiae* strains are hampered by inefficiencies in the metabolism of xylose. Evidence exists that a native xylose utilising pathway is present in *S. cerevisiae* (Thomson 1993; Rodriguez-Peña 1998), making this the ideal organism to manipulate for xylose/xylan utilisation. Introduction of the xylose reductase/xylitol dehydrogenase pathway (XR/XDH) in *S. cerevisiae* resulted in a redox imbalance (due to the respective NADPH and NAD^+ co-factor requirements), which led to carbon being channelled into glycerol and xylitol production under oxygen limitation. The use of xylose isomerases is more advantages since no co-factors are required. Unfortunately many of the early attempts at expression of xylose-isomerases in *S. cerevisiae* had failed.

1.2. AIMS OF THE STUDY

This study focuses on the utilisation of the xylan portion of the lignocellulose using engineered *S. cerevisiae* strains. The aims of the study are:

1.1. To construct and evaluate a *S. cerevisiae* strain that co-expresses the major endo-β-1,4-xylanase II (*xyn2*) from *Trichoderma reesei*, a β-xylosidase (*xlnD*) from *Aspergillus niger*, a xylose isomerase (*xylA*) from *Bacteroides thetaiotaomicron*, and the *Scheffersomyces stipitis* xylulose kinase gene (*xyl3*) on xylose or xylan as the sole carbohydrate source.

1.2. To improve xylose metabolism by the recombinant *S. cerevisiae* strain using evolutionary adaptation methodologies.

1.3. To elucidate some of the underlying principles of xylose metabolism by a xylose isomerase-expressing *S. cerevisiae* strain using a metabolomics approach.

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CHAPTER 2

Literature review

2.1 INTRODUCTION

Plant material (predominantly cell walls) is the largest source of biomass and renewable energy on earth (Reiter 2002). The interest in renewable energy is driven by the finite fossil fuel supply. During cell growth, a large portion of photosynthetically produced organic carbon becomes incorporated into plant cell walls, which are typically composed of cellulose, hemicellulose, lignin (collectively known as lignocellulose), waxes and proteins (Huber et al. 2006; Lerouxel et al. 2006). The function of this intricate network of polymers is to provide plants with structural support and protection against pathogenic attack (Hématy et al. 2009). The lignocellulose composition varies substantially between different plant species (Fig. 2.1).

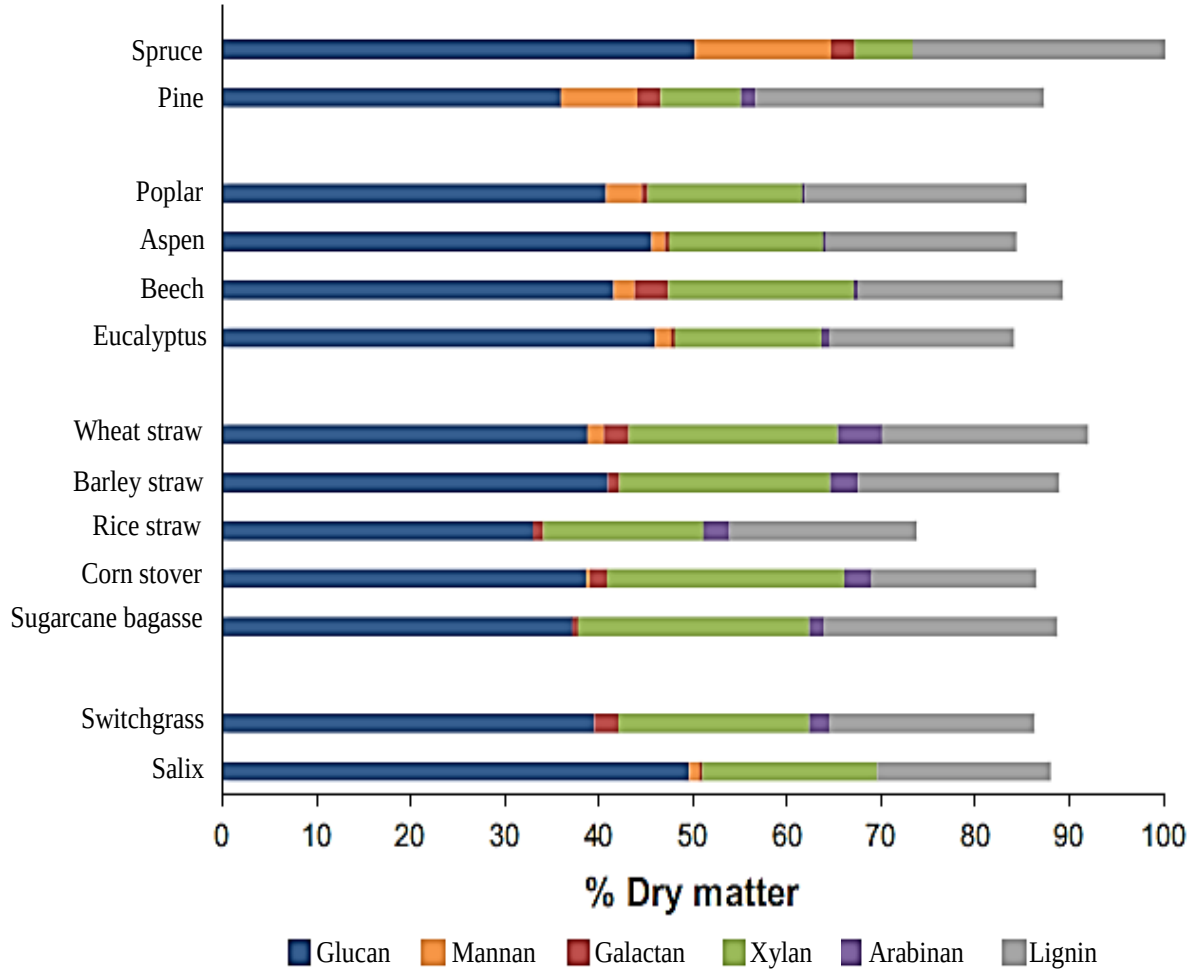

Fig. 2.1 Composition of various agro-industrial residues that can be used for lignocellulose conversion to bioethanol. Total values below 100% depend on limitations in carbohydrate analysis (Nogué and Karhumaa 2014).

2.2 PLANT CELL WALL STRUCTURE

The plant cell wall is a heterogeneous complex consisting of a primary and secondary cell wall. The middle lamella is a thin lignin rich layer that connects the plant cells. Cell migration is not possible due to the hardiness of plant structures and therefore plant growth relies on cell expansion. The primary cell wall that is deposited during cell growth is mechanically robust to support the structure as well as pliable enough to allow expansion of the growing cell (Cosgrove 2005; Reiter 2002). The primary wall contains mainly cellulose, hemicellulose and small amounts of pectins and structural proteins (Fig. 2.2).

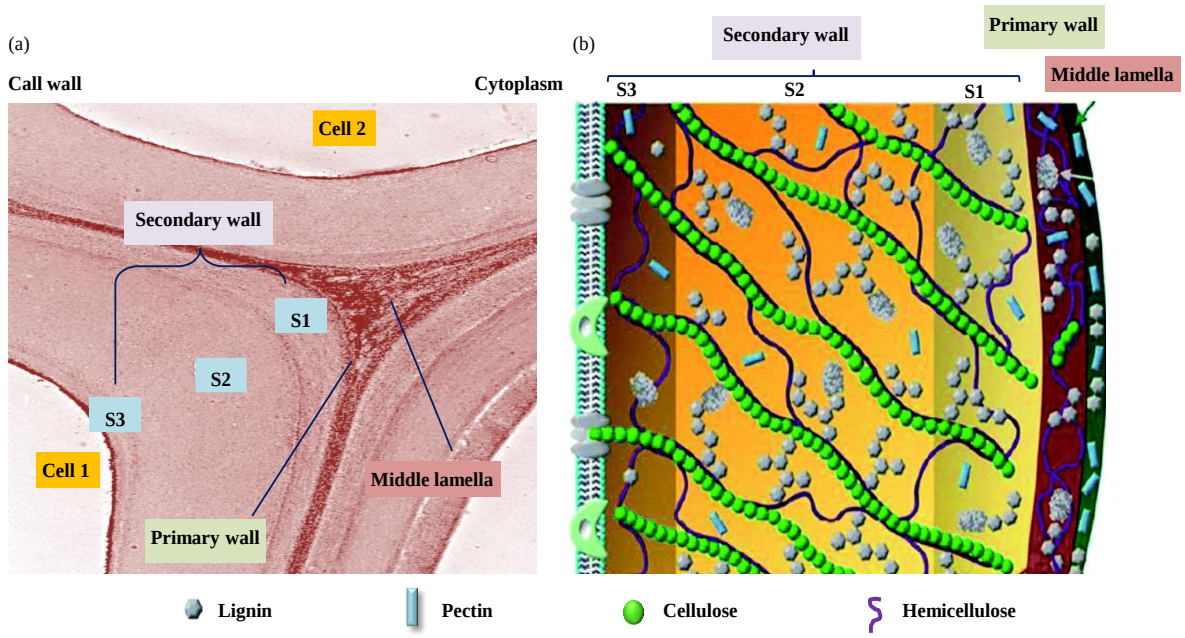

Fig. 2.2 (a) Photograph and (b) schematic representation of the primary and secondary cell walls, indicating the complex network of polysaccharides present. (adapted from Tabet and Aziz 2013; Cheng et al. 2015).

After cell growth terminates, a secondary wall is deposited on the inside of the primary wall, which provides additional strength to the plant structure. Secondary walls are much thicker than primary walls and account for the majority (e.g. 70-80% in corn stover) of lignocellulosic biomass (Jordan et al. 2012). Secondary walls contains mostly cellulose, hemicellulose and lignin (Lerouxel et al. 2006; Pauly and Keegstra 2008) and is divided into S1, S2 and S3 layers based on the structure and chemical composition of the polymers present (Mathews et al. 2015). The S1 layer contains the highest percentage of lignin. The S2 layer is thicker than the S1 layer

and therefore contains the highest amount of lignin, cellulose and hemicellulose per cell. The S3 layer forms the boundary of the middle lamella and contains the least amount of lignin.

Plant cell wall material plays an important role in the African community as a source of heat (wood for fire), building material (timber), raw materials for various industries (paper and textile) and is a constituent of foods such as fruits and vegetables (Doblin et al. 2013; Pauly and Keegstra 2008). The insoluble lignocellulose fraction (referred to as dietary fibre) stimulates bowel movement, speeding up the passage of waste through the digestive tract (Liu et al. 2014). Dietary fibre can effectively reduce the risk of diabetes, cardio-vascular diseases, stroke, coronary heart disease, hypertension, hypercholesterolemia, hyperlipidaemia, gastrointestinal cancer, constipation, diverticulosis and haemorrhoids.

A relatively small portion of the total plant material produced annually is currently utilised in industrial processes, leaving an underutilised source of biomass (Schubert 2006). Industrial plant waste, agricultural residues (corn stover, wheat straw and bagasse), garden refuse (plant material), forest residues (sawdust, mill waste) and municipal waste (paper) can be applied as feedstock for the production of liquid biofuels and chemicals (Fig. 2.1). Using waste as feedstock is advantageous since it does not conflict with agricultural use or human food supplies (Mathews et al. 2015).

2.2.1 Cellulose

Cellulose represents the most abundant polysaccharide in plant cell walls and contributes to approximately 35-50% of the total dry weight (Lynd et al. 2002). It is produced at an estimated 180 Gtons per annum (Vermerris and Abril 2014). Cellulose is a linear polymer comprised exclusively of glucopyranose units linked by β-1,4 glycosidic bonds (Fig. 2.3). The β-glucans present in the primary walls of grasses have a structure similar to that of cellulose, but contain β-1,3 and / or β-1,4 -D-glucopyranosyl units in a non-repeating order, along with side chains of varying lengths. Glucans are typically absent in the biomass by the end of the harvesting season and is therefore not considered a major source for bioethanol production (Ten and Vermerris 2013).

Glycosidic bonds are some of the most stable bonds in nature and have a half-life of over five million years (Knob et al. 2010). The basic component of cellulose is the disaccharide cellobiose, repeating itself to produce long chains of cellulose containing 5000-10000 glucose

units (Buruiana et al. 2013). Several of these cellulose chains (20-300) are then clustered together through intra/inter-molecular hydrogen bonds and van der Waals forces to form microfibrils, which in turn assemble into cellulose fibres (Agbor et al. 2011; Pérez et al. 2002). Cellulose fibres can present itself either as organised (crystalline cellulose) or non-organised (amorphous cellulose) regions (Pérez et al. 2002). Crystalline cellulose is more compact due to the organised layering of the fibrils, while amorphous cellulose is less organised in this manner. The structural differences between these types of cellulose affect the enzymatic hydrolysis of the polymer, as crystalline cellulose is less susceptible to the action of enzymes. Cellulose is insoluble in most solvents, which is attributed to the high degree of crystallinity and rigid intra/inter-molecular hydrogen bonds (Liu et al. 2014). Yet, the cellulose fibres encountered in industrial applications are typically altered from their native structure since the organisation of crystalline and amorphous regions can be disrupted by sample preparation.

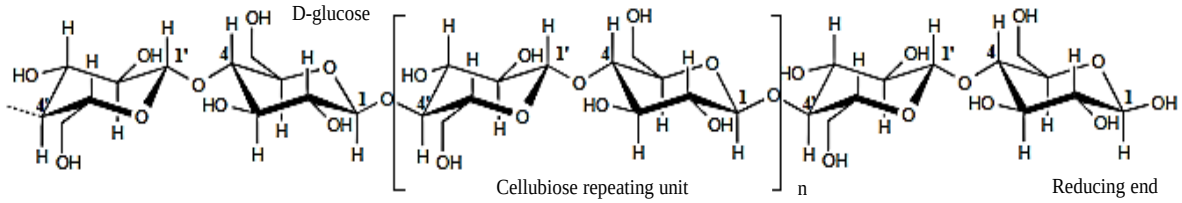

Fig. 2.3 The polymeric cellulose structure is comprised of β-1,4 linked glucose units (Jordan et al. 2012).

Cellulose fibres are mainly used for the production of fibre for textiles (flax, cotton, jute) and paper (cotton, trees) (Vermerris and Abril 2014), but also has various applications in the pharmaceutical industry. More recently, cellulose and cellulose derivatives have been used for the production of absorbent materials, insulation, fillers, films, membranes, lactic acid, furfural and other chemicals (Ten and Vermerris 2013; Vermerris and Abril 2014; Mathews et al. 2015). Cellulose has also gained considerable interest as a source of glucose for the microbial conversion to biofuels and bio-based chemicals.

2.2.2 Hemicellulose

Hemicellulose can be defined as the alkaline-extractable polysaccharides from plant cell walls (Jordan et al. 2012). It represents the second most abundant polysaccharide in plant cell walls and can contribute to approximately 20-35% of the total dry weight (Lynd et al. 2002). Unlike cellulose, hemicellulose is a highly branched, heterogeneous complex of polysaccharides composed of hexose and pentose sugars (Fig. 2.4). Hemicellulose can be classified as xylan, xyloglucan (heteropolymer of xylose and glucose), (fucogalacto) xyloglucan (heteropolymer of xylose, fucose, galactose and glucose), glucomannan (heteropolymer of glucose and mannose), galactoglucomannan (heteropolymer of galactose, glucose and mannose) and arabinogalactan (heteropolymer of galactose and arabinose) (Ten and Vermerris 2013). Xylan represents the major component of hemicellulose in herbaceous and hardwood biomass, while mannan represents the major component of hemicellulose in soft or coniferous wood (Juturu and Wu 2014). Hemicellulose interacts with cellulose and lignin via hydrogen bonds and ionic interactions, respectively, contributing to the intricate structure of cell walls (Mathews et al. 2015).

Xylan is composed mainly of β-1,4 linked xylopyranose units (Fig. 2.4) with a chain length of 70-130 and 150-200 xylopyranose residues in softwood and hardwood xylans, respectively (Collins et al. 2005). The majority of xylans is highly branched hetero-polysaccharides, although linear unsubstituted xylan has also been reported in esparto grass, tobacco and certain marine algae (Collins et al. 2005). The latter also contains β-1,3-linkages in addition to the β-1,4 linkages. The xylopyranosyl backbone chain can be substituted to varying degrees (depending on its origin) with glucuronopyranosyl, 4-*O*-methyl-D-glucuronopyranosyl, α-L-arabinofuranosyl, acetyl, feruloyl and/or *p*-coumaroyl side-chain groups (Juturu and Wu 2012). Xylans obtained from grasses and annual plants are typically arabinoxylans, whereas hardwood and softwood contains mostly *O*-acetyl-4-*O*-methylglucuronoxylan and arabino-4-*O*-methyl-glucuronoxylan, respectively (Collins et al. 2005).

Galactoglucomannan is mainly found in softwoods and glucomannan in hardwoods. Both consists of a β-1,4 linked mannose and glucose backbone, but galactoglucomannan contains galactose substituents. Xyloglucans are found in the primary cell walls of higher plants (Liu et al. 2014). The backbone chain consists of β-1,4 linked glucan with three out of four glucose residues substituted with α-D-(1-6) xylose. The xylose can contain β-1,2 linked galactose, depending on the origin of the xyloglucans. The D-galactose residues can be substituted with

α-fucosyl residues (Liu et al. 2014). Hemicelluloses are generally used for the production of ethanol and lactic acid and have various applications in the pharmaceutical industry. It is also used for the production of xylitol (artificial sweetener), packaging films and hydrogels (Ten and Vermerris 2013).

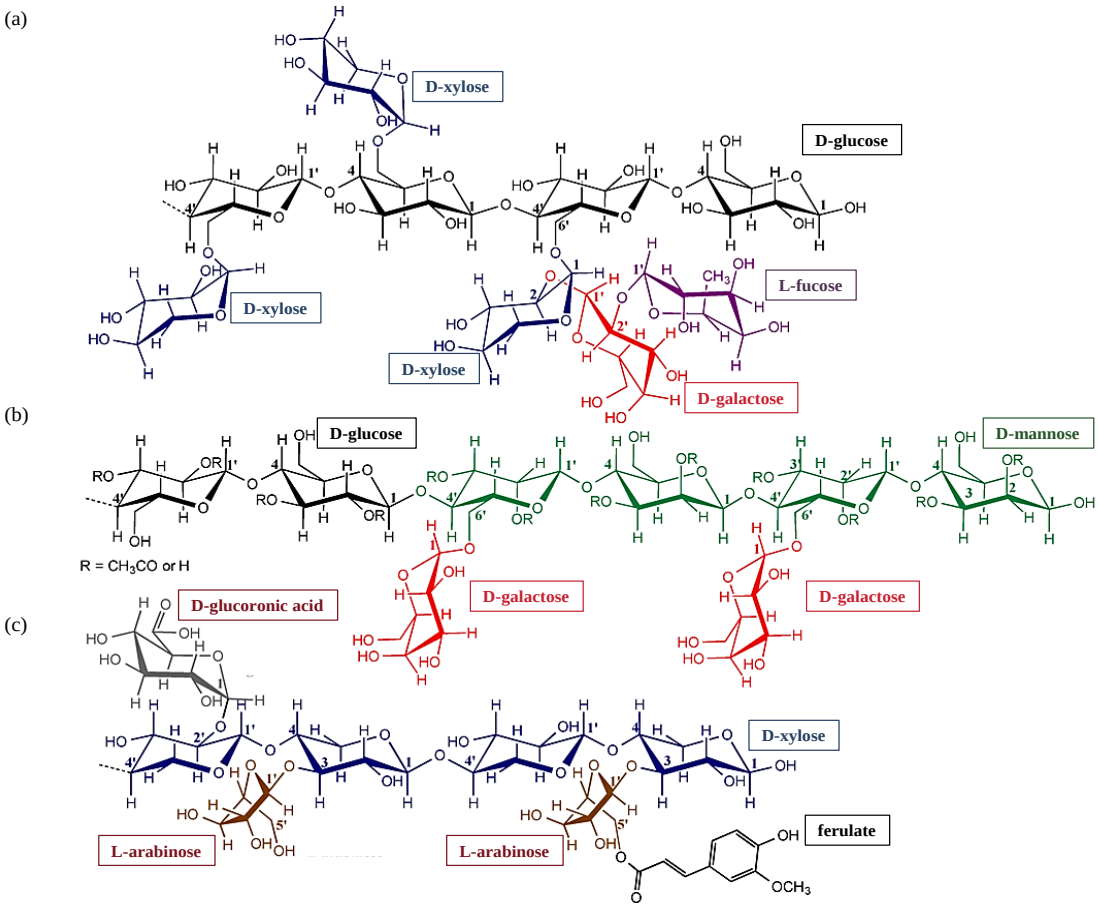

Fig. 2.4 Hemicelluloses are present as linear or highly branched heteropolysaccharides and includes (a) (fucogalacto) xyloglucan, (b) galactoglucomannan and (c) glucuronoarabinoxylan. (adapted from Ten and Vermerris 2013).

2.2.3 Lignin

Unlike cellulose and hemicellulose, lignin is a non-carbohydrate polymer synthesised from three fundamental phenylpropionic alcohol precursors; coniferyl alcohol (guaiacyl), sinapyl alcohol (syringyl) and *p*-coumaryl alcohol (*p*-hydroxyphenyl) (Fig. 2.5), which form lignin dimers that polymerise to form the lignin polymer. These phenylpropane units are linked via radical chemistry coupling reactions (β-aryl ether, diaryl propane, biphenyl, diaryl ether, and phenylcoumaran, spirodienone and pinoresinol bonds).

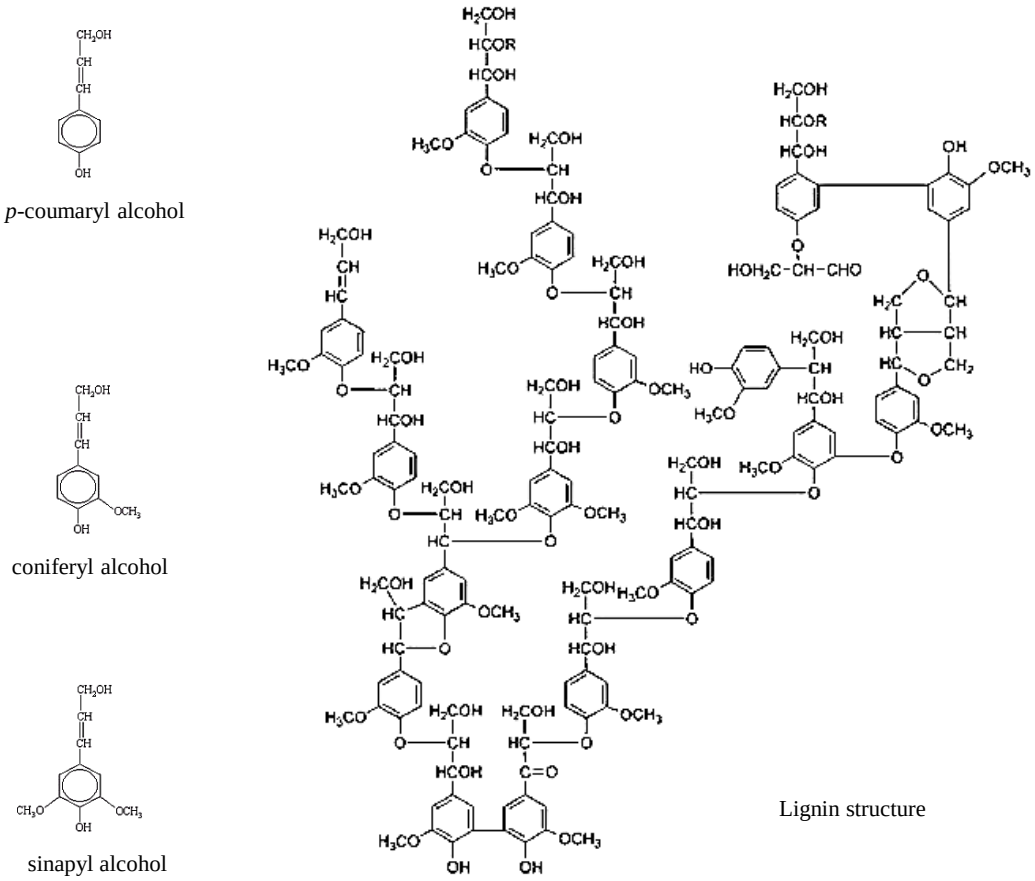

Fig. 2.5 Chemical structures of the three lignin monomer precursors and a hypothetical lignin structure (Vivekanandhan et al. 2013).

Lignin is an amorphous, water-insoluble and optically inactive polymer that can constitute approximately 5-30% of plant total dry weight. Lignin is deposited in the void between the cellulose and hemicellulose to create a water-impermeable layer around the cell, which provides a physical barrier to pests and pathogens, contributing to structural support and facilitating water transport through the vascular tissue due to its hydrophobic nature (Ten and Vermerris 2013; Vermerris and Abril 2014). It is resistant against microbial attack and oxidative stress, so much so that it can be detected in soil long after other plant materials have decayed (Agbor et al. 2011; Mathews et al. 2015). Softwood typically contains more lignin than grass and hardwood species (Mathews et al. 2015). Softwood lignin contains mainly coniferyl alcohols (80%), whereas hardwoods typically contain 56% coniferyl alcohols and 40% sinapyl alcohols. Grass lignin contains equal amounts of coniferyl and sinapyl alcohols and more *p*-coumaryl alcohol than softwoods and hardwoods (up to 10%). Lignin is used in the production of adhesives, phenolic compounds and food additives.

2.2.4 Biomass pretreatment (bioethanol perspective)

In bioprocess engineering, pretreatment refers to the process whereby any source of lignocellulose material is converted from its native form of resistance (to hydrolytic attack) to a form of sufficient accessibility to enzymatic hydrolysis (Agbor et al. 2011). The intricate plant cell wall architecture has evolved to withstand this enzymatic hydrolysis (Vermerris and Abril 2014). Pretreatment methods are employed to remove some of the hemicellulose and lignin that form a physical barrier around the cellulose chains (Öhgren et al. 2007; Behera et al. 2014). Lignin prevents the binding of hydrolytic enzymes to the substrates, thus limiting product yield. Pretreatment is the most expensive process in bioethanol production due to the additional energy requirement and chemical input, while the cellulolytic enzymes also contribute substantially to the cost. However, pretreatment is obligatory to ensure maximum utilisation of plant polysaccharides and reduces the amount of enzyme needed.

The pretreatment process enhances the ability to convert lignocellulose and can include chemicals, heat or mechanical force. Mechanical pretreatment increases the surface area for enzymatic or chemical reactions by decreasing particle size and the degree of polymerisation. Wet oxidation is an oxidative pretreatment method in which oxygen or air is employed as a catalyst at temperature above 120°C to solubilise the hemicellulose, remove the lignin and fractionate the cellulose (Badiei et al. 2014; Behera et al. 2014). Acid pretreatment includes the use of inorganic acids (nitric, sulfuric, phosphoric, hydrochloric acid, etc.) and organic acids (maleic, para-acetic acid, etc.) in order to solubilise the hemicellulose and precipitate the lignin. With strong acid hydrolysis, a high glucose yield is obtained without the need for subsequent enzymatic hydrolysis (Badiei et al. 2014). Alkaline pretreatment employs alkali compounds (sodium hydroxide, calcium hydroxide (lime), potassium hydroxide, aqueous ammonia, ammonium hydroxide and hydrogen peroxide or combination of these) to solubilise the lignin and reduce crystallinity of the cellulose (Badiei et al. 2014; Behera et al. 2014).

Oxidative delignification pretreatment uses oxidising agents (ozone, hydrogen peroxide, oxygen, etc.) with aromatic rings to convert lignin to carboxylic acids at ambient temperature and atmospheric pressure (Badiei et al. 2014; Behera et al. 2014). The organosolvation method uses a mixture of organic solvents (ethanol, methanol, acetone, ethylene, etc.) with inorganic acid catalysts (sulphuric or hydrochloric) in aqueous form to break down hemicellulose and remove lignin (Badiei et al. 2014; Behera et al. 2014). Ionic liquids are used as selective solvents of lignin and cellulose (Badiei et al. 2014). The structure of lignin and hemicellulose

is unaltered after treatment with ionic liquids, allowing the selective extraction of unaltered lignin as waste stream.

CO_2 can be used as a supercritical fluid under high pressure for the effective solubilisation of lignin. Ammonia fibre explosion (AFEX) is a physio-chemical pretreatment process that uses exposure to liquid ammonia at high temperature and pressure, followed by a sudden reduction in pressure. Steam pre-treatment uses steam at a high temperature, whereas liquid hot water pretreatment uses water in the liquid state at elevated temperatures (>160°C). Chemical pretreatment is currently the method of choice when the process is followed by anaerobic fermentation (Behera et al. 2014). It does however, require a large amount of chemicals and water, recycling of chemicals and waste disposal. The process can be combined with high temperatures, which results in a high facility investment and increase in process cost.

At high severity pretreatment, the hemicellulose is hydrolysed to monomeric sugars and then further degraded to compounds that are inhibitory to subsequent fermentation processes (Behera et al. 2014). The nature and concentration of the inhibitory compounds can vary significantly depending on the feedstock (agricultural and forestry residues), the pretreatment method and conditions employed (temperature, residence time, pressure, pH, etc.). The inhibitory compounds are grouped into furan derivatives (furfural and 5-hydroxymethyl furfural (HMF), carboxylic acids (acetic, formic, levulinic acids), phenol derivatives (syringaldehyde, vanillic acid) and heavy metal ions (released by corrosion of the digester). In *S. cerevisiae*, these compounds result in the inhibition of RNA and protein synthesis of alcohol dehydrogenase (ADH), pyruvate dehydrogenase (PDH) and aldehyde dehydrogenase (ALDH). Furan derivatives damage the cell walls and the cell membranes, whereas the presence of both furfural and HMF suppress cell growth (Behera et al. 2014). Therefore, inhibitor tolerant microorganisms need to be used (isolated, adapted or constructed) or the hydrolysate needs to be detoxified prior to fermentation. Detoxification adds to the production cost, making strain construction a more viable option. Inhibition compounds can be converted into inert materials or their concentration reduced by means of physical, chemical and biological processes. The effectiveness of a detoxification method depends on the type of inhibitors present in the hydrolysate and the fermentative organism to be used for the ethanol production. Not all microorganisms are affected equally by the inhibitor composition and concentration, therefore the fermentative microorganisms to be used for the sugar to ethanol conversion plays a role in determining the detoxification method (Behera et al. 2014). Physical processes such as evaporation are effective in the removal of volatile compounds (furfural, vanillin and acetic

acid). Chemical detoxification methods include calcium hydroxide over liming, neutralisation, activated charcoal addition, use of ion exchange resins or tin oxides. Biological detoxification involves the enzymatic treatment of the hydrolysate using laccases and peroxidases derived from lignolytic fungi (Behera et al. 2014).

2.3 ENZYMATIC HYDROLYSIS OF XYLAN

Hemicellulose can be degraded by either chemical or biological means (using hemicellulases as catalysts). Chemical degradation is not environmentally friendly, as it requires strong acids and harsh conditions. The process also results in the formation of various sugar degradation products such as HMF and furfural, and lignin degradation products such as phenolics (den Haan et al. 2013). These by-products are toxic to microbes and have a negative impact on the subsequent fermentation processes.

With low severity pretreatments, the recovery of hemicellulose sugars is high, but the remaining hemicellulose hinders cellulase access to cellulose (Öhgren et al. 2007). The glucose yield can be improved by the addition of xylanases to the hydrolysis process to hydrolyse and assist in the removal of the hemicellulose fraction that remains associated with the cellulose after the pretreatment, thereby increasing accessibility of cellulose to cellulases and releasing xylose for conversion to ethanol (Öhgren et al. 2007). The enzymatic hydrolysis takes place at mild conditions without the accumulation of toxic components.

2.3.1 Endoxylanases

The complete hydrolysis of xylan requires a large variety of cooperatively acting enzymes due to the heterogeneity and complexity of the xylan structure (Fig. 2.6) (Collins et al. 2005). Hemicellulases contain mainly glycoside hydrolases (EC 3.2.1-3.2.3) that hydrolyse the glycosidic bonds between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety (Knob et al. 2010). Endo-1,4-β-D-xylanases (EC 3.2.1.8) cleave the xylan backbone at random, whereas the β-D-xylosidases (EC 3.2.1.37) cleave xylose monomers from the non-reducing end of xylo-oligosaccharides and xylobiose units. A large number of xylanases have been identified, studied and classified in glycoside hydrolase (GH) families 5, 7, 8, 10, 11 and 43 according to the CAZy database [http://www.cazy.org/]. Each of these families are characterised by a particular fold and mechanism of action. Family 10 and 11

xylanases have been extensively studied, whereas studies on the xylanase members of the other families are lacking with only a small number of xylanases belonging to families 5, 7, 8 and 43.

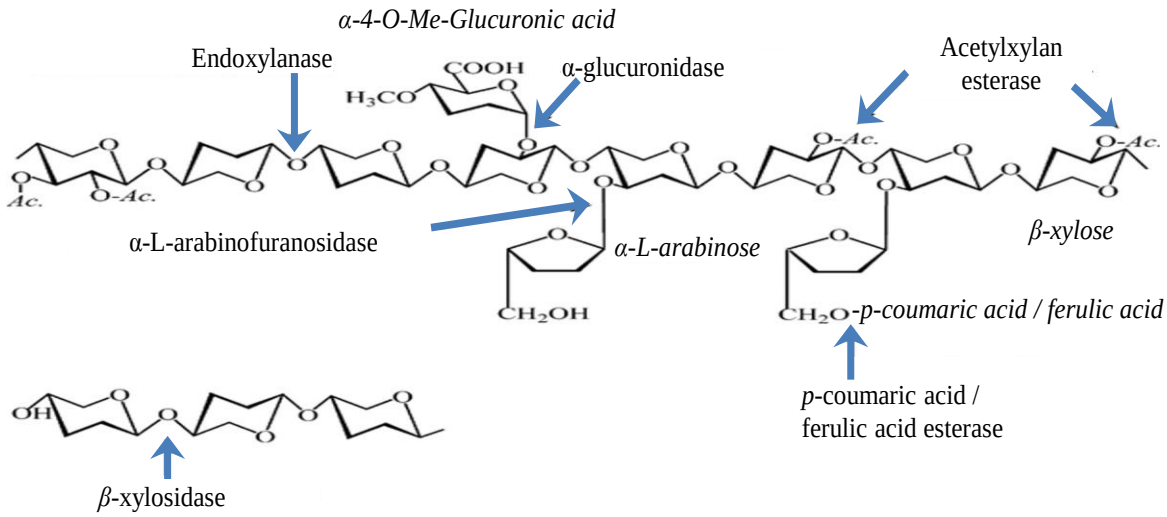

Fig. 2.6 The structure of a hypothetical xylan strand is illustrated and the enzymes involved in its hydrolysis indicated (Collins et al. 2005).

2.3.2 Xylosidases

According to Biely (1985), β-D-xylosidases can be classified as either xylobiases or exo-1,4-β-xylanases based on their affinities for xylobiose and larger xylooligosaccharides, respectively. Currently, β-xylosidases are defined as enzymes that catalyse the removal of xylose from xylobiose and from the non-reducing ends of short xylooligosaccharides (Knob et al. 2010). Xylobiases prefers xylobiose as substrate. The β-xylobiases play an important role in xylan degradation by preventing product inhibition through the removal of the xylobiose that inhibits the endoxylanases. The β-xylosidases also display transglycosylation activities wherein monosaccharide units or alcohols can be transferred to xylose units, increasing their potential biotechnological applications.

Exo-xylanases hydrolyse the xylan backbone from the reducing-end, producing short-chain oligomers or hydrolysing oligomers with a degree of polymerisation of 2-3 or more xylose residues (Juturu and Wu 2014). They are inert on pure polymeric xylan and inactive on xylobiose. The β-xylosidases are divided into families 3, 30, 39, 43, 52 and 54 (Knob et al. 2010) with filamentous fungal β-xylosidases only grouped into families 3, 43 and 54.

2.3.3 Accessory enzymes

The accessory enzymes remove the side groups, making the backbone more accessible to enzymatic hydrolysis. In general, the accessory enzymes exist as homo- and hetero-dimers and includes the α-L-arabinofuranosidases (EC 3.2.1.55) that hydrolyse the glycosidic bonds between arabinofuranosyl substitutions and the xylopyranosyl backbone residues of arabinoxylan (Juturu and Wu 2014). They typically belong to GH families 3, 43, 51, 54 and 62. The α-D-glucuronidases (EC 3.2.1.139), belonging to GH family 67 and 115, is responsible for the removal of the α-1,2 linked α-(4-*O*-methyl)-D-glucuronic acid from the xylan backbone. Acetyl xylan esterases (EC 3.1.1.72) are responsible for hydrolysing acetyl substitutions on xylan side chains and are classified into families 1, 2, 3, 4, 5, 6, 7, 12 and 15. Ferulic acid esterases (EC 3.1.1.73) release ferulic acid from the arabinose side chains of the xylan backbone whereas the *p*-coumaric acid esterases (EC 3.1.1.x) release *p*-coumaric acid. The activity, stability and substrate diversity of the accessory enzymes have been improved by genetic engineering, yet the expression levels for both homologous and heterologous expression remain low (mg/L) (Juturu and Wu 2014).

2.3.4. Industrial applications of hemicellulases

The applications of hemicellulose enzymes have been well documented for the improvement of animal feed quality in poultry and dairy industries; and in the food industry for baking, coffee bean processing, coconut oil extraction, fruit juice, wine and vinegar production (Juturu and Wu 2014). They are used for the production of biomedical compounds such as antiglycemic, antimetastatic, anticarcinogenic agents and probiotics production. Other applications include oil and gas well stimulation, paper pulp processing and detergent formulation etc. The application and contribution to the depolymerisation of hemicellulose to release sugars for chemical production, is expected to increase owing to the rapid development of second-generation biofuels.

2.4 CARBON CONVERSION

Life can be explained as the set of processes involved in the exchange of material through an organism and its environment (referred to as metabolism) to enable the organism to maintain and procreate itself (Benner 2010). These processes involve pathways of central carbon metabolism and incorporate both anabolic and catabolic reactions for the production of precursor intermediates and energy. Some of the more central reactions in *S. cerevisiae* involves those of glycolysis, gluconeogenesis, tricarboxylic acid cycle (TCA), glyoxylate cycle, pentose phosphate pathway (PPP) and the electron transport chain.

Glycolysis is used for the conversion of hexoses to trioses, mainly pyruvate, ATP and NADH. Gluconeogenesis is in essence the reverse of glycolysis and is required for the production of hexoses from non-sugar carbon sources. The tricarboxylic acid cycle and glyoxylate cycles are important for the supply of precursors for biosynthesis of amino acids, metabolism of two- and three-carbon substrates and $FADH_2$ and NADH generation. The PPP comprises several carbon-shuffling reactions that provide precursors for biosynthesis of nucleic acids, amino acids and reducing power in the form of NADPH. The electron transport chain plays a crucial role for the generation of ATP via oxidative phosphorylation of $FADH_2$/NADH by ATP synthetase (Rodrigues et al. 2006; McKee and McKee 2011).

Metabolic pathways are intimately connected and share several intermediates, cofactors and compartments in the cell. Pathways are either negatively or positively regulated depending on the extracellular resources available and cellular demands for energy and precursors (Wills 1990). This phenomenon is referred to as metabolic regulation and is a remarkably complex system, since the regulation of central carbon pathways is tightly supervised and fine-tuned by the cell to ensure optimal utilisation of carbon (Wills 1990).

The regulation of *S. cerevisiae* central carbon metabolism has gained much attention due to its industrial relevance and ability to ferment glucose (and other hexoses) to ethanol under fully aerobic conditions, which has popularised this yeast as a eukaryotic model organism (Pronk et al. 1996). The understanding of these regulatory mechanisms has been a point of interest for several decades and despite a large reservoir of information, a global view of central carbon metabolism and regulation in *S. cerevisiae* is not yet fully understood.

2.4.1 Glucose transport in *S. cerevisiae*

Glucose is by far the preferred energy source for *S. cerevisiae* and can be utilised both aerobically and anaerobically (Carlson 1999; Kim et al. 2013a). This yeast is considered one of the most effective glucose utilising organisms and possesses a complex system for the translocation of glucose across the plasma membrane. The *RGT2*/*SNF3* glucose signalling pathway is responsible for glucose uptake, while the *SNF1*/*MIG1* glucose repression pathway negatively regulates the genes involved in the glucose oxidation (Fig. 2.7) (Bisson et al. 1987; Celenza et al. 1988; Özcan and Johnston 1995; Özcan et al. 1996; Kim et al. 2013a).

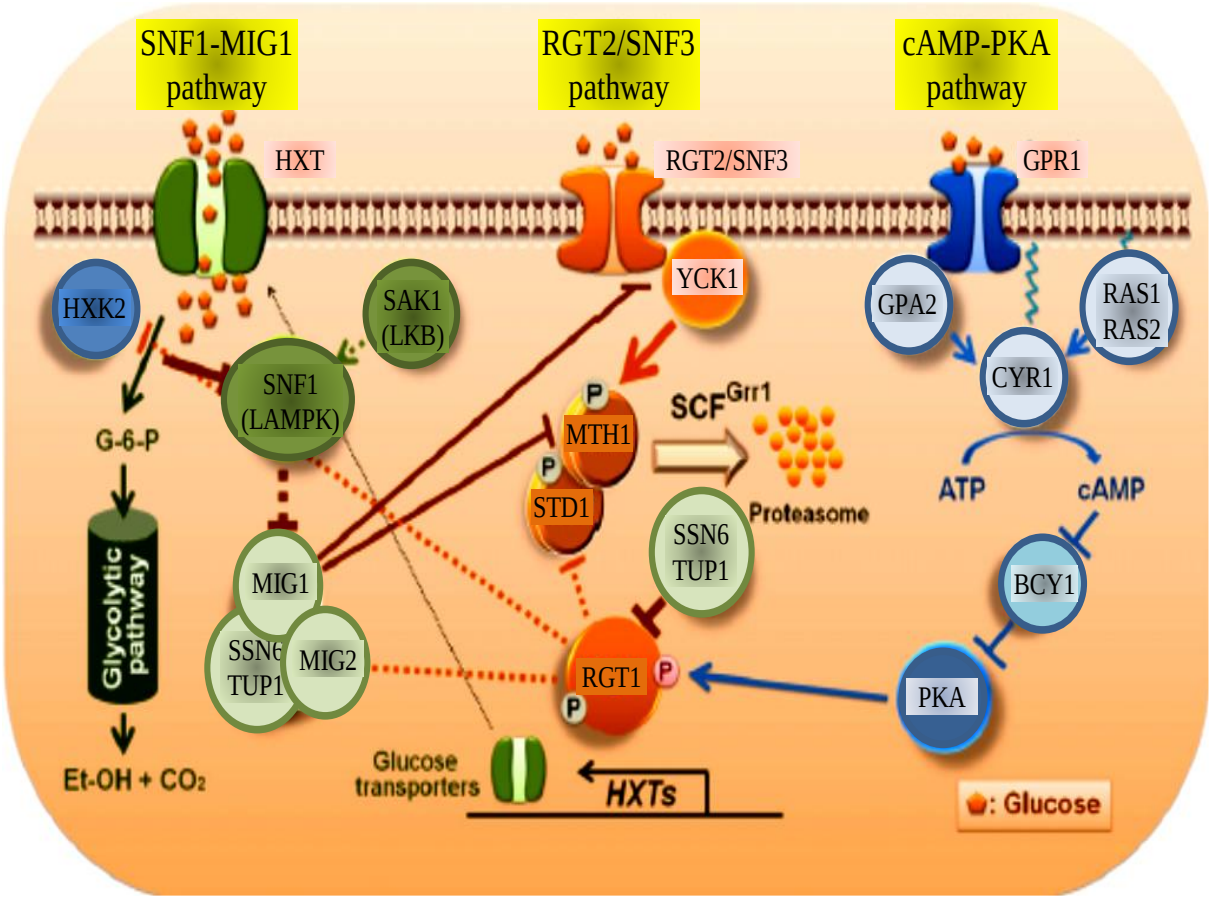

Fig. 2.7 Schematic representation of the three pathways involved in the regulation of the hexose transporters (Kim et al. 2013a).

At least 18 transporters are involved in passive hexose transport in *S. cerevisiae* (Wieczorke et al. 1999; Gonçalves et al. 2014). The HXT1, 2, 3, 4, 6, 7 and GAL2 are the main hexose transporters and inactivation of all six results in a strain that is unable to consume or ferment

glucose effectively (Gonçalves et al. 2014). The transporters display different affinities for different hexose sugars, enabling the yeast to cope with the vast range of sugars and to respond swiftly to changes in glucose availability. The low-affinity glucose transporter (HXT1) is only expressed when glucose levels are high (≥1%), whereas the high-affinity glucose transporters (HXT2, HXT4, HXT6 and HXT7) are expressed when glucose levels are low (~0.2%) (Kim et al. 2013a). HXT6 and HXT7 are also subjected to glucose-induced degradation. The HXT3 and HXT5 are intermediate-affinity glucose transporters that are expressed in both low and high glucose concentrations.

In *S. cerevisiae*, three isozymes of hexokinases are responsible for the phosphorylation of glucose, namely hexokinase I (HXK1), hexokinase II (HXK2) and glucokinase (GLK1). HXK1 and HXK2 are non-specific kinases and play a role in the phosphorylation of mannose and fructose (Albig and Entian 1988; Diderich et al. 2001; Rodríguez et al. 2001). Various levels of transcriptional regulation of the hexose phosphorylation encoding genes exist, which is dependent on the source of the carbon and its relative abundance (Diderich et al. 2001). The function of HXK2 has been reported to be the key regulatory hexokinase in *S. cerevisiae* as it is involved in the glucose-repression response (Rodríguez et al. 2001). In the presence of glucose, the *HXK1* and *GLK1* genes are repressed and become rapidly de-repressed with a diauxic shift to non-fermentable carbon source. In contrast, the *HXK2* gene is induced in the presence of glucose and becomes repressed with a diauxic shift to non-fermentable carbon source. In addition, hexokinases are subject to allosteric regulation, for example, ATP allosterically inhibits HXK2 activity (Larsson et al. 2000). Apart from the function of HXT2 in glycolysis, the protein has also been shown to be involved in the nucleus where it is involved in positive transcriptional regulation of itself and negative regulation of *HXT1* and *GLK1* gene expression (Rodríguez et al. 2001).

2.4.2 Glucose conversion to ethanol in *S. cerevisiae*

The initial reactions of glycolysis rely on the uptake and phosphorylation of glucose to glucose 6-phosphate (G6P). The addition of a phosphate group is believed to prevent glucose from being transported back out of the cell (McKee and McKee 2011). G6P can be converted to fructose 6-phosphate (F6P) and 6-phosphogluconate (6PG). The latter is involved in the oxidative branch of the pentose phosphate pathway, which is discussed elsewhere in this review. The G6P is a key regulatory intermediate in glycolysis, playing a role in the induction

of the lower glycolytic reactions (Müller et al. 1995). F6P subsequently gets phosphorylated to fructose 1,6-bisphosphate (F1,6P), via the activities of phosphoglucose isomerase (PGI) and phosphofructokinase (PFK), respectively (Rodrigues et al. 2006). As in the case of HXK, PFK is also subject to allosteric inhibition by ATP (Larsson et al. 2000). Under physiological conditions, the activity of PFK only catalyses the forward reaction. A distinctively different enzyme, fructose 1,6-bisphosphatase, is responsible for the reverse reaction that is required during gluconeogenisis (Fothergill-Gilmore and Michels 1993). PGI is not known to be susceptable to allosteric regulators or cofactors and its regulation is therefore dependant on the rates of substrate and product formation (van den Brink et al. 2008). On the other hand, PFK is strongly susceptable to regulation by ADP, AMP, fructose 2,6-bisphosphate (F2,6P), ATP and various intermediates (van den Brink et al. 2008).

The final steps following F1,6P leads to the production of pyruvate through the sequential reactions of aldolase (FBA), triosephosphate isomerase (TPI), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase (PGK), phosphoglycerate mutase (PGM), enolase (ENO) and pyruvate kinase (PYK). F1,6P is cleaved by the reversible FBA producing glyceraldehyde 3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP) (Fothergill-Gilmore and Michels 1993; Rodrigues et al. 2006). Under osmotic stress, *S. cerevisiae* will catalyse the reduction of DHAP to glycerol (that acts as an osmolyte and osmoprotectant) via the NAD-dependant glycerol 3-phosphate dehydrogenase 1 (GDP1), whilst NAD-dependant glycerol 3-phosphate dehydrogenase 2 (GDP2) is used as a redox sink for the production of glycerol to maintain a redox balance in the cytosol under anaerobic conditions (Overkamp et al. 2002; Murray et al. 2011). In the absence of osmotic and redox stress, DHAP is converted back to GAP (via the cofactor-independent TPI). The following reactions catalysed by GAPDH, PGK, PGM, ENO and PYK systematically converts GAP to pyruvate (Fothergill-Gilmore and Michels 1993). Pyruvate can be converted to acetyl-CoA catalysed by the thiamine pyrophosphate (ThPP)-dependant pyruvate dehydrogenase (PDH) (Pronk et al. 1996). Alternatively, pyruvate can be converted to acetaldehyde (catalysed by the ThPP- and magnesium-dependant pyruvate decarboxylase, PDC) for alcoholic fermentation or oxaloacetate (via the magnesium- and ATP-dependant pyruvate carboxylase) (Pronk et al. 1996).

2.4.3 Crabtree effect

Ethanol is toxic to most microbes and therefore its accumulation provides the host with an advantage over its competitors. Yeasts such as *S. cerevisiae* and *Schizosaccharomyces pombe* are classified as Crabtree positive yeasts due to their ability to convert six-carbon (C6) sugars (such as glucose) to two-carbon (C2) components (such as ethanol) without complete oxidation to CO_2, even in the presence of oxygen. In Crabtree positive yeasts, the energy for growth is provided by glycolysis and fermentation pathways rather than by the oxidative respiration pathway. This effect is more prominent with cultivation on glucose and is reduced with growth on carbon sources that is less avidly utilised (Martínez et al. 2014). Cultivation of *S. cerevisiae* on galactose results in a mild Crabtree effect and a mixed respiro-fermentative phenotype.

In S. cerevisiae, the Crabtree effect relies mostly on carbon catabolite repression to prevent expression of the genes involved in the respiration pathway (Piškur et al. 2006). Crabtree positive yeasts switch its metabolism upon depletion of glucose and accumulation of ethanol, resulting in diauxic growth (diauxic shift). The ethanol is converted back to acetaldehyde (if oxygen is present) by the alcohol dehydrogenase. In *S. cerevisiae*, the cytoplasmic *ADH1* gene is expressed constitutively and is involved in the production of ethanol. The *ADH2* gene is expressed only when the internal sugar concentration drops. The ADH2 has a high affinity for ethanol and favours the reverse reaction, converting ethanol back to acetaldehyde.

In Candida albicans (Crabtree negative yeast), the expression of genes encoding mitochondrial ribosomal proteins (MRPs), cytoplasmic ribosomal proteins (CRPs) and rRNA processing proteins are all correlated, whereas the MRP expression in *S. cerevisiae* is correlated with the expression of stress genes induced during the slower respiratory growth in non-fermentable carbon sources, such as glycerol and ethanol (Ihmels et al. 2005). In addition, the regulatory rapid growth element (RGE) motif was found to be absent in the MRP encoding genes of *S. cerevisiae*, the stress genes and promoter regions of genes that should not be expressed when glucose is abundant. It appears that *S. cerevisiae's* anaerobic growth capacity is associated with a global rewiring of the yeast transcriptional network involving dozens of genes.

The mechanism underlying the Crabtree effect remains to be determined and is subject to controversy (Martínez et al. 2014; Rosas-Lemus et al. 2014). Martínez et al. (2014) compiled the most recent model for the regulatory mechanisms involved in the Crabtree effect (Fig. 2.8). The BAS1, PHO2 and GCN4 transcription factors play a central role in the regulatory events causing the Crabtree effect. High glucose levels result in the up-regulation of *BAS1*. BAS1 is

part of the BAS1-PHO1 complex that acts as mediator of the change in growth phenotype. A mutation in *RAS2* results in a lower Crabtree effect and down-regulation of *GCN4*. Meanwhile, a higher growth rate on galactose is ascribed to the reduced activity of the SOK2 transcriptional repressor.

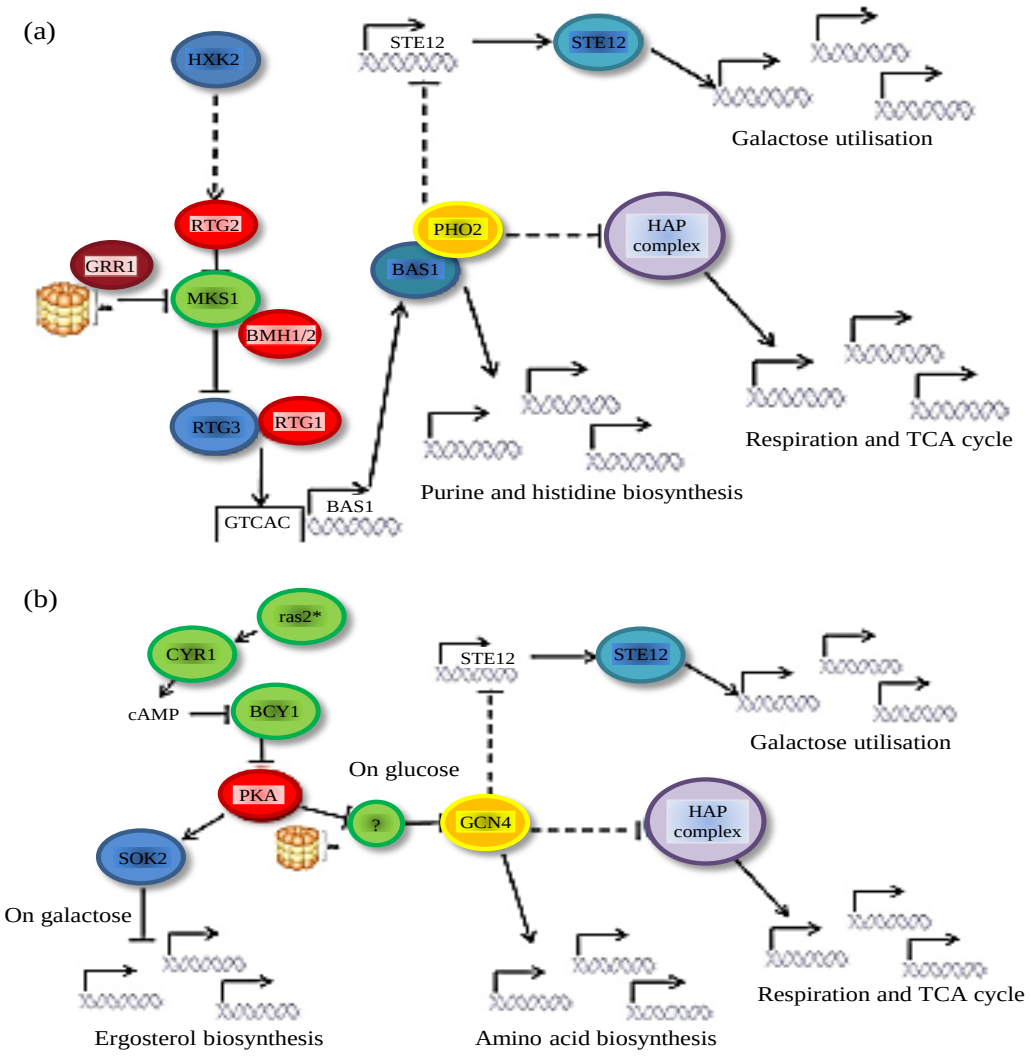

Fig. 2.8 Schematic representation of the regulatory cascade involving the (a) BAS1, PHO2 and (b) GCN4 (with a mutated *RAS2*, ras*). Dashed lines indicate proven interaction, but unclear mechanism (Martínez et al. 2014).

The accumulation of glucose 6-phosphate (G6P) and fructose-1,6-biphosphate (F1,6P) accompanied by the inhibition of oxygen consumption in the presence of glucose, had recently been identified as triggers of the Crabtree effect (Rosas-Lemus et al. 2014). In *S. cerevisiae*, oxidative phosphorylation is strongly regulated by the mitochondrial unspecific channel

(MUC). The G6P and F1,6P accumulate during glycolysis and interact with the mitochondrial MUC (Rosas-Lemus et al. 2014). G6P promotes partial opening of MUC, leading to proton leakage and uncoupling, which results in accelerated oxygen consumption through the uncoupling of oxidative phosphorylation. The F1,6P closes MUC and thus inhibits the rate of oxygen consumption even in the presence of G6P. F1,6P is proposed to be an important modulator of MUC, whose closure contributes to the Crabtree effect through the tight coupling of mitochondria. Furthermore, additional mitochondrial effects are observed at 5 mM F1,6P including the inhibition of complex III and IV activities.

Aerobic fermentation is a problem for some industries (including industries where heterologous protein production is the primary objective) because it lowers the yield of the desired product by channelling carbon to ethanol production. Several successful attempts had been made at controlling ethanol yield and production (Crabtree effect) through genetic engineering. The overexpression of the glycerol 3-phosphate dehydrogenase (*GPD1*) or the lactate dehydrogenase (*LDH*) resulted in the divergence of the carbon toward glycerol and lactate, respectively (Heux et al. 2006). However, the accumulation of one particular by-product may affect the sensory properties of the final product if it is targeted for human consumption (such as wine). An alternative strategy would involve the redirection of the carbon flux to multiple metabolites rather than a single product. Reduction in ethanol yield had been obtained through cofactor engineering through the expression of a water-forming NADH oxidase from *Lactococcus lactis* and *Streptococcus pneumonia* (Heux et al. 2006; Vemuri et al. 2007) as well as the *Histoplamsa capsulatum* alternative oxidase (*AOX1*). This strategy results in a metabolic redistribution pattern due to the decreased NADH levels affecting numerous oxido-reduction reactions (and many metabolic pathways), including ethanol production. In a separate study, Elbing et al. (2004) was able to abolish the Crabtree effect by expressing a chimeric hexose transporter (fusion between *HXT1* and *HXT7*) in a *S. cerevisiae* strain defective in the major hexose transporter genes (*HXT1-7*).

2.4.4 Xylose metabolism

Pretreatment methods employed by the cellulosic bioethanol industry traditionally focused on the removal of the two main protective coats around cellulose, hemicellulose and lignin, in order to increase acid or enzyme accessibility to cellulose (Öhgren et al. 2007; Behera et al. 2014). Lignin is ideally solubilised by a pretreatment method and the remaining

polysaccharides collected by screening (filtering) of the resulting slurry. Currently, the waste stream contains a significant amount of xylose, which can also be utilised for conversion to ethanol. Several naturally occurring bacterial and fungal species are capable of xylose assimilation (Jeffries 1983; Skoog and Hahn-Hägerdal 1988; Lin and Tanaka 2006), but are not suitable for the bioconversion of lignocellulose to ethanol on commercial scale.

The baker's yeast *S. cerevisiae* possesses several traits that makes it a more suitable candidate for the fermentation of lignocellulose-derived sugars compared to other yeasts. These traits include amongst others: (i) rapid sugar consumption; (ii) ability to grow anaerobically; (iii) high resistance to ethanol and other (iv) inhibitory compounds found in lignocellulosic hydrolysates; (v) insensitivity to bacteriophage contamination; (vi) overall high ethanol yields (vii) and its ability to grow well at low pH (Olsson and Hahn-Hägerdal 1993; van Maris et al. 2007). Despite the fact that evidence of a native xylose utilising pathway in *S. cerevisiae* exists (Rodriguez-Peña et al. 1998; Toivari et al. 2004), this yeast has generally been regarded as being incapable of xylose metabolism. The main bottleneck in the construction of a xylose-utilising strain is the lack of an effective xylose transport system and lack of a xylose utilising pathway, which will be discussed in more detail in following sections.

2.4.5 Pentose transport in *S. cerevisiae*

Xylose transmembrane transport is the first step in xylose metabolism, however, evolution has not paved a dedicated transport system for pentose sugars in *S. cerevisiae*. Natural xylose metabolising yeasts such as *Scheffersomyces stipitis* and *Candida shehatae* take up xylose by active proton symport. Passive uptake of xylose in *S. cerevisiae* is mediated by the hexose transporters and is competitively inhibited by glucose (Runquist et al. 2009). Mostly the high-affinity glucose transporters (HXT4, HXT5, HXT7 and GAL2) are responsible for transporting xylose in *S. cerevisiae* (Hamacher et al. 2002), but these hexose transporters have a low affinity for xylose ranging from 137 mM-1.5 M (Busturia and Lagunas 1986; Kötter and Ciriacy 1993). This low affinity is presumably the rate-limiting step in efficient xylose metabolism (Wahlbom et al. 2003; Kuyper et al. 2005), whereas competitive inhibition prevents simultaneous utilisation required for lignocellulose conversion. Therefore, more efficient xylose transporters or dedicated xylose transporters are required for efficient xylose metabolism.

Many pentose transporters have been obtained from native pentose-utilising organisms (such as *Candida intermedia*, *S. stipitis*, *Arabidopsis thaliana*, *Debaromyces hansenii*, *Neurospora crassa* and *Ambrosiozyma monospora*) and expressed in *S. cerevisiae* with limited success. The complications include inefficient translation, posttranslational modification, incorrect folding and targeting to the cell membrane. The Mgt05196 transporter from *Meyerozyma guilliermondii* performed almost equal to the GAL2 with fermentation of the strains on xylose as sole carbon source, but the Mgt05196 indicated a higher preference for xylose than glucose (Wang et al. 2015). Molecular modification of this transporter resulted in a mutant that was able to transport xylose without glucose inhibition, making this the most promising xylose transporter to date (Wang et al. 2015).

The use of a native transporter is a promising alternative to circumvent the problems associated with the expression of foreign transporters. The GAL2 is the most efficient endogenous xylose transporter in *S. cerevisiae*, but also act as a high-affinity glucose transporter (Wang et al. 2015). The competitive inhibition of the GAL2 (and other native hexose transporters) prevents simultaneous utilisation of glucose and xylose since xylose is only transported upon depletion of glucose. GAL2 mutants have recently been generated (through random mutagenesis) with enhanced uptake capacity for xylose and decreased glucose affinity, resulting in improved co-consumption of glucose and xylose (Reznicek et al. 2015). In addition, xylose metabolism shares the lower part of the glycolysis pathway with glucose metabolism. In the absence of glucose, the G6P levels remain low because the xylose pathway links onto F6P and GAP. GAP, however, is required for the induction of the lower glycolysis enzymes for improved flux (Müller et al. 1995), hence the need for co-consumption of glucose and xylose.

Mutants deficient in MTH1, showed constitutive expression of the major hexose transporters due to the prevention of binding of the RGT1 to the promoter areas (Kim et al. 2013a). Therefore mutation in the *MTH1* gene should therefore benefit both glucose and xylose transport. The YCK1 phosphorylates MTH1, which results in targeted degradation of MTH1 (Fig. 2.7). Therefore, the overexpression of the *YCK1* gene should also result in constitutive expression of the hexose transporters. IRA1 and IRA2 are negative regulators of RAS1 (the cAMP-PKA) and therefore, mutations in these genes result in the activation of RAS signalling (Tamanoi 2011) which should also benefit transport.

2.4.6 Conversion of xylose to xylulose

Xylulose, the isomer of xylose, can be metabolised by *S. cerevisiae*. As a result, efforts for the expression of heterologous pathways in *S. cerevisiae* seem to be ideal for the development of xylose-assimilating yeasts. Over the past three decades, research focussed on the development of recombinant xylose-utilising *S. cerevisiae* strains through the heterologous expression of one of two biochemical pathways for the conversion of xylose to xylulose (Fig. 2.9).

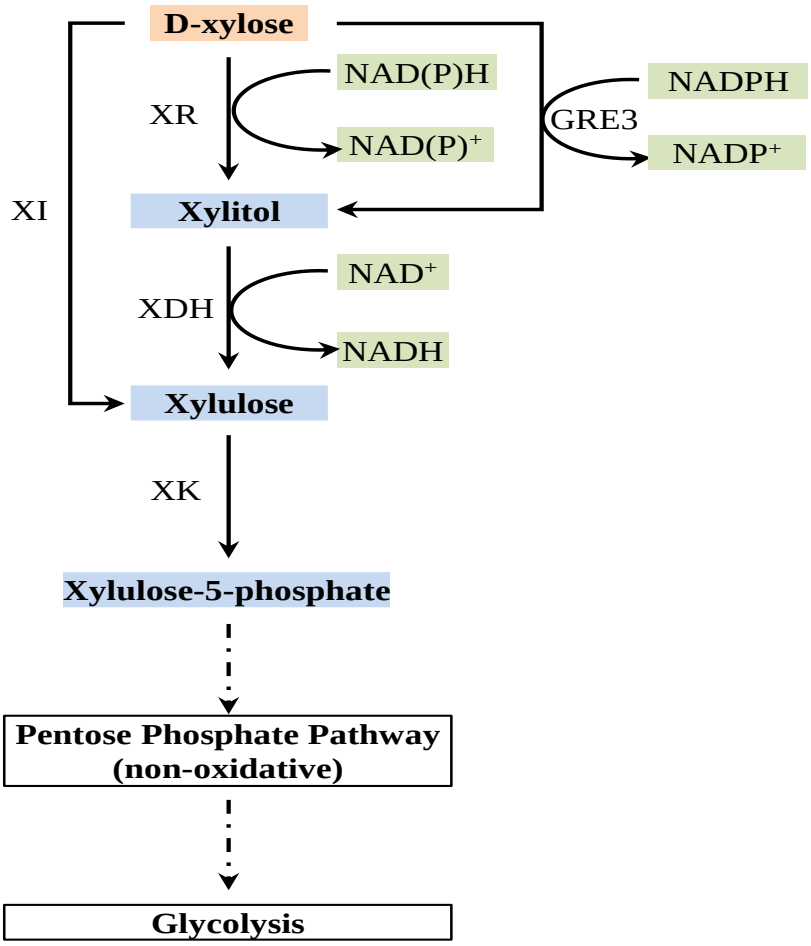

Fig. 2.9 Schematic representation of the pathways employed for xylose conversion to xylulose 5-phosphate, which then enters the PPP and glycolysis. (adapted from Dellomonaco et al. 2010; Kricka et al. 2014).

2.4.6.1 Xylose reductase and xylitol dehydrogenase pathway

Common to eukaryotic organisms, this pathway involves the reduction of xylose to xylitol via xylose reductases (XR, EC 1.1.1.21) (Bolen et al. 1986; Rizzi et al. 1988) and the subsequent oxidation of xylitol to xylulose via xylitol dehydrogenases (XDH, EC 1.1.1.9) (Fig. 2.9). Although XR generally has dual coenzyme specificity for NADPH and NADH, its preference favours NADPH (van Maris et al. 2007). In addition, XDH is strictly NAD^+-dependant. This mismatch in cofactor specificities results in a "cofactor imbalance" whereby $NADP^+$ and NADH accumulate (and NADPH and NAD^+ are depleted). The accumulation of NADH is especially problematic under industrially relevant anaerobic conditions. NADH cannot be efficiently re-oxidised to NAD^+ without oxygen as a terminal electron acceptor, thus severely inhibiting xylose metabolism (Bruinenberg et al. 1983; Bruinenberg et al. 1984).

The redox imbalance generally has the consequence of excess xylitol and even glycerol production under anaerobic conditions, compromising ethanol yield. However, in the presence of oxygen, some of the xylose can be directed via the oxidative PPP to regenerate NADPH. This, however, results in the loss of carbon as CO_2 and consequently lowers ethanol yields (Bruinenberg et al. 1983; Jeffries and Jin 2004; van Maris et al. 2006). In spite of the problems associated with redox imbalance, the XR/XDH strategy has been used with much success in the construction of xylose-assimilating *S. cerevisiae* strains (Ho et al. 1998; Kötter and Ciriacy 1993; Jeppsson et al. 2002; Sonderegger and Sauer 2003; Jeffries and Jin 2004).

The GRE3 is the nonspecific aldose reductase that reduces xylose to xylitol, using NADPH as cofactor. Deletion of the *GRE3* gene has proven advantages due to the removal of the principal native route of xylitol production (Träff and Cordero 2001). Therefore, overexpression of the *GRE3* (as a replacement of the xylose reductase) has been regarded as an undesirable approach because the NADPH requirement would aggravate redox imbalance with xylitol dehydrogenases using NAD^+. Yet, Kim et al. (2013b) demonstrated that replacement of the xylose reductase with overexpression of *GRE3* in XR/XDH *S. cerevisiae* strains resulted in higher ethanol yields and productivities with cultivation on xylose and a mixture of glucose and xylose under oxygen-limited conditions. The study suggests that the xylitol accumulation might be due to insufficient XDH activity and not the redox imbalance. The redox imbalance was recently addressed by overexpression of the NADPH-dependent *GRE3* gene with the $NADP^+$-dependent *XDH* (*S. stipitis*), resulting in an increase in ethanol yield and reduction in

xylitol formation compared to the reference strain (Khattab and Kodaki 2014). This improvement is attributed to the effective regeneration of NADPH/$NADP^+$ cofactors.

2.4.6.2 Xylose isomerase pathway

The xylose isomerase pathway is common to most prokaryotic organisms and involves the direct isomerisation of xylose to xylulose via xylose isomerases (XI, EC 5.3.1.5) (Fig. 2.9) (Chen 1980). XIs do not require pyridine nucleotide cofactors, but do require divalent cations such as Co^{2+}, Mn^{2+} and Mg^{2+} (Bhosale et al. 1996). Regardless of the difficulties involved in expressing XIs in *S. cerevisiae*, this strategy has the major advantage of evading redox imbalance complications observed with XR/XDH metabolism.

Several fungal and bacterial xylose isomerases have been cloned into *S. cerevisiae* to date, but many resulted in the expression of malfunctioning enzymes (Briggs et al. 1984; Sarthiy et al. 1987; Amore et al. 1989; Moes et al. 1996; Schründer et al. 1996; Gárdonyi and Hahn-Hägerdal 2003). In several cases, putative transcripts were detected with northern blot analysis, but putative XI protein products were insoluble and inactive (Sarthiy et al. 1987; Amore et al. 1989; Gárdonyi and Hahn-Hägerdal 2003). It has been speculated that differences in internal pH amongst bacteria and yeasts, incorrect enzyme folding, inter/intra-molecular disulphide bridge formation and the absence of essential cofactors or ions, could be features contributing towards the non-functional expression of this enzyme. Since the first reported functionally expressed XI in *S. cerevisiae*; sourced from the extreme thermophilic bacterium *Thermus thermophiles*, (Walfridsson et al. 1996); research efforts has further identified a few more promising XIs (Table 2.1) (Harhangi et al. 2003; Kuyper et al. 2003; van Maris et al. 2007; Brat et al. 2009; Madhavan et al. 2009; Zha et al. 2014).

2.4.7 The pentose phosphate pathway

The pentose phosphate pathway (PPP) is virtually present in all forms of life and has a central role to play in carbon metabolism. The biochemical reactions of this pathway can be divided into two sections, the oxidative branch and the non-oxidative branch. The oxidative branch converts the glycolytic intermediate, G6P, to ribulose 5-phosphate (Ru5P) via the sequential reactions of glucose 6-phosphate dehydrogenase (G6PDH/SWF1), 6-phosphoglucono-lactonase (6PGL) and 6-phosphogluconate dehydrogenase (6PGDH) (Figure 2.10). The

biochemical reactions of the oxidative branch leads to the synthesis of two NADPH per G6P metabolised, which is believed to be the major source of cellular reducing power (Nogae and Johnston 1990; Stincone et al. 2014).

Table 2.1 Reported specific activity of recombinant xylose isomerases secreted by *Saccharomyces cerevisiae* and maximum specific growth rate of the strains. (adapted from Parachin and Gorwa-Grauslund 2011).

Source of XI	**Activity (U/mg protein)**	**Aerobic growth rate on xylose (h^{-1})**	**Identity with *Piromyces* XI**
Metagenome *xym1*[a]	0.33 ± 0.05	0.02	61%
Metagenome *xym2*[a]	0.20 ± 0.04	0.02	63%
Piromyces sp.[a,b]	0.35	0.07	100%
Piromyces sp.	0.3 to 1.1	0.22	100%
Piromyces sp.[b]	0.5 to 0.8	0.02	100%
Piromyces sp.[b]	0.05	0.056	100%
Orpinomyces sp.	1.6	0.01	94%
Xanthomonas campestris	0.016	0.02	61%
Bacteroides thetaiotaomicron[c]	0.14	n.m.	83%
Clostridium phytofermentans[b]	0.03	0.057	54%
Clostridium thermosulfurogenes	n.m.	n.d.	49%
Escherichia coli	n.m.	n.d.	48%
Bacillus subtilis	n.m.	n.m.	47%
Lactobacillus pentosus	n.d.	n.d.	46%
Thermus thermophilus	0.0007 to 0.012	n.m.	29%
Prevotella ruminicula[c]	0.81	n.m.	n.m.
Metagenome *Ru-xylA*[c]	1.31	n.m.	n.m.
Metagenome *Ru-xylA*[d]	1.45	n.m.	n.m.

n.m., not measured; n.d., not detected. Sequence identity with *Piromyces* XI gene is also given at the protein level. [a]Glucose-grown cells, [b]Codon-optimised gene, [c](Hou et al. 2015), [d]mutated gene.

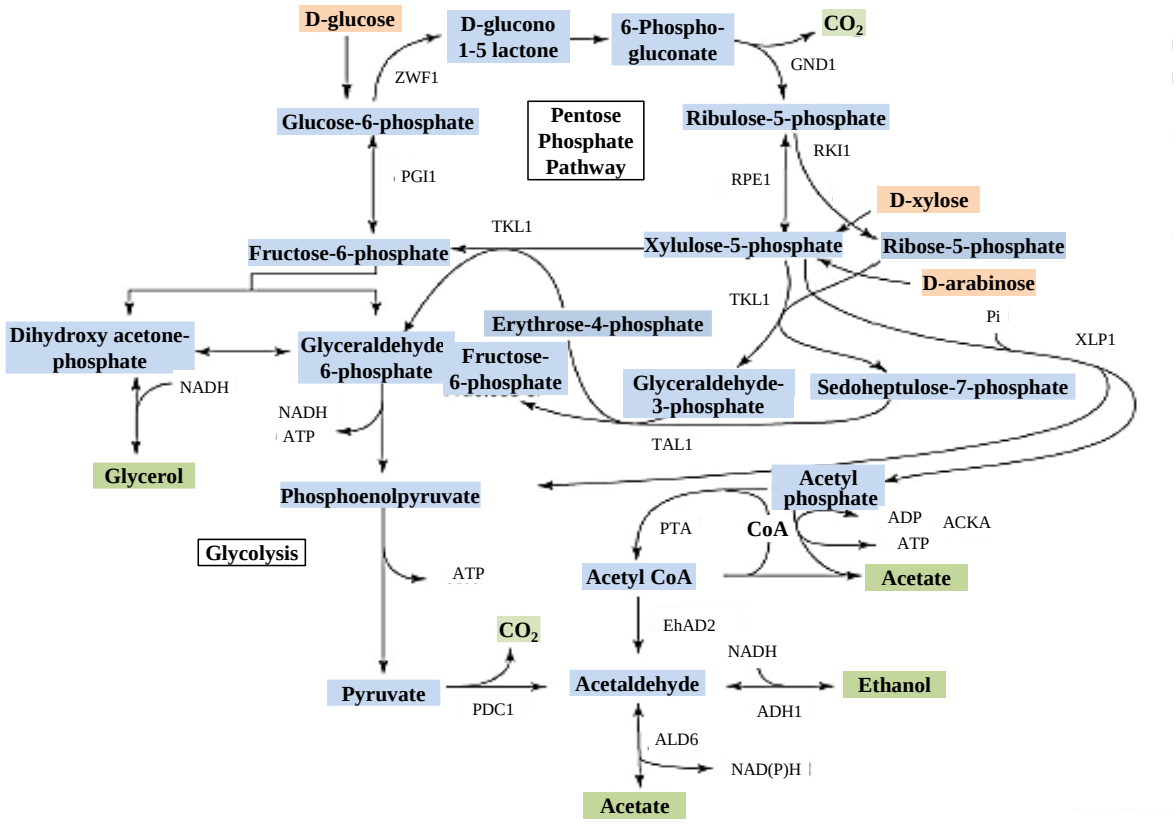

Fig. 2.10 The schematic representation of the carbon flow from xylose through the PPP and glycolysis. (adapted from Jeffries 2006).

The Ru5P is channeled into the reactions of the non-oxidative branch where it is converted to either ribose 5-phosphate (R5P) via ribose 5-phosphate isomerase (RPI) or xylulose 5-phosphate (Xu5P) via ribulose 5-phosphate epimerase (RPE). R5P is an important precursor for the synthesis of RNA/DNA molecules. Further downstream reactions involving the multi-substrate transketolase (TKL) and transaldolase (TAL) enzymes forms the core of the non-oxidative PPP where several carbon reshuffling reactions take place (Stincone et al. 2014). The TKL catalysis the transfer of two-carbon fragments from a ketose donor to an aldose acceptor to produce aldose and ketose products. Thus, for the first two reactions of the non-oxidative PPP, TKL cleaves off a two-carbon fragment (glycolaldehyde moiety) from X5P (resulting in the three-carbon aldose product GAP) and transfers it to R5P to produce the seven-carbon ketose product sedoheptulose 7-phosphate (S7P).The xylulose 5-phosphate enters the non-oxidative PPP and is converted by the TKL and TAL to F6P and GAP, which may then further be metabolised via glycolysis (Braus 1991; Stincone et al. 2014). The pathway is in principle identical to glucose metabolism.

Glucose induces a strong carbon catabolite repression (CCR) response in *S. cerevisiae* (Wills 1990), whereas xylose fails to fully activate the CCR (Jin et al. 2004; Salusjärvi et al. 2008). The G6P is produced from glucose and is believed to play a role in the induction of the genes of the lower glycolysis pathway (Müller et al. 1995). The lack of induction (when glucose is absent) results in the accumulation of GAP due to a downstream bottle neck. The GAP is then channelled into glycerol. Glycerol production was ascribed to the redox imbalance that is generated in strains carrying the XR/XDH pathway. Yet, GAP will still accumulate in strains carrying the XI due to the sub-optimal flux through the lower glycolysis, emphasising the need for co-cultivation on xylose and glucose.

The capacity of the non-oxidative PPP in *S. cerevisiae* is understood to be a limiting factor for the metabolism of xylose via the PPP (Fiaux et al. 2003). This limiting factor has been attributed as the main reason that S7P accumulates during xylulose fermentations, instead of producing glycolytic intermediates (Ciriacy and Porep 1986; Senac and Hahn-Hägerdal 1990). Several efforts have been made to develop recombinant xylose-utilising *S. cerevisiae* strains with enhanced non-oxidative PPP capacity. Overexpression of native TAL in recombinant XR/XDH *S. cerevisiae* showed enhanced growth on xylose, however, overexpression of native TKL had no influence, suggesting that conversion of sedoheptulose 7-phosphate to glycolytic intermediates instead of synthesis of sedoheptulose 7-phosphate, is limiting the capacity of the non-oxidative PPP (Walfridsson et al. 1995). In later experiments, overexpression of all four native non-oxidative PPP enzymes (*RPI1*, *RPE1*, *TAL1*, *TKL1*) resulted in enhance growth on xylulose, but had no effect on xylose metabolism by a recombinant XR/XDH *S. cerevisiae* (Johansson and Hahn-Hägerdal 2002). Furthermore, the authors suggest that the PPP only controls xylulose fermentation, but it would seem more as if other factors such as xylose transport, XR and/or XDH resulted in the data indicating no enhanced PPP capacity during xylose fermentations. In contrast, work done by Zaldivar et al. (2002) showed no accumulation of S7P in a recombinant XR/XDH *S. cerevisiae*. However, industrial strains with additional sets of chromosomes were used in the study and could potentially be the reason for non-limiting conversion of xylose to xylulose and subsequent high non-oxidative PPP capacity.

2.5 THIS STUDY

Fossil fuel supply is controlled by a relatively small number of countries (Ten and Vermerris 2013) who generate large revenues for the oil and gas industry and tax revenues to the governments involved. Bioethanol can be produced simultaneously from cellulose and hemicellulose hydrolysis and fermentation (Öhgren et al. 2007) if the pretreatment step could be such that it maximises the sugar yield by improving hemicellulose and cellulose accessibility to hydrolytic enzymes while avoiding substrate degradation (Öhgren et al. 2007). Cellulolytic yeast strains have already been constructed (la Grange et al. 2010), therefore the first part of the study involved the construction of a xylan-utilising *S. cerevisiae* strain through the co-expression of the endoxylanase (*xyn2*) of *Trichoderma reesei*, the β-xylosidase (*xlnD*) of *Aspergillus niger*, the *Scheffersomyces stipitis* xylulose kinase (*xyl3*) together with the codon-optimised xylose isomerase (*xylA*) from *Bacteroides thetaiotaomicron*. The native non-specific aldose reductase (*GRE3*) was deleted to ensure minimal production of xylitol.

This study focussed on the construction of a xylose- and xylan-utilising *S. cerevisiae* strain and its evaluation on xylan as sole carbohydrate source. A diploid xylose utilising *S. cerevisiae* strain has been constructed for the analysis of the intracellular metabolite pools generated at different time points during the cultivation on glucose and xylose. The aim was to reveal novel physiological responses to glucose and xylose metabolism and consequently aid our understanding of how xylose metabolism is regulated.

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CHAPTER 3

Engineering of *Saccharomyces cerevisiae* to utilize xylan as a sole carbohydrate source by co-expression of an endoxylanase, xylosidase and a bacterial xylose isomerase

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Engineering of *Saccharomyces cerevisiae* to utilize xylan as a sole carbohydrate source by co-expression of an endoxylanase, xylosidase and a bacterial xylose isomerase

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3.1 ABSTRACT

Xylan represents a major component of lignocellulosic biomass, and its utilization by *Saccharomyces cerevisiae* is crucial for the cost effective production of ethanol from plant biomass. A recombinant xylan-degrading and xylose-assimilating *Saccharomyces cerevisiae* strain was engineered by co-expression of the xylanase (*xyn2*) of *Trichoderma reesei*, the xylosidase (*xlnD*) of *Aspergillus niger*, the *Scheffersomyces stipitis* xylulose kinase (*xyl3*) together with the codon-optimized xylose isomerase (*xylA*) from *Bacteroides thetaiotaomicron*. Under aerobic conditions, the recombinant strain displayed a complete respiratory mode, resulting in higher yeast biomass production and consequently higher enzyme production during growth on xylose as carbohydrate source. Under oxygen limitation, the strain produced ethanol from xylose at a maximum theoretical yield of ~90%. This study is one of only a few that demonstrates the construction of a *S. cerevisiae* strain capable of growth on xylan as sole carbohydrate source by means of recombinant enzymes.

Keywords xylan degradation · hemicellulose · xylose isomerase · bioethanol · *S. cerevisiae*

3.2 INTRODUCTION

Alternative fuels need to be generated to complement or replace fossil based fuels in order to meet the growing energy demands of the world. One such alternative, bioethanol, has been the focus since the early 70s due to its contribution to energy security and its mostly positive environmental impact, with countries such as Brazil and the USA being the world's biggest producers of bioethanol. Microbial fermentation of lignocellulose (including agricultural residues, municipal solid waste, wood and dedicated energy crops) remains one of the most attractive options for the production of bioethanol [7, 43]. However, a high percentage of lignocellulose (fermentable carbon) conversion to ethanol is required for financial feasibility of bioethanol technologies implying that cellulose conversion alone will not be sufficient.

Xylan constitutes the major fraction of hemicellulose and can comprise up to 35% of the total dry weight of plants [12, 24]. Xylan is a heteropolysaccharide consisting of a β-D-1,4-linked xylopyranoside backbone, substituted with various sugar units and *O*-acetyl groups [12]. Enzymatic hydrolysis of xylan requires at least an endo-β-xylanase and a β-d-xylosidase, which hydrolyses xylan to free xylose units and substituent-linked xylooligos. Xylan-degrading *S. cerevisiae* strains have been reported that produce xylan-degrading enzymes extracellularly (free enzymes) and cell wall tethered [14, 18, 30, 34]. Prior to microbial fermentation, pretreatment techniques are applied to the lignocellulose which yields water insoluble solids (WIS) containing mostly cellulose and a liquid fraction containing mostly xylose, oligosaccharides and furans [35]. The baker's yeast *S. cerevisiae* is well suited for bioethanol production and would therefore be ideal for the fermentation of pretreated lignocellulosic fractions. Unfortunately, this yeast is incapable of degrading xylan and cannot metabolize xylose.

The development of xylose-assimilating *S. cerevisiae* strains have been achieved largely through the installation of the fungal oxidoreductive pathway using xylose reductase (XR) to reduce xylose to xylitol and subsequently xylitol dehydrogenase (XDH) which oxidizes xylitol to xylulose [28]. Xylulose is then phosphorylated to xylulose 5-phosphate via a xylulokinase [29] and is converted into glyceraldehyde 3-phosphate and fructose 6-phosphate via the pentose phosphate pathway (PPP). XR and XDH have different nucleotide co-factor preferences, NADPH and NAD respectively, which results in a redox imbalance in XR/XDH strains and leads to the accumulation of xylitol and glycerol under oxygen limitation [2, 10, 17, 39]. As a result, the bacterial pathway has been considered more redox profitable since it converts xylose

to xylulose in a single enzymatic reaction using a xylose isomerase (XI), which does not require nucleotide cofactors [40]. Early attempts at functional expression of xylose isomerases in *S. cerevisiae* have failed. Walfridsson et al. [42] was the first to report the functional expression of a xylose isomerase (thermophilic bacterium, *Thermus thermophiles*) in *S. cerevisiae*. More recently, xylose isomerases from bacteria [1, 9, 16, 40] and fungi [8, 16, 23] have been functionally expressed in *S. cerevisiae*, with various degrees of success.

In this study, a xylan utilizing strain of *S. cerevisiae* had been constructed by co-expressing the *Trichoderma reesei* endoxylanase encoding gene (*xyn2*) [18], the *Aspergillus niger* xylosidase encoding gene [18], the *Bacteroides thetaiotaomicron* xylose isomerase encoding gene (*xylA*) [5] and the *Scheffersomyces stipitis* xylulose kinase gene (*xyl3*) [11]. Furthermore, to ensure minimal production of xylitol, the native *S. cerevisiae* non-specific aldose reductase (*GRE3*) was deleted [36]. The *S. cerevisiae* Y294[XYN2 XLO2] strain [18], expressing the *xlnD* and *xyn2* genes, were used as benchmark. This study demonstrated growth on xylose and xylan as well as ethanol production on xylose under anaerobic conditions.

3.3 MATERIALS AND METHODS

3.3.1 Media and culture conditions

Bacterial cultivation (37°C) took place in Luria-Bertani liquid medium or agar (5 g/L yeast extract, 10 g/L NaCl and 10 g/L tryptone) supplemented with 100 µg/mL ampicillin for selection. *S. cerevisiae* transformants were initially plated onto synthetic complete (SC) medium (3.4 g/L yeast nitrogen base without amino acids [Difco], 5 g/L $(NH_4)_2SO_4$, 20 g/L glucose and yeast synthetic dropout medium supplements [Sigma-Aldridge, Steinheim, Germany]). Liquid cultivation took place at 30°C on an orbital shaker at 200 rpm and *S. cerevisiae* strains were routinely cultured in YP medium (10 g/L yeast extract, 20 g/L peptone) containing either 20 g/L glucose (YPD) or 20 g/L xylose (YPX) at 30°C.

3.3.2 Strains and plasmid constructs

The relevant genotypes of all the bacterial and yeast strains, along with the plasmids used in this study, are listed in Table 3.1.

Table 3.1 Microbial strains and plasmids used in this study

Strains or plasmids	Genotype	Source/ Reference
Strains		
E. coli DH5α	*supE44 ΔlacU169 (ϕ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Life Technologies, Rockville, Md.
S. cerevisiae Y294	*α leu2-3,112 ura3-52 his3 trp1-299*	ATCC 201160
S. cerevisiae Y294[YMXR]	*bla URA3 fur1::LEU2 gre3::TRP1*; $ADH2_{PT}$	This study
S. cerevisiae Y294[YMX1]	*bla Sh Ble URA3 fur1::LEU2 gre3::TRP1*; $ADH2_P$-*xlnD*-$ADH2_T$[a]; *URA3* $ADH2_P$-*xyn2*-$ADH2_T$[a]; $ENO1_P$-*xylA*-$ENO1_T$[b]; $ENO2_P$-*xyl3*-$ENO2_T$[b]	This study
S. cerevisiae Y294[XYN2 XLO2]	*bla URA3 fur1::LEU2 gre3::TRP1* $ADH2_P$-*xlnD*-$ADH2_T$; $ADH2_P$-*xyn2*-$ADH2_T$[b]	[18]
S. cerevisiae Y294[YMX1-A1]	*bla Sh Ble URA3 fur1::LEU2 gre3::TRP1*; $ADH2_P$-*xlnD*-$ADH2_T$[a]; *URA3* $ADH2_P$-*xyn2*-$ADH2_T$[a]; $ENO1_P$-*xylA*-$ENO1_T$[b]; $ENO2_P$-*xyl3*-$ENO2_T$[b]	This study
S. cerevisiae Y294[YMX1-A2]	*bla Sh Ble URA3 fur1::LEU2 gre3::TRP1*; $ADH2_P$-*xlnD*-$ADH2_T$[a]; *URA3* $ADH2_P$-*xyn2*-$ADH2_T$[a]; $ENO1_P$-*xylA*-$ENO1_T$[b]; $ENO2_P$-*xyl3*-$ENO2_T$[b]	This study
S. cerevisiae Y294[YMX1-A3]	*bla Sh Ble URA3 fur1::LEU2 gre3::TRP1*; $ADH2_P$-*xlnD*-$ADH2_T$[a]; *URA3* $ADH2_P$-*xyn2*-$ADH2_T$[a]; $ENO1_P$-*xylA*-$ENO1_T$[b]; $ENO2_P$-*xyl3*-$ENO2_T$[b]	This study
Plasmids		
YEp352	*bla URA3*	ATCC® 37673™
pDLG1	*bla URA3* $ADH2_{PT}$	[18]
pDLG56	*bla URA3* $ADH2_P$-*xyn2*-$ADH2_T$; $ADH2_P$-$MF\alpha1_s$-*xlnD*-$ADH2_T$	[18]
pDLG115	*bla δ-site* $ENO1_P$-*xylA*-$ENO1_T$; $ENO2_P$-*xyl3*-$ENO2_T$ *KanMX δ-site*	This study
pMJM121	*bla δ-site URA3* $ENO1_P$-*xylA*-$ENO1_T$ *Sh ble* $ENO2_P$-*xyl3*-$ENO2_T$ *δ-site*	This study
pPICZ_A	*bla Sh ble*	Invitrogen™

[a] δ-integrated expression, [b] Episomal multicopy expression

3.3.3 Plasmid construction

Standard protocols were followed for DNA manipulations [31]. The restriction enzymes and T4-ligase were purchased from Rosche Applied Science (Germany) and used according to manufacturer's instructions. Zymoclean Gel Recovery Kit (Zymo Research Corporation, USA) was used to elute DNA from agarose gel. Polymerase chain reactions (PCR) were carried out with a Perkin Elmer GeneAmp® PCR System 2400 (Perkin Elmer, USA) using Phusion DNA polymerase (Finnzymes) according to the supplier's specifications. The *B. thetaiotaomicron xylA* (Accession number AA075900.1) has previously been codon optimized and synthetically manufactured (GenScript Corporation) for expression in *S. cerevisiae* [4]. The *xylA* and the *xyl3* were cloned onto the YEp352 backbone to produce pDLG115 (Table 1). The *Sh ble* gene was amplified from pPICZ_A by means of PCR using the oligo primers ZCN-R (5′-GATCGGATCCT AATTCAGCTTGCAAATTAAA GCC-3′) and ZCN-L (5′-CTAGGGATCCGAATTCC CCACACACCATAGC-3′) and subsequently cloned into the *Eco*RI and *Spe*I sites of pDLG115, to create pMJM121.

3.3.4 Yeast transformations

The laboratory *S. cerevisiae* Y294 strain was transformed with the recombinant plasmids using electroporation as described by Cho et al. [3]. The pMJM121 was digested with *Cla*I to produce the expression cassette and *Sh ble* gene fragment flanked by the delta (δ) DNA sequences. This DNA fragment was then conferred to Y294[*XYN2 XLO2*] to generate *S. cerevisiae* Y294[YMX1]. Over-expression of the relevant cloned genes was facilitated by integration of the expression cassettes via homologous recombination with native δ-sequences distributed throughout the yeast genome [21]. Transformants were selected for growth on YPD plates containing 200 mg/L zeocin. The reference strain, *S. cerevisiae*Y294[YMXR] was generated by transferring pDLG1 to *S. cerevisiae* Y294. All relevant strains had been transformed to disrupt the *FUR1* and *GRE3* genes with the *LEU2* [18] and the *TRP1* marker genes, respectively [36].

3.3.5 Strain adaptation

The *S. cerevisiae* Y294[YMX1] strain was cultivated in YPX medium until the stationary phase was reached. Serial-transfer cultivation was maintained for ~200 generations (3 months) in which 5 µl of the culture was repeatedly transferred to fresh YPX medium. Samples of the culture were taken after ~60 generations (1 month) (*S. cerevisiae* Y294[YMX1-A1]), ~120 generations (2 months) (*S. cerevisiae* Y294[YMX1-A2]) and ~200 generations (3 months) (*S. cerevisiae* Y294[YMX1-A3]) of adaptation and stored as cryocultures. The *S. cerevisiae* Y294[YMX1-A3] strain was streaked out and single colonies isolated and evaluated for growth on YPX.

3.3.6 Enzyme activity assays

The endoxylanase activity was determined using the reducing sugar assay according to la Grange et al. [19]. The XylA activity was determined by harvesting *S. cerevisiae* Y294[YMX1] after 48-72 h of cultivation in 100 mL YPD. The cells were washed twice with 0.5 volumes cold 50 mM N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES)/NaOH (pH 7.5) before resuspending in 2 mL of the same buffer. Mechanical disruption of the cells was achieved using 0.4 µm glass beads and a FastPrep FP120 (Savant) at a speed of 50 oscillations/sec for 3 × 20 s with 1 min cooling intervals on ice. Cell debris was removed by centrifugation at 17,000×*g* for 10 min at 4°C and the supernatant kept on ice.

Xylose isomerase activity assays were performed according to the resorcinol method described by Kulka [15] and modified as described by Schenk and Bisswanger [33]. The substrate was prepared in 50 mM citrate buffer (pH 7) and the assay performed at various temperatures to determine the temperature preference of the enzyme. The optimum pH was determined by using substrate prepared in 50 mM citrate buffer at varying pH values.

3.3.7 SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 7.5% polyacrylamide gel according to the method described by Laemmli [20]. Intracellular protein samples were prepared for electrophoresis by adding 5 µL of cell extract to 7 µL loading buffer (60 mM Tris.HCl (pH 6.8), 25% glycerol, 2% SDS, 0.1% bromophenol blue, and 14 mM

β-mercaptoethanol) and subsequently boiled at 100°C for 3 min before loading the gel. After electrophoresis at 120 V, the gel was washed with dH_2O and subsequently stained with Coomassie brilliant blue R-250 (Bio-Rad) to visualize the protein species.

Zymogram analysis was carried out by the method described by Sapunova et al. [32]. Intracellular protein samples were prepared for electrophoresis as described above, with the exception that the loading buffer lacked β-mercaptoethanol. The samples were incubated at 80°C for 3 min prior to loading the gel. After electrophoresis at 120 V, the gel was washed with dH_2O and subsequently submerged in 50 mM TES buffer (pH 7.5) containing 10 mM $MgSO_4 \cdot 7H_2O$ and 100 mM xylose for 20 min. The gel was washed with dH_2O and submerged in 0.1% 2,3,5-triphenyltetrazolium chloride solution in 1 M NaOH at 30°C for 1 min in darkness. The xylulose, product of the xylose isomerization reaction, oxidized the colorless 2,3,5-triphenyltetrazolium chloride to formazan, which results in a pink-red band on the polyacrylamide gel.

3.3.8 Aerobic growth on glucose and xylose as sole carbohydrate source

The recombinant strains were precultured in 5 mL of YPD at 30°C overnight and inoculated into 100 mL of YPD or YPX in 500 mL baffled Erlenmeyer flasks to an initial optical density of 0.1 at OD_{600}. Samples were taken at regular intervals. Cell growth was measured spectrophotometrically at 600 nm. To obtain dry cell weight (DCW) measurements, calibration curves were setup that correlated OD_{600} with DCW. The relevant extracellular metabolites (ethanol, glycerol, xylitol and xylose) were quantified using HPLC. All shake flask experiments were performed on an orbital shaker at 200 rpm.

3.3.9 Anaerobic growth on xylose as the sole carbohydrate source

The recombinant strains were precultured in 5 mL of YPD at 30°C overnight and inoculated into 100 mL of YPX in 120 mL serum bottles to an initial OD_{600} of 0.5. The anaerobic fermentation took place at 30°C with shaking at 200 rpm with regular sampling over a period of 40 h. Cell growth was measured spectrophotometrically and converted to DCW, and the relevant extracellular metabolites (ethanol, glycerol, xylitol and xylose) quantified using HPLC, as described above.

3.3.10 Aerobic growth on xylan as the sole carbohydrate source

A 10% (v/v) preculture (overnight culture in YPD) was used to inoculate 50 mL YPBX medium (10 g/L yeast extract, 20 g/L peptone, 50 g/L of Beechwood xylan as the sole carbohydrate source). Samples were obtained over a period of 28 days and cell numbers determined with the aid of a haemocytometer. Xylose, xylitol, glycerol, acetic acid, ethanol, xylotriose, xylobiose and oligosaccharide peaks [aldobiuronic acid (U4m2X) and aldotriuronic acid (U4m2XX)] were noted.

3.3.11 Analysis of extracellular metabolites

The concentrations of glucose, xylose, ethanol, glycerol, xylitol and acetic acid were determined using high performance liquid chromatography (HPLC), with a Waters 717 injector (Milford, MA, USA) and Agilent 1100 pump (Palo Alto, CA, USA). The compounds were separated on an Aminex HPX-87H column (Bio-Rad, Richmond, CA), at a column temperature of 60°C with 5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 mL/min and subsequently detected with a Waters 410 refractive index detector.

The concentrations of xylobiose, xylotriose, aldotriuronic acid and aldobiuronic acid were determined using a Dionex Ultimate 3000 system equipped with a Coulochem III electrochemical detector with a working gold electrode operating in the pulsed amperometric mode. Separation of the oligosaccharides was achieved by elution on a CarboPac PA1 (4 × 250 mm) analytical column coupled to a PA1 (4 × 50 mm) guard column and using 250 mM NaOH as mobile phase at a flow rate of 1 mL/min.

3.4 RESULTS

3.4.1 Strain construction

The *xylA* and *xyl3* genes were obtained by means of PCR and cloned onto expression vectors where expression was directed by the *ENO1* and *ENO2* promoter sequences, respectively. The *S. cerevisiae*Y294[YMX1] strain, was constructed to harbour the *xlnD* and *xyn2* genes on an episomal plasmid whereas the *xylA* and the *xyl3* were integrated into native δ-sequences (low copy expression). The *GRE3* (nonspecific aldose reductase) gene was disrupted, in order

to minimize the production of xylitol from xylose [36]. Autoselection of the *URA3* plasmid was obtained by disruption of the *FUR1* gene to allow for growth in complex media without the risk of losing the episomal plasmid [22]. The *S. cerevisiae* Y294[YMXR] reference strain lacked the *xlnD*, *xyn2*, and *xylA* genes and contained both *GRE3* and *FUR1* disruptions, whereas the *S. cerevisiae* Y294[XYN2 XLO2] strain harboured the *xlnD* and *xyn2* on an episomal plasmid with disrupted *GRE3* and *FUR1* genes.

3.4.2 Partial characterization of the B. thetaiotaomicron XylA

The recombinant XylA showed maximum activity at a temperature of 65°C (Fig. 3.1a) and pH 7.5 (Fig. 3.1b) using resorcinol as substrate. SDS-PAGE revealed a protein species of approximately 50 kDa corresponding to the predicted molecular weight of the XylA monomer (Fig. 3.1c). Under non-denaturing conditions, an approximate 110 kDa species is apparent. Furthermore, zymogram analysis verified the approximate 110 kDa species to be the active XylA dimer (Fig. 3.1d).

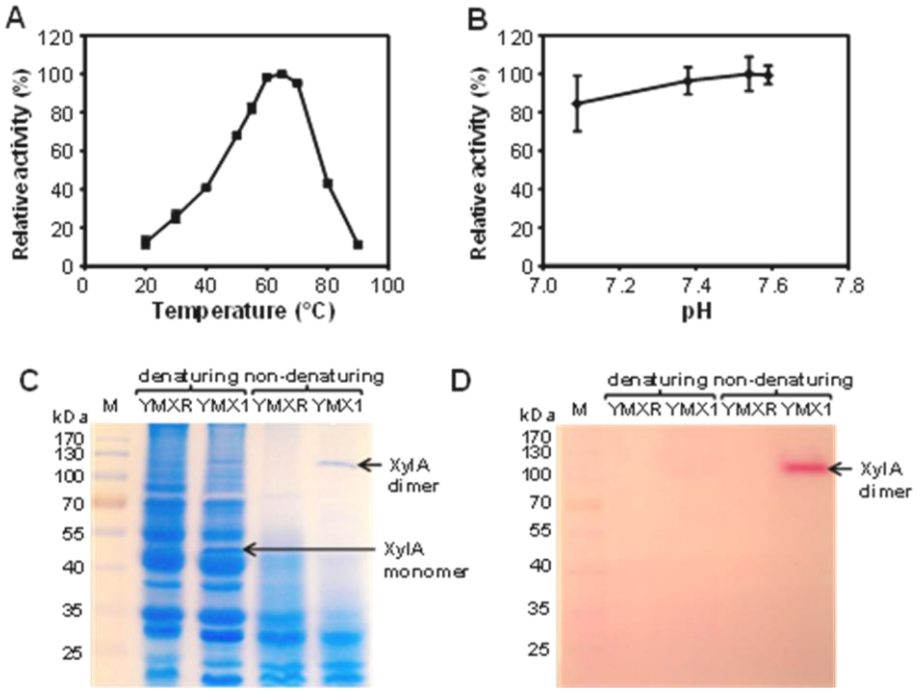

Fig. 3.1. The *B. thetaiotaomicron xylA* was expressed in *S. cerevisiae* Y294 and the (A) optimum temperature and (B) pH of the recombinant XylA determined using resorcinol as substrate. The (C) SDS-PAGE was performed on the intracellular protein fraction of the *S. cerevisiae* Y294[YMXR] and Y294[YMX1] strains with the corresponding (D) zymogram indicating the active XylA dimer. Lane M contains the protein molecular weight marker with the sizes depicted on the left hand side.

3.4.3 Aerobic growth on glucose and xylose

The *S. cerevisiae* Y294[YMXR] and *S. cerevisiae* Y294[YMX1] strains were cultivated in YPD and YPX. Growth and concentration of the relevant metabolites were monitored over time (Fig. 3.2a, b, c, d). Sustained growth was achieved by the recombinant *S. cerevisiae* Y294[YMX1] strain when xylose was used as the sole carbohydrate source, though it was evident that the growth rate on xylose was slow compared to growth on glucose (Fig. 3.2c, d). Low levels of xylitol were produced (~0.9 g/L) despite the deletion of the *GRE3*. A higher biomass yield was obtained during cultivation on xylose compared to cultivation on glucose.

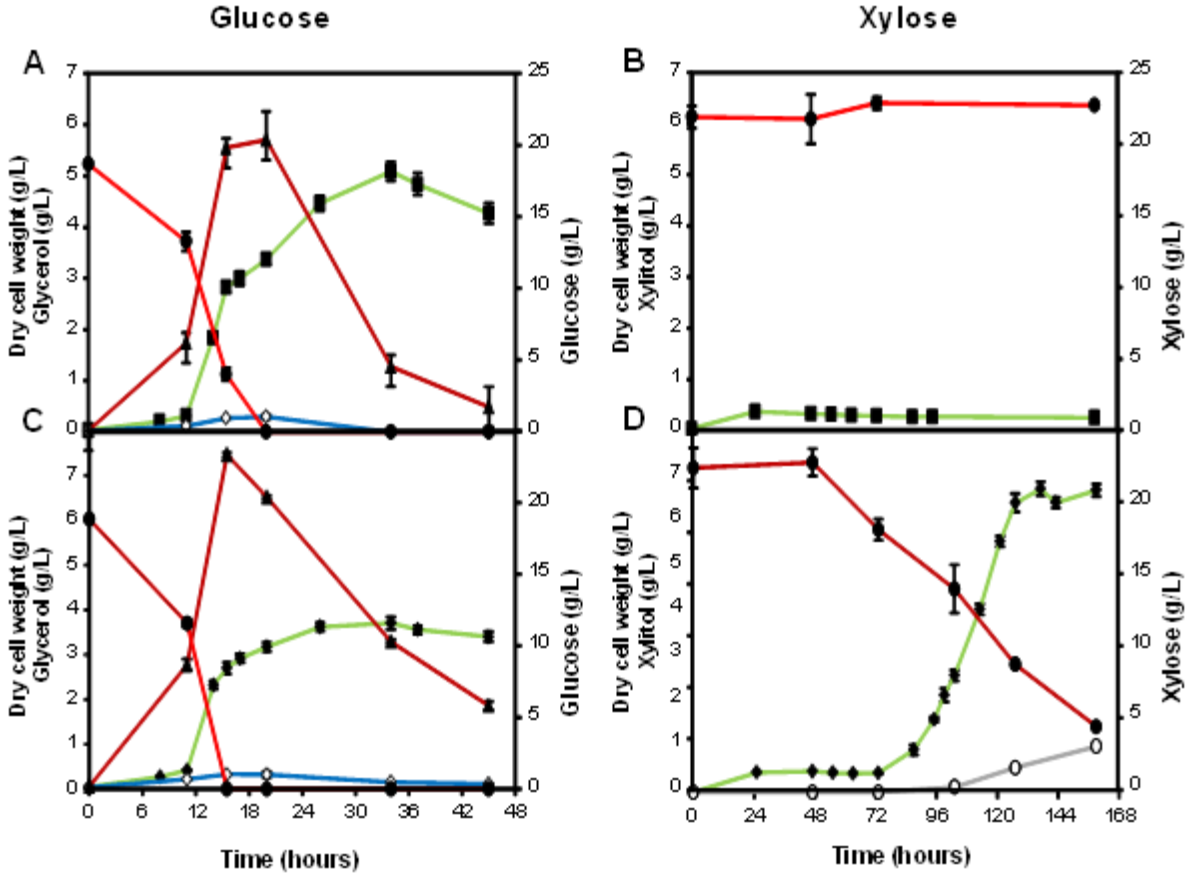

Fig. 3.2. The (A and B) (—■—) *S. cerevisiae* Y294[YMXR] and (C and D) (—♦—) *S. cerevisiae* Y294[YMX1] strains were cultivated on glucose (YPD, left hand side) and xylose (YPX, right hand side) as sole carbohydrate source under aerobic conditions. The extracellular (—▲—) ethanol, (—◊—) glycerol and (—○—) xylitol accumulation as well as the residual (—●—) glucose/ xylose was monitored over time.

The biomass yield of *S. cerevisiae* Y294[YMX1] at the end of 32 h on glucose (~ 0.21 g DCW/g glucose, Fig. 3.3a) was significantly lower than on xylose at 159 h (~0.52 g DCW/g xylose, Fig. 3.3b), similar to the results obtained in Fig. 3.2. A significant increase in Xyn2 activity was observed which corresponds with the increase in cell mass (Fig. 3.3c, d). The *S. cerevisiae* Y294[YMX1] strain produced ~198 and ~234 nkat/mL/g biomass on glucose after 32 h and xylose after 159 h, respectively. The Xyn2 activity was ~422 and ~404 nkat/mL/g biomass on glucose (after 82 h) and xylose (after 345 h), respectively.

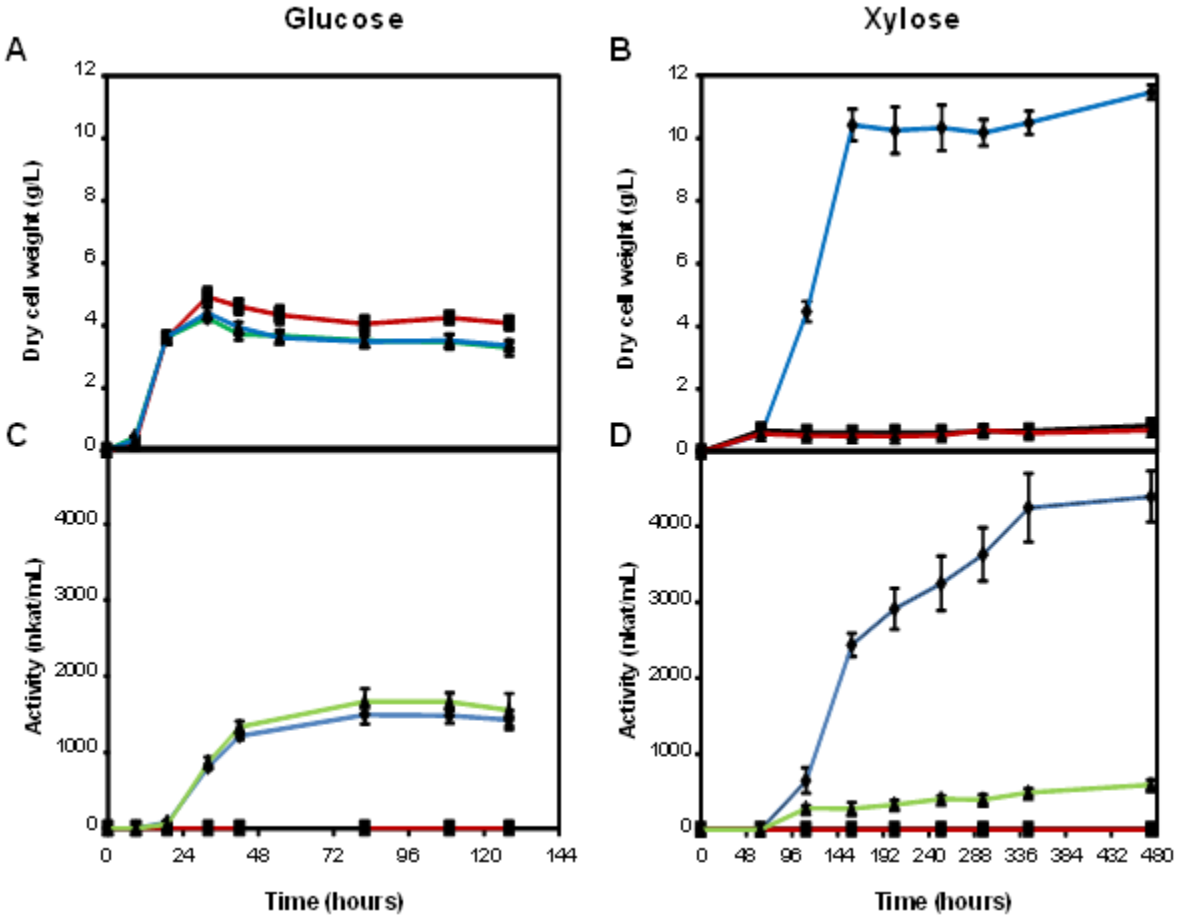

Fig. 3.3. The (—♦—) *S. cerevisiae* Y294[YMX1], (—■—) *S. cerevisiae* Y294[YMXR] and (—▲—) *S. cerevisiae* Y294[XYN2 XLO2] strains were cultivated on glucose (YPD, left hand side) and xylose (YPX, right hand side) as sole carbohydrate source under aerobic conditions. The (A and B) cell density and (C and D) extracellular endoxylanase activity was measured at regular intervals.

3.4.4 Anaerobic fermentation on xylose as the sole carbohydrate source

The *S. cerevisiae* Y294[YMX1] and *S. cerevisiae* Y294[YMXR] strains were evaluated for xylose conversion under anaerobic conditions (Fig. 3.4). The *S. cerevisiae* Y294[YMX1] strain was able to consume the xylose (~20 g/L) and produce ~9 g/L of ethanol after 40 days. The formation of by-products i.e. xylitol, glycerol and acetic acid, were relatively low following xylose fermentation.

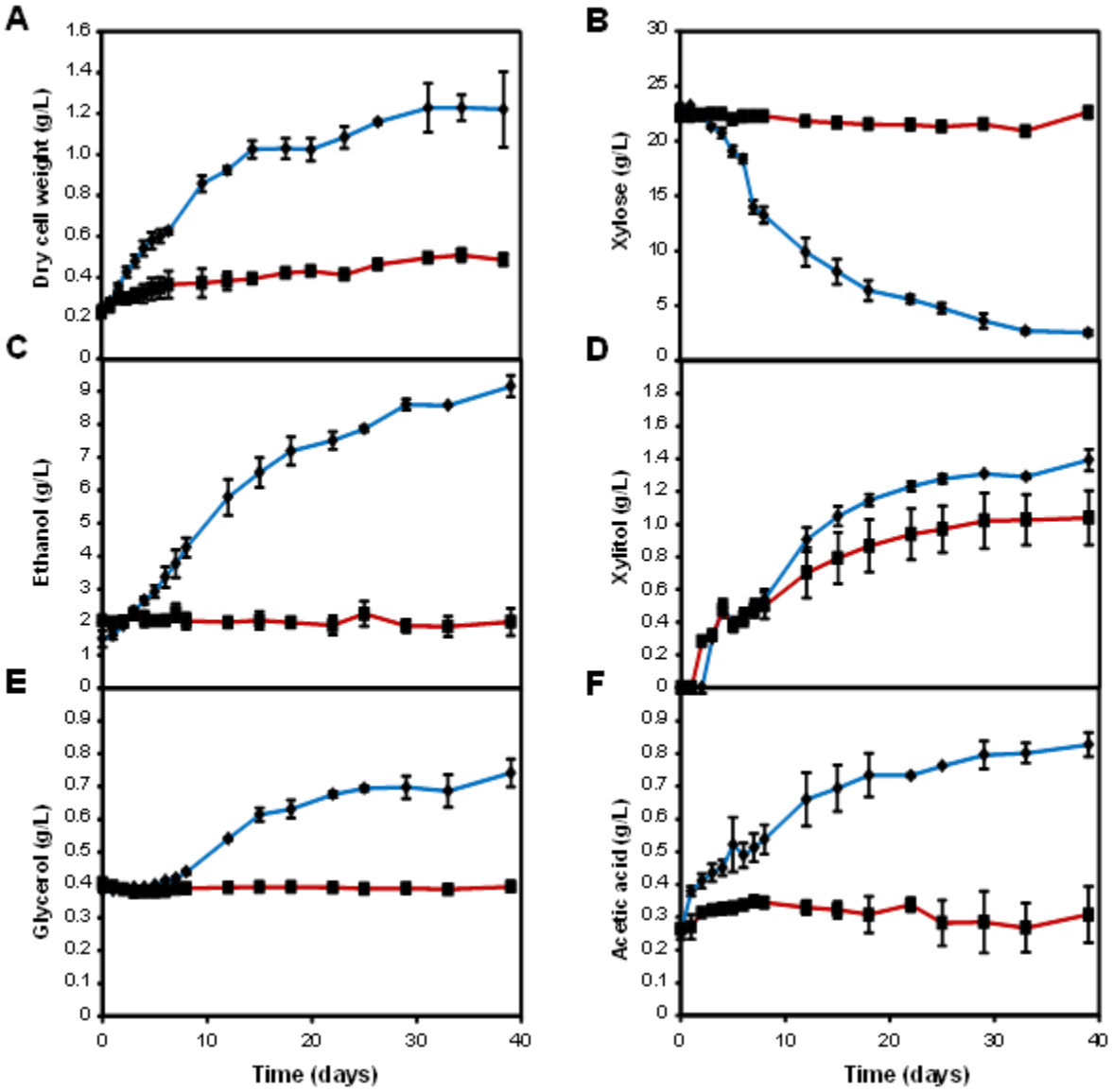

Fig. 3.4. The (—♦—) *S. cerevisiae* Y294[YMX1] and (—■—) *S. cerevisiae* Y294[YMXR] strains were cultivated on xylose as sole carbohydrate source (YPX) under oxygen-limited conditions and the (A) cell density, the extracellular (B) xylose, (C) ethanol, (D) xylitol, (E) glycerol and (F) acetic acid concentrations were monitored over time.

3.4.5 Aerobic growth on xylan as the sole carbohydrate source

The *S. cerevisiae* Y294[YMX1], *S. cerevisiae* Y294[YMXR] and *S. cerevisiae* Y294[XYN2 XLO2] strains were aerobically cultivated on YPBX. The *S. cerevisiae* Y294[YMX1] strain was able to grow on polymeric xylan as sole carbohydrate source, although slow (Fig. 3.5).

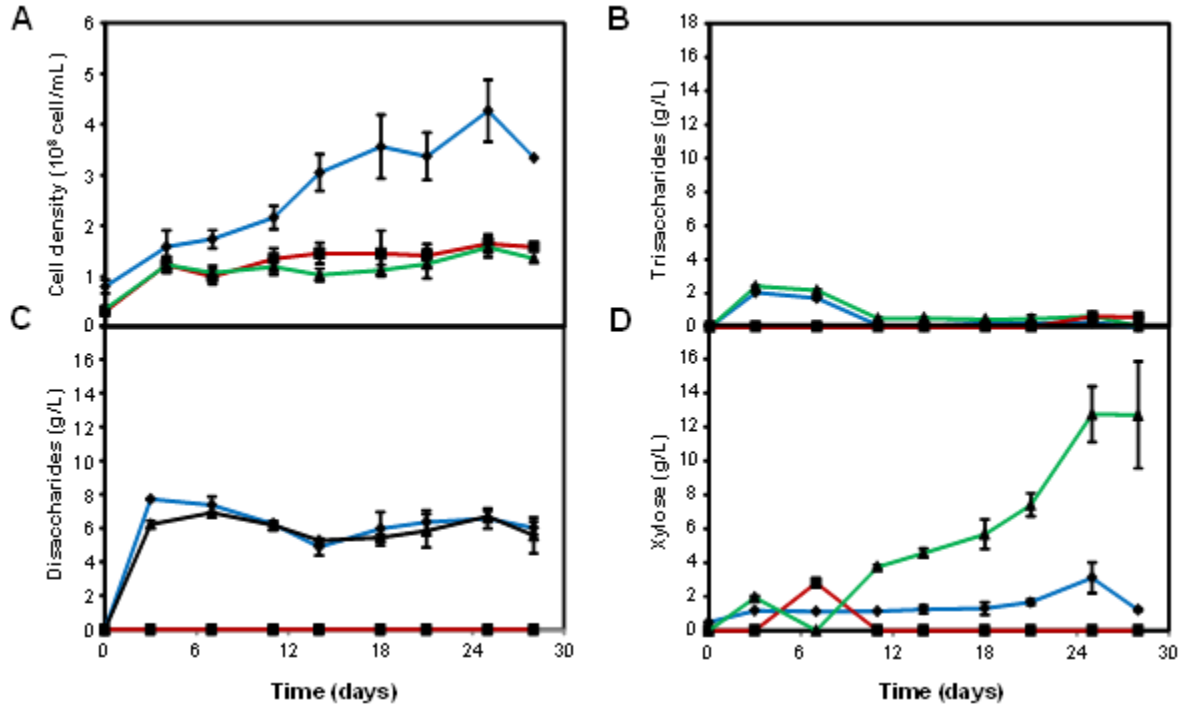

Fig. 3.5. The (—♦—) *S. cerevisiae* Y294[YMX1], (—■—) *S. cerevisiae* Y294[YMXR] and (—▲—) *S. cerevisiae* Y294[XYN2 XLO2] strains were aerobically cultivated on beechwood xylan (YPBX) as sole carbohydrate source and the (A) cell density, (B) trisaccharides, (C) disaccharides and (D) xylose concentrations monitored over time.

Xylan degradation profile reveals high initial release of trisaccharides (xylotriose and aldotriuronic acid (U4m2XX) [38]) and disaccharides (xylobiose, and aldobiuronic acid (U4m2X) [38]) for the *S. cerevisiae*Y294[YMX1] and Y294[XYN2 XLO2] strains (Fig. 3.5b, c). Trisaccharides indicated a gradual decrease over time; however, the concentration remained slightly lower for that produced by *S. cerevisiae* Y294[YMX1] compared to *S. cerevisiae* Y294[XYN2 XLO2]. An increase in endoxylanase activity can be expected (as was the case in Fig. 3.3d) as a result of the biomass increase of *S. cerevisiae* Y294[YMX1] (Fig. 3.5a). On the other hand, it appears that the release of disaccharides was similar for both *S. cerevisiae* Y294[YMX1] and *S. cerevisiae* Y294[XYN2 XLO2] and accumulated in the medium (Fig. 3.5c). Xylose accumulated to ~12.7 g/L for *S. cerevisiae* Y294[XYN2 XLO2],

while low levels could be detected for *S. cerevisiae* Y294[YMX1], suggesting that xylose was consumed for biomass production (Fig. 3.5d). As expected, no ethanol was detected and the production of xylitol, glycerol and acetic acid were negligible (data not shown).

3.4.6 Strain adaptation

The growth profiles of the *S. cerevisiae* Y294[YMX1] strain was compared to the adapted Y294[YMX1-A1], Y294[YMX1-A2] and Y294[YMX1-A3] populations on xylose as sole carbohydrate source. After 3 months of adaption the *S. cerevisiae* Y294[YMX1-A3] population was able to utilize the xylose much faster than the unadapted *S. cerevisiae* Y294[YMX1] counterpart and reached stationary phase after 70 hours of cultivation. Single colonies were obtained from the *S. cerevisiae* Y294[YMX1-A3] population and their growth monitored on xylose over time (Fig. 3.6b, not all the strains are displayed). The *S. cerevisiae* Y294[YMX1-A3.1] strain clearly outperformed the other adapted strains by reaching the stationary phase after 60 hours of cultivation.

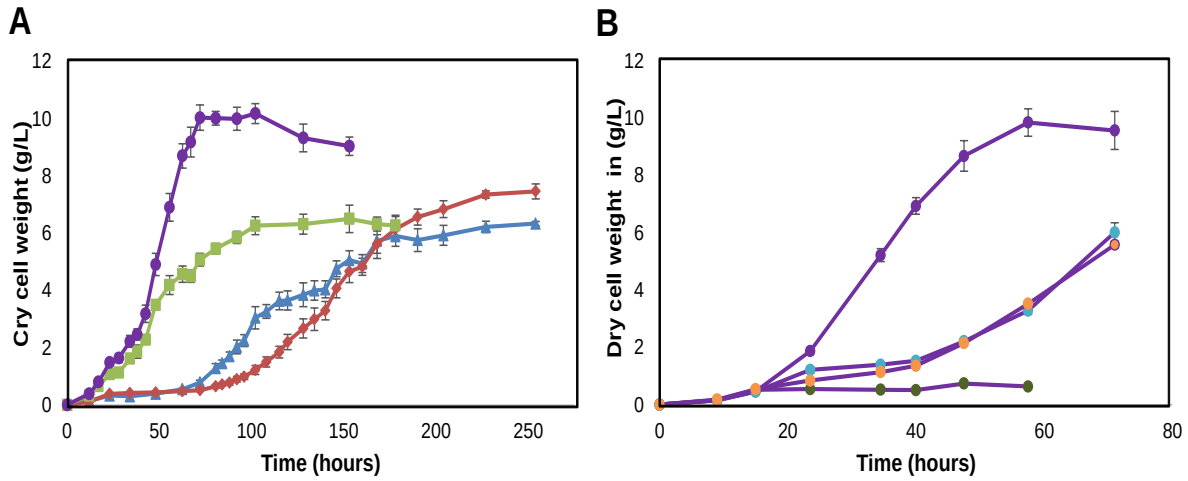

Fig. 3.6. (A) The growth profiles of the and (—▲—) *S. cerevisiae* Y294[YMX1-A1], (—■—) Y294[YMX1-A2] and (—●—) Y294[YMX1-A3] populations were compared to the (—♦—) *S. cerevisiae* Y294[YMX1] strain in YPX medium under aerobic conditions. (B) Single colonies were obtained from the *S. cerevisiae* Y294[YMX1-A3] population ((—●—) Y294[YMX1-A3.1], (—●—) Y294[YMX1-A3.2], (—●—)Y294[YMX1-A3.3] and (—●—) Y294[YMX1-A3.4] and evaluated for growth on xylose as sole carbohydrate source (YPX).

3.5 DISCUSSION

In order to obtain maximum xylose yields from hemicellulose fractions, harsh pretreatment methods should be avoided due to negative effect on xylose degradation. Unfortunately, milder pretreatment methods lead to residual xylan, which reduces the accessibility of the cellulose portion to hydrolytic enzymes [25]. Therefore, developing xylan metabolizing *S. cerevisiae* strains would be ideal to reduce pretreatment inputs, maximize intact xylose recovery for metabolic conversion as well as providing a more accessible cellulose portion. To date, only a few *S. cerevisiae* strains had been documented that can utilize xylan [14, 30, 34] primarily due to the difficulties involved in the utilization of xylose [13, 40]. Xylose isomerases directly convert xylose to xylulose, which can be utilized by *S. cerevisiae* using the pentose phosphate pathway. The *B. thetaiotaomicron* xylose isomerase [40] is one of only a few xylose isomerases that had been successfully expressed in *S. cerevisiae* [24].

In this study, a laboratory *S. cerevisiae* Y294 strain was engineered for co-expression of the *T. reesei* endoxylanase encoding gene (*xyn2*) and the *A. niger* xylosidase encoding gene (*xlnD*) for xylan hydrolysis, and the *B. thetaiotaomicron* xylose isomerase encoding gene (*xylA*) and the *Scheffersomyces stipitis* xylulose kinase gene (*xyl3*) for xylose metabolism. The previous attempt at expression of the native *xylA* was suboptimal [40], hence the decision to codon optimize the gene for expression in *S. cerevisiae* for this study. The resulting *S. cerevisiae* Y294[YMX1] strain was evaluated for its ability to utilize xylose as sole carbohydrate source and subsequent ethanol production. The μ_{max} of ~0.06 g/g/h on xylose compares well relative to other reported *xylA*-expressing *S. cerevisiae* [26]. The recombinant *S. cerevisiae* Y294[XYN2 XLO2] strain, constructed by la Grange et al. [18], was used as benchmark strain.

The *GRE3* gene encodes for the major aldose reductase in *S. cerevisiae*. It was therefore disrupted to prevent loss of carbon due to the formation of xylitol. Despite the disruption of the *GRE3* in the *S. cerevisiae*Y294[YMX1] strain, xylitol could still be detected (Figs. 2.4) suggesting the existence of additional aldose reductases in *S. cerevisiae* [27, 37]. Cultivation on xylose also resulted in more biomass being produced than with cultivation on glucose. The lack of ethanol production under aerobic conditions suggests that *S. cerevisiae* does not recognize xylose as a Crabtree positive sugar making more carbon available for the biomass production. In addition, the utilization of the xylose is preceded by a long lag phase. This might be explained by the low-level expression of the *xylA* resulting in a slow accumulation of the

XylA monomers preventing dimer formation. The lengthy lag phase might be addressed by multicopy expression of the *xylA* gene. Under oxygen-limited conditions the *S. cerevisiae* Y294[YMX1] strain produced 90% of the theoretical ethanol yield after 40 days of cultivation on xylose (Fig. 3.4). This time consuming fermentation is most probably also related to the XylA activity and should benefit from overexpression of the *xylA* gene.

The overexpression of multiple genes has a negative impact on biomass production (Figs. 3.2a, c, 3.3a) probably due to a metabolic burden [6, 41]. The Xyn2 activity was not affected by the choice of carbon source, but rather increased proportionately with the increase in cell mass (Fig. 3.3d). This study demonstrated the successful growth of *S. cerevisiae* Y294[YMX1], expressing xylan-degrading and xylose-assimilating genes, on polymeric glucuronoxylan (Fig. 3.5). The initial high levels of disaccharides and trisaccharides suggest the accumulation of aldotribiuronic acid and aldotriuronic acid [38], (Fig. 5.5b, c). For complete utilization of beechwood xylan, the *S. cerevisiae* Y294[YMX1] strain would benefit from the addition of an α-glucuronidase to remove the glucuronic acid substituents from the main chain [12].

This study contributes to the repertoire of *S. cerevisiae* strains capable of utilizing xylose as sole carbohydrate source. Sun et al. [34] was the first to report on xylan conversion to ethanol using a *S. cerevisiae* strain that produces a minihemicellulosome and using the XR and XDH combination. Yet, this is the first report of the efficient xylose fermentation and xylan utilization by *S. cerevisiae* secreting free enzymes. To our knowledge, this has been the first report of the use of the codon-optimized *xylA* of *B. thetaiotaomicron* in *S. cerevisiae* for the production of bioethanol from beechwood xylan. The XylA proved to work well and provides an alternative to the xylose reductase and xylulose dehydrogenase combinations traditionally used. This is also the first report of a xylan utilizing *S. cerevisiae* strain that is an improvement on the xylan hydrolysing *S. cerevisiae* Y294[XYN2 XLO2] strain originally constructed by la Grange et al. [18].

The rate of bioethanol production is directly influenced by the level of xylose and the rate of xylose conversion. The xylose isomerase is required to prevent xylose accumulation, which would result in product inhibition of the xylosidase enzyme. It is therefore paramount that the xylose isomerase activity needs to be high enough to prevent it from being the rate-limiting step. Therefore, much still needs to be done in terms of improving the *S. cerevisiae* Y294[YMX1] strain. Future work will focus on bioreactor studies (using xylose or xylan feeds) to improve the cell growth rate and the xylose consumption rate of the

*S. cerevisiae*Y294[YMX1] strain through directed evolution strategies. It remains, however, a good candidate for further manipulation directed toward the conversion of lignocellulosic substrates to ethanol.

3.6 ACKNOWLEDGEMENTS

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CHAPTER 4

Metabolic flux analysis of a xylose utilising *Saccharomyces cerevisiae* strain expressing the *Bacteroides thetaiotaomicron* xylose isomerase

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Metabolic flux analysis of a xylose-utilising *Saccharomyces cerevisiae* strain expressing the *Bacteroides thetaiotaomicron* xylose isomerase

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4.1. ABSTRACT

The utilisation of all polymers present in lignocellulose is required to make second-generation bioethanol economically feasible. Ethanol production from xylan is hampered by the metabolic inability of *S. cerevisiae* to utilise xylan (xylose). In this study, a diploid *S. cerevisiae* S288c[2n YMX1] strain was constructed expressing the xylose isomerase encoding gene (*xylA*) from *Bacteroides thetaiotaomicron* and the xylulose kinase gene (*xyl3*) from *Scheffersomyces stipitis*. The native aldose reductase gene (*GRE3*) was deleted to minimise xylitol production, but cultivation on xylose resulted in xylitol accumulation despite the disruption of the *GRE3* gene. Metabolomic data was obtained for this strain at different growth phases during cultivation on glucose or xylose as sole carbohydrate sources. Cultivation on xylose resulted in an increase in the pool size of the metabolites of the pentose phosphate pathway and an accumulation of sedoheptulose 7-phosphate, suggesting low levels of transaldolase 1 activity. The data suggested that pyruvate kinase 1controls the flux through glycolysis. Glucose 6-phosphate was seven-fold lower with cultivation of xylose and therefore insufficient for activation of the Crabtree effect. The UDP-D-glucose levels were eight-fold higher with cultivation on xylose compared to glucose, indicating that the carbon was channelled towards biomass production. The adenylate and guanylate energy charges did not control the metabolic state and were tightly regulated by the cultures, whilst the catabolic- and anabolic-reduction charges appeared to fluctuate between metabolic states and may provide insight into to the Crabtree effect.

Keywords metabolomics · xylose isomerase · bioethanol · *S. cerevisiae*

4.2. INTRODUCTION

The baker's yeast *Saccharomyces cerevisiae* is well suited for use in industrial processes due to its high fermentation capacity, tolerance to low pH and high ethanol concentrations and overall robustness (Smith et al. 2014). These characteristics make this yeast ideal for bioethanol production, which can be used as a replacement for fossil fuels. Bioethanol production requires pretreatment of the biomass and genetic engineering of the yeast for utilisation of the lignocellulosic components. Much progress has been made in the utilisation of starch and cellulose for bioethanol production, but the utilisation of xylan (the main component of hemicellulose) is limited due to the difficulties related to the metabolism and transport of xylose.

The yeast *S. cerevisiae* cannot utilise xylose and therefore the xylose isomerase (XI) or oxido-reductive pathway (xylose reductase/xylitol dehydrogenase; XR/XDH) have been introduced into *S. cerevisiae* to confer the ability to convert xylose to xylulose, which is utilised through the pentose phosphate pathway (PPP). Most xylose-consuming *S. cerevisiae* strains have been constructed using the XR/XDH pathway due to the initial difficulties encountered in the expression of functional XI genes (Wasylenko and Stephanopoulos 2015). The bacterial XI pathway neither requires nucleotide co-factors nor result in the redox imbalance that typically arises from the use of the XR/XDH pathway, resulting in the accumulation of xylitol. Yet, the study by Kim et al. (2013) indicated that xylitol accumulation might be the result of insufficient XDH activity (in at least some strains) and not necessarily the redox imbalance. Khattab and Kodaki (2014) recently addressed the redox imbalance issue by using the native NADPH-dependent aldose reductase (GRE3) in combination with the $NADP^+$-dependent XDH (*Scheffersomyces stipitis*). An increase in ethanol yield and reduction in xylitol formation was observed, which is attributed to the effective regeneration of the NADPH/$NADP^+$ cofactors.

Although significant progress has been made in the engineering of xylose-consuming *S. cerevisiae* strains, the ethanol productivities achieved on xylose are still significantly lower than those observed on glucose (Wasylenko and Stephanopoulos 2015). The use of metabolite profiles has proven to be a useful tool in elucidating the flux through metabolic pathways. Previous analysis of central carbon metabolite pool sizes and metabolic fluxes of a xylose-utilising *S. cerevisiae* strain indicated that the flux through the non-oxidative PPP is high, but the flux through the oxidative PPP is low (Klimacek et al. 2010; Bergdahl et al. 2012; Matsushika et al. 2013; Wasylenko and Stephanopoulos 2015). Unlike glucose, xylose is not

recognised as a fermentable carbon source and does not activate the full carbon catabolite repression response in *S. cerevisiae.* Therefore, bottlenecks downstream of glyceraldehyde 3-phosphate (GAP) can be anticipated, such as inefficient re-oxidation of NADH. Under industrially relevant anaerobic conditions, the accumulation of NADH inhibits xylose metabolism because NADH cannot be efficiently re-oxidised to NAD^+ without oxygen as electron accepter (Bruinenberg et al. 1983; Bruinenberg et al. 1984).

The purpose of this study was the construction of a stable diploid *S. cerevisiae* strain with increased xylose conversion capacity using the codon-optimised XI encoding gene (*xylA*) from *Bacteroides thetaiotaomicron* and the xylulose kinase (*xyl3*) from *Scheffersomyces stipitis.* The native *S. cerevisiae* non-specific aldose reductase gene (*GRE3*) was deleted to minimise carbon loss due to the production of xylitol. The changes in gene expression is ultimately reflected in the metabolome and therefore the metabolomic data was obtained from this strain at different time points during cultivation on glucose or xylose as sole carbon sources. The metabolic profiles were used to elucidate the main pathways involved in carbon conversion including glycolysis, the PPP and the Krebs cycle.

4.3. MATERIALS AND METHODS

4.3.1. Media and culture conditions

The *S. cerevisiae* strains were routinely cultured in YP medium (10 g/L yeast extract, 20 g/L peptone) containing either 20 g/L glucose (YPD) or 20 g/L xylose (YPX) at 30°C. Zeocin and hygromycin B (Calbiochem) was added at a concentration of 500 and 200 mg/L, respectively, when required.

4.3.2. Strains and plasmid constructs

All microbial strains and plasmids used in this study are listed in Table 4.1.

4.3.3. DNA amplification

Standard protocols were followed for PCR amplification reactions using the oligonucleotide primers listed in Table 4.2. The *Pfu* polymerase was used according to the supplier's specifications. Genomic DNA was prepared using the ZR Fungal/Bacterial DNA MiniPrep Kit (Zymo Research, USA) and used as template for the amplification of the *HO*, *MAT*α and *MAT***a** DNA regions. The pBHD1 vector was used as template for the amplification of the *gre3'-hphNT-gre5'* fragment required for the disruption of the *GRE3* gene (*hphNT* accession nr AAA92252).

Table 4.1. Microbial strains and plasmids used in this study

***S. cerevisiae* strains or and plasmids**	**Genotype**	**Source or rReference**
Haploid strains		
S288c(α)	*MAT*α *SUC2 gal2 mal2 mel flo1 flo8-1 hap1 ho bio1 bio6*	ATCC® 204508™
S288c(a)	*MAT***a** *SUC2 gal2 mal2 mel flo1 flo8-1 hap1 ho bio1 bio6*	Johan Thevelein
S288c(a)[MJM121]	*MAT***a** *Sh Ble ho* $ENO1_P$*-xylA-*$ENO1_T$; $ENO2_P$*-xyl3-*$ENO2_T$	This study
S288c(α)[MJM121]	*MAT*α *Sh Ble ho* $ENO1_P$*-xylA-*$ENO1_T$; $ENO2_P$*-xyl3-*$ENO2_T$	This study
S288c(a)[XylA-Xyl3]	*MAT***a** *Sh Ble ho gre3:: hphNT* $ENO1_P$*-xylA-*$ENO1_T$; $ENO2_P$*-xyl3-*$ENO2_T$	This study
S288c(α)[XylA-Xyl3]	*MAT***a** *Sh Ble ho gre3:: hphNT* $ENO1_P$*-xylA-*$ENO1_T$; $ENO2_P$*-xyl3-*$ENO2_T$	This study
Diploid strain		
S288c(2n)[YMX12]	*SUC2 gal2 mal2 mel flo1 flo8-1 hap1 ho bio1 bio6*; *Sh Ble gre3:: hphNT* $ENO1_P$*-xylA-*$ENO1_T$; $ENO2_P$*-xyl3-*$ENO2_T$	This study
Plasmids		
YEp352	*bla URA3*	ATCC® 37673™
pMJM121	*bla δ-site URA3* $ENO1_P$*-xylA-*$ENO1_T$ *Sh ble*; $ENO2_P$*-xyl3-*$ENO2_T$ *δ-site*	This study
pBHD1	*hphNT*	This laboratory

4.3.4. Strain construction

Transformation of the prototrophic *S. cerevisiae* S288c(a) and S288c(α) haploid strains was achieved by means of electroporation as described by Cho et al. (1999). Transformants were selected on YPD agar plates containing either 500 mg/L zeocin or 200 mg/L hygromycin. Transformants of opposite mating type phenotypes displaying the fastest growth on xylose were selected for mating as described by Hasunuma et al. (2011), to generate the diploid *S. cerevisiae* S288c(2n)[YMX12] strain. Individual haploid strains were cultivated in liquid YPD medium for 24 h. Cell cultures were combined, harvested and spread plated onto YPD plates. Diploid cells were confirmed by means of PCR according to Huxley et al. (1990), using the *MAT* locus primers listed in Table 4.2.

Table 4.2. The DNA sequences of the oligonucleotide primers used in the study

Gene	Primer name	DNA sequence of primers in a 5' to 3' orientation
hphNT[a]	GRE3-hphNT-L	AAGCGATATGGGACTTGATTATTTAGACCTGTATTATATTC GTTTAGCTTGCCTCGTCCC
	GRE3-hphNT-R	GTTTTTGCCAACTGTAAGTCCATCTCAATGAATGATTGAGG ACGGATCTGATATCACCTAATAACTTCGTATAGC
GRE3[b]	GRE3-Con-L	CTTCACTGGTTACTCTTAATAACGGTCTG
	GRE3-Con-R	CAAATCCTAACCTTTTATATTTCTCTACAGGG
Mat locus[c]	MAT**a**	ACTCCACTTCAAGTAAGAGTTTG
	MATα	GCACGGAATATGGGACTACTTCG
	Mat overlap	AGTCACATCAAGATCGTTTATGG
XylA[b]	XylA-L	CGCTGACGCTATTCAAGCTG
	XylA-R	GCCAATTCGTGTTCGAAAGTG
Xyl3[d]	Xyl3-L	GATCGAATTCATGACCACTACCCCATTTG
	Xyl3-R	TCGACTCGAGTTAGTGTTTCAATTCACTTTCC

[a] amplification of the *hphHT* marker with *gre3* flanking regions
[b] confirmation of disruption of the *GRE3* gene or presence of *xylA* gene
[c] confirmation of mating type according to Huxley et al. (1990)
[d] cloning and confirmation of *Xyl3* gene. Restriction sites (for cloning purposes) are underlined.

4.3.5. Aerobic batch cultivations

Aerobic batch cultivations were performed using computer-controlled 1.3 L glass bioreactors (New Brunswick Scientific BioFlo 110) with a working volume of 0.9 L. The bioreactors were equipped with two disc impellers and an exhaust gas condenser (maintained at 5°C) to prevent evaporation of media components and fermentation products. The culture temperature was maintained at 30°C using a heating jacket and cooling water. The pH of the cultures were was monitored through a pH electrode (Mettler Toledo, Halstead, UK) and maintained at a constant of pH 5.5 by automatic addition of 2 M KOH. The dissolved oxygen concentration was measured by means of a polarographic oxygen electrode (Ingold AG, Urdorf, Switzerland) and controlled by a cascade system, which coupled a constant air flow of 1.0 vvm with adjustable agitation (250-1000 rpm) to maintain a minimum of 30% air saturation.

The inoculums were prepared by inoculating 15% (v/v) glycerol cryo-stock cultures into 5 ml YPD or YPX. The cultures were allowed to grow to mid-exponential phase before transferring to 50 ml fresh media in 500 ml Erlenmeyer flasks. Mid-exponential phase pre-cultures were used to inoculate the bioreactors to an initial optical density (OD_{600}) of 0.1. Samples were obtained at regular intervals through a sampling port for the measurement of extracellular metabolite (ethanol, glycerol, xylitol, xylose and acetic acid) concentrations and cell growth. Calibration curves were used to correlate OD_{600} with dry cell weight (DCW). All fermentations were repeated at least four times.

4.3.6. HPLC analysis of extracellular metabolites

The extracellular metabolite concentrations were determined as previous described (Chapter 3). High performance liquid chromatography (HPLC) was used to quantify the ethanol, glycerol, xylitol, xylose and acetic acid concentrations using a Waters 717 injector (Milford, MA, USA), an Aminex HPX-87H column (Bio-Rad, Richmond, CA) at 60°C and an Agilent 1100 pump (Palo Alto, CA, USA). The mobile phase (5 mM H_2SO_4) was set at a flow rate of 0.6 ml/min and the compounds detected with a Waters 410 refractive index detector. All HPLC standards were supplied by Sigma-Aldrich (Steinheim, Germany).

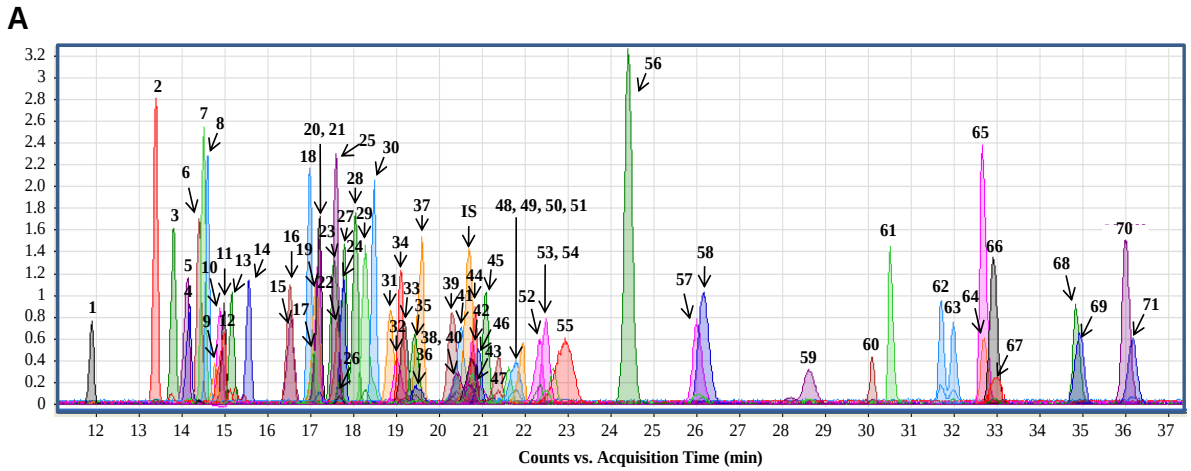
4.3.7. Sample quenching and extraction of intracellular metabolites

Culture samples were obtained through leakage-free quenching (Canelas et al. 2008) with glucose-repressed growth (7 and 8 hours) and glucose-derepressed growth (16 and 20 hours) during glucose cultivation, or with glucose-derepressed growth (30, 42, 54 and 66 hours) during xylose cultivation, and prepared for metabolome analysis. Briefly, 5 ml of culture samples were injected directly from the bioreactor sampling port (<1 seconds) into 25 ml pure methanol (pre-cooled to -40°C), briefly mixed and placed back in the cryostat (final methanol concentration ≥83.33%). The yeast cells were obtained by centrifugation at 4,000 × *g* and -9°C for 3 minutes and washed once with equal volume of an 83.33% methanol solution (pre-cooled to -40°C). The supernatants were decanted and the internal standards (7.5 µl of 1 mM 1,4-piperazine-diethanesulfonic acid (PIPES) and 10 µl of 17 µM 10-camphorsulfonic acid) added. PIPES was used as an internal standard in CE-MS in accordance to Hasunuma et al. (2011) and 10-camphorsulfonic acid used as an internal standard for LC-MS as described by Suga et al. (2013). Cell pellets were stored at -80°C till further analysis.

Extraction of intracellular metabolites was obtained by the boiling ethanol method described by Lange et al. (2001). Briefly, five 5 ml of a 75% (v/v) ethanol solution was preheated in a water bath to 95°C and rapidly poured over the frozen cell pellet (stored on dry ice). The samples were mixed (vortex) and placed back into the water bath for three 3 minutes. Extracts were transferred to clean tubes and the ethanol solution allowed to evaporate under vacuum using a CentriVap centrifugal vacuum concentrator (Labconco, Kansas City, MO). Dried residues were stored at -80°C until further analysis.

4.3.8. CE/TOF-MS analysis of intracellular metabolites

The dried metabolites, stored at -80°C, were dissolved in 20 µl of Milli-Q water prior to CE/TOF-MS analysis. CE/TOF-MS analysis was performed in the positive mode to profile cationic metabolites using an Agilent CE capillary electrophoresis system, an Agilent 6224 TOF LC/MS system and an Agilent 1200 series isocratic HPLC pump according to the method described by Ho et al. (2014). The Agilent Chem-Station software was used for system control and data acquisition. All standards were of analytical grade and supplied by Sigma-Aldrich (Steinheim, Germany) (Fig. 4.1).



1. NAD^+
2. cAMP
3. cGMP
4. Thiamine pyrophosphate (ThPP)
5. Flavin adenine dinucleotide (FAD) $(M–2H)^{2–}$
6. D-Glucuronic acid (D-Galacturonic acid)
7. D-Gluconic acid
8. Shikimic acid
9. NADH $(M–2H)^{2–}$
10. ADP-Glu
11. GDP-Glu
12. Flavin mononucleotide (FMN)
13. ADP-Rib
14. UDP-Glu
15. GMP
16. AMP
17. $NADP^+$
18. 3-Hydroxybutyric acid
19. CMP
20. dTMP
21. D-Sedoheputulose 7-phosphate (S7P)
22. Inosine 5'-monophosphate (IMP)
23. D-Glucose 6-phosphate (G6P)
24. UMP
25. Nicotinic acid
26. Acetoacetyl CoA $(M–2H)^{2–}$
27. D-Fructose 6-phosphate (F6P)
28. D-Glucose 1-phosphate (G1P)
29. Acetyl-CoA $(M–2H)^{2–}$
30. D-Glyceric acid
31. D-Ribose 5-phosphate (R5P)
32. CoA $(M–2H)^{2–}$
33. GDP
34. Lactic acid (Lac)
35. ADP
36. D-Erythrose 4-phosphate (E4P)
37. D-Ribulose 5-phosphate (Ru5P)
38. NADPH $(M–2H)^{2–}$
39. dTDP
40. GTP $(M–2H)^{2–}$
41. CDP
42. DL-Glyceraldehyde 3-phosphate (GAP)
43. dATP $(M–2H)^{2–}$
44. ATP $(M–2H)^{2–}$
45. UDP
46. Glyoxylic acid
47. Succinyl-CoA $(M–2H)^{2–}$
48. dTTP $(M–2H)^{2–}$
49. Glycerol-phosphate (Glycerol-P)
50. CTP $(M–2H)^{2–}$
51. dCTP $(M–2H)^{2–}$
52. UTP $(M–2H)^{2–}$
53. Dihydroxyacetone phosphate (DHAP)
54. Glycolic acid
55. Pyruvate
56. 2-Isopropylmalic acid
57. D-Fructose 1,6-biphosphate (FBP) $(M–2H)^{2–}$
58. 6-Phosphogluconic acid (6PG)
59. D-Ribulose-1,5-bisphosphate (RuBP)
60. Phosphoribosyl pyrophosphate (PRPP)
61. Phosphoric acid
62. D-2-Phosphoglyceric acid (2PGA)
63. D-3-Phosphoglyceric acid (3PGA)
64. Phosphoenolpyruvic acid (PEP)
65. Succinic acid (Suc)
66. Malic acid (Mal)
67. 2-Ketoglutaric acid (2KG)
68. Fumaric acid (Fum)
69. Citric acid (Cit)
70. *cis*-Aconitic acid (*cis*-Aco)
71. Isocitric acid (Iso-cit)

IS. PIPES (IS)

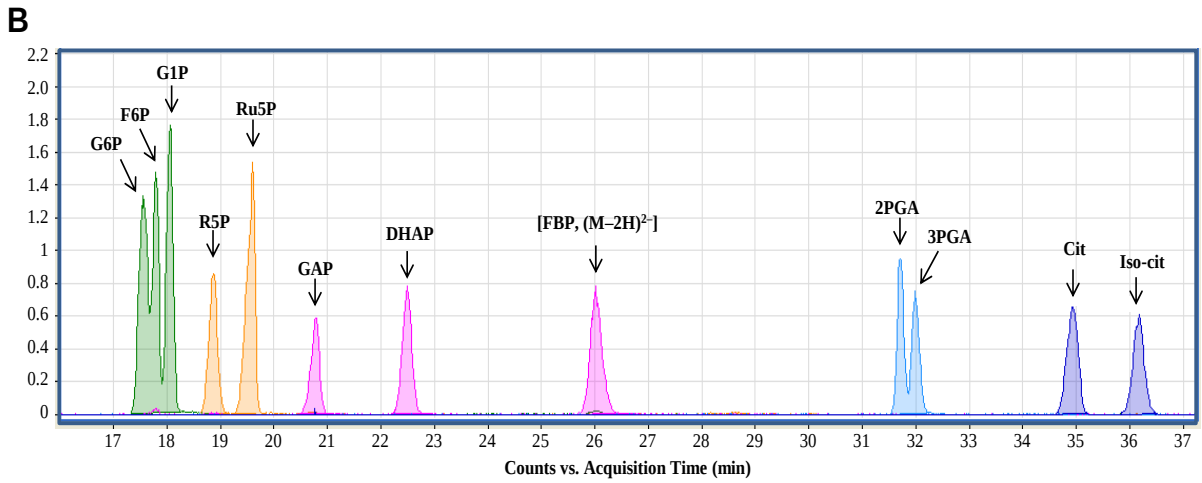


Fig. 4.1. Separation profile (standards) obtained from CE/TOF-MS of (A) all relevant metabolites and (B) the five most relevant isomers expected in the sample preparations.

4.4. RESULTS

4.4.1. Strain construction

The mating type of the *S. cerevisiae* S288c(a) and S288c(α) haploid parental strains were was confirmed by PCR (Huxley et al. 1990) prior to transformation (data not shown). The pMJM121 plasmid (previously constructed using the YEp352 backbone, (see Chapter 3) was digested with *Cla*I to produce a linear fragment, containing the *xylA* and *xyl3* cassettes as well as the *Sh ble* marker gene, flanked by the delta (*δ*) DNA sequences. This DNA fragment was introduced into the *S. cerevisiae* S288c(a) and S288c(α) strains by means of homologous recombination using the *δ*-sequences distributed throughout the yeast genome (Lee and da Silva, 1997), generating the *S. cerevisiae* S288c(a)[MJM121] and S288c(α)[MJM121] strains. The resultant transformants were able to grow on YPD plates containing 500 mg/L zeocin. Positive transformants were selected based on growth rates and retransformed with the *gre3'-hphNT-gre5'* PCR product (*hphNT* flanked with the *gre3* DNA sequence) for disruption of the native *GRE3* gene, generating the *S. cerevisiae* S288c(a)[XylA-Xyl3] and S288c(α)[XylA-Xyl3] strains with selection on 200 mg/ml hygromycin B.

4.4.2 Growth profiles on xylose (shake flasks)

Several *S. cerevisiae* S288c(a)[XylA-Xyl3] and S288c(α)[XylA-Xyl3] transformants were cultivated in YPX and their growth profiles compared with the parental strains (data not shown). The fastest growing strain of each mating type, phenotype (*S. cerevisiae* S288c(a)[XylA-Xyl3.6] and S288c(α)[XylA-Xyl3.5]), were used for mating to obtain *S. cerevisiae* S288c(2n)[YMX12]. The presence of the *gre3* disruption, *xylA*, *xyl3*, *MAT***a** and *MAT*α loci was confirmed with amplification of the regions using the appropriate oligonucleotide primers (Table 4.2, results not shown). The aerobic growth profile of the *S. cerevisiae* S288c(2n)[YMX12], *S. cerevisiae* S288c(a)[XylA-Xyl3.6] and S288c(α)[XylA-Xyl3.5] in YPX medium (Fig. 4.2) were similar for the three strains, but the diploid strain reached the stationary phase 24 hours earlier.

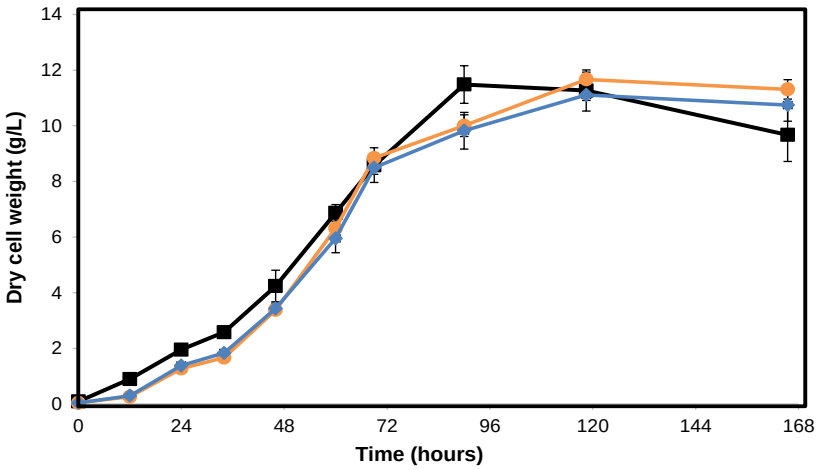


Fig. 4.2. The growth patterns of the (—●—) *S. cerevisiae* S288c(a)[XylA-Xyl3.6], and (—●—) S288c(α)[XylA-Xyl3.5] haploid strains were used to generate the (—■—) *S. cerevisiae* S288c(2n)[YMX12] diploid strain and the growth of the strains compared on YPX. Error bars represent the standard deviation of three independent biological repeats.

4.4.3 Extracellular fluxes

Bioreactors containing YPD or YPX were inoculated to an initial OD_{600} of 0.1 with mid-exponential phase *S. cerevisiae* S288c(2n)[YMX12] pre-cultures, and aerobic batch cultivations were performed. Culture samples were obtained at different growth phases. The extracellular metabolite concentrations were determined (Fig. 4.3A and B) and medium conditions (pH and oxygen concentration (in arbitrary units)) monitored (Fig. 4.3C and D). The *S. cerevisiae* S288c(2n)[YMX12] strain was able to utilise the glucose approximately ten 10 times faster than xylose (Fig. 4.3A and B). The glycerol, ethanol and acetic acid accumulated with cultivation on glucose, but not on xylose. The biomass increased steadily upon depletion of the glucose, coinciding with the utilisation of the glycerol, ethanol and acetic acid that accumulated during the first ten 10 hours of glucose metabolism. The decrease in acetic acid coincided with the increase in pH of the medium (Fig. 4.3C) and the utilisation of the proteins and peptone present in the medium, releasing NH_4^+ in response to carbon starvation (Ljungdahl and Daignan-Fornier, 2012). (Bioreactors were only equipped with KOH to maintain pH.). The cultivation on glucose and xylose resulted in a typical drop in dissolved oxygen over the first ten 10 hours (Fig. 4.3C), which increased once the culture reached the stationary phase (cessation of metabolism). Cultivation on xylose resulted in an increase in dissolved oxygen between 10 and 30 hours, which is due to the delayed growth rate of the culture (and the

accompanied low oxygen demand). The increase in growth rate after 36 hours resulted in an increase in oxygen demand which led to a drop in the dissolved oxygen concentration. Xylitol accumulated extracellularly with cultivation on xylose (Fig. 4.3B). Intracellular metabolites were identified based on the migration patterns and mass/charge ratios (m/z) of the standards displayed in Fig. 4.1.

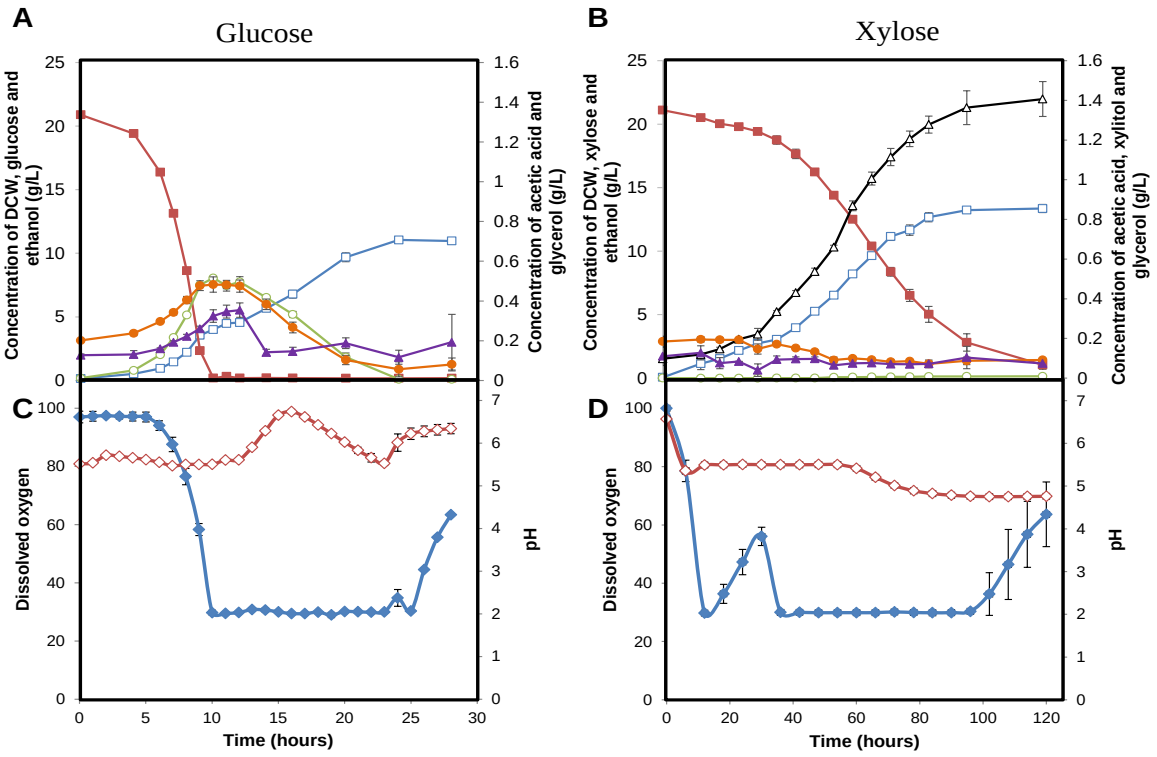


Fig. 4.3. The *S. cerevisiae* S288c(2n)[YMX12] strain was cultivated on glucose (A, YPD, left hand side) and xylose (B, YPX, right hand side) as sole carbohydrate source under aerobic conditions. The (A and B) (—□—) biomass, extracellular (—○—) ethanol, (—●—) glycerol, (—▲—) acetic acid, (—△—) xylitol, (—■—) glucose/xylose (C and D), (—♦—) dissolved oxygen (arbitrary units) and (—◊—) pH was monitored over time. Error bars represent the standard deviation of four independent biological repeats.

4.4.4 Intracellular fluxes

Central carbon metabolite pool sizes of *S. cerevisiae* S288c(2n)[YMX12] were measured at different time points during the cultivation on glucose or xylose. The sampling times were chosen according to the growth phase of the cultures in order to compare the different culture conditions. The metabolic pool of all the metabolites involved in glycolysis was high after seven and eight hours of cultivation on glucose with the exception of PEP (Fig. 4.4). In general,

the cultivation on xylose resulted in a significantly smaller metabolic pool for most of the metabolites (with the exception of phosphoenol pyruvate (PEP) and 2-phosphoglycerate (2PG)).

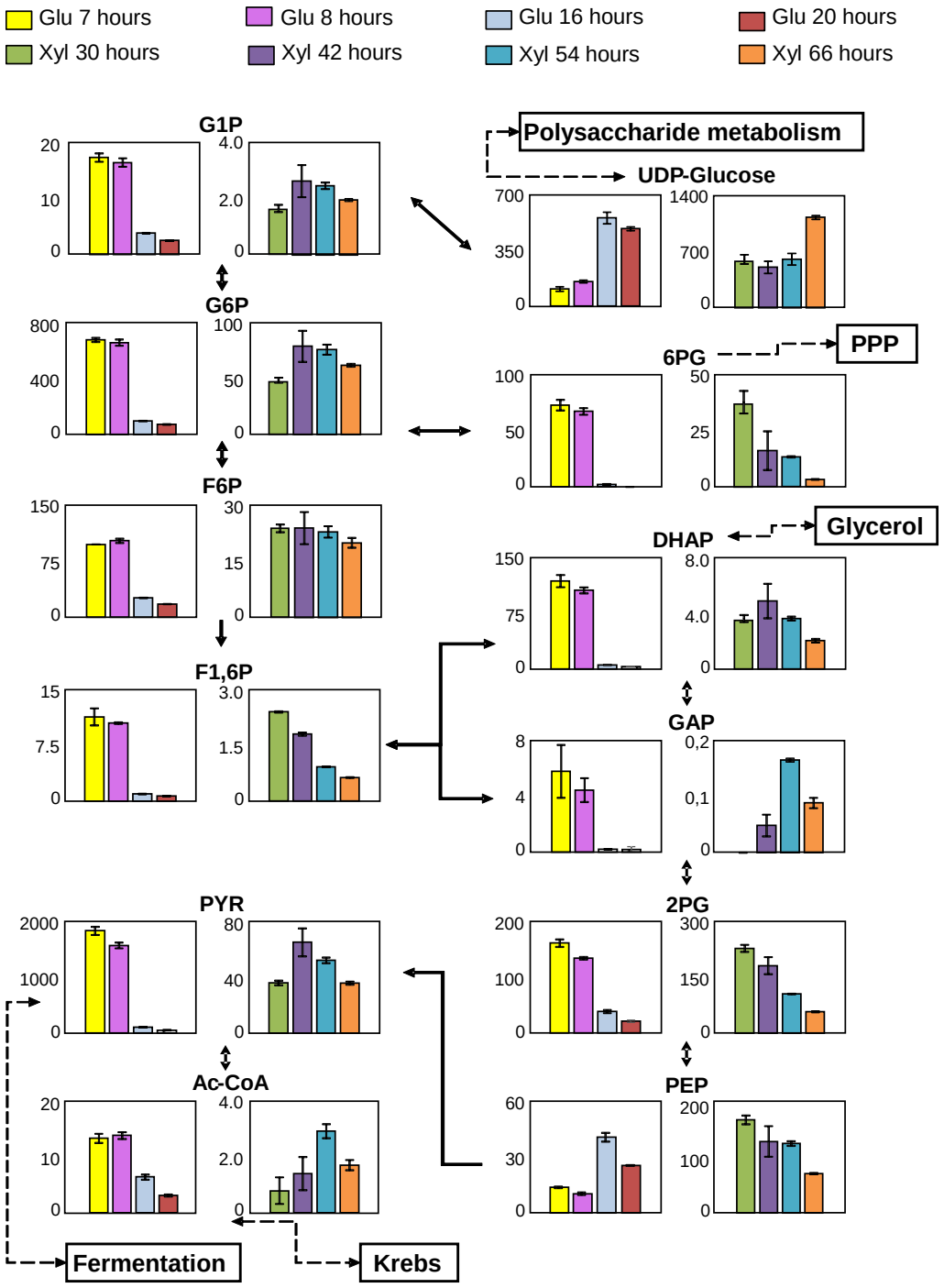


Fig. 4.4. The pool sizes (nmol/mg DCW) for the metabolites of glycolysis. Data obtained from the samples taken after 7, 8, 16 and 20 hours of cultivation on glucose is are depicted on the left while the data obtained from the samples taken after 30, 42, 54 and 66 hours of cultivation on xylose, are to the right. Error bars represent the standard error of four independent biological repeats.

During growth on xylose, the concentration of the glucose 1-phosphate (G1P), glucose 6-phosphate (G6P) and fructose 6-phosphate (F6P) remained the same over time, whereas the remainder of the metabolites indicated a steady decrease. The carbon obtained from xylose through the non-oxidative PPP enters glycolysis at F6P and glyceraldehyde-3-phosphate (GAP). Yet, a significant amount of G6P was detected with cultivation on xylose.

Cultivation on glucose resulted in a small metabolic pool for the metabolites of the PPP, whereas the cultivation on xylose resulted in a significant increase in the pool sizes of ribulose 5-phosphate (Ru5P), ribose 5-phosphate (R5P), xylulose 5-phosphate (X5P) and sedoheptulose 7-phosphate (S7P) (Fig. 4.5). R5P and X5P are converted to S7P and GAP.

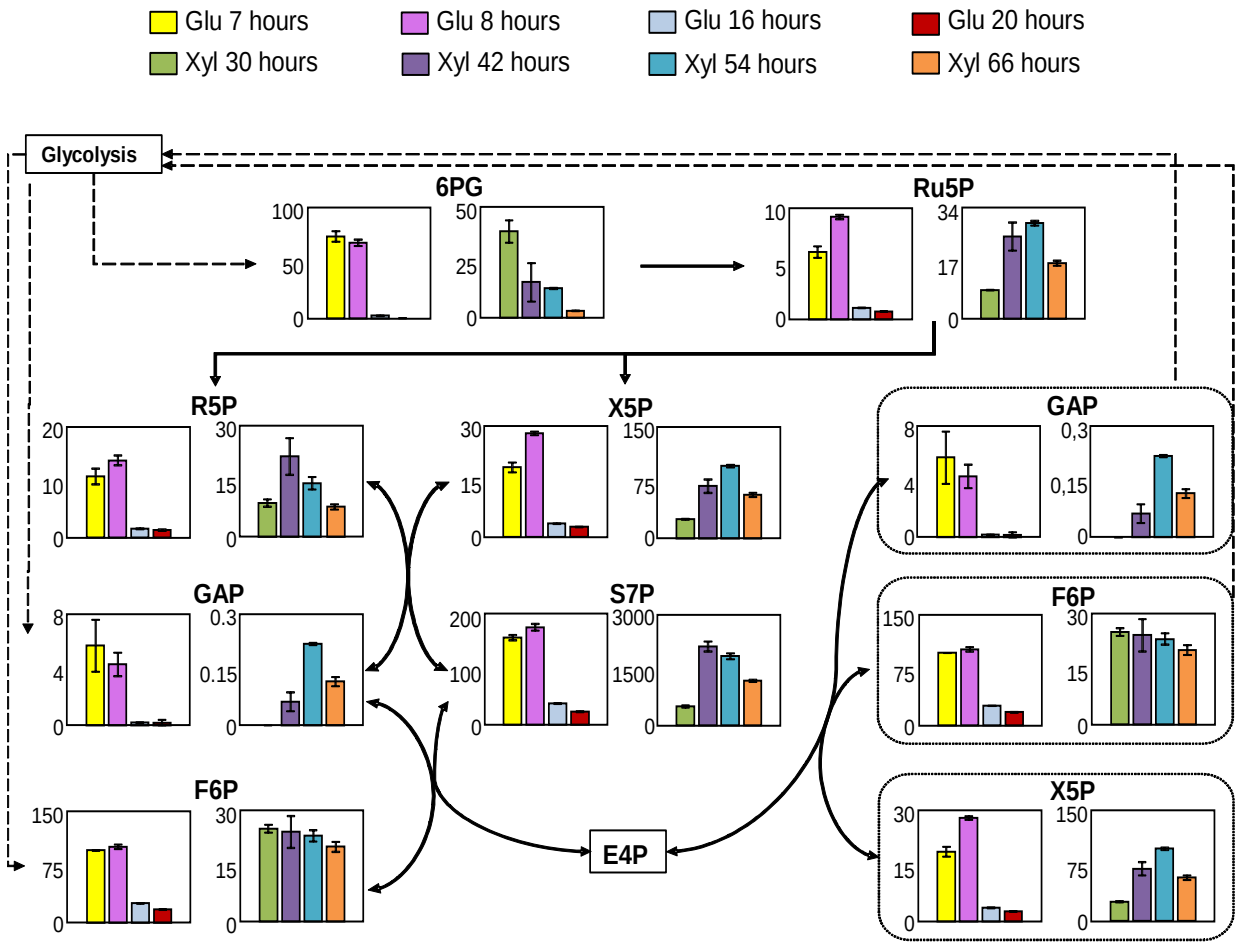


Fig. 4.5. The pool sizes (nmol/mg DCW) for the metabolites of the PPP. Data obtained from the samples taken after 7, 8, 16 and 20 hours of cultivation on glucose are depicted on the left, while the data obtained from the samples taken after 30, 42, 54 and 66 hours of cultivation on xylose, are to the right. Error bars represent the standard error of four independent biological repeats.

The pool sizes of metabolites of the Krebs cycle were generally smaller with cultivation on xylose compared to glucose. The metabolite pools of the Krebs cycle decreased over time with the exception of fumarate (FUM), isocitrate (ICIT) and *cis*-aconitate (ACON), which increased with cultivation on glucose (Fig. 4.6).

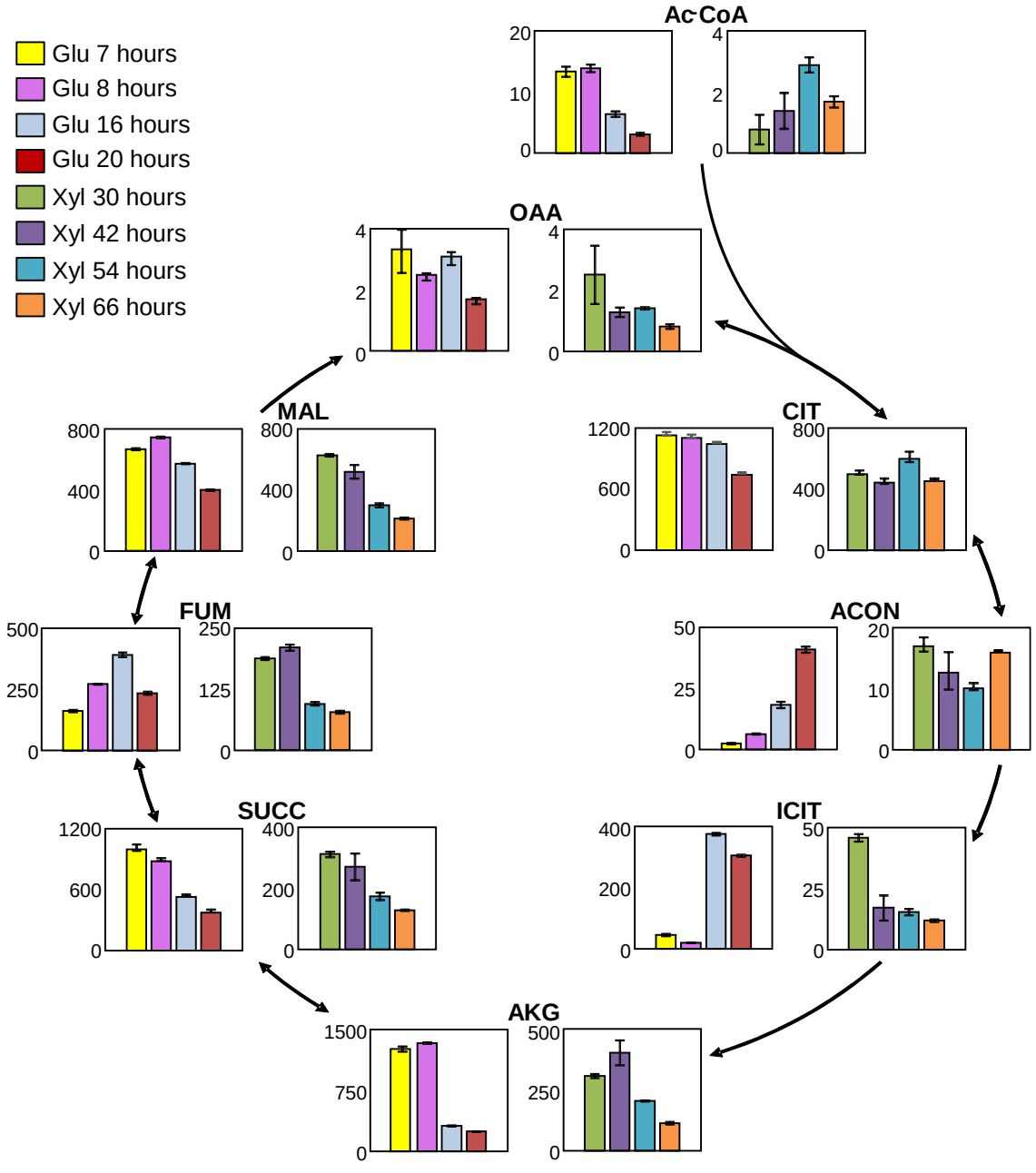


Fig. 4.6. The pool sizes (nmol/mg DCW) for the metabolites of the Krebs cycle. Data obtained from the samples taken after 7, 8, 16 and 20 hours of cultivation on glucose are depicted on the left while the data obtained from the samples taken after 30, 42, 54 and 66 hours of cultivation on xylose, are to the right. Error bars represent the standard error of four independent biological repeats.

The catalytic reduction charge (CRC: [NADH]/[NADH]+[NAD^+]), the anabolic reduction charge (ARC: [NADPH]/[NADPH]+[$NADP^+$]), the adenylate energy charge (AEC: [ATP]+0.5[ADP]/[ATP]+[ADP]+[AMP]) and the guanylate energy charge (GEC: [GTP]+0.5[GDP]/[GTP]+[GDP]+[GMP]) were calculated (Fig. 4.7) (Klimacek et al. 2010; Bergdahl et al. 2012; Matsushika et al. 2013). The metabolic pools for the different co-factors showed a similar profile (trend) for the cultivation on glucose and xylose (data not shown). The AEC and GEC remained between 0.7 and 0.9 throughout the cultivation, while the CRC and the ARC indicated an increase in NADH and NADPH over time with cultivation on glucose (Fig. 4.7).

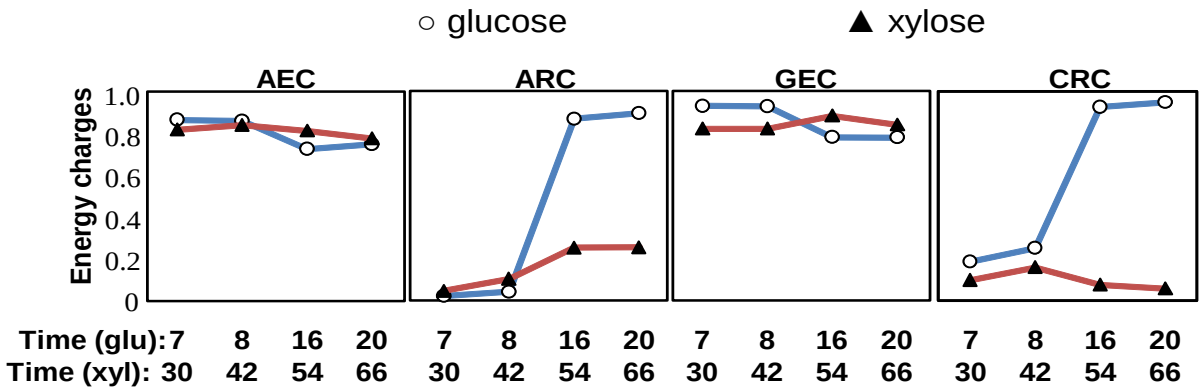


Fig. 4.7. The intracellular charge (ratios) of the culture was calculated using the pool sizes with cultivation on glucose (in blue) or xylose (in red) as carbohydrate source. Data points represent averages of four biological repeats (standard deviation ≤10).

4.5 DISCUSSION

Plant biomass (lignocellulose) can be used as feedstock for conversion to biofuels such as bioethanol. Xylose utilisation is a critical step towards xylan utilisation, but differences in strain backgrounds and growth conditions hamper the interpretation of the data accumulated through numerous studies involving the central carbon conversion pathways over the past three decades. Microbial growth requires careful coordination between nutrient assimilation, energy generation, redox balancing and biomass production. Therefore, this study examined the different pathways as a whole in order to find the optimal balance (or bottleneck) of catalytic activities for a given host at defined growth conditions. Haploid xylose-utilising *S. cerevisiae* S288c(a)[XylA-Xyl3] and S288c(α)[XylA-Xyl3] strains were constructed (expressing the *xylA* and *xyl3* genes) and cultivated on xylose as sole carbohydrate source. The fastest growing

strains of opposite mating types were hybridised to obtain the *S. cerevisiae* S288c(2n)[YMX12] strain (Fig. 4.2), since diploid strains typically display higher metabolic vigour and metabolite levels than haploid strains (Ding et al. 2010)

This study focused on determining the growth phase-dependent changes of the intracellular and extracellular metabolites produced by a diploid *S. cerevisiae* S288c(2n)[YMX12] strain harbouring the XI pathway. The strain was cultivated aerobically on glucose or xylose and the extracellular metabolite concentrations monitored over time (Fig. 4.3). The strain indicated a typical diauxic growth pattern on glucose. Ethanol was produced despite the presence of oxygen due to the high concentration of glucose (Crabtree effect). The ethanol was metabolised once the glucose was depleted and converted back to pyruvate (PYR), which then entered the Krebs cycle. Glycerol production coincided with the production of ethanol and acetic acid with cultivation on glucose. Glycerol acted as a protective agent that is synthesised in times of environmental stress and can function in maintaining a redox balance (Overkamp et al. 2004, Murray et al. 2011). In this study, it appears as if the glycerol acted as a defence against ethanol and acetic acid toxicity. The decrease in acetic acid and ethanol toxicity led to a decrease in glycerol demand and the excess glycerol was used for lipid metabolism. The glycerol concentration remained low with cultivation on xylose, presumably because little acetic acid and ethanol are produced.

The biomass concentrtion in the bioreactor (Fig. 4.3) was not as profound as in the shake flask experiments where double the amount of biomass was previously observed (see Chapter 3). Oxygen becomes limited during rapid growth on glucose in shake flasks (batch cultivations) while in the bioreactor the oxygen is constantly maintained. Therefore, the difference in biomass yields is more profound with cultivation in shake flasks due to oxygen limitation (Pronk et al. 1996). Despite the disruption of the *GRE3* gene, the xylitol accumulated extracellularly with cultivation on xylose. This might indicate the involvement of additional putative aldose reductases that had previously been identified in *S. cerevisiae* (Petrash et al. 2001, Träff et al. 2002).

Quantitative metabolomics provided the basis for comprehensive analysis of the main metabolic pathways involved in carbon conversion and energy/redox metabolism. The intracellular metabolome of the *S. cerevisiae* S288c(2n)[YMX12] strain was compiled at four time points (representing different growth phases) during aerobic cultivation on glucose or xylose (Fig. 4.4-4.6). The high ratio of G6P to F6P during growth on glucose suggested that

metabolism was satisfactory quenched during sample preparation (Wasylenko and Stephanopoulos 2015). An increase in PEP was noticed at 16 and 20 hours of cultivation, which coincided with the depletion of glucose after 12 hours of cultivation (Fig. 4.4) (Xu et al. 2012). The conversion of PEP to PYR is a rate-limiting step in glycolysis with the conversion of PEP to PYR being accelerated in the presence of glucose and slowing down when glucose is depleted. The data support the finding that pyruvate kinase I (PYK1) controls the flux through glycolysis (Pearce et al. 2001). The concentration of PYR was much lower with cultivation on xylose and coincided with the levels from samples taken after glucose depletion (16 h and 20 h). This indicates that xylose did not induce the expression of the *PYK1* gene, which negatively affected the flux through the rest of glycolysis. The lack of induction of the *PYK1* resulted in the low levels of metabolites detected throughout glycolysis with cultivation on xylose. The initial high levels of glucose result in the accumulation of PYR, which might indicate that the PYR conversion to ethanol might be rate-limiting (Fig. 4.4). The decrease in internal glucose concentration resulted in the production of the carbon catabolite repressed alcohol dehydrogenase 2 (ADH2) protein, which has a high affinity for ethanol and converts ethanol back to acetaldehyde.

In *S. cerevisiae*, the accumulation of G6P is a known trigger for the increase in turnover in the lower part of glycolysis (Boles et al. 1993, Boles et al. 1996). The level of G6P accumulation was about seven-fold lower with cultivation on xylose compared to that obtained on glucose (Fig. 4.4) and was therefore insufficient for the activation of the Crabtree response. High levels of fructose 1,6-phosphate (F1,6P) has recently been proposed as an important modulator of mitochondrial unspecific channel (MUC), whose closure contributes to the Crabtree effect (Rosas-Lemus et al. 2014). The levels of F1,6P was substantially lower with cultivation on xylose contributing to the incomplete activation of the Crabtree effect.

The significant amount of G6P that was detected with cultivation on xylose is due to the reverse reaction of the phosphoglucose isomerase enzyme (Fig. 4.4). The G6P is used for the synthesis of carbohydrates resulting in depletion of the G6P pool (Bergdahl et al. 2012, François et al. 2001). G1P is used for the production of nucleotide sugars and is obtained solely from the G6P pool with cultivation on xylose. The intracellular concentration of G1P is therefore lower than with cultivation on glucose due to the smaller G6P pool. Yet, the UDP-D-glucose levels were approximately eight times higher with cultivation on xylose compared to glucose (at 16 and 20 h), indicating that the carbon is channelled towards biomass production.

The accumulation of PEP (with cultivation on xylose) resulted in product inhibition of the preceding enzyme reactions, leading to the accumulation of 2PG and GAP etc. Yet, low levels of GAP (compared to the other metabolite pools) were detected with cultivation in glucose or xylose (Fig. 4.4), suggesting a high throughput at this point in glycolysis. This observation support the finding of Shestov et al. (2014) that glyceraldehyde 3-phosphate dehydrogenase is one of the main determinants of flux control through glycolysis. In the present study, the GAP is channelled to glycerol production through DHAP (Fig. 4.4).

Xylose is converted through the non-oxidative PPP, which links onto glycolysis at F6P and GAP. Cultivation on xylose resulted in an increase in the pool size of the metabolites of the PPP (Fig. 4.5), which is consistent with previous observations during pentose utilisation (in *S. cerevisiae*) (Matsushika et al. 2013). The GAP (in combination with S7P) were converted to erythrose 4-phosphate (E4P) and F6P, hence the low levels of GAP. The S7P, however, accumulated possibly due to the insufficient transaldolase (TAL1) activity. Surprisingly, when the TAL and TKL genes were overexpressed in a XR/XDH strain, only the overexpression of TAL resulted in enhanced xylose metabolism capability (Walfridsson et al. 1995) supporting our findings that TAL activity is the rate-limiting step in the PPP. E4P and R5P serve as precursors for amino acid and nucleotide biosynthesis and therefore did not accumulate.

The elevated levels of citrate (CIT), α-ketogluterate (AKG) and succinate (SUCC) were probably due to accumulation of the malate (MAL) resulting in the feedback inhibition of the Krebs cycle (Fig. 4.6). The accumulation of MAL and lower levels of FUM might be due to the influx of carbon to MAL through the glyoxylate pathway (from SUCC). The AKG is channelled into the pyrimidine pathway (for biomass production), preventing the accumulation of ICIT, which is especially noticeable with cultivation on xylose. The low levels of oxaloacetate (OAA) are either due to the direct conversion of OAA to PYR through pyruvate decarboxylase or to its use in gluconeogenesis.

The intracellular adenylate charge (AEC) is a common method for evaluating the overall energy state of a culture and has also been proposed to be an important regulatory signal for controlling the energy balance of a cell. In exponentially growing *S. cerevisiae*, the AEC reaches ~0.8 and may drop below 0.5 in response to carbon starvation. The *S. cerevisiae* S288c(2n)[YMX12] strain was able to maintain a constant AEC (Fig. 4.7), indicating that AEC is tightly regulated by the cell, which is consistent with previous findings by Bergdahl et al.

(2012) obtained with a xylose-utilising strain grown on glucose or xylose. The data suggests that AEC released the same signal irrespective of the carbon source.

In addition to AEC, the guanylate energy charge (GEC) can also be used as a method for evaluating the energy state of a culture although GEC is not related to any biological process. Bergdahl et al. (2012) suggested that GEC is a more sensitive technique compared to AEC for evaluating the energy state based on the fast and distinct response that GEC exhibits when the metabolism is switched from glucose to xylose utilisation. GEC is based on the GTP and GDP concentrations, which act as important regulatory factors for biomass and protein synthesis. The GEC remained consistent throughout the study, indicating that the GEC is also tightly regulated by the cell (Fig. 4.7). Exponentially growing cells have been shown to have a GTP/GDP ratio ≥ 4, whilst a GTP/GDP ratio < 1 indicates stationary phase cultures grown on glucose (Rudoni et al. 2001). The GTP/GDP ratios were calculated for each of the conditions (data not shown). For glucose metabolism at 7 and 9 hours, the ratios were ~6.2, indicating sturdy exponential growth. After switching to ethanol metabolism, the ratios were reduced to ~1.3, which indicated poor exponential growth. For xylose metabolism, the ratios increased from ~ 1.8 (30 and 42 hours) to ~3.3 (54 hours). Despite the consistent GEC, the GTP/GDP ratios suggested that the rate of protein and biomass synthesis during xylose metabolism was about 3.4 to 1.9 times slower than that for glucose growth. The data is accordance to that presented by Bergdahl et al. (2012), who demonstrated that the rate of cell growth resulted in xylose being sensed as a non-fermentable carbon source.

The biomass production increased the demand for NADPH produced by the oxidative PPP, which explains the increased ARC (higher ratio of NADPH:$NADP^+$) for later time points. An increase in the CRC was also observed for cultivation on glucose, which coincided with the abolishment of the Crabtree effect. The drop in glucose levels resulted in the PYR being channelled through the Krebs cycle which led to the production of NADH.

Although many xylose-utilising strains had been constructed to date, few studies make use of the expression of an XI. The XI expressing strain (multicopy episomal vector) used by Wasylenko and Stephanopoulos (2015) is able to consume xylose or glucose at the same rate and represents the benchmark of recombinant xylose-consuming yeasts. It had been improved upon by overexpression of the genes of the non-oxidative branch of the PPP and using a three step process of evolutionary engineering to obtain a growth rate of the 0.23 /hour on xylose (and glucose), which is attributed to the elevated levels of expression of the XI. The

S. cerevisiae S288c(2n)[YMX12] strain used in this study contains the XI integrated into the genome (low copy integration) and displayed a growth rate of 0.04 /hour, and can therefore benefit from an increase in expression levels of the XI in addition to the overexpression of the *TAL1* gene. Overexpression of PPP genes especially that of *TAL*, should indeed enhance the flux through the PPP and consequently improve xylose metabolism. In general strain adaptation can also be used to further improve growth on xylose. Overexpression of PYK could increase the flux through PEP to PYR, potentially enhancing the growth rate of S288c(2n)[YMX12] on xylose. This study identified a novel rate-limiting step for xylose metabolism; the enhanced flux from G1P to UDP-D-glucose. Therefore, reducing this flux should increase the glycolytic and PPP activities for S288c(2n)[YMX12], resulting in enhanced xylose metabolism and reduced biomass production.

4.6 CONCLUSIONS

The glycolytic PYK1 protein is induced by high glucose levels and controls the flux through glycolysis. The low levels of G6P and F1,6P results in incomplete inactivation of the Crabtree effect. In this study it was observed that G1P levels accumulate during xylose cultivation indicating a novel finding that more carbon is used for biomass production compared to glucose cultivation (Crabtree growth). Also, the accumulation of the PYR might indicate that the conversion of pyruvate to ethanol is a potential rate limiting step in ethanol production, which warrants further investigation.

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CHAPTER 5

General discussion and conclusions

5.1. GENERAL INTRODUCTION

The interest in renewable energy is driven by fuel security, the finite fossil fuel supply and the increase in atmospheric greenhouse gases (leading to climate change). Bioethanol production is the closest to an economically viable option among the liquid fossil fuel alternatives (Sarkar et al. 2012). First-generation bioethanol production from starch (corn, USA) and sucrose (sugar cane, Brazil) are well-established processes, but both these industries had to overcome significant challenges. It is expected that similar technical challenges will arise in second-generation bioethanol production from lignocellulosic feedstocks. Additional challenges include: (1) a variety of enzymes required for the complete hydrolysis of biomass; (2) detoxification of the biomass after pretreatment (or development of resistant strains); and (3) strain development for the utilisation of pentoses (Nogué and Karhumaa 2014).

Although the low cost of biomass as feedstock has its obvious advantages for biofuel production, it is outweighed by the efficient and cost-effective process of petroleum refinery. The low bulk density of biomass (such as grasses) affects transportation and storage, thus increasing the input cost. Pre-processing of biomass to higher density should benefit its transport and will lead to job creation in rural areas (Ten and Vermerris 2013). In addition, biofuels waste streams can be used for the production of bio-based chemicals to generate extra revenue to reduce the cost of biofuels, making them more competitive with fossil fuels. Maximum use of the waste stream will enhance the economic sustainability of biofuel production. The biomass pretreatment method will determine the quality and quantity of the waste products and hence the potential value-added products that can be derived.

The yeast *S. cerevisiae* has a successful industrial history due to properties such as robust fermentation capabilities (and Crabtree positive nature), efficient glucose repression, fast growth rate, ability to consume ethanol, tolerance to high ethanol concentration and low oxygen and pH levels (Piškur et al. 2006). These properties are widely distributed among different microorganisms, but are uniquely combined in *S. cerevisiae*. Much progress has been made on the conversion of cellulose to ethanol in recent years. Where xylan or xylose is concerned, research efforts have focused on the development of xylose-metabolising *S. cerevisiae* strains, rather than studying the effects that xylose has on central carbon and energy metabolism in *S. cerevisiae*. As a result, our understanding of the mechanisms involved in the regulation of xylose metabolism in recombinant xylose-utilising *S. cerevisiae* strains is limited, although some efforts have been made to understand xylose regulatory mechanisms (Salusjärvi et al.

2008; Klimacek et al. 2010; Wasylenko and Stephanopoulos 2015). Despite the development of several recombinant xylose-metabolising *S. cerevisiae* strains, xylose consumption remains slow with lower growth rates and fermentation capacity compared to glucose metabolism (Souto-Maior et al. 2009; Du et al. 2010). In order to make second-generation bioethanol a reality, challenges regarding efficient xylan metabolism need to be addressed.

5.2. ENGINEERING XYLAN METABOLISM

This study focussed on the hydrolysis of xylan and the utilisation of the resulting xylose by *S. cerevisiae* and demonstrated the benefit of combining genetic engineering with adaptive evolutionary techniques. The study involved the genetic engineering of *S. cerevisiae* for the expression of the *B. thetaiotaomicron xylA* gene and the partial characterisation of the recombinant XylA enzyme. In addition, the *Scheffersomyces stipitis* xylulokinase (*xyl3*) was overexpressed and the native aldose reductase (*GRE3*) gene disrupted to minimize xylitol formation. The xylose-utilising *S. cerevisiae* Y294[YMX1] and reference Y294[YMXR] strains were constructed and evaluated for growth on xylose and xylan as sole carbohydrate sources. The results indicated that aerobic growth on xylose as sole carbohydrate source elicits a Crabtree negative response in Y294[YMX1], resulting in biomass instead of ethanol being produced whereas the reference Y294[YMXR] strain exhibited no growth. The Y294[YMX1] strain exhibited satisfactory growth and was used for further adaptation on xylose to obtain a faster growing strain (due to faster xylose conversion).

The *Trichoderma reesei* xylanase (*xyn2*) and *Aspergillus niger* (*xlnD*) were co-expressed with the *xylA* gene. The secreted Xyn2 activity was not affected by the choice of carbon source and showed a linear increase with biomass (relative to glucose growth). In addition to the high biomass and lack of ethanol production observed, the data supported the assumption that xylose elicits a Crabtree negative response in *S. cerevisiae*. The recombinant enzyme activities in *S. cerevisiae*Y294[YMX1] were sufficient to allow growth on Birchwood xylan, albeit with substantially lower growth rates compared to xylose. The slow growth rate can be attributed to poor xylan hydrolysis due to steric hindrance by glucuronic acid side-chains, evident by the accumulation of aldobiuronic and aldotriuronic acids during growth on xylan. Xylan-fermenting *S. cerevisiae* strains have been reported in the literature, however, all fermentations were carried by high density inocula (e.g. OD_{600} of ~50 or ~100 g/L wet weight). This study focussed more on the development of xylan utilising strains from a proof-of-concept

perspective (Katahira et al. 2004; Sakamoto et al. 2012; Sun et al. 2012) and is one of only a few *S. cerevisiae* strains reported that is capable of growth on xylan from low inoculum densities (OD_{600} ~0.5).

Strain adaptation resulted in a strain that is capable of enhanced growth on xylose. It is difficult to speculate on the origin of the improvement since a combination of factors could have played a role. It is most likely that a change on genomic level affected the whole xylose conversion rate and would most possibly include a change in the first part of the xylose utilisation pathway that would result in a general increase in the flux through glycolysis or the PPP. These changes would include a general increase in xylose transport and/or an increase in xylose isomerase activity. An increase in the levels of activity of transaldolase (TAL) would alleviate the bottleneck in the pentose phosphate pathway (PPP) and increase the flux through it.

A diploid *S. cerevisiae* S288c(2n)[YMX12] strain was constructed from haploid prototrophic strains expressing the *S. stipitis xyl3* gene, *B. thetaiotaomicron xylA* gene and containing the *GRE3* gene disruption. Growth performance on either glucose or xylose was evaluated using computer-controlled bioreactors. The purpose was to minimise variations in metabolite concentration data as a result of the expression of *xyn2*, *xlnD* and other laboratory auxotrophic mutations, and to maintain well-controlled growth for physiological analysis. The *S. cerevisiae* S288c(2n)[YMX12] outperformed the original Y294[YMX1] by far, proving the benefits of employing prototrophic, diploid strains for enhanced xylose metabolism.

5.3. ELUCIDATING MECHANISMS OF XYLOSE METABOLISM

The inhibition of respiration (Crabtree effect) in the presence of excess glucose under fully aerobic conditions has mostly been attributed to the regulatory signalling mechanisms of glycolysis (Souto-Maior et al. 2009). In the current study, the high concentrations of glucose induced the expression of *HXK2* in *S. cerevisiae* S288c(2n)[YMX12] presumably via the Rgt2/Snf3 pathway, leading to the accumulation of G6P. Since elevated levels of G6P is responsible for controlling overall activation of glycolysis (Müller et al. 1995; Kim et al. 2013), several intermediates involved in upper glycolysis also accumulated in comparison with xylose metabolism. The data suggested that G6P production during xylose metabolism was insufficient to activate a high flux through upper glycolysis and ultimately couldn't induce the glucose repression response. The redox-balanced synthesis of Xu5P by the *B. thetaiotaomicron*

XylA was converted through the non-oxidative PPP to GAP and F6P during xylose metabolism. The levels of S7P accumulated substantially higher during xylose-metabolism, indicating that TAL activity is a rate-limiting step in the conversion of GAP/S7P to F6P/E4P. As a result, the flux of F6P through the PPP into glycolysis would be low since the E4P pool size was reduced by weak TAL activity and additionally channelled to nucleic acid synthesis, indicating that carbon entered glycolysis mainly at the GAP branch.

Another regulatory fate for G6P in *S. cerevisiae* is its conversion to UDP-D-glucose, synthesised from G1P via the reaction of uridylyl glucose pyrophosphorylase (UDPase), and plays a key role in central carbon metabolism. It is involved in protein glycosylation, galactose metabolism and the synthesis of storage compounds such as trehalose and glycogen. In particular, UDP-D-glucose is involved in polysaccharide metabolism, especially β-glucan, which constitutes more than 50% of cell wall carbohydrates (Daran et al. 1995; Thevelein and Hohmann, 1995). Therefore, *S. cerevisiae* requires G6P for biomass production. Since carbon exited the PPP mainly in the form of GAP, it can be speculated that gluconeogenesis was employed using fructose 1,6-bisphosphatase (F1,6Pase) since the conversion of F1,6P to F6P via glycolysis is essentially irreversible. During growth on xylose, the concentration of F6P remained consistent, opposed to gradually decreasing over time as is the case of most metabolites, whilst GAP levels were low. The data agreed with the assumption that large amounts of carbon is channelled from GAP to biomass production rather than ethanol, evident by higher UDP-D-glucose levels on xylose, and that a tight balance between energy generation and precursor metabolites exists as indicated by the consistent levels of F6P and G6P. Thus, the flux through the PPP during growth on xylose is not sufficient to promote G6P accumulation and hence activate carbon catabolite repression of respiration.

Trehalose 6-phosphate (T6P), a compound synthesised from UDP-D-glucose during the production of trehalose, is crucial for regulating glucose metabolism by inhibiting HXK2 in *S. cerevisiae* (Blázquez et al. 1993; Gancedo and Flores, 2004). Our data indicates that UDP-D-glucose levels were substantially low during the glucose growing phase, but rapidly increased when metabolism was switched to ethanol. This regulatory signal contributes to *S. cerevisiae's* ability to repress respiration in abundant glucose concentrations and switch to a more conservative mode of metabolism (synthesise trehalose and glycogen) when its preferred carbon source becomes limited. In contrast, UDP-D-glucose remains high throughout xylose metabolism (as well as the ethanol metabolism phase).

In *S. cerevisiae*, pyruvate (PYR) represents a major branch point in glycolysis, ultimately between respiratory dissimilation of sugars versus alcoholic fermentation (Pronk et al. 1996). The metabolomics data for glycolysis indicated that low levels of PYR are detected with cultivation on xylose (irrespective of the concentration), suggesting that the *PYK1* gene is not fully induced during cultivation on xylose as sole carbohydrate source. In contrast, the PYR levels are high with cultivation on high levels of glucose and drops upon glucose depletion. This indicates that the *PYK1* gene requires high levels of glucose for induction of optimal levels of expression and is not affected by the presence of oxygen. The accumulation of PYR during aerobic cultivation on glucose suggests that the conversion of PYR to Ac-CoA is negatively affected under high glucose concentrations, resulting in a diminished flux through the Krebs cycle. The accumulated PYR is then forced into conversion to ethanol. This PYR bottleneck might regulate the switch from respiration to Crabtree. The accumulation of PYR also indicates that the conversion to ethanol is rate-limiting under aerobic conditions, indicating that this pathway has not yet been optimised through evolution. There is a possibility that the pyruvate decarboxylase I (*PDC1*) gene and/or the *ADH1* gene are expressed at suboptimal levels in the presence of oxygen, because they originally evolved to produce ethanol under fermentative conditions.

The purpose of the Krebs cycle is to generate GTP and $FADH_2$ for protein synthesis and oxidative phosphorylation, respectively (Rudoni et al. 2001; Rodrigues et al. 2006). During glucose repression, the activity of the Krebs cycle is low since fermentative metabolism results in substrate-level phosphorylation for ATP synthesis. MAL and OAA indicate similar accumulation levels during the metabolism of glucose and xylose despite the differences in intermediate concentrations earlier on in the Krebs cycle (CIT to FUM). Pyruvate is directed towards alcoholic fermentation under glucose repression (respiro-fermentative metabolism) via PDC and ADH, but depending on the energy demands, seems to be able to bypass the PDH (respiration) and allow carbon to flow into the Krebs cycle for respiration. However, if respiration is not needed anymore, OAA is converted back to pyruvate where it becomes available again for PDC and ethanol production. This study suggests that the Krebs cycle was used simultaneously for respiration and fermentation during cultivation on glucose and that the lack of carbon catabolite repression during xylose metabolism could not activate the PDC and PDH by-pass complex, as evident by the lower levels of intermediates.

5.4. FUTURE WORK

Strains of *S. cerevisiae* has long been used and studied as cellular factories for the production of foreign proteins and other compounds of high value (Porro et al. 2005; Çelik and Çalık 2012). The development of xylose-utilising *S. cerevisiae* strains through recombinant and adaptive evolutionary techniques has made slow progress over recent years. Despite all the time and energy invested in this field, the desired rates of xylose consumption and fermentation remain unsatisfactory. Xylose utilisation by recombinant xylose-utilising *S. cerevisiae* strains remains limited compared to glucose metabolism (Souto-Maior et al. 2009; Du et al. 2010). It is generally acknowledged that the effective utilisation of xylose will require complex global changes in cellular processes and their regulatory mechanisms.

The identification of xylose isomerases that are well expressed in *S. cerevisiae* shifted the focus to elucidate the underlying principles of xylose metabolism in recombinant *S. cerevisiae.* The metabolomic studies have revealed novel responses regarding xylose metabolism. Yet, despite numerous studies performed on different aspects of xylose utilisation by *S. cerevisiae,* much remains unclear since the interpretation of the data is complicated by strain background and growth conditions.

The *S. cerevisiae* Y294[YMX1] strain is one of only a few *S. cerevisiae* strains reported to be capable of growth on xylan. Yet, much genetic engineering can still be done to improve the strain for xylose and xylan conversion. Overexpression of genes usually entails the use of promoters obtained from genes that are involved in glycolysis. Cultivation on xylose indicated a bottleneck in lower glycolysis (Jeffries 2006), implying that the choice of promoters should be revisited. All glycolytic promoter regions should be indirectly evaluated by using a reporter gene and a host strain capable of growth on xylose such as the *S. cerevisiae* Y294[YMX1] strain.

The strongest promoter region should be used for the overexpression of the *xylA.* It was not possible to tell from this study whether the XylA in the *S. cerevisiae* S288(2n)[YMX12] strain was rate-limiting. Yet, overexpression of the *xylA* gene is recommended to prevent accumulation early on in the xylose-utilising pathway, because the bottleneck will negatively affect the rest of the pathway. Overexpression can be accomplished by increasing the copy number of the *xylA* gene in the haploid counterparts through repeated cycles of transformation using different dominant markers or recycling of the markers. Integration into the rDNA sequences on the genome can also result in the integration of multiple copies of the desired

gene, although instability of the recombination area has been observed, leading to a loss in copy number over time.

The PYK1 has inefficient activity with cultivation on xylose (but not glucose), which slows down the flux of glycolysis. Therefore, the xylose-utilising strain would benefit from overexpression of the *PYK1* gene using a promoter that is not negatively affected by xylose. Similarly, the strain should be genetically manipulated to overexpress the native *TAL1* gene to prevent the accumulation of S7P. Yet, the repeated use of a promoter may decrease transcription from this promoter due to saturation of the cellular expression machinery (although the effects are strain dependent). Therefore, it is recommended that different promoter regions be used for overexpression of the different genes.

Deletion or inactivation of the *GRE3* gene is standard practise to minimise carbon loss through xylitol production and also to prevent inhibition of XylA by xylitol (Kovalevsky et al. 2012). Yet, xylitol is still detected, indicating that there might be other aldose reductase involved in xylitol production. The identification and disruption of these genes might therefore benefit xylulose production. Also, the genome sequencing of evolved strains revealed that a loss of function mutation in the *p*-nitrophenyl phosphatase (*PHO13*) gene improved growth on xylose (Kim et al. 2013). It is suggested that PHO13 generates a futile cycle with xylulokinase overexpression and warrants further investigation. However, the DNA sequence of the Y294[YMX1-A3.1] *PHO13* gene revealed an intact ORF, suggesting that the evolutionary changes took place elsewhere in the strain. Therefore, genome sequencing of the evolved *S. cerevisiae* Y294[YMX1-A1] to Y294[YMX1-A3] strains could reveal novel gene targets to aid the understanding, and therefore develop improved xylose-metabolising *S. cerevisiae* strains.

The pyruvate accumulated with cultivation on high levels of glucose suggested that the conversion to ethanol is slower than the production of pyruvate under Crabtree conditions. This phenomenon might be strain dependent, but is worth investigating since it is problematic for bioethanol production. The *S. cerevisiae* S288(2n)[YMX12] strain should be cultivated on glucose under anaerobic conditions to determine if the presence or lack of oxygen still results in the accumulation of PYR. The accumulation of PYR (irrespective of the levels of oxygen) would indicate that the activities of the pyruvate decarboxylase (PDC1) and/or the ADH1 are lacking. Accumulation of PYR under aerobic conditions (and no accumulation under anaerobic

conditions) will suggest that the levels of PDC1 and/or ADH1 are negatively affected by the presence of oxygen.

Xylose transport by the xylose-utilising strain was not addressed in the study, but is also worth investigating. Xylose transport can be enhanced through overexpression of all the native *S. cerevisiae* hexose transporters by deletion or disruption of the native *MTH1* or *IRA2* genes. Xylose utilisation can then be further improved upon by generating a diploid strain, followed by strain adaptation. Once the ideal xylose utilising strain had been constructed, the metabolomics study needs to be repeated using chemically defined medium (unlike this study that used a complex medium) and cultivation under aerobic versus anaerobic conditions. It is evident that elucidating the mechanisms of xylose regulation will not only significantly boost the development of more efficient xylose fermenting *S. cerevisiae* strains, but also provide new insights for the development of Crabtree negative strains. The next step would then entail the construction of a xylan-fermenting strain through the addition of xylanase genes and the genes encoding for the accessory enzymes.

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Appendix

Published review article

Engineering *Saccharomyces cerevisiae* for next generation ethanol production.

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Engineering *Saccharomyces cerevisiae* for next generation ethanol production

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Abstract

Conversion of cellulose, hemicellulose or starch to ethanol via a biological route requires enzymatic conversion of these substrates to monosaccharides that can be assimilated by a fermenting organism. Consolidation of these events in a single processing step via a cellulolytic or amylolytic microorganism(s) is a promising approach to low-cost production of fuels and chemicals. One strategy for developing a microorganism capable of such consolidated bioprocessing involves engineering *Saccharomyces cerevisiae* to expresses a heterologous enzyme system enabling (hemi)cellulose or starch utilization. The fundamental principle behind consolidated bioprocessing as a microbial phenomenon has been established through the successful expression of the major (hemi)cellulolytic and amylolytic activities in *S. cerevisiae*. Various strains of *S. cerevisiae* were subsequently enabled to grow on cellobiose, amorphous and crystalline cellulose, xylan and various forms of starch through the combined expression of these activities. Furthermore, host cell engineering and adaptive evolution have yielded strains with higher levels of secreted enzymes and greater resistance to fermentation inhibitors. These breakthroughs bring the application of CBP at commercial scale ever closer. This mini-review discusses the current status of different aspects related to the engineering of *S. cerevisiae* for next generation ethanol production.

Keywords: second-generation biofuels, strain engineering, bioethanol, cellulose, raw starch

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INTRODUCTION

The increasing priority that governments place on energy security and environmental concerns, the emphasis on bio-energy from the industrial perspective due to increasing pressures to reduce carbon footprints, and the steadily increasing demand for and cost of crude oil, has led to the development of an active biofuels industry. [1] First generation biofuels such as ethanol from corn starch or sugarcane already contribute considerable amounts of liquid fuels in several countries. World-wide production of bioethanol was 106 billion litres in 2011 and was estimated to reach 113 billion litres in 2012 (OECD-FAO Agricultural Outlook 2011-2020; http://stats.oecd.org/viewhtml.aspx?QueryId=30104&vh=0000&vf=0&l&il=blank&lang=en) . However, the first generation technologies suffer from some technical inefficiencies and a shortage in the availability of feedstock in order to displace a more significant amount of petroleum-based fuels. Lignocellulosic biomass is the most abundant source of renewable carbon on Earth and is the only feedstock that could possibly be sufficient to satisfy the world's energy and chemical needs in a sustainable and renewable manner.[1,2] Second-generation biofuels therefore seek to overcome the problem of feedstock supply shortage by utilizing the energy contained in total plant biomass.

Conversion of biomass to ethanol via a biological route commences with physical and/or chemical pre-treatment processes to render the polymeric fractions more accessible to enzymatic hydrolysis.[3] Four biologically mediated events are then required to convert pre-treated lignocellulose to ethanol, namely: (i) production of depolymerising enzymes, (ii) hydrolysis of the polysaccharides components of biomass, (iii) fermentation of the resulting hexose and (iv) pentose sugars present.[4] This four-stage process is termed separate hydrolysis and fermentation (SHF, Fig. 1(a)). Improvements in biomass conversion technologies involve combining two or more of these steps. Hydrolysis and fermentation steps are combined in either simultaneous saccharification and fermentation (SSF, Fig. 1(b)) of hexoses or simultaneous saccharification and co-fermentation (SSCF, Fig. 1(c)) of both hexoses and pentoses if a suitable fermentative organism is available. These processes avoid the feedback inhibition effect that the release of sugars during polymer hydrolysis has on the enzymes. However, when a mesophilic process organism is used, it requires lowering the operating temperature to a level that is suboptimal for enzymatic activity. Ultimately, one-step consolidated bioprocessing (CBP, Fig. 1(d)) of lignocellulose to bioethanol is envisioned, where all steps occur in a single reactor and a single microorganism or microbial consortium

converts pretreated biomass to a commodity product such as ethanol with no requirement for additional exogenous enzymes. CBP would represent a breakthrough for low-cost biomass processing due to the economic benefits of process integration[1,5,6] and avoiding the high costs of enzymes that make the biological conversion route unattractive.[7] Most of the fuel ethanol currently produced worldwide is based on starch substrates.[8] However, as the liquefaction and saccharification of starch requires heating and the addition of enzymes, the development of an organism that could ferment raw starch to ethanol in one step would similarly represent a low-cost processing alternative.

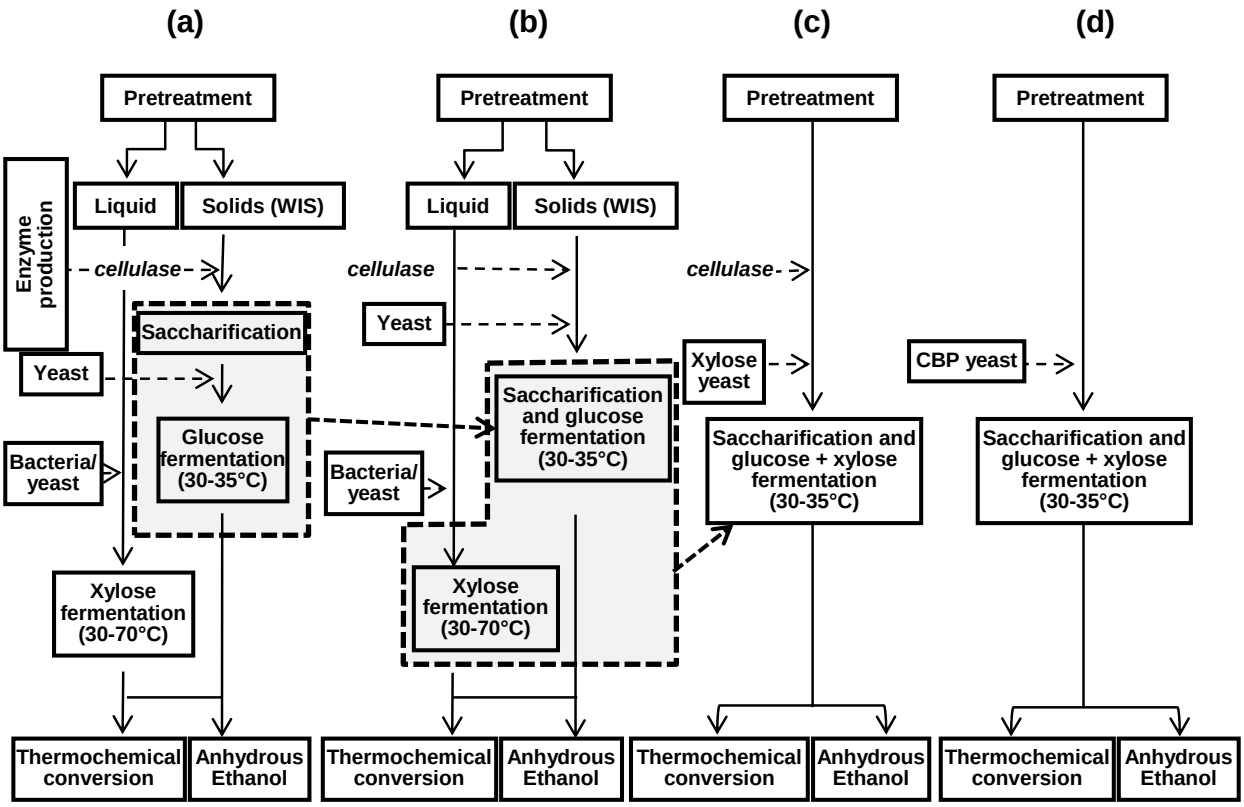

Fig. 1. Schematic representations of the processes involved in conversion of lignocellulosic biomass to ethanol and how steps can be combined for process improvement. (a) Separate hydrolysis and fermentation (SHF). (b) Simultaneous saccharification and fermentation (SSF). (c) Simultaneous saccharification and co-fermentation (SSCF) (d) Consolidated bioprocessing (CBP). WIS = water insoluble solids. "Thermochemical conversion" refers to further processing done on remaining solids consisting mainly of lignin. "Xylose yeast" refers to a yeast strain enabled to ferment xylose.

Organisms that simultaneously hydrolyze the cellulose and hemicelluloses in biomass or directly ferment raw starch (Fig. 2) and produce a valuable product such as ethanol at a high rate and titer, would therefore significantly reduce the costs of current biomass conversion technologies. The yeast *S. cerevisiae* has long been used as an industrial ethanologen due to its high rate of ethanol production from glucose, high ethanol tolerance, general robustness and favorable GRAS (generally regarded as safe) status.[9-11] Furthermore its genome can be rapidly and stably manipulated and it is thus a good candidate to develop for CBP technologies. However, the drawbacks that must be overcome include the engineering of pentose sugar utilization and the production of the enzymes required to hydrolyze cellulose and hemicellulose or starch.[12] This review will look at some of the progress made and improvements required to develop *S. cerevisiae* as an optimal organism for next generation ethanol production from both starch and lignocellulosic feedstocks.

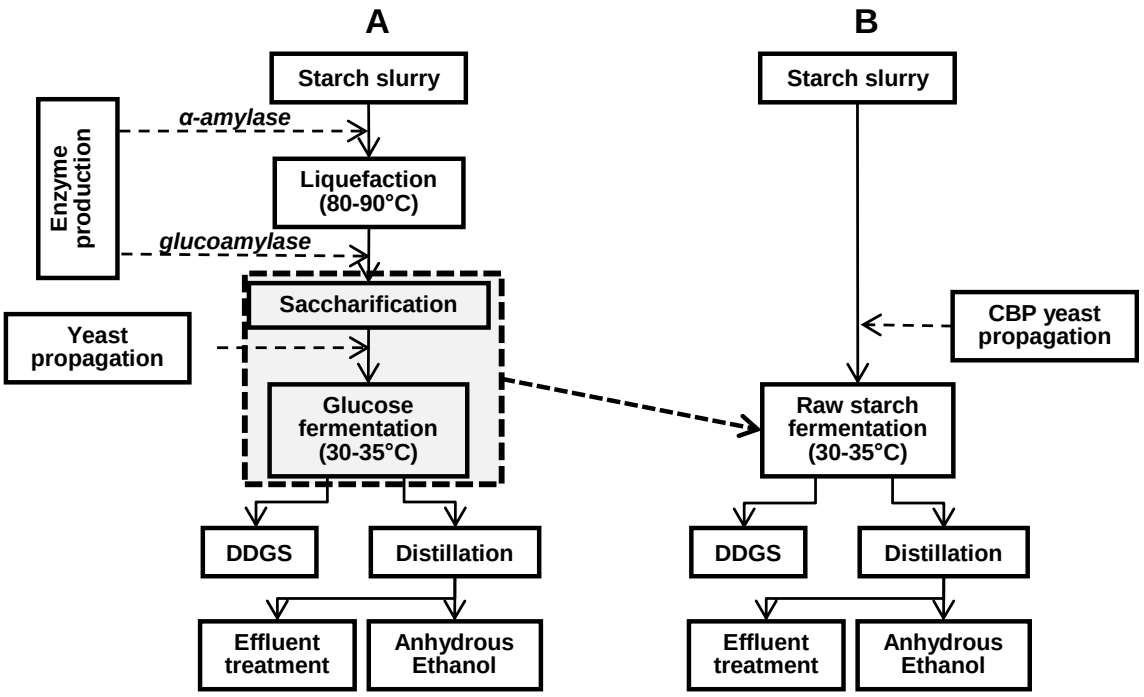

Fig. 2. Schematic representations of the processes involved in conversion of starch to ethanol and how they can be combined for process improvement. (a) A currently employed process. (b) The streamlined process avoiding separate heating and saccharification steps if a raw starch utilizing yeast is employed. DDGS = dried distillers grains with soluble.

STARCH CBP CONVERSION

After cellulose, starch is the most abundant hexose polymer in plants[8] and the commercial production of biofuels from cornstarch through liquefaction, hydrolysis and fermentation (Fig. 2) is a relatively mature technology (reviewed in[8]). However, the excess energy demand for heating of the starch slurry, and the high cost of adding large quantities of exogenous enzymes have a negative impact on the current first generation starch-to-ethanol processes. Both these limitations can be addressed if a single organism could produce the starch-hydrolyzing enzymes and ferment the resulting sugars to ethanol, i.e. perform CBP. The cost-effective conversion of raw starch therefore requires the heterologous expression of starch-hydrolyzing enzymes (e.g. α-amylases and glucoamylases) in the fermenting yeast strain to enable liquefaction, hydrolysis and fermentation.

The two main strategies for the development of amylolytic yeast strains to date focused on the secretion of amylases into the fermentation broth and tethering of enzymes on the cell surface. The efficiency of secreted enzymes has been demonstrated at low (1-2%) starch loadings[13-17], but the *de novo* production of these enzymes significantly delays the conversion of raw starch at high substrate loadings. An amylolytic *S. cerevisiae* strain expressing multiple integrated copies of the *Aspergillus awamori* glucoamylase and *Debaryomyces occidentalis* α-amylase was able to convert 79% of 200 g L^{-1} raw corn starch to 10.3% (v/v) ethanol after 144 h, but this required a rich growth medium (YP, 2% yeast extract and 2% Bacto-peptone) and relatively high inoculum (a ten-fold dilution of a cell culture entering stationary phase on YP with 2% soluble starch).[18,19]

Tethered enzymes displayed on the surface of recombinant *S. cerevisiae* have shown good potential, but the tethered enzymes can only interact with substrate in the immediate vicinity of the cell surface. thus requiring high cell loadings.[20-24] Increased ploidy of the host strain significantly increased enzyme titres.[25-26] A tetraploid *S. cerevisiae* host expressing the *Rhizopus oryzae* glucoamylase and *Streptococcus bovis* α-amylase produced 70 g L^{-1} ethanol from 150 g L^{-1} raw starch after 72 h at a cell loading of 10 – 100 g L^{-1} wet cell mass.[25] This implies a 85% conversion of the available starch, but it remains difficult to compare this with other reports as substrate and cell loading are not standardised.

Amidst good progress, the major challenge remains the production of α-amylases and glucoamylases with high substrate affinities and specific activities that can effectively convert raw starch to ethanol concentrations in excess of 10% (w/v) within 48 to 72 hours.[27] Recently, Mascoma Corporation (Lebanon, NH, USA) developed a "drop-in" yeast strain that produces glucoamylase, which could save on the addition of commercial glucoamylase during the classical high temperature conversion of grain starches to ethanol. The yeast will be marketed by Lallemand Ethanol Technology as TransFerm™ yeast and according to Mascoma Corporation more than 400 million litres of ethanol have been produced with this strain to date.[28] However, a commercial CBP yeast producing α-amylase and glucoamylase enzymes for raw starch utilisation is not yet available. It might also be beneficial in the future to construct CBP yeasts that produce both α-amylase and glucoamylase together with an endoglucanase, cellobiohydrolase, β-glucosidase and auxiliary enzymes to ensure full utilization of both the starch and (hemi)cellulolytic residues, which could account for up to 30% of the dry weight in the case of cassava pulp.[29]

CBP LIGNOCELLULOSE CONVERSION

Engineering CBP strains for cellulose bioconversion

Lignocellulosic plant biomass consists of 40–55% cellulose, 25–50% hemicellulose and 10–40% lignin, depending on the source.[30] The main polysaccharide present is water-insoluble crystalline cellulose, which also represents the major fraction of fermentable sugars. As wild-type *S. cerevisiae* strains do not produce secreted hydrolytic activities active on cellulose, these activities have to be engineered into cellulose-CBP strains. The hydrolysis of crystalline cellulose is achieved by the co-operative action of (i) endoglucanases (EGs), which act in the amorphous regions of cellulose to release cellodextrins and provide free chain ends; (ii) exoglucanases, including cellodextrinases and cellobiohydrolases (CBHs), which act on crystalline cellulose in a processive manner starting at the free chain ends and releasing mainly cellobiose; and (iii) β-glucosidases (BGLs), which hydrolyze cellobiose and small cello-oligosaccharides to glucose.[4,31]

In the last 25 years, several authors have reported the expression of cellulase encoding genes in *S. cerevisiae*.[11] Several researchers have sought to produce cellulases in an organism that would not yield interfering activities, so as to gain insight into the mechanism of the original

cellulolytic enzyme, whereas others have sought to enable the yeast to hydrolyze cellulosic substrates. Recombinant strains of *S. cerevisiae* were created through the expression of a *β*-glucosidase, which could grow on and ferment cellobiose, the main product of cellobiohydrolase action, at roughly the same rate as on glucose.[32] This strains expressing *Saccharomycopsis fibuligera* β-glucosidase (*bgl1*) had a similar growth rate when grown on cellobiose or glucose, reached similar biomass and obtained a final ethanol yield of 2.3 g/L from 5 g/L cellobiose, compared to the 2.1 g/L when glucose (5.26 g/L) was used as substrate. Recently, the high affinity cellodextrin transport system of the model cellulolytic fungus *Neurospora crassa* was reconstituted into *S. cerevisiae.*[33] This allowed a recombinant strain also producing intracellular *β*-glucosidase to grow on cellodextrins up to cellotetraose. Sadie *et al*.[34] similarly showed that heterologous production of the *Kluyveromyces lactis* lactose permease facilitated transport of cellobiose into a recombinant *S. cerevisiae* strain. The report also showed the successful expression of a *Clostridium stercorarium* cellobiose phosphorylase (*cepA*) and that strains co-producing the heterologous CEPA and LAC12 were able to grow on cellobiose as sole carbohydrate source. Recently a strain was engineered that produced a xylose isomerase and cellobiose phosphorylase from *Ruminococcus flavefaciens* along with several other native gene manipulations that enabled fermentation of glucose, xylose, and cellobiose under anaerobic conditions.[35]

Cho *et al*.[36] demonstrated with SSF experiments using a strain co-producing a *β*-glucosidase and an exo/endocellulase, that loadings of externally added cellulase could be reduced. This paved the way for engineering strains that could grow on and ferment cellulosic substrates without the need for external enzyme addition. Fujita *et al*.[37,38] reported co-expression and surface display of cellulases in *S. cerevisiae*. High cell density suspensions of a strain displaying the *Trichoderma reesei* EG2 and CBH2, and the *Aspergillus aculeatus β*-glucosidase, were able to directly convert 10 g L^{-1} phosphoric acid swollen cellulose (PASC) to approximately 3 g L^{-1} ethanol. An *S. cerevisiae* strain co-expressing the *T. reesei* EG1 (*cel7B*) and *S. fibuligera β*-glucosidase (*cel3A*) was able to grow on and convert 10 g L^{-1} PASC to ethanol up to 1 g L^{-1}.[39] A similar strain that produced significantly higher endoglucanase activity displayed improved conversion of PASC to ethanol.[40] More recently, Yamada *et al*.[41] developed an ingenious method to integrate a cocktail of cellulase genes through multi-copy δ-integration, to optimize expression levels. Different cellulase expression cassettes encoding the three main cellulase activities were integrated into *S. cerevisiae* chromosomes in one step, and strains expressing an optimum ratio of these cellulases were selected for by growth on

media containing PASC as carbon source. Although the total integrated gene copy numbers of an efficient "cocktail" δ-integrant strain was roughly half that of a conventional δ-integrant strain, the PASC degradation activity (64.9 mU g^{-1}-wet cells) was higher than that of a conventional strain (57.6 mU g^{-1} wet cells). This suggested that optimization of the cellulase expression ratio improved PASC degradation activity more than simply overexpression of cellulase genes. . Using a high cell loading of this strain, they obtained 3.1 g L^{-1} ethanol from PASC after 72h, accounting for 75% of the theoretical ethanol yield form the hydrolyzed fraction.

The fungal cellulases discussed above are part of so-called free enzyme systems in their native hosts. However, some bacteria such as *Clostridium thermocellum* have been shown to produce cellulase complexes, named cellulosomes, on their cell wall surfaces.[42] Multiple catalytic components are assembled on a scaffoldin subunit through strong non-covalent protein–protein interactions between cohesin modules on the scaffoldin and dockerin modules on the enzymes [43] This highly ordered structure of multiple enzymes in close proximity to the substrate results in a high level of enzyme–substrate–microbe synergy.[44,45] Several groups have attempted to reconstruct a mini-cellulosome on the *S. cerevisiae* cell surface.[46-48] Ito *et al*.[46] constructed a chimeric scaffoldin to allow cell surface display of both *T. reesei* EG2 and *A. aculeatus* BGL1, yielding yeast strains capable of hydrolyzing *β*-glucan. *S. cerevisiae* strains were also engineered to display a tri-functional mini-cellulosome consisting of a mini-scaffoldin containing a cellulose binding domain and three cohesin modules, anchored to the cell surface, as well as three types of cellulases, namely the *T. reesei* EG2 and CBH2 and *A. aculeatus* BGL1 , each bearing a C-terminal dockerin.[48] This strain was able to hydrolyze and ferment PASC to ethanol with a titer of 1.8 g L^{-1}.

As exoglucanase activity is required for the successful hydrolysis of crystalline cellulosic substrates, the addition of high-level expression of cellobiohydrolases to strains actively hydrolyzing amorphous cellulose substrates should enable effective conversion of crystalline cellulose to ethanol. While high-titer production of CBHs in yeast has proven problematic, the production of relatively high levels of CBH1 and CBH2 in *S. cerevisiae* was recently reported for the first time.[49,50] Under high cell density fermentations, recombinant CBH1 and CBH2 could be secreted up to 0.3 g L^{-1} and 1 g L^{-1}, respectively. Through this route a yeast strain was constructed that was able to convert most of the glucan available in paper sludge to ethanol and displace ~60% of the enzymes usually required to convert the sugars available in pretreated

hardwood to ethanol in an SSF configuration.[50] A similar strain expressing three alternative cellulases produced ethanol from pretreated corn stover in one step without the addition of exogenous enzymes.[51] This strain fermented 63% of the available cellulose to 2.6% (v/v) ethanol in 96 hours. These results demonstrate that cellulolytic *S. cerevisiae* strains can be used as a platform for developing an economically viable biofuel process, but that further optimization of the strains are required.

Strains for hemicellulose conversion

Unlike cellulose and starch, which are predominantly homogenous polymers of *β*-glucans and *α*-glucans,[52,53] hemicelluloses are heterogeneous polysaccharides with diverse structural compositions. The extents to which the structures of hemicelluloses differ depend on the plant species, but four major groups may be distinguished based on polysaccharide content: (i) xyloglucan, (ii) xylans, (iii) mixed-linkage glucans, and (iv) mannans (reviewed in[54,55]). In the context of lignocellulose to ethanol conversion technologies, it is important that the hydrolysis of hemicelluloses are realized, not only to add carbon value to the production chain, but also to expose more cellulose to enzymatic attack, since hemicellulose binds cellulose fibers within lignocellulosic structures and xylo-oligomers strongly inhibits the activities of cellulase enzymes.[56,57] This could significantly lower cellulase loadings and hence enhance process economics.[58,59]

As mentioned previously, *S. cerevisiae* lacks the ability to assimilate pentose sugars such as xylose and arabinose which may be prevalent in hemicellulose depending on the feedstock. Over the past three decades, considerable research efforts have focused on the development of recombinant xylose and arabinose utilizing strains. Engineering xylose utilization has largely been done by the heterologous expression of either xylose reductase/xylitol dehydrogenase (XR/XDH) or xylose isomerase (XI) encoding genes (reviewed in[5,60,61]). Expression of XI's in *S. cerevisiae* has the advantage of evading redox imbalance complications observed with the XR/XDH pathway. In addition, overexpression of pentose phosphate pathway enzymes had positive effects on xylose metabolism and deletion of the *S. cerevisiae* non-specific aldose reductase (*GRE3*) significantly limited xylitol formation.[62] More recently, Zhou *et al.*[63] developed a *S. cerevisiae* strain overexpressing the *Piromyces* spp. XI, *Scheffersomyces* (*Pichia*) *stipitis* XK, and genes of the non-oxidative PPP. In combination with evolutionary

adaptation techniques, they were able to report a recombinant yeast strain displaying an anaerobic xylose consumption rate of 1.866 g g^{-1} h^{-1} with ethanol yields of 0.41 g g^{-1}. This represents a remarkable breakthrough in engineering xylose utilization in yeasts.

For the complete hydrolysis of the hemicellulose xylan, the actions of six enzymes are required, namely endoxylanase, *β*-xylosidase, *α*-arabinofuranosidase, *α*-glucuronidase, acetylxylan esterase and ferulic acid esterase.[54] Since xylans represent the most abundant hemicellulose of most biomass materials targeted for ethanol production, the greater part of research efforts to create hemicellulolytic *S. cerevisiae* strains have focused on this substrate. Initial attempts involved the expression of extracellular *β*-xylosidase and xylanase II genes from *Aspergillus niger* and *T. reesei*, respectively.[64] The engineered strain was capable of converting birchwood xylan to short-chain xylo-oligomers, with xylose being the major end product (more than 20 g L^{-1} xylose released from 50 g L^{-1} xylan). Katahira *et al*.[65] developed a yeast strain co-displaying the *A. oryzae β*-xylosidase and *T. reesei* xylanase II on the cell-surface of *S. cerevisiae* and showed that xylose remained the major end product. Furthermore, application of this technology in a recombinant xylose fermenting yeast resulted in the direct conversion of birchwood xylan to ethanol with a productivity rate of 0.13 g L^{-1} h^{-1}, and a yield of 0.30 g g^{-1} sugar consumed. More recently the same group developed similar yeast strains with the addition of the *A. aculeatus β*-glucosidase that was capable of direct conversion of rice straw hydrolysate to ethanol with a productivity rate of 0.37 g L^{-1} h^{-1} and ethanol yields of 0.32 g g^{-1} of total sugars consumed.[66] As it is known that synergistic interactions amongst cellulases and hemicellulases may exist, engineering these activities onto the cell-surface of yeasts in a well-controlled and ordered manner could result in higher synergism. Enhanced synergism of aribinoxylan and birchwood xylan hydrolysis was indeed shown with the development of *S. cerevisiae* strains displaying mini-hemicellulosome's on the cell-surface consisting of a miniscaffoldin harboring xylanase II, arabinofuranosidase, and *β*-xylosidase enzymes.[67] However, a maximum ethanol yield of only 0.31 g g^{-1} birchwood xylan consumed could be produced when anchoring a bi-functional (xylanase II and *β*-xylosidase) hemicellulosome to a *S. cerevisiae* strain expressing the *S. stipitis* XR/XDH xylose fermenting pathway. This strain produced ~1 g L^{-1} ethanol in 80 hours in high cell density fermentations. A similar study further supported the fact that engineering hemi-cellulosomes result in enhanced synergy, but also highlights the importance of engineering various carbohydrate binding domains.[68]

STRAIN IMPROVEMENT FOR CBP

Adaptation strategies to create more inhibitor tolerant strains

As stated earlier, the conversion of lignocellulosic materials to bio-ethanol requires physical-chemical pretreatment prior to enzymatic hydrolysis and fermentation, to overcome the natural recalcitrance of lignocellulose to biological conversion.[69] Steam- or acid-catalyzed pretreatment methods are preferred due to acceptable cost and similarity to industrial technologies for lignocellulose processing. However, these methods also result in the co-production of a number of degradation products from the hemicellulose (furfural, acetic acid), lignin (phenolic compounds) and cellulose (5-hydroxmethyl-2-furaldehyde, HMF) fractions.[70,71] These pretreatment by-products are present in both the hydrolysate liquor and water insoluble solid (WIS) fractions of the pretreatment slurry and have been shown to inhibit microbial fermentation, resulting in reduced ethanol yields and/or productivity through synergistic action, which has negative cost implications. These two components of the pretreatment slurry should be co-fermented in a single reaction step by the ideal lignocellulose-CBP organism, capable of enzymatic hydrolysis of the WIS together with simultaneous fermentation of the glucose and xylose released by pretreatment and subsequent hydrolysis, in the presence of inhibitors. Such an organism must be capable of performing this fermentation without the aid of the various chemical and biological detoxification strategies that have been proposed[72,73] as these strategies add to the cost of ethanol production from lignocellulose.[74] However, while selecting strains with improved inhibitor resistance is important, care should be taken not to select strains where resistance is dependent on higher biomass production, which would be at the expense of ethanol production. The same limitation will apply to engineering yeasts for higher levels of hydrolytic enzymes that are dependent on higher biomass yields.

Engineering of a lignocellulose-CBP strain of *S. cerevisiae* should therefore include the development of robust yeast strains with increased resistance towards inhibitors formed during pretreatment.[70] *S. cerevisiae* has significant capacity for such development, based on intrinsic robustness and hardiness towards various environmental stresses.[75,76] The selection of host strains for CBP development should therefore include screening for inhibitor resistance.[77] Furthermore, the so-called “hardening” of selected yeast strains for improved robustness and metabolic capability for detoxification of pretreatment by-products has been demonstrated through a combination of rational metabolic engineering[78,79], directed evolution[80] and

adaptation through long-term cultivation under selection pressures.[81,82] Long-term continuous cultivation of *S. cerevisiae* in the presence of inhibitors has been demonstrated as a means to develop improved physiological traits,[9,83,84] including improvement of resistance to inhibitors formed during lignocellulose pretreatment.[81,82,85,86] The efficacy of such continuous cultivation towards the improvement of metabolic characteristics is enhanced when combined with both mutagenesis and metabolic engineering.[87] The use of directed evolution of yeast strains as a method to produce inhibitor resistant yeasts has been applied with great success.[88] The authors were able to obtain yeast strains that were significantly more resistant to the inhibitory effect of spruce hydrolysate after 429 and 97 generations, by applying selective pressure in batch cultures and chemostat, respectively. In a relatively short time the specific growth rate in spruce hydrolysate was increased from 0.18 h^{-1} to 0.33 h^{-1} and ethanol yields increased up to a 50% under anaerobic conditions. Pinel *et al.*[89] combined random UV mutagenesis and genome shuffling to obtain yeast strains resistant to inhibitors in hardwood spent sulfite liquor. Superior yeast strains were then selected by evolutionary engineering to obtain strains capable of growing in undiluted spent sulfite liquor.

An example of metabolic engineering for improved resistance to inhibitors is the overexpression of oxido-reductases to catalyze the reduction of HMF and furfural, resulting in significant improvements in ethanol productivity.[89,90] Other metabolic targets for improved detoxification capability have also been identified.[91,92] A better understanding of yeast metabolism and physiology brought about by accumulating genomic, transcriptomic and metabolic data provides the tools for rational design of industrially relevant organisms.[93] Identifying gene products responsible for desired phenotypes remains the key step to strain engineering strategies; and possibly the most elusive at this point, considering the polygenic nature of many desired phenotypes.[94] Applying a phenomics approach on the analysis of mutants' phenotypes lacking genes of interest, Endo *et al.*[95] predicted the involvement of two clusters of genes involved in the cells' resistance to vanillin, an inhibitor in the lignin fraction of pretreated lignocellulose. They further identified ergosterol synthesis mutants that had decreased resistance to vanillin, suggesting a link between vanillin resistance and ergosterol content in cellular membranes.[95] Gorsich *et al.*[78] identified 62 genes whose deletion resulted in lowered resistance to furfural. Overexpression of four of the genes identified, namely *ZWF1*, *GND1*, *RPE1* and *TKL1*, showed that only the *ZWF1* overexpression resulted in increased resistance to furfural at levels toxic to the wild type strain.[78] To engineer resistance to phenolic inhibitors, the overexpression of *PAD1* resulted in a 50 to 100% improvement in ethanol

productivity, in the presence of ferulic and cinnamic acid[80], while heterologous expression of a *Trametes versicolor* laccase allowed growth in media containing coniferyl aldehyde at concentrations that completely inhibited the growth of the control strain.[80]

Using SHF strategies, several researchers have shown that fermentation inhibitors may also have a dramatically negative impact on added hydrolytic enzymes.[96] The presence of tannic, gallic, hydroxy-cinnamic, and 4-hydroxybenzoic acids, as well as vanillin lead to 20– 80% deactivation of cellulases and β-glucosidases, varying with the specific enzymes, the source they were derived from and the particular compound.[97] The effect of inhibitors on heterologous hydrolases has not been well researched but this should clearly be a future research focus to help create CBP yeasts that produce heterologous enzymes that are inhibitor tolerant and therefore active in process conditions.

Strategies to enhance secretion

The expression of the three major cellulolytic activities in pioneering *S. cerevisiae* cellulose-CBP strains allowed for partial degradation of cellulosic substrates, or complete degradation with the addition of commercial enzyme cocktails.[49,50] Similarly, amylase production in starch-CBP yeasts was not sufficient to allow full saccharification in a short time period. It is thus apparent that higher levels of these enzymes must be produced, perhaps in addition to other cellulolytic activities to enhance substrate hydrolysis. Since yeasts with different genetic backgrounds display apparent dissimilarity in secreted heterologous protein titers, tolerance to inhibitors, growth rates, biomass and ethanol yields, careful selection of the basal strain should be prioritized. A clear example of the difference in secretion capacity was the significant variation in secreted levels of *S. fibuligera* BGL1 among seven genetically diverse *S. cerevisiae* strains, with enzyme activities ranging from 73 mU/ml to 250 mU/ml.[98]

Enhancing the secretion capacity of *S. cerevisiae* is of particular importance for CBP-yeasts, since a plethora of different amylases or (hemi)cellulases and accessory enzymes will have to be expressed in one organism. However, similar to yeasts' capability for enhanced resistance to fermentation inhibitors, there is considerable room for improving its secretion capacity, as it is estimated that secretory expression is 100- to 1000-fold lower than the possible theoretical yield.[99] To optimize protein secretion, Wentz and Shusta[100] developed a high-throughput screening technique to rapidly screen yeast cDNA overexpression libraries by displaying the

reporter proteins on the cell surface and measuring relative secreted quantities with rapid flow cytometry. With this approach they identified five putative secretion enhancing genes, obtaining a 1.5-fold to 7.9-fold increased secretion of two different secreted reporter proteins and identified *CCW12* and *ERO1* as the most 'general' enhancers.[100] However, many studies emphasize the reporter protein-specific nature of these secretion enhancements.[93,101-103] Another factor that complicates the discovery of novel secretion and inhibitor tolerance genes is the polygenic nature of these phenotypes and synergies that exists between different genes with no apparent metabolic relationship.[94,102,104]

With recent advances in high-throughput sequencing, genetic mapping of quantitative trait loci (QTL) has become an efficient tool for identifying multiple genes responsible for a single complex phenotype where each QTL contributes a fraction of the phenotype.[94] "Pooled-segregant whole-genome sequencing analysis" combines genome shuffling and sequencing to identify chromosomal regions responsible for a specific phenotype. It was successfully employed to identify the specific alleles that were responsible for heat[105] and ethanol[106] tolerance of a Brazilian bioethanol strain. Not only did Swinnen *et al.*[106] identify two genes responsible for high ethanol tolerance, namely *MKT1* and *SWS2*, but also described the positive effect of lowered *APJ1* transcription levels on this phenotype; since this technique provides information on gene regulatory elements as well (Table 1).

Table 1. Some *S. cerevisiae* gene targets involved in improvements of CBP yeasts

Gene	Function in cell	Yeast strain improvement	Reference
ZWF1	Catalyzes the first step of the pentose phosphate pathway and adaption to oxidative stress	Increased resistance to furfural: Overexpression allowed growth at toxic furfural concentrations of 50mM	[78]
PAD1	Decarboxylates aromatic carboxylic acids to its vinyl derivatives and mRNA binding activity	In the presence of dilute acid spruce hydrolysate, *PAD1* overexpressing strains had up to 29% faster ethanol production rate	[80]
CCW12	Cell wall maintenance at sites of cell wall synthesis	Improved scTCR[a] secretion by 2.5-fold at 20°C	[100]
ERO1	Redox balance in ER and oxidative protein folding	Improved scTCR secretion by 3.2-fold at 20°C	[100]
MKT1	Involved in mitochondrial genome stability and posttranscriptional regulation of HO[b]	Presence of a specific *MKT1* allele improved ethanol tolerance	[106]
SWS2	Mitochondrial ribosomal protein subunit involved in sporulation efficiency	Presence of a specific *SWS2* allele improved ethanol tolerance	[106]
APJ1	A heatshock protein that forms nuclear foci upon DNA replication stress	Lowered transcription of a specific *APJ1* allele improved ethanol tolerance	[106]
PSE1	Import receptor interacting with nuclear pore	The overexpression of *PSE1* increased the secretion of heterologous Cel3A[c] with 3.7-fold	[104]
SOD1	Reduce reactive oxygen species in cell cytoplasm	The combined overexpression with *PSE1* enhanced Cel3A secretion with 4.5-fold	[104]

[a] scTCR = single-chain T-cell receptor

[b] HO = Site-specific endonuclease required for gene conversion at the *MAT* locus

[c] Cel3A = *S. fibuligera* β-glucosidase

DISCUSSION AND PERSPECTIVES

Since the late twentieth century, CBP using a cellulolytic or amylolytic yeast has progressed significantly from an idea to proof of concept at pilot scale due to concerted efforts by researchers around the globe[1,8,11,21,50] and startup companies, such as Mascoma Corporation.[107] The use of CBP yeasts will clearly bring about operational cost savings by replacing expensive enzymes and reducing operational units,[108,109] but new challenges have also been brought to light. Although the major cellulases have been successfully expressed in yeast, additional enzymes are required for efficient conversion of complex lignocelluloses.[109] In the future, fine-tuning of cellulolytic yeasts to express the major cellulases in optimal ratios could improve conversion rates; however, total replacement of exogenous enzymes in the foreseeable future seems remote. Because CBP requires integration of pretreatment with the hydrolysis-fermentation process, the required attributes of the CBP yeast will most likely differ from feedstock to feedstock. This exposes the second limitation of the development of a lignocellulose-CBP yeast: it might be impossible to develop a strain that has equal efficiency on all pretreated feedstocks. Re-engineering of alternative CBP yeasts for different feedstocks can be very costly, compared to SSF or SSCF where yeasts at most have to be engineered for pentose utilization. This also applies to starch feedstocks that differ in the actual starch content and composition thereof, with varying levels of non-starch material also associated with the feedstock. However, considering the relative ease with which *S. cerevisiae* strains can be genetically manipulated relative to the filamentous fungi used for the production of enzymes, CBP yeasts may still hold the be advantage. A further concern is that high yeast biomass yields, achieved at the expense of ethanol production, may be required to achieve near-complete replacement of exogenous enzymes, which may turn out to be a sub-optimal strategy.

Despite great advantages in the development of cellulosic ethanol technologies including CBP processes, the projected capital and operational costs, and by extension investment risk, for green field commercial plants remain high. Therefore, the preferred route for deployment of cellulosic ethanol technologies would be integration with first generation bio-based industries, such as sugar and ethanol production for sugarcane juices and bagasse, or sugar and fiber rich waste streams of the paper and pulp industry.[1] Combining biological and thermochemical processes, such as combustion, pyrolysis and gasification, could help to maximize the energy efficiency and overall economics of biofuels production. [110,111] To complete the carbon and energy cycles, biofuels production using both 1st generation and advanced cellulosic

technologies can be complemented with anaerobic digestion to lower organic loadings in waste streams[112], as well as the production of electricity from excess steam production.[113,114]

In summary, the application of recombinant *S. cerevisiae* strains expressing different hydrolases for CBP processes clearly presents an attractive alternative to SSF and SSCF for lignocellulose or starch bioconversion. However, careful attention should be given to further challenges such as production of optimal enzyme mixes required for efficient substrate conversion, tolerance to pretreatment inhibitors, and integration of CBP processes with 1st generation industries to minimize the capital investment and improve overall economics. Combining different favorable features of CBP yeasts, as highlighted in this review, will be fundamental in bringing CBP to commercial reality.

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