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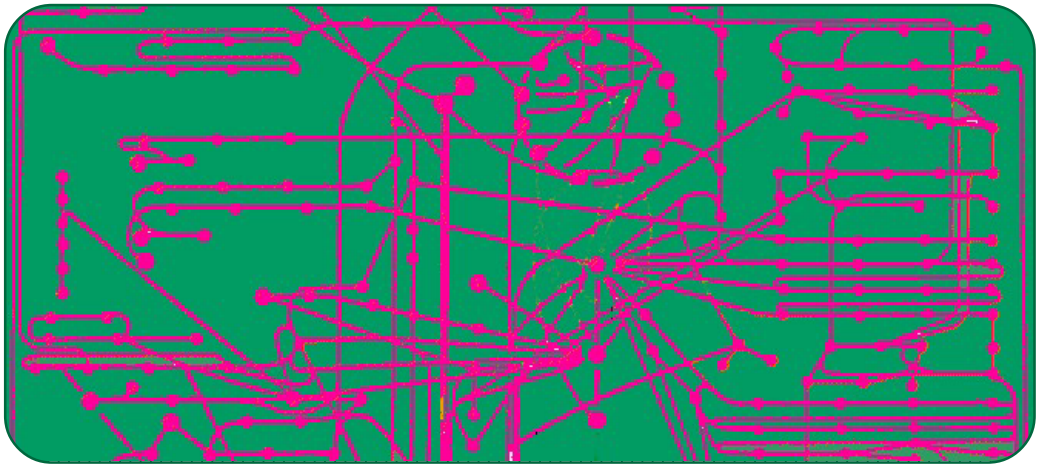

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Transcriptome and proteome analysis of xylose-metabolising *Saccharomyces cerevisiae*

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Abstract

Increasing concern about global climate warming has accelerated research into renewable energy sources that could replace fossil petroleum-based fuels and materials. Bioethanol production from cellulosic biomass by fermentation with baker's yeast *Saccharomyces cerevisiae* is one of the most studied areas in this field. The focus has been on metabolic engineering of *S. cerevisiae* for utilisation of the pentose sugars, in particular D-xylose that is abundant in the hemicellulose fraction of biomass. Introduction of a heterologous xylose-utilisation pathway into *S. cerevisiae* enables xylose fermentation, but ethanol yield and productivity do not reach the theoretical level.

In the present study, transcription, proteome and metabolic flux analyses of recombinant xylose-utilising *S. cerevisiae* expressing the genes encoding xylose reductase (XR) and xylitol dehydrogenase (XDH) from *Pichia stipitis* and the endogenous xylulokinase were carried out to characterise the global cellular responses to metabolism of xylose. The aim of these studies was to find novel ways to engineer cells for improved xylose fermentation. The analyses were carried out from cells grown on xylose and glucose both in batch and chemostat cultures. A particularly interesting observation was that several proteins had post-translationally modified forms with different abundance in cells grown on xylose and glucose. Hexokinase 2, glucokinase and both enolase isoenzymes 1 and 2 were phosphorylated differently on the two different carbon sources studied. This suggests that phosphorylation of glycolytic enzymes may be a yet poorly understood means to modulate their activity or function.

The results also showed that metabolism of xylose affected the gene expression and abundance of proteins in pathways leading to acetyl-CoA synthesis and altered the metabolic fluxes in these pathways. Additionally, the analyses

showed increased expression and abundance of several other genes and proteins involved in cellular redox reactions (*e.g.* aldo-ketoreductase Gcy1p and 6-phosphogluconate dehydrogenase) in cells grown on xylose. Metabolic flux analysis indicated increased NADPH-generating flux through the oxidative part of the pentose phosphate pathway in cells grown on xylose.

The most importantly, results indicated that xylose was not able to repress to the same extent as glucose the genes of the tricarboxylic acid and glyoxylate cycles, gluconeogenesis and some other genes involved in the metabolism of respiratory carbon sources. This suggests that xylose is not recognised as a fully fermentative carbon source by the recombinant *S. cerevisiae* that may be one of the major reasons for the suboptimal fermentation of xylose. The regulatory network for carbon source recognition and catabolite repression is complex and its functions are only partly known. Consequently, multiple genetic modifications and also random approaches would probably be required if these pathways were to be modified for further improvement of xylose fermentation by recombinant *S. cerevisiae* strains.

Preface

This study was carried out at VTT Biotechnology (Technical Research Centre of Finland) in the Metabolic Engineering team. Financial support from the Academy of Finland and Tekes – Finnish Funding Agency for Technology and Innovation is gratefully acknowledged. The work was part of the research programme "VTT Industrial Biotechnology" (Academy of Finland; Finnish Centre of Excellence programme 2000–2005, Project no. 64330). I also thank the University of Helsinki for a grant for writing this thesis. I am grateful to Vice President R&D, Prof. Juha Ahvenainen, Vice President Prof. Hans Söderlund and Vice President Richard Fageström for the possibility to prepare this thesis and for creating the excellent working facilities. Technology Managers Sirkka Keränen and Tiina Nakari-Setälä are thanked for their supportive attitude towards this work.

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I am grateful to my whole family for the attention and support that they have given through my life. Thank you Tuomas, Akseli and Olavi for your love and care and for remaining me daily about the most important things in life.

Laura

Espoo, April 2008

List of publications

This thesis is based on the following four studies, which are referred to in the text by their Roman numerals I–IV. In addition, some unpublished data is presented.

I Pitkänen, J.-P., Aristidou, A., Salusjärvi, L., Ruohonen, L. and Penttilä, M. 2003. Metabolic flux analysis of xylose metabolism in recombinant *Saccharomyces cerevisiae* using continuous culture. *Metab. Eng*. **5**: 16–31.

II Salusjärvi, L., Poutanen, M., Pitkänen, J.-P., Koivistoinen, H., Aristidou, A., Kalkkinen, N., Ruohonen, L. and M. Penttilä. 2003. Proteome analysis of recombinant xylose-fermenting *Saccharomyces cerevisiae*. *Yeast* **20**: 295–314.

III Salusjärvi, L., Pitkänen, J.-P., Aristidou, A., Ruohonen, L. and Penttilä, M. 2006. Transcription analysis of recombinant *Saccharomyces cerevisiae* reveals novel responses to xylose. *Appl. Biochem. Biotechnol*. **128**: 237–261.

IV Salusjärvi, L., Kankainen, M., Koivistoinen, H., Soliymani, R., Pitkänen, J.-P., Penttilä, M. and Ruohonen, L. 2008. Regulation of xylose metabolism in recombinant *Saccharomyces cerevisiae*. In press.

List of abbreviations

1,3-BPG	1,3-bisphosphoglycerate
2-DE	two-dimensional electrophoresis
2-PG	2-phosphoglycerate
3-PG	3-phosphoglycerate
6-Pglnate	6-phosphogluconate
AcCoA	acetyl-CoA
ANOVA	analysis of variance
bp	base pair
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CDW	cell dry weight
cRNA	complementary ribonucleic acid
Cyt C	cytochrome C
DHAP	dihydroxyacetone phosphate
E 4-P	erythrose 4-phosphate
FAD	flavin adenine dinucleotide, oxidised form
$FADH_2$	flavin adenine dinucleotide, reduced form
G 3-P	glycerol 3-phosphate
GA 3-P	glyceraldehyde 3-phosphate

HPLC	high-performance liquid chromatography
HXT	hexose transporter encoding gene
MALDI-TOF	matrix-assisted laser desorption/ionisation time of flight mass spectrometric analysis
mRNA	messenger ribonucleic acid
MudPIT	multidimensional protein identification technology
Oaa	oxaloacetate
PCR	polymerase chain reaction
PEP	phosphoenolpyruvate
PKA	cAMP-dependent protein kinase A
PPP	pentose phosphate pathway
Ru 5-P	ribulose 5-phosphate
Su 7-P	sedoheptulose 7-phosphate
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TCA	tricarboxylic acid
UQ	ubiquinone
XDH	xylitol dehydrogenase of *Pichia stipitis*
XI	xylose isomerase
XK	xylulokinase
XR	xylose reductase of *P. stipitis*

Contents

Appendix B: Publications I–IV of this publication is not included in the PDF version. Please order the printed version to get the complete publication (http://www.vtt.fi/publications/index.jsp)

1. Introduction

Microorganisms are utilised for the production of large numbers of biochemical products in the chemical, food and pharmaceutical industries. However, the native microorganisms are often not fully optimised for these tasks and consequently metabolic engineering strategies *e.g.* deletion, insertion, amplification and mutation of specific genes are applied to modify their metabolism for better process performance and yield of target molecules. The availability of the whole genome sequences of microorganisms and the advent of "omic" methodologies such as transcriptomics, proteomics and metabolomics has extended the information of cellular metabolism at the organism-wide level and brought insight into the complexity of metabolic networks. This novel data should facilitate the identification of target genes and pathways of a microorganism for modification to more efficient production of target products.

The yeast *Saccharomyces cerevisiae* is one of the most widely used microorganisms in industrial applications. In addition to its classical applications *i.e.* production of fermented foods and beverages, a number of processes are aimed at the production of yeast biomass itself, heterologous proteins, or low molecular weight metabolites (Walker, 1998). Being one of the simplest eukaryotes, *S. cerevisiae* possesses several features that make it particularly useful for both industrial and research use. It has a relatively short generation time, it can be easily cultivated under controlled conditions, and it has the ability to adapt to differences in availability of nutrients, temperature, osmolarity, osmotic pressure and acidity of the environment (Gasch *et al.*, 2000). *S. cerevisiae* is also able to grow in the complete absence of oxygen, which makes it an attractive organism for bioprocesses. Moreover, the techniques for its genetic manipulation were developed early and are well established (Beggs, 1978; Johnston, 1994). *S. cerevisiae* has also been at the leading edge in the development of genome-wide analysis methods (Castrillo and Oliver, 2004). Its genome was the first eukaryotic genome to be sequenced (Goffeau *et al.*, 1996), and since many of the basic biological processes are conserved throughout eukaryotes, *S. cerevisiae* is widely used as a model organism in biomedical research and even for studies of human molecular biology (Bassett *et al.*, 1996). Open reading frames of *S. cerevisiae* have been deleted in order to determine the function of all its proteins (Giaever *et al.*, 2002; Shoemaker *et al.*, 1996), the first

microarray studies were reported 1997 (DeRisi, 1997; Lashkari *et al.*, 1997) and yeast microarrays are currently in routine use (see, http://transcriptome.ens.fr/ymgv/) (Hayes *et al.*, 2002). Furthermore, the first whole-proteome microarray was developed for yeast (Michaud *et al.*, 2003) and the protein-protein interactions within the whole proteome have been studied (Uetz *et al.*, 2000). In addition, methods for the analysis of yeast metabolites and metabolic fluxes have been set up (Nissen *et al.*, 1997; Maaheimo *et al.*, 2001; Allen *et al.*, 2003; Castrillo *et al.*, 2003). In current studies, the information of metabolite levels and metabolic fluxes is increasingly integrated to transcription and proteome data (see, Kresnowati *et al.*, 2006; Tai *et al.*, 2007 as examples).

In recent years, increasing concern about global climate warming and the negative environmental penalty of fossil fuels has directed efforts towards the use of plant matter for production of fuels, chemicals and materials. The most commonly utilised renewable fuel today is ethanol produced from sugar cane or starch of *e.g.* corn grain or wheat. In the future, however, the large-scale production must rely more on low-cost lignocellulosic biomass such as agricultural and forestry residues that are not used for nutrition (Farrell *et al.*, 2006; Hahn-Hägerdal *et al.*, 2006; van Maris *et al.*, 2006). Lignocellulosic biomass is rich in hemicellulose that consists of heterogeneous polymers of pentoses, hexoses and sugar acids. The efficient simultaneous fermentation of all sugars present is a prerequisite for a cost-effective production of fuel ethanol. Significant portion of the hemicellulose fraction may consist of xylans, making its constituent D-xylose the second most abundant sugar in nature. *S. cerevisiae* is not able to utilise xylose or other pentose sugars, but since it has an exceptional capability to ferment hexose sugars and good tolerance towards ethanol and inhibitors present in lignocellulosic hydrolysates, considerable efforts have been made to develop genetically engineered *S. cerevisiae* strains capable of fermenting xylose (for reviews see, Ho *et al.*, 1999; Aristidou and Penttilä, 2000; Hahn-Hägerdal *et al.*, 2001; Hahn-Hägerdal *et al.*, 2006; Jeffries, 2006; van Maris *et al.*, 2006; Chu and Lee, 2007; Hahn-Hägerdal *et al.*, 2007).

1.1 Sugar metabolism in *S. cerevisiae*

S. cerevisiae is one of the few yeasts that are able to grow anaerobically when sterols and unsaturated fatty acids are provided (Andreasen and Stier, 1954;

Visser *et al.*, 1990). It is a facultatively fermentative yeast, meaning that both oxidative and substrate level phosphorylation can be the source of adenosine 5-triphosphate (ATP) that functions as the free-energy carrier in the cells (van Dijken *et al.*, 1993). Under anaerobic conditions alcoholic fermentation is the only mode of ATP production, whereas under aerobic conditions *S. cerevisiae* may exhibit either fully respiratory or at high glucose concentrations and growth rates mixed respiro-fermentative metabolism, making it a Crabtree-positive yeast (Postma *et al.*, 1989; Verduyn *et al.*, 1984). The Crabtree effect *i.e.* alcoholic fermentation under aerobic conditions in the presence of excess sugar is a consequence of intracellular pyruvate accumulation that results in enhanced flux via pyruvate decarboxylase (Pdc1p) and further in ethanol formation (Fig. 1). This overflow metabolism at the pyruvate branch point has been postulated to be due to an increased glycolytic rate exceeding that of the pyruvate dehydrogenase (Pdh) reaction or/and limited capacity of the respiratory system to oxidise mitochondrial NADH, possibly due to glucose repression of genes encoding respiratory enzymes (van Dijken *et al.*, 1993). The latter hypothesis is supported by a recent study in which the aerobic ethanol production was reduced by increasing the respiratory capacity of *S. cerevisiae* via overexpression of the gene encoding alternative oxidase from *Histoplasma capsulatum* (Vemuri *et al.*, 2007).

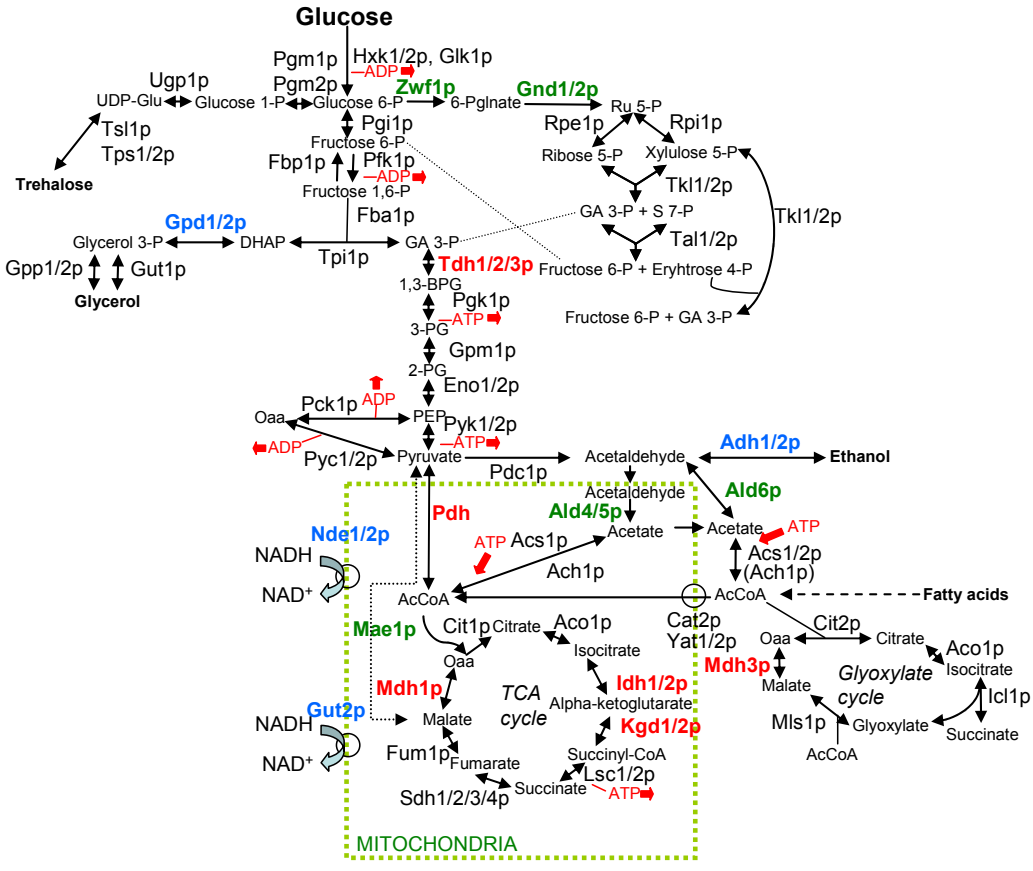

Figure 1. Schematic presentation of central energy-providing carbon catabolic pathways in S. cerevisiae. Red, green and blue colours indicate reactions producing NADH, NADPH and NAD$^+$, respectively. Dissimilation of one molecule of glucose results in the formation of two molecules of glyceraldehyde 3-phosphate (GA 3-P) that are further converted to pyruvate with concomitant generation of four ATPs. During growth on non-fermentable carbon sources genes for gluconeogenesis (PCK1 and FBP1) and glyoxylate and TCA cycles are derepressed in order to generate reducing equivalents and synthesise the intermediates for gluconeogenesis.

The nicotinamide nucleotides NADH and NADPH exist in both reduced and oxidised forms and they function as electron carriers in multiple enzymatic reactions that are an indispensable part of the metabolism of living cells. In the case of both co-factors, it is essential that a balance between the oxidised and

reduced forms exists in each compartment of the cell. Depending on whether the metabolism is respiratory or fermentative, the reduction and oxidation take place in different metabolic pathways. *S. cerevisiae* lacks a transhydrogenase activity that could catalyse the interconversion of NADH and NADPH (Bruinenberg *et al.*, 1985). For a long time redox co-factors were not believed to be able to pass through the mitochondrial membrane, but recently mitochondrial NAD^+-transporters transporting NAD^+ into mitochondria were characterised (Todisco *et al.*, 2006). Moreover, redox equivalents are shuttled between the compartments in the form of reduced or oxidised metabolites (Rigoulet *et al.*, 2004) (Fig. 2). For example, the glycerol 3-phosphate shuttle shown in Fig. 2 is involved in reoxidation of cytosolic NADH. However, the physiological conditions in which it functions are not exactly known (Bakker *et al.*, 2001). The ethanol-acetaldehyde shuttle, in turn, has been suggested to play a role in the reoxidation of mitochondrial NADH under anaerobic conditions (Bakker *et al.*, 2000) (Fig. 2). The intracellular redox potential is determined to a lesser extent by the ratio of $NADPH/NADP^+$, whereas the $NADH/NAD^+$-ratio plays a more important role (van Dijken and Scheffers, 1986). NADPH is preferentially used in assimilatory pathways and is mainly produced by the pentose phosphate pathway (PPP), although cytosolic $NADP^+$-dependent isocitrate dehydrogenase and acetaldehyde dehydrogenases as well as NADH-kinases may also contribute to its production (Bieganowski *et al.*, 2006; Grabowska and Chelstowska, 2003; Minard and McAlister-Henn, 2005).

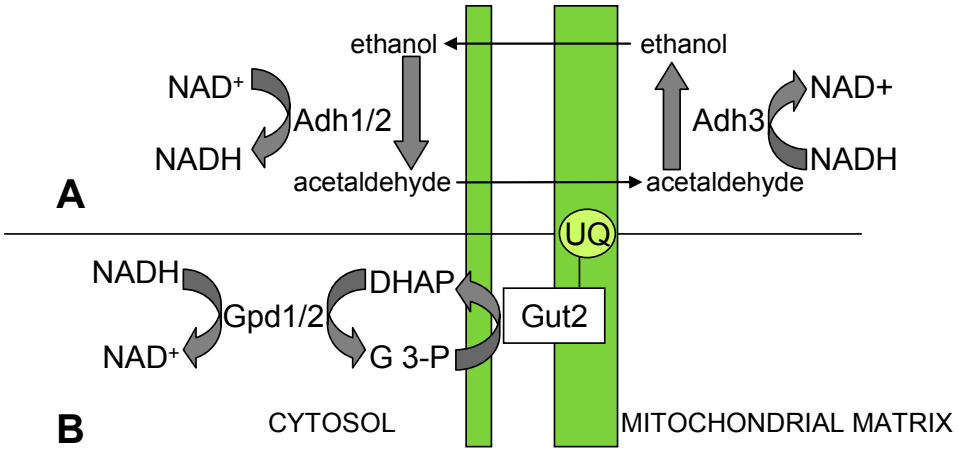

Figure 2. Shuttling of oxidised and reduced metabolites between cytosol and mitochondria by (A) the ethanol-acetaldehyde shuttle that is in principle reversible and (B) the glycerol 3-phosphate shuttle. Gut2p transfers the electrons to ubiquinone in the respiratory chain. Abbreviations: Adh1/2; cytosolic alcohol dehydrogenase isoenzymes, Adh3; mitochondrial alcohol dehydrogenase, Gpd1/2; glycerol 3-phosphate dehydrogenase isoenzymes, Gut2; mitochondrial membrane-bound glycerol 3-phosphate dehydrogenase, DHAP; dihydroxyacetone phosphate, G 3-P; glyceraldehyde 3-phosphate. The figure is adapted from Bakker et al., (2001).

1.1.1 Fermentative and respiratory metabolism

Under anaerobic conditions during fermentation the only source of ATP is substrate-level phosphorylation in glycolysis, where one mole of glucose is converted to two moles of pyruvate. At the same time two ATP and NADH molecules are formed. A closed redox balance is achieved by decarboxylation of pyruvate to acetaldehyde that is further reduced to ethanol by alcohol dehydrogenase (Pronk *et al.*, 1996) (Fig. 1). In addition, glycerol is produced in order to reoxidise the surplus NADH that is mainly generated in the synthesis of biomass but may also originate from the production of oxidised metabolites such as acetate, acetaldehyde or pyruvate (van Dijken and Scheffers, 1986) (Fig. 1).

Under aerobic conditions assimilatory NADH can be oxidised by respiration, which is energetically a more favourable way of sugar utilisation. Acetyl-CoA formed by the decarboxylation of pyruvate in mitochondria by the pyruvate dehydrogenase complex replenishes the tricarboxylic acid (TCA) cycle. Acetyl-CoA can also be formed through the cytosolic pyruvate dehydrogenase bypass that involves the enzyme activities of pyruvate decarboxylase (Pdc1p), acetaldehyde dehydrogenase (Ald6p) and acetyl-CoA synthetase (Acs1p) (Pronk *et al.*, 1994) (Fig. 1). Acetyl-CoA is a precursor for several biosynthetic processes *e.g.* lipid synthesis (Daum *et al.*, 1998), and pyruvate dehydrogenase bypass plays an important role in the supply of acetyl-CoA in the cytosol (Pronk *et al.*, 1994). Some of the intermediates of the TCA cycle are withdrawn for the biosynthetic reactions of amino acids, and in order to compensate for the loss, pyruvate is also used for the synthesis of oxaloacetate in the reaction catalysed by the cytosolic pyruvate carboxylase (Pyc1/2p) (Pronk *et al.*, 1994) (Fig. 1). The transfer of oxaloacetate into mitochondria and the use of pyruvate for the synthesis of acetyl-CoA via acetaldehyde elevate the cytosolic NADH/NAD^+ -ratio since carbon is leaving the cytosol without regeneration of NAD^+ that was utilised in the synthesis of pyruvate. The cofactor shuttles such as glycerol 3-phosphate shuttle or ethanol acetaldehyde shuttle, discussed in section 1.1, can be used to transfer the reducing potential of NADH across the mitochondrial membrane (Bakker *et al.*, 2001).

In addition to sugars, various other organic compounds *e.g.* fatty acids, glycerol, ethanol and acetate can support aerobic growth of *S. cerevisiae*. Utilisation of these compounds involves gluconeogenesis and the glyoxylate cycle (Haarasilta and Oura, 1975) (Fig. 1). The pathway of gluconeogenesis is in essence a reversal of glycolysis and it uses otherwise common enzymes with glycolysis except that the reaction carried out by pyruvate kinase (Pyk1/2p) is replaced with reactions catalysed by pyruvate carboxylase (Pyc1/2p) and phosphoenolpyruvate carboxykinase (Pck1p), and the reaction carried out by phosphofructokinase (Pfk1p) is replaced by the reaction catalysed by fructose bisphosphatase (Fbp1p) (Fig. 1). In *S. cerevisiae* gluconeogenesis starts most often from oxidation of ethanol to acetaldehyde by alcohol dehydrogenase 2 (Adh2p) (Fig. 1). Acetaldehyde is further oxidised to acetate that is converted to acetyl-CoA in a reaction catalysed by acetyl-CoA synthetase (Acs1p). Acetyl-CoA may enter either the mitochondrial TCA and/or cytosolic glyoxylate cycles, resulting in formation of malate, which is either converted to pyruvate by oxidative

decarboxylation carried out by mitochondrial malic enzyme (Mae1p) or oxidised to oxaloacetate in cytosol by malate dehydrogenase (Mdh2/3p). Pyruvate produced in the former reaction can be further converted to oxaloacetate by Pyc1/2p and subsequently to phosphoenolpyruvate by Pck1p. In addition, oxaloacetate can be used to replenish the TCA and glyoxylate cycles (Fig. 1) (Voet and Voet, 1995).

NADH and $FADH_2$ produced in the reactions of the TCA cycle are reoxidised and oxygen is reduced to water via the electron transport chain on the inner mitochondrial membrane (Fig. 3). The energy released by the transfer of electrons is used to synthesise ATP by oxidative phosphorylation. *S. cerevisiae* lacks complex I of the respiratory chain, present in many other fungi, but has instead an internal (Ndi1p) and two external NADH dehydrogenases (Nde1p and Nde2p) (Joseph-Horne *et al.*, 2001). Internal and external NADH dehydrogenases are not proton-translocating but participate in oxidation of mitochondrial and cytosolic NADH, respectively (Marres *et al.*, 1991; Luttik *et al.*, 1998; Bakker *et al.*, 2001). In addition, the glycerol 3-phosphate shuttle is an indirect mechanism to oxidise cytosolic NADH and transfer electrons to the respiratory chain via the FAD-linked glycerol 3-phosphate dehydrogenase (Gut2p) (Fig. 2) (Larsson *et al.*, 1998). Mitochondria of *S. cerevisiae* mitochondria also have the ability to oxidise lactate to pyruvate via L-lactate cytochrome-c oxidoreductase (Cyb2p) that is located in the intermembrane space and transfers electrons to cytochrome c (Cénas *et al.*, 2007).

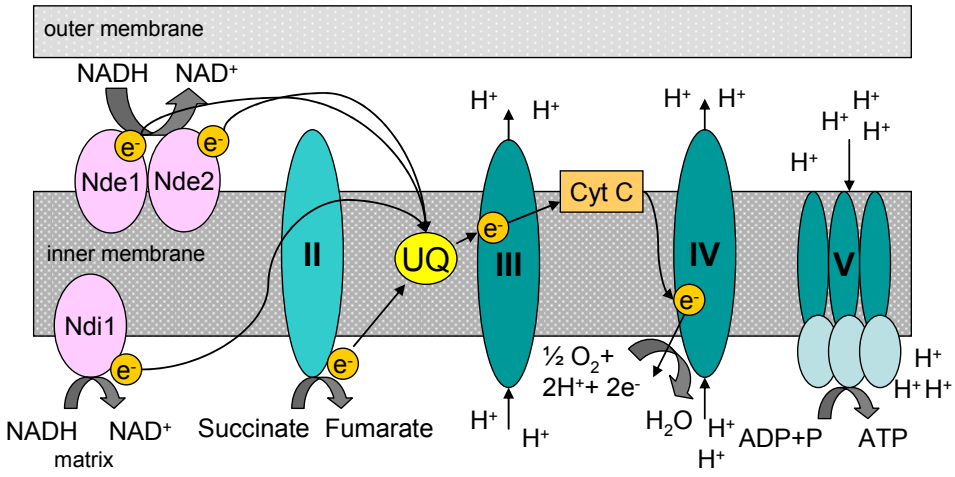

Figure 3. The electron transport chain of S. cerevisiae. The electrons donated by NADH or $FADH_2$ are passed through the enzyme complexes of the electron transport chain to the terminal electron acceptor oxygen. Ubiquinone (UQ) and cytochrome C mediate the electron transfer. Energy obtained from the transfer of electrons is used to create a transmembrane proton gradient, resulting in a membrane potential difference that is used to synthesise ATP by ATP synthase. In S. cerevisiae NADH-dehydrogenase encoded by NDI1 replaces complex I. The complexes II to V are succinate dehydrogenase (II), cytochrome bc1 (III), cytochrome c oxidase (IV) and ATP synthase (V). This figure is adapted from Veiga et al., (2003) and Bakker et al., (2001).

1.1.2 Regulation of carbon utilisation

S. cerevisiae prefers glucose or fructose as carbon sources but is also able to grow on a range of other carbon sources (Schüller, 2003). Hexose sugars are predominantly transported into yeast cells by the members of the hexose transporter family that comprises 20 proteins (Boles and Hollenberg, 1997). In the presence of glucose, the transcription of genes that are essential for utilisation of non-fermentable carbon sources and other sugars than glucose are repressed (for review see, *e.g.* Gancedo, 1998). In addition, the presence of glucose affects turnover of messenger RNAs (mRNA) (Scheffler *et al.*, 1998), triggers post-translational modification of some proteins (*e.g.* Fbp1p and Mdh2p) leading to their degradation (Görts, 1969; Hung *et al.*, 2004; Müller *et al.*, 1981),

and inhibits the activity of some enzymes *e.g.* maltase (Siro and Lovgren, 1978). The key enzymes of glycolysis and gluconeogenesis are moreover under allosteric regulation. As an example, fructose 2,6-bisphosphate activates the glycolytic phosphofructokinase enzyme but inhibits the activity of fructose 1,6-bisphosphatase catalysing the reverse reaction in gluconeogenesis (Voet and Voet, 1995).

One of the first responses of *S. cerevisiae* to glucose is induction of the genes encoding the hexose transporters. The signal is mediated via the glucose transporter-like proteins Snf3p and Rgt2p that function as sensors for the extracellular glucose. Snf3p and Rgt2p respond to low and high glucose concentrations, respectively (Liang and Gaber, 1996; Özcan, 2002). C-terminal tails of the Snf3p and Rgt2p interact with the transcriptional corepressors of transcription factor Rgt1p, Mth1p and Std1p, and with protein kinase Yck1/2p, which catalyses phosphorylation of Mth1p and Std1p in the presence of glucose. Phosphorylated Mth1p and Std1p become degraded via Grr1p-dependent ubiquitination that results in relief of *HXT* repression by the transcription factor Rgt1p (Moriya and Johnston, 2004) (Fig. 4).

Protein kinase Snf1p, transcription factor Mig1p and hexokinase Hxk2p are the key mediators in the glucose-repression pathway (Carlson, 1999; Westergaard *et al.*, 2007). Snf1p-kinase forms a complex with an activating subunit Snf4p and one of the proteins Sip1p, Sip2p or Gal83p (for review, see Carlson, 1999). At low levels of glucose Snf1p is activated by phosphorylation for which at least three upstream kinases Pak1p, Tos3p and Elm3p are involved (Hong *et al.*, 2003). Once activated, Snf1p further phosphorylates Mig1p, causing translocation of Mig1p from the nucleus to the cytoplasm (DeVit and Johnston, 1999) and thus leading to derepression of glucose-repressible genes (Westergaard *et al.*, 2007). At high levels of glucose, Reg1p/Glc7p-phosphatase complex facilitates the conversion of Snf1p to its autoinhibited state (Sanz *et al.*, 2000) (Fig. 4).

Hxk2p participates in the signalling of glucose repression by interacting with Mig1p and Snf1p and inhibiting the phosphorylation of Mig1p at high levels of glucose (Ahuatzi *et al.*, 2006). Mig1p and Hxk2p also contribute to glucose induction by repressing *SNF3* and *MTH1* (Kim *et al.*, 2006). In addition to its function in repressing genes of hexose transporters, Rgt1p is required for repression of *HXK2* at low levels of glucose together with transcription cofactor

Med8p (Palomino *et al.*, 2005). The repressor function of Rgt1p is regulated by phosphorylation by Snf1p and Tpk3p at low and high levels of glucose, respectively (Kim and Johnston, 2006; Palomino *et al.*, 2006) (Fig. 4).

TPK3 is one of the three genes encoding catalytic subunits of the cyclic AMP-dependent (cAMP) protein kinase A (PKA) (Toda *et al.*, 1987). The addition of glucose to cells growing on a non-fermentable carbon source causes a rapid increase of cAMP levels due to activation of adenylate cyclase via the G-protein coupled receptor complex, Gpr1p/Gpa2p, and Ras1p/Ras2p pathways (Kraakman *et al.*, 1999). The activity of adenylate cyclase also depends on phosphorylation of glucose either by the hexokinases 1 and 2 or glucokinase (Colombo *et al.*, 2004). cAMP activates the protein kinase activity of PKA by binding to its inhibitory subunit Bcy1p and causing its dissociation from the complex (Toda *et al.*, 1987). Active PKA regulates by phosphorylation a variety of proteins involved in transcription, energy metabolism, reserve carbohydrate synthesis, cell cycle, stress resistance and pseudohyphal growth, enabling a rapid cellular response to the availability of a fermentative carbon source (for review see, Thevelein and de Winde, 1999).

For more detailed information of the Snf3p/Rgt2p-Rgt1p glucose induction pathway (Özcan, 2002), the Mig1p-Hxk2p glucose repression pathway (Ahuatzi *et al.*, 2006) and the cAMP/PKA pathway (Rolland *et al.*, 2000) and their interplay with each other see, Kim and Johnston, (2006), Kim *et al.*, (2006) and Santangelo, (2006) for review.

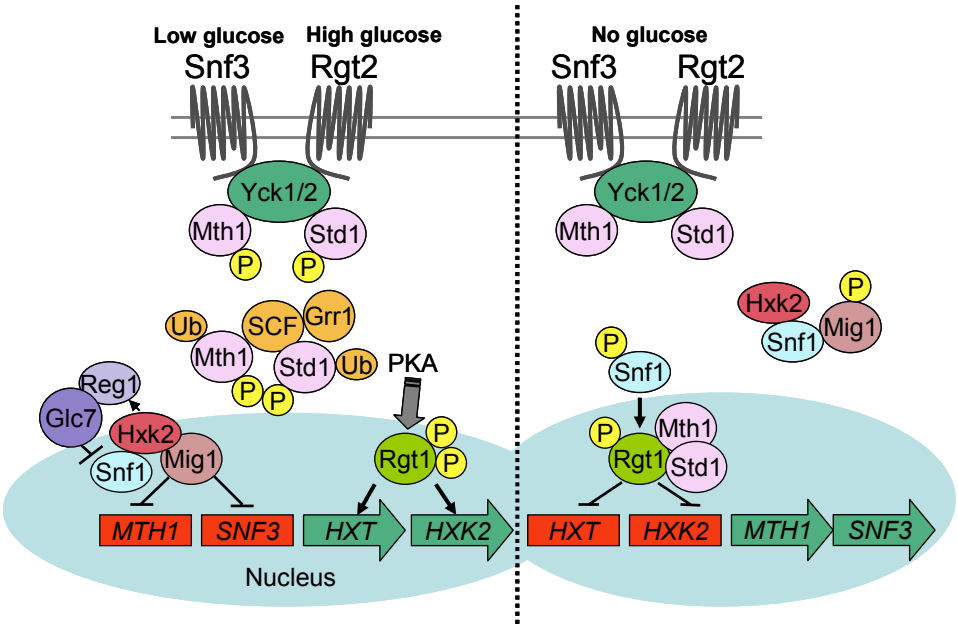

Figure 4. Mechanisms for glucose recognition and repression. In the presence of glucose, Snf3p and Rgt2p glucose sensors stimulate the phosphorylation of Mth1p and Std1p by Yck1/2p. Phosphorylated Mth1p and Std1p are ubiquitinated by SCF/Grr1p and subsequently degraded. This and additionally hyperphosphorylation by PKA releases Rgt1p from its upstream binding sites and results in derepression of HXTs and HXK2. At the same time Mig1p represses MTH1 and SNF3. Hxk2p participates in this process by interacting with both Snf1p and Mig1p and inhibiting the phosphorylation of Mig1p by Snf1p. Hxk2p also regulates the phosphorylation status of Reg1p and consequently influences Glc7-phosphatase to negatively regulate the activity of Snf1p. In the absence of glucose, Mth1p and Std1p are bound to Rgt1p that is additionally phosphorylated in an Snf1p-dependent manner and HXTs and HXK2 are repressed. Snf1p also phosphorylates Mig1p, resulting in its export from the nucleus to the cytoplasm and in derepression of its target genes. The figure is adapted from Santangelo (2006).

1.1.3 Regulation of growth on non-fermentable carbon sources

During growth on glucose, the metabolic building blocks are derived from glycolysis, the TCA cycle and the pentose phosphate pathway. When glucose becomes exhausted, the cells prepare to utilise ethanol formed during fermentation or other non-fermentable carbon sources possibly present (*e.g.* acetate, glycerol, lactate and oleate) by switching from fermentative to oxidative metabolism during the transition phase called the diauxic shift. Major changes occur in the overall gene expression that are due to the release from glucose repression of genes encoding proteins needed for growth on carbon sources other than glucose (DeRisi, 1997). The derepression affects in particular the genes encoding proteins for gluconeogenesis, the glyoxylate and TCA cycles, respiration, peroxisomal biogenesis, β-oxidation and the genes encoding proteins needed for utilisation of other sugars than glucose *e.g.* galactose, sucrose and maltose (Gancedo, 1998; Schüller, 2003).

The transcriptional activator Cat8p is essential for growth on non-fermentable carbon sources (Hedges *et al.*, 1995), and it is required for the derepression of genes encoding enzymes of gluconeogenesis and the glyoxylate cycle (*e.g.* *PCK1*, *FBP1*, *ICL1* and *MLS1*; see Fig. 1) and also some other genes encoding proteins needed for utilisation of non-fermentable carbon sources (Haurie *et al.*, 2001). Mig1p represses the expression of *CAT8* and its activation on non-fermentable carbon sources is dependent on phosphorylation by Snf1p and another yet unidentified protein kinase (Randez-Gil *et al.*, 1997).

Adr1p is another transcription factor that plays an important role in growth on non-fermentable carbon sources. The expression of *ADR1* does not depend on Snf1p (Dombek *et al.*, 1993), but Snf1p promotes its binding to promoter areas of the genes it regulates (Young *et al.*, 2002). Additionally, many of the Adr1p-dependent genes are dependent on Snf1p for their expression. The genes regulated by Adr1p (*e.g*. *ADH2*, *ACS1*, *GUT2*, *CYB2, FDH1, POX1*) encode primarily proteins in pathways leading from ethanol (*ADH2*, *ACS1*), glycerol (*GUT2*), lactate (*CYB2*), formate (*FDH1*) and β-oxidation of fatty acids (*POX1*) to the formation of NADH and acetyl-CoA that may enter the TCA cycle and be used for the cell's energy supply (Young *et al.*, 2003).

Transcription of genes encoding proteins in the TCA cycle, the electron transport chain and mitochondrial biogenesis are regulated by the Hap2/3/4/5-protein complex. *HAP4* is repressed by glucose via the Mig1p pathway (DeRisi, 1997). Its repression is released on non-fermentable carbon sources leading for activation of respiration. In addition, a subset of genes of the TCA and glyoxylate cycles (*CIT1*, *CIT2*, *ACO1*, *IDH1* and *IDH2*; see Fig. 1) is under the so-called retrograde control by Rtg1p-3p. This transcriptional activator complex ensures that under conditions in which the respiratory function of the cell is reduced or eliminated, sufficient glutamate is synthesised for biosynthetic processes and that the glyoxylate cycle provides an adequate supply of metabolites for the TCA cycle to support anabolic pathways (Liu and Butow, 1999).

1.2 Genome-wide analysis methods in metabolic engineering

Cells are robust and thus frequently oppose modifications and try to maintain their metabolic state and functions constant. In metabolic engineering trials, this may lead to production of unwanted side products at the expense of product yield. Transcription profiling, proteomics and metabolite profiling allow the identification of global cellular effects of the genetic modifications at the level of gene expression, proteins, metabolites and metabolic fluxes. This information can be utilised to identify new targets for genetic manipulation and redesign of metabolic pathways for an improved phenotype.

As an example, transcription analysis of a wild-type *S. cerevisiae* and two engineered strains with improved galactose uptake rates resulted in the identification of *PGM2*, encoding the major isoform of phosphoglucomutase as a target for metabolic engineering of the galactose utilisation pathway. Overexpression of *PGM2* resulted in 70% increase in the galactose uptake rate and in a three-fold higher specific ethanol production rate compared with the parent strain (Bro *et al.*, 2005). Recently, microarray analysis of brewer's yeast variants obtained by UV-mutagenesis and spontaneous selection, possessing improved fermentation capability and viability under high-gravity fermentation conditions, resulted in identification of genes affecting the fermentation performance. Of these, overexpression of *LEU1* resulted in faster fermentation

under high-gravity conditions compared with the control strain (Blieck *et al.*, 2007). On the other hand, transcript comparison of a mutated xylose-utilising *S. cerevisiae* strain exhibiting an improved growth rate on xylose with its parent strain did not result in identification of successful targets for further manipulations (Wahlbom *et al.*, 2003b). The genome-wide analyses do not always turn into successful metabolic engineering applications due to still existing limitations in the analysis, integration and understanding of large amounts of complex data. The continuously improving mathematical modelling and computational analysis will certainly help to interpret the role of different components and their interactions in the cell (Kitano, 2002; Vemuri and Aristidou, 2005).

1.2.1 Microarray analysis

Transcription analysis by microarrays can be applied to any organism of which the genome has been sequenced or a large collection of cDNA clones exists. Microarrays are usually manufactured by spotting either short oligonucleotides or longer DNA fragments that are complementary to sequences of individual genes on a solid support such as coated glass surface or a nylon membrane. The short oligonucleotides can also be synthesised directly on a surface by the photolithography technique developed by Affymetrix Inc. (Santa Clara, CA, USA) (Pease *et al.*, 1994) (Fig. 5). The expression levels of the genes are measured by hybridising cDNA, which is generally fluorescently labelled, with the spotted gene fragments. The transcriptional profile under given conditions reflects the genes and pathways that are induced or repressed relative to a reference sample(s).

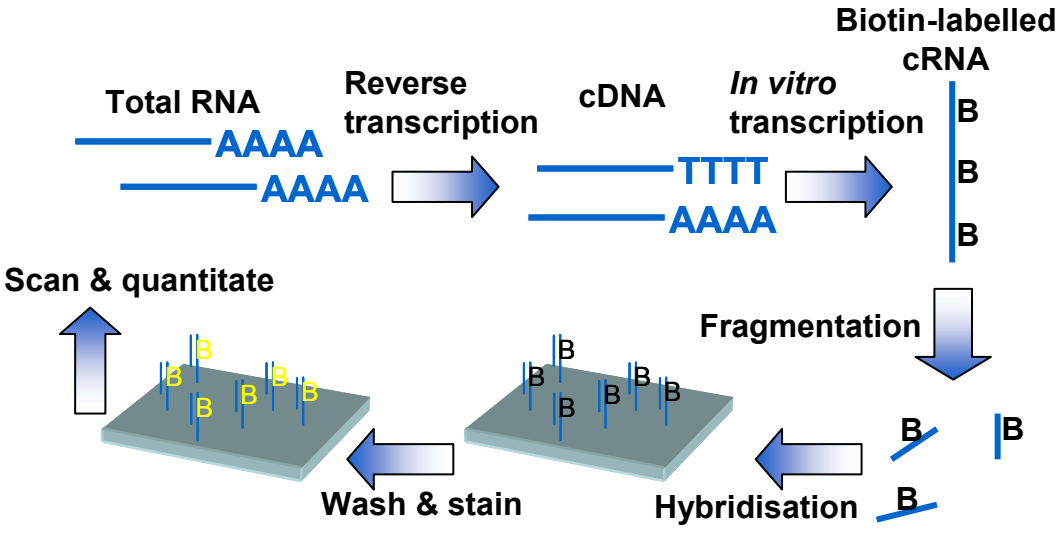

Figure 5. The principle of microarray analysis with Affymetrix GeneChips. Total RNA is isolated from the cells and mRNAs are converted into double-stranded cDNA by reverse transcription. The cDNA is converted to cRNA by in vitro transcription and simultaneously labelled with biotin. The cRNA is subsequently fragmented and hybridised on the array containing probes for the different genes. Any unbound cRNA is washed away and the hybridised cRNA is stained with the fluorescent Cy5 conjugated with streptavidin that attaches to biotin. Finally the arrays are scanned to quantitate the hybridisation signals.

1.2.2 Proteome analysis

Proteome analysis aims at the quantification of all proteins in a cell. This information is particularly valuable since the amount of mRNA does not necessarily reflect the amount of functional protein molecules (Gygi *et al.*, 1999b; Ideker *et al.*, 2001). However, compared with transcription analysis, proteome analysis usually provides information relating to only a limited number of gene products. On the other hand, information concerning post-translational modifications, subcellular localisation, turnover or interaction with other proteins may be obtained. The conventional methodology in proteome analysis employs two-dimensional gel electrophoresis to separate cellular proteins, and mass spectrometry to identify them (Guerrera and Kleiner, 2005;

Patton, 2002) (Fig. 6). For visualisation and quantification, the proteins can be labelled either before the isoelectric separation by fluorescent or radioactive labels (Alban *et al.*, 2003) or after the second dimension by staining the sodium dodecyl sulphate polyacrylamide (SDS) gels *e.g.* with silver or fluorescent dyes (Patton, 2002; Thompson *et al.*, 2003).

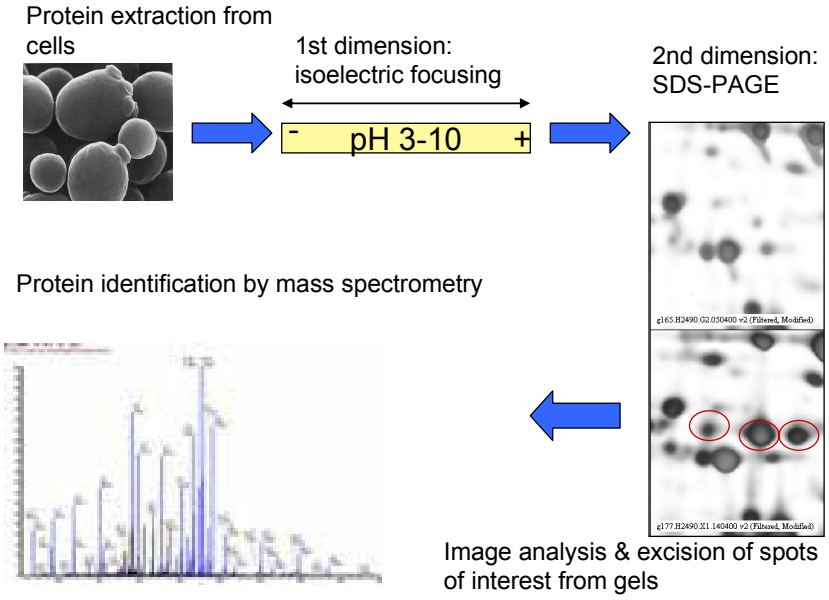

Figure 6. The principle of proteome analysis by 2-DE gels. Proteins, extracted from the cells, are first separated according to their isoelectric points and subsequently according to their molecular weights in SDS-PAGE. Protein spot patterns from different samples are compared and quantified and the protein spots of interest are identified by mass spectrometric methods.

Drawbacks of 2-DE gel-based proteome analysis are poor reproducibility, limited sensitivity and dynamic range, and the limitation to detect only abundant and hydrophilic proteins that are not highly basic or acidic (Gygi *et al.*, 2000). In order to overcome these limitations, new prefractionation techniques, better solvents for sample preparation and new staining methods for protein visualisation and quantification are constantly emerging (van den Bergh and Arckens, 2005). In recent years, different proteomics methods that couple liquid chromatography to mass spectrometry have, on the other hand, become increasingly popular (Flory *et al.*, 2006; Shi *et al.*, 2004; Thompson *et al.*, 2003).

These methods allow automated separation of complex peptide mixtures with high speed, sensitivity and resolution. As an example, multidimensional protein identification technology (MudPIT) was used to analyse yeast proteome with high protein coverage and the method allowed identification of both low-abundance and membrane proteins (Washburn *et al.*, 2001; Wei *et al.*, 2005). Quantitative proteome analysis strategies by mass spectrometry generally make use of stable isotope labels that differentiate peptides by mass between different samples studied. The stable isotopes can be introduced into proteins or peptides by chemical, enzymatic or metabolic incorporation (Gygi *et al.*, 1999a; Ross *et al.*, 2004; Washburn *et al.*, 2003).

Post-translational modifications (*e.g.* phosphorylation, acetylation, glycosylation, methylation, ubiquitination) are an important way to regulate the activity and cellular function of proteins. For example, phosphorylation of proteins is the major player in most if not all signalling cascades regulating the cellular metabolism. Various strategies *e.g.* protein microarrays or different affinity-based enrichment methods such as immunoprecipitation and immobilized metal affinity columns in combination with mass spectrometry have been applied to quantitate and characterise the post-translational modifications (Mann and Jensen, 2003; Ptacek *et al*., 2005). Phosphorylated proteins can also be visualised by autoradiography of 2-DE gels after metabolic incorporation of radiolabelled phosphate (^{32}P), by Western analysis with antibodies against specific phosphorylation sites or by comparison of a phosphatase-treated sample with the non-treated control sample either with 2-DE gels or by mass spectrometric methods (Mann and Jensen, 2003). Studying and understanding the relationship between the modifications and functional changes is, however, still a laborious analytical challenge and has become as one of the most active research areas in proteomics (see, Jensen, (2004), Mann and Jensen, (2003), Seo and Lee, (2004) for reviews on the topic).

1.2.3 Metabolome analysis

The metabolites comprise a range of different molecules that participate in metabolic reactions required to generate energy and building blocks for growth and cellular functions. The metabolic flux is the rate of turnover of metabolites through a metabolic pathway or an enzyme. The metabolic fluxes reflect closely

the metabolic state of the cell. The metabolome of *S. cerevisiae* comprises approximately 600 metabolites (Forster *et al*., 2003) and it is more diverse in chemical properties compared with the transcriptome or proteome. Large variations in the nature and concentrations of metabolites make the analysis of metabolites challenging. Gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), and nuclear magnetic resonance spectroscopy (NMR) are commonly used to measure metabolite pools within the cells but no single method is presently available for the measurement of all the metabolites present (Dunn *et al*., 2005). Metabolic fluxes can be roughly calculated based on the reaction stoichiometry and data of the extracellular metabolite concentrations, but more exact estimation of fluxes is obtained by feeding cells with a ^{13}C-labelled carbon source that proceeds through the metabolic network (Stephanopoulos *et al*., 1998; Schmidt *et al*., 1998). At steady state, the fraction of labelled carbon in a given precursor metabolite pool can be used to calculate the flux through that pathway. The intracellular fluxes, including those that are cyclic and reversible, may be calculated by following the distribution of the positionally labelled isotopomers through the different pathways by GC-MS or by NMR (Schmidt *et al*., 1997). The rate-limiting enzymatic steps that control the carbon flow in a pathway can be calculated by taking into account the activity of individual enzymes in the given pathway. The flux control coefficient represents the relative change in flux through a pathway divided by the relative change in activity of an enzyme that was responsible for a flux change (Stephanopoulos *et al*., 1998). It has, however, been shown that in most cases the flux control is distributed over all steps in a pathway rather than that only one enzyme would be responsible for a change of a flux (Fell and Thomas, 1995).

1.3 Bioethanol and engineering of *S. cerevisiae* for xylose metabolism

Lignocellulosic feedstocks from agriculture and forestry provide a cheap and sustainable resource for production of transportation fuels and chemicals. As a significant proportion of the hemicellulose fraction of lignocellulosics is pentose sugars, the ability to metabolise them along with hexoses is a prerequisite for organisms to be used in processes based on plant materials. The most abundant pentose sugar is D-xylose, the primary constituent of xylans that comprise the

bulk of hemicellulose in plant cell walls (Zaldivar *et al.*, 2001). Xylose can be fermented to ethanol by bacteria, yeasts and filamentous fungi (Jeffries, 1983; Olsson and Hahn-Hägerdal, 1996; Singh *et al.*, 1992).

Several bacteria *e.g. Escherichia coli* and *Klebsiella oxytoca* are able to utilise a variety of sugars including xylose, and the efficient ethanol producer *Zymomonas mobilis* has been successfully engineered for xylose metabolism (Dien *et al.*, 2003; Zhang *et al.*, 1995). Major disadvantages associated with using bacteria in fermentation processes are narrow pH range, more stringent nutritional requirements compared with yeast, sensitivity to high ethanol concentrations and sensitivity to acetic acid and other inhibitors present in hydrolysates (Dien *et al.*, 2003). Filamentous fungi are potential microorganisms to be used for ethanol production from different sugar sources and especially from wood hydrolysates due to their ability to produce cellulases and ferment cellulose directly to ethanol. As an example, *Mucor indicus* produces ethanol from hexoses with a comparable yield and productivity to *S. cerevisiae*, although its industrial applicability suffers from a tendency to switch to filamentous growth (Karimi *et al.*, 2005). Common problems with filamentous fungi are also slow productivity and formation of by-products such as acetic acid (Olsson and Hahn-Hägerdal, 1996; Panagiotou *et al.*, 2005).

Two main routes for metabolism of xylose have been described in microorganisms. Numerous bacteria, including *E. coli* (Lawlis *et al.*, 1984) and *Bacillus* and *Lactobacillus* species (Lokman *et al.*, 1991; Rygus *et al.*, 1991), use xylose isomerase to convert xylose to xylulose, which is then phosphorylated to xylulose 5-phosphate, an intermediate of pentose metabolism. Although some fungi are also known to possess a xylose isomerase (Harhangi *et al.*, 2003), yeasts and filamentous fungi generally use xylose reductase and xylitol dehydrogenase for conversion of xylose to xylulose (Jeffries, 1983). In almost all yeasts capable of xylose utilisation, ethanolic fermentation of xylose is absent or extremely slow (Toivola *et al.*, 1984). Only a few yeast species, namely strains of *Brettanomyces naardenensis*, *Pachysolen tannophilus*, *P. stipitis*, *Candida shehatae*, *Pichia segobiensis* and some *Candida tenuis* strains are able to ferment xylose slowly under anaerobic conditions (Bruinenberg *et al.*, 1984; Toivola *et al.*, 1984), but none of these yeasts is able to grow under anaerobic conditions on either xylose or glucose (Visser *et al.*, 1990).

S. cerevisiae is not able to metabolise xylose, although some strains that are able to co-utilise it along other substrates or obtained ability to grow on xylose at extremely slow rate under aerobic conditions have been reported (van Zyl *et al.*, 1989; Attfield and Bell, 2006). The extension of substrate range for fermentation of xylose is one of the most active fields in metabolic engineering of *S. cerevisiae* (Ostergaard *et al.*, 2000). Xylose-fermenting *S. cerevisiae* strains have been constructed by over-expression of the genes of *P. stipitis* encoding NAD(P)H -dependent xylose reductase (XR) and NAD^+ -dependent xylitol dehydrogenase (XDH) (Kötter and Ciriacy, 1993; Kötter *et al.*, 1990). The xylose utilisation and ethanol production were further improved by over-expression of the endogenous gene encoding xylulokinase (Ho *et al.*, 1998; Eliasson *et al.*, 2000; Toivari *et al.*, 2001). However, the xylose pathway with XR and XDH results in a redox cofactor imbalance due to different cofactor requirements of XR and XDH enzymes (Bruinenberg *et al.*, 1983). In recombinant *S. cerevisiae*, this has been proposed to be one of the major reasons for low ethanol yields from xylose, production of xylitol as a side product and dependence of oxygen for growth on xylose (Bruinenberg *et al.*, 1983). These limitations have also been ascribed to inefficient xylose uptake (Gárdonyi, *et al.*, 2003), low flux through the PPP (Walfridsson *et al*., 1995; Karhumaa *et al.*, 2005) and limited rate of ATP production (Sonderegger *et al*., 2004b).

Several studies have focused on improving the xylose fermentation by *S. cerevisiae*. These include different strategies for relieving the redox imbalance *e.g.* by introducing a phosphoketolase pathway (Sonderegger *et al.*, 2004a), by expression of a $NADP^+$ -dependent D-glyceraldehyde 3-phosphate dehydrogenase (Verho *et al.*, 2003; Bro *et al.*, 2006), by modifying the cofactor preference of the ammonium assimilation pathway from NADPH to NADH (Roca *et al.*, 2003), by altering the cofactor affinity of XR and XDH (Jeppsson *et al.*, 2006; Hou *et al.*, 2007; Watanabe *et al.*, 2007a; Watanabe *et al.*, 2007b), or by disruption of the oxidative PPP (Jeppsson *et al.*, 2002). In order to improve co-fermentation of glucose and xylose, the *MIG1* or both *MIG1* and *MIG2* were deleted in a *S. cerevisiae* strain with the xylose pathway from *P. stipitis* (Roca *et al*., 2004). However, ethanol formation by these strains was not remarkably improved although the specific xylose uptake rate in chemostat cultivation increased compared with the parental strain (Roca *et al*., 2004) (for more examples see *e.g.* the following reviews: Aristidou and Penttilä, 2000; Hahn-Hägerdal *et al.*, 2001; Hahn-Hägerdal *et al.*, 2007).

Recently, the redox imbalance was completely overcome by functional expression of the xylose isomerase (XI) from the anaerobic fungus *Piromyces* sp E2 (Kuyper *et al.*, 2003). This did not alone result in the expected improvement in growth and fermentation of xylose, confirming that other limitations in xylose metabolism, in addition to redox imbalance, also exist. Xylose fermentation by *S. cerevisiae* strain RWB202 expressing XI from *Piromyces* sp E2 was subsequently considerably improved by evolutionary engineering, by overexpressing the genes encoding the enzymes of the non-oxidative PPP, and by deleting *GRE3* encoding a major aldose reductase activity in *S. cerevisiae* (Kuyper *et al.*, 2004; Kuyper *et al.*, 2005a; Kuyper *et al.*, 2005b). The resulting strain RWB218 produced ethanol in anaerobic batch fermentation on 2% xylose with a yield of 0.41 g g^{-1} and growth rate of 0.12 h^{-1} (Kuyper *et al.*, 2005b).

Overexpression of the genes for the non-oxidative PPP and deletion of *GRE3* also improved xylose utilisation in strains with the oxidoreductive xylose pathway (Johansson and Hahn-Hägerdal, 2002; Karhumaa *et al.*, 2005; Ni *et al.*, 2007). These results suggest that the rate of the flux downstream from xylulose into central carbon metabolism is important for xylose fermentation by *S. cerevisiae*. In addition, in strains with the oxidoreductive xylose pathway, high activity of XR and XDH has been shown to improve xylose fermentation (Karhumaa *et al.*, 2005; Karhumaa *et al.*, 2007). In strains with a high XR activity (Gárdonyi *et al.*, 2003) or in strains with XI (Kuyper *et al.*, 2003), the xylose uptake may also limit the rate of fermentation. This is supported by the observation that in the evolutionary engineered strain RWB218 with the XI from *Piromyces* sp E2 the xylose uptake kinetics were substantially improved compared with the parental strain (Kuyper *et al.*, 2005b). *S. cerevisiae* lacks a specific xylose transporter and it takes up this sugar by hexose transporters that have a low affinity for xylose (Hamacher *et al.*, 2002; Lee *et al.*, 2002; Saloheimo *et al.*, 2007). Recently, two glucose/xylose transporters from *Candida intermedia* were characterised and functionally expressed in a *S. cerevisiae* strain carrying the oxidoreductive xylose pathway and lacking all hexose transporter genes. However, these transporters did not notably support growth on xylose (Leandro *et al.*, 2006). Heterologous expression of a xylose transporter homologue, *xlt1*, from *Trichoderma reesei* in a similar *S. cerevisiae* host supported growth on xylose only after a prolonged cultivation that obviously also resulted in adaptive mutation(s) in the host strain (Saloheimo *et al.*, 2007).

The properties of *S. cerevisiae* with the oxidoreductive xylose pathway have also been improved by mutagenesis (Wahlbom *et al.*, 2003a) and by evolutionary engineering (Sonderegger and Sauer, 2003; Pitkänen *et al.*, 2005). Transcription analyses and metabolic modelling and flux analyses have been carried out to characterise these (Wahlbom *et al.*, 2003b; Sonderegger *et al.*, 2004b; Pitkänen *et al.*, 2005) and non-mutated *S. cerevisiae* strains possessing the xylose pathway (Wahlbom *et al.*, 2001; Jin and Jeffries, 2004; Jin *et al.*, 2004). These studies indicate that the improved properties of mutated or evolved xylose-utilising *S. cerevisiae* strains appear to rely on increased xylose uptake (Pitkänen *et al.*, 2005), increased expression of *XKS1* (Wahlbom *et al.*, 2003b), increased expression of *SOL3*, *GND1*, *TAL1* and *TKL1* in the oxidative and non-oxidative PPP (Wahlbom *et al.*, 2003b; Sonderegger *et al.*, 2004b), increased activity of transketolase, transaldolase, and glucose 6-phosphate dehydrogenase (Pitkänen *et al.*, 2005). The evolved C1 strain derived from TMB3001 (Eliasson *et al.*, 2000) with enhanced xylose catabolism had increased carbon fluxes through the PPP and glycolysis, further leading to the conclusion that ultimately the rate of ATP formation limits anaerobic growth on xylose (Sonderegger *et al.*, 2004b).

The studies with non-mutated xylose-utilising *S. cerevisiae* strains showed increased expression of genes encoding gluconeogenic, TCA cycle and respiratory enzymes (Jin *et al.*, 2004), and increased flux from glucose 6-phosphate to ribulose 5-phoshate through the PPP (Wahlbom *et al.*, 2001) in cells grown on xylose compared with cells grown on glucose. These studies have provided valuable information concerning xylose metabolism in recombinant xylose-metabolising *S. cerevisiae*, but overall the multiple changes observed have proved to be difficult to interpret and the results have not hitherto led to great improvements of xylose fermentation by *S. cerevisiae*. The recently published genome sequence of the yeast *P. stipitis* naturally utilising xylose may turn out to be useful for further understanding and improvement of recombinant xylose metabolism in *S. cerevisiae* (Jeffries *et al.*, 2007). Additionally, the future challenge remains to construct xylose-fermenting production strains with good fermentation performance on lignocellulosic hydrolysates under industrial conditions.

1.4 Aims of the study

In the present study, metabolic flux, proteome and transcription analyses were carried out in order to study the xylose metabolism of recombinant xylose-utilising *S. cerevisiae* expressing the genes encoding xylose reductase (XR) and xylitol dehydrogenase (XDH) from *P. stipitis* and the endogenous gene encoding xylulokinase. The aim was to identify new targets for genetic modifications in order to improve the yield and rate of ethanol production from xylose.

Since very little is known about how xylose is sensed and what signalling pathways are involved in its metabolism in *S. cerevisiae* cells, the aim of the work presented here was to gain further insight into the regulation of cellular metabolism during xylose utilisation. Additionally, the different cofactor preferences of XR and XDH offered a model to study cellular redox metabolism and to obtain understanding of the flexibility or robustness of cellular metabolism.

The metabolic flux, proteome and transcription analyses of the xylose-utilising *S. cerevisiae* grown in chemostat cultures were among the first initiatives for genome-wide analyses in our laboratory, with the objective to establish the use of these analysis techniques for yeast. The long-term aim was to build up analytical techniques and data analysis methods for integration of information from multiple levels of cellular metabolism.

2. Materials and methods

All materials and methods are described in detail in the original publications I–IV.

2.1 Strains

Table 1. The S. cerevisiae strains used in studies I–IV and in unpublished studies.

Strain	Description	Reference
H1346	CEN.PK2-1D (*MATα*, *leu2*-3/112, *ura3-52*, *trp1-289*, *his3Δ1*, *MAL2-8*^{c}, *SUC2*)	(Boles *et al.*, 1996)
H2446	Derivative of CEN.PK2-1D; *XYL1* and *XYL2* of *Pichia stipitis* chromosomally integrated into the *URA3* locus. *XYL1* is expressed under the *PGK1* promoter and *XYL2* under the modified *ADH1* promoter. *XKS1* of *S. cerevisiae* is present on a multicopy plasmid YEplac195 under the modified *ADH1* promoter. Additionally, the strain contains the empty multicopy plasmid YEplac181 with *LEU2* marker.	I
H2490	Derivative of H2446. Histidine and tryptophan auxotrophies of H2446 are cured by integrating *HIS3* and *TRP1* back to their respective loci.	I
H2217	VTT-C-99318. (CEN.PK2-1D; *ura3*::*XYL1 XYL2 his3*::*XKS1 kanMX*). *XYL1* and *XYL2* of *P. stipitis* are chromosomally integrated into the *URA3* locus. *XYL1* is expressed under the *PGK1* promoter and *XYL2* under the modified *ADH1* promoter. *XKS1* of *S. cerevisiae* under the modified *ADH1* promoter is integrated into the *HIS3* locus.	(Verho *et al.*, 2003)
H3094	H2217 harbouring the empty multicopy plasmid YEplac181 with *LEU2* marker.	This study (unpublished results)
H3095	H2217 harbouring *GCY1* under the *PGK1* promoter on the multicopy plasmid YEplac181 with *LEU2* marker.	This study (unpublished results)
H3127	H2217 harbouring *TYE1* under the *TPI1* promoter on the multicopy plasmid pYX212 (Ingenius, UK) with *URA3* marker.	This study (unpublished results)
H3128	H2217 harbouring the empty multicopy plasmid pYX212 (Ingenius, UK) with *URA3* marker.	This study (unpublished results)

2.2 Fermenter cultivations

Table 2. Fermenter cultivations carried out in studies I–IV and in unpublished studies.

Culture	Analyses	Study
Aerobic chemostat cultivations with H2446 on 200 mM xylose (30 g/l) and on 200 mM xylose + 5.6 mM, 2.8 mM or 0.56 mM glucose (1, 0.5 and 0.1 g/l).	Metabolic flux analysis of samples from steady states.	I
Aerobic chemostat on 56 mM (10 g/l) glucose with H2490. The culture was switched to anaerobic chemostat conditions after six residence times.	Metabolic flux analysis of samples from steady states.	I
	Proteome analysis of samples from steady states and from 5, 30 and 60 minutes after the switch to anaerobic cultivation.	II
	Transcription analysis of the aerobic steady state samples.	III
	Transcription analysis of the anaerobic steady state samples.	Unpublished results
Aerobic chemostat on 17 mM (3 g/l) glucose + 180 mM (27 g/l) xylose with H2490. The culture was switched to anaerobic chemostat conditions after six residence times.	Metabolic flux analysis of samples from steady states.	I
	Proteome analysis of samples from steady states and from 5, 30 and 60 minutes after the switch to anaerobic cultivation.	II
	Transcription analysis of the aerobic steady state samples.	III
	Transcription analysis of the anaerobic steady state samples.	Unpublished results
Aerobic batch cultures on 278 mM (50 g/l) glucose with H2217.	Proteome and transcription analyses of samples at 5 and 24 hours.	IV
Aerobic batch cultures on 333 mM (50 g/l) xylose with H2217.	Proteome and transcription analyses of samples at 72 hours.	IV

2.2.1 Chemostat cultures

H2490 was grown in aerobic and anaerobic chemostat cultures (dilution rate (D) 0.05 h^{-1}) on YNB (Yeast nitrogen base, w/o amino acids) (Sigma, USA) with 56 mM (10 g/l) glucose and 180 mM (27 g/l) xylose + 17 mM (3 g/l) glucose as described in I. Yeast for the proteome analysis was harvested from the aerobic and anaerobic steady states of the cultures and from time points of 5, 30 and 60 minutes after the change to anaerobic conditions (II). Yeast for the transcriptome analysis (III) was harvested from the aerobic and anaerobic steady states of the cultures with H2490 (anaerobic data is unpublished). H2446 was grown in aerobic chemostat cultures on YNB supplemented with L-histidine and L-tryptophan and either 200 mM (30 g/l) xylose or 200 mM xylose + varying amounts of glucose (5.6 mM, 2.8 mM or 0.56 mM) (I). Yeast for the metabolic flux analyses was harvested after two residence times on each carbon source (I).

2.2.2 Aerobic batch cultures

In study IV, H2217 was grown in aerobic batch fermentations on synthetic complete (SC) medium (modified from Sherman *et al.*, 1983) supplemented with 333 mM (50 g/l) xylose or 278 mM (50 g/l) glucose. Three separate fermentations (F0, F1 and F2) were carried out on both carbon sources. Optical density at 600nm (OD_{600}) and cell dry mass were measured from all cultivation samples as described in study I. Yeast for the transcriptome analyses was harvested at 5 h and 24 h of the glucose fermentations F0, F1 and F2 and at 72 h of the xylose fermentations F0, F1 and F2. Samples from the fermentations F2 were hybridised on Affymetrix Yeast Genome S98 arrays three times and the samples from the fermentations F0 and F1 once. Proteome analysis was carried out from the same time points as the transcriptome analysis of all three glucose and xylose fermentations (F0, F1 and F2). Extracellular concentrations of glucose, xylose, xylitol, ethanol, acetate and glycerol were analysed by high-performance liquid chromatography (HPLC) as described in study I.

2.3 Metabolic flux analysis

The metabolic fluxes on xylose of strains H2490 and H2446 were estimated by flux balancing analysis (I). Accumulation rates of extracellular metabolites were

measured from steady states of the chemostat cultivations. The metabolic model used consisted of 71 metabolites, and 38 and 39 reactions for anaerobic and aerobic conditions, respectively.

2.4 Two-dimensional polyacrylamide gel electrophoresis

Proteome analysis of soluble *S. cerevisiae* proteins was carried out by two-dimensional polyacrylamide gel electrophoresis (2-DE) (II and IV). Yeast for the analysis was harvested by centrifugation (2 min., 5000g) and cells were subsequently frozen in liquid nitrogen. Cells were disrupted by shaking with glass beads, either directly in solubilisation buffer containing urea and detergent (II) or in 10% trichloroacetic acid (Merck, USA) (IV). The first dimension, isoelectric focusing, was carried out in pH range 3–10 and the second dimension in either 12% (II) or 11% polyacrylamide gel (SDS-PAGE) (IV). The SDS-PAGE gels were stained either with silver (II) or fluorescent Sypro Ruby or phosphoprotein specific Pro-Q Diamond (IV). The images of the 2-DE gels were processed, analysed and compared with each other with the PDQuest software (BioRad, USA) (II, IV). The protein spot quantities were normalised to the total optical density of each gel image. In study II, the proteins with significant changes in quantity between the aerobic and anaerobic steady-state samples from glucose and xylose + glucose chemostat cultures were selected by using Student's *t*-test. In study IV, the differences in abundance of proteins were determined using the one-way ANOVA at a *p*-value of 0.01 (Zar, 1999). The abundance values were then mean centred and the replicate gels were averaged and the signal intensity values for identified protein spots were clustered using hierarchical clustering with Euclidean distance and average linkage. The protein spots of interest were excised from the gels and identified with matrix-assisted laser desorption/ionisation mass spectrometric analysis (MALDI-TOF) (II and IV) (Poutanen *et al.*, 2001).

2.5 Transcription analysis with microarrays

Yeast for the microarray analyses (III and IV) was harvested and frozen as for the proteome analysis described above. Cells were disrupted with glass beads and RNA was extracted by using Trizol reagent (Invitrogen, USA). In study III, [^{33}P] CTP-labelled cDNA was hybridised onto Yeast Gene Filters (ResGen^{TM},

Invitrogen, USA) and the signal was detected by scanning the exposed phosphorimager screens on a Typhoon instrument (GE Healthcare, USA). The transcription data from the aerobic chemostat cultures on 56 mM glucose and 180 mM xylose + 17 mM glucose in study III, and the unpublished transcription data from the anaerobic chemostat cultures on 56 mM glucose and 180 mM xylose + 17 mM glucose, were analysed by using ArrayVision and ArrayStat softwares (Imaging Research, Canada).

In study IV, the transcription analysis was carried out with Affymetrix YG-S98 microarrays (Affymetrix, USA). The cDNA synthesis, synthesis of biotinylated cRNAs, and hybridisations were carried out at the Finnish DNA Microarray Centre (Turku Centre for Biotechnology) according to protocols provided by Affymetrix. The resulting data was analysed as described in study IV.

2.6 Strain construction (unpublished results)

GCY1 encoding an aldo-keto reductase of *S. cerevisiae* was amplified by PCR from the genomic DNA of *S. cerevisiae* strain H1346 with primers 5'-GTCTGGATCCAAAATGCCTGCTACTTTACAT-3' and 5'-GCTAGGATCC TTACTTGAATACTTCGAA-3'. Both primers contained *Bam*HI restriction sites to facilitate the cloning. The PCR product was cloned into the TOPO vector (Invitrogen), and the 940 bp *Bam*HI fragment from the resulting vector was further ligated to the *Bam*HI site of the yeast expression vector YEplac181 with the *PGK1* promoter. The resulting plasmid p2160 and the empty control plasmid p1184 were transformed into *S. cerevisiae* strain H2217 using the lithium acetate transformation method (Gietz *et al.*, 1992).

TYE7 encoding a putative transcription factor of *S. cerevisiae* was amplified by PCR from the genomic DNA of *S. cerevisiae* strain H1346 with primers 5'-GTCTGAATTCAAAATGAACTCTATTTTAGAC-3' and 5'-GCAAGAATTC TTATTTTTGGTCTTGTTTCA-3'. Both primers had *Eco*RI restriction sites to facilitate cloning of the PCR product into the TOPO-vector (Invitrogen). The 880 bp *Eco*RI fragment from the resulting vector was further ligated to the *Eco*RI site of the yeast expression vector pYX212 with the *TPI1* promoter. The resulting plasmid, p2238, and the empty control plasmid p2159 were transformed into *S. cerevisiae* strain H2217 as described above.

2.7 Shake flask cultures (unpublished results)

H3095 with *GCY1* in the multicopy plasmid and the control strain H3094 with the empty plasmid were cultured on an orbital shaker (250 rpm) at 30°C in 250 ml shake flasks containing 50 ml SC medium (modified from Sherman *et al.*, 1983) lacking leucine for selection and supplemented either with 333 mM (50 g/l) xylose or 333 mM xylose + 3 μg/ml antimycin A (Sigma). Cultures were inoculated to an initial OD_{600} of 0.5 with cells cultured overnight on SC medium without leucine and supplemented with 111 mM glucose and harvested by centrifugation. Growth was measured as OD_{600}, and four cultures were carried out with both strains. Extracellular metabolites were measured from culture samples taken at regular intervals by HPLC as described in study I. H3127 with *TYE7* in the multicopy plasmid and the control strain H3128 with the empty plasmid were cultured similarly to H3095 and H3094 except that the medium lacked uracil for the selection of the plasmid.

2.8 Enzyme activity assays (unpublished results)

The activity of Gcy1p was assayed from crude cell extracts of H3095 by testing several different substrates: 100 mM glycerol, 40 mM D-L-glyceraldehyde, 50 mM xylose, 83 mM acetate, 300 mM formaldehyde, 25 mM glyoxylic acid, 25 mM glyoxylate and 250 mM methylglyoxal. The cells of H3095 with *GCY1* in YEplac181 and the cells of the control strain H3094 were disrupted with glass beads in 50 mM Hepes pH 7.0, containing 1 mM $MgCl_2$ and 0.1 mM EDTA. The protease inhibitors phenylmethylsulphonyl fluoride (final concentration 1 mM) and pepstatin A (final concentration 0.01 mg/ml) were added to the extraction buffer. Assays were carried out in buffers containing 33 mM Tris-HCl (pH8.8), 1 mM $MgCl_2$ or 100 mM Na-phosphate pH 7.0 or 50 mM Hepes pH 7.0, 1 mM $MgCl_2$, 0.1 mM EDTA with 1 mM PMSF and 0.01 mg/ml pepstatin A by using either 0.5 mM NADPH, $NADP^+$, NADH or NAD^+ as cofactors. No activity towards substrates other than methylglyoxal was detected.

3. Results and discussion

3.1 Cultivations and the experimental background

3.1.1 Aerobic and anaerobic chemostat cultivations

The studies I–IV describe the results of metabolic flux (I), proteome (II and IV) and transcription (III and IV) analyses of recombinant *S. cerevisiae* with the oxidoreductive xylose-utilisation pathway. In studies I–III, the analyses were carried out with the strain H2490 (XR and XDH encoding genes integrated, XK encoding gene on a multicopy vector) grown in aerobic and anaerobic chemostat cultures on 56 mM glucose and on 180 mM xylose + 17 mM glucose. Due to the low growth rate of the H2490 strain on xylose, the growth medium of xylose cultivation contained 10% glucose of the total sugar amount (I). This allowed the maintenance of identical growth rates in the glucose and xylose cultures, and consequently growth rate-dependent changes in the gene expression pattern and protein abundances were minimised. However, the residual glucose was zero throughout both the glucose and xylose chemostat cultures and the cells were in a glucose-derepressed state (I). On the other hand, the residual xylose was ~ 147 mM (22 g/l) and ~ 167 mM (25 g/l) in aerobic and anaerobic xylose cultures, respectively (I). Consequently, this experimental set-up allowed comparison of the effect of residual xylose on metabolic fluxes (I) and on the gene expression and protein abundance patterns with the glucose derepressed cells (II, III). As a result of proteome and transcription analyses, 22 proteins spots and 225 genes with different abundances and expression, respectively, between glucose and xylose steady state samples were identified (II, III). Several of these proteins also responded to the switch from aerobic to anaerobic cultivation (II, Table 3). Moreover, 224 genes had different expression levels in the cells from the anaerobic steady states of glucose and xylose + glucose chemostat cultures (unpublished data, Appendix V).

Additionally, aerobic chemostat cultures on 200 mM (30 g/l) xylose + varying glucose concentrations (5.6 mM, 2.8 mM or 0.56 mM) were carried out with the strain H2446 for metabolic flux analysis that was applied to study the effect of decreasing, small concentrations of glucose on metabolism of xylose (I). Samples were harvested after two residence times on each carbon source and the effect of the glucose concentration in the feed on selected carbon fluxes is shown in Fig. 3 of study I.

3.1.2 Aerobic batch cultivations

In study IV, the strain H2217 (XR, XDH and XK encoding genes integrated) was grown in three replicate aerobic batch fermentations on 278 mM glucose or 333 mM xylose as carbon sources. The first sample was harvested at 5 h from the glucose cultures, when the residual sugar was about 205 mM (37 g/l) and the cells were in glucose repressed state. At the time of the second sample at 24 h, all glucose was consumed and cells were in glucose derepressed state. In xylose cultures, about 213 mM (32 g/l) of xylose was present at 72 h, when samples were harvested (IV, Fig. 1). Volumetric profiles of xylose and glucose consumption, biomass formation and xylitol and ethanol production (g/l) of the batch cultures are shown in Fig. 1 of study IV.

Aerobic batch fermentations allowed a comparison of the yeast proteome and transcriptome on xylose with both glucose repressed and glucose derepressed cells. The aim was to determine how signalling and carbon catabolite repression differed in cells grown on either glucose or xylose. Comparison of xylose-grown cells with glucose repressed and derepressed cells using transcriptional and proteome analyses enabled responses solely due to the absence of glucose repression to be distinguished from those directly linked to metabolism of xylose. As a result, 70 protein spots (see Fig. 7) and 1439 genes with different abundances and transcription levels, respectively, in the cells growing on xylose and in glucose repressed and derepressed cells were identified. Moreover, proteome analyses showed distinct patterns in phosphorylation of hexokinase 2, glucokinase and enolase isoenzymes in the xylose- and glucose-grown cells (IV).

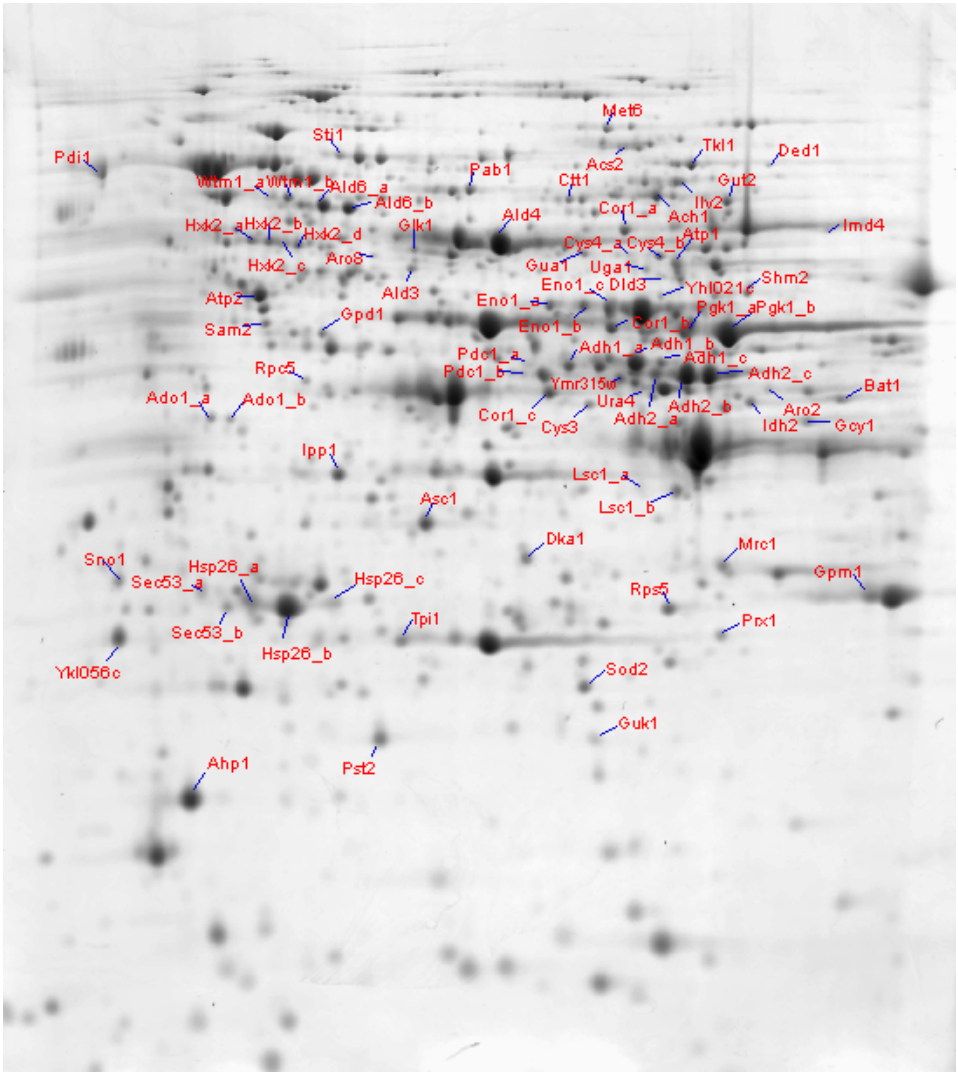

Figure 7. Image of the 11% SDS-PAGE 2-DE-gel showing the locations of the protein spots of H2217 with different abundances in glucose repressed and derepressed cells and in cells grown on xylose in the aerobic batch fermentations (pI range 3–10 from left to right) (IV).

3.2 Metabolism on xylose has both respiratory and fermentative features

Results from studies II–IV suggest that xylose is neither a fully fermentative nor a respiratory carbon source for *S. cerevisiae* with the recombinant oxidoreductive xylose pathway. In cells grown on xylose many genes repressed via the Snf1p/Mig1p pathway were only partially repressed compared with the glucose-grown cells. The analysis of the aerobic chemostat cultures on glucose and xylose + glucose indicated decreased abundance of Atp7p and several proteins of the TCA cycle (Fum1p, Mdh1p, Idh1p and Idh2p) in cells grown on xylose + glucose compared with the cells grown on glucose (I, Table 2). The transcript analysis of corresponding cells showed consistently lower expression of *MDH1*, *KGD1* and *2*, *IDH2*, *FUM1* and *ICL1* (Fig. 8) (III). Moreover, *HXK1* and several genes encoding proteins involved in trehalose synthesis had lower expression levels on xylose + glucose (Fig. 8) (III). All of these genes are normally repressed by glucose (Klein *et al.*, 1998), and were consequently derepressed in the glucose-limited chemostat culture. Thus, in chemostat cultures on xylose + glucose the residual xylose present seemed able to repress these genes. In accordance with gene expression and protein levels also fluxes in the TCA cycle were reduced compared with the glucose-grown cells (I).

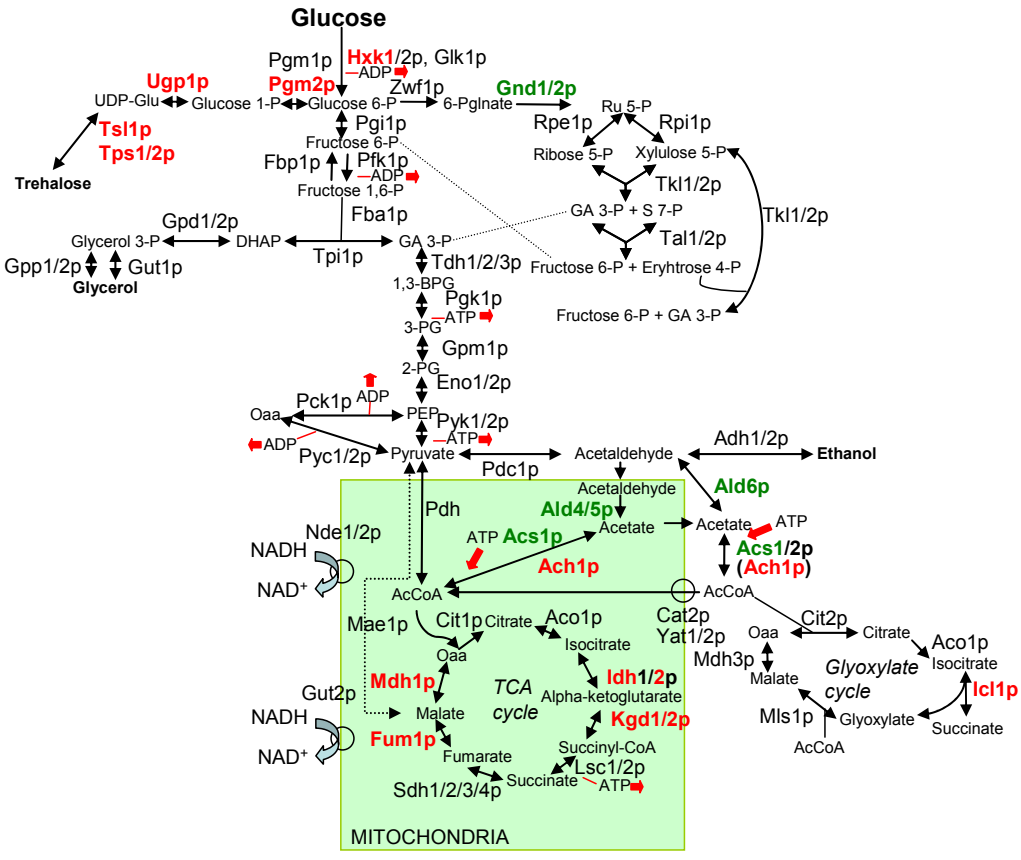

Figure 8. The central carbon metabolism of H2490 grown in aerobic chemostat cultures on 180 mM xylose + 17 mM glucose or on 56 mM glucose. The proteins encoded by genes with higher and lower expression in cells grown on xylose + glucose compared with the glucose-limited culture are coloured green and red, respectively.

Additionally, the transcript analysis of the aerobic batch cultures on xylose and glucose (IV) revealed several glucose repressible genes that had higher expression levels on xylose compared with the levels measured from the glucose repressed, but lower compared with the glucose derepressed cells (Fig. 9, cluster 2) (IV). These genes encoded proteins of respiration (*e.g. COX5a, QCR7*), gluconeogenesis (*e.g. FBP1, PCK1*), tricarboxylic acid and glyoxylate cycles (*e.g. MDH1, KGD1, ICL1*), alcohol catabolism (*e.g. ADH2*) and trehalose and glycogen synthesis pathways (*e.g. TSL1, GLG1* and *2, GSY2* and *GLC3*). Furthermore, genes encoding the transcriptional regulators of these genes such

as *ADR1*, *CAT8*, *HAP4*, *SIP1-2* and *4* and *REG2* (Johnston, 1999) were expressed in an analogous manner (Fig. 9, cluster 2) (IV). The abundance of proteins with respiratory function and proteins of the TCA cycle such as ATP synthases Atp1p, Atp2p, ubiquinol-cytochrome-c reductase (Cor1p), isocitrate dehydrogenase Idh2p and the α-subunit of succinyl-CoA ligase Lsc1p also had a similar trend in their abundances (Fig. 10). The results were in agreement with the proteome and transcript changes observed in the analysis of the aerobic chemostat cultures (II, III). In study by Belinchón and Gancedo, (2003) with a recombinant xylose-metabolising *S. cerevisiae* strain, xylose similarly attenuated the derepression of *ICL1* and *FBP1*. The authors hypothesised that the extent of repression caused by xylose or other carbon sources would depend on the intracellular metabolite pattern, which is different during growth on different carbon sources owing to different rates of glycolytic flux. In study I, the glycolytic flux to pyruvate was shown to be significantly decreased in the cells grown on xylose + glucose in the chemostat culture compared with the glucose-grown cells (I, Fig. 1).

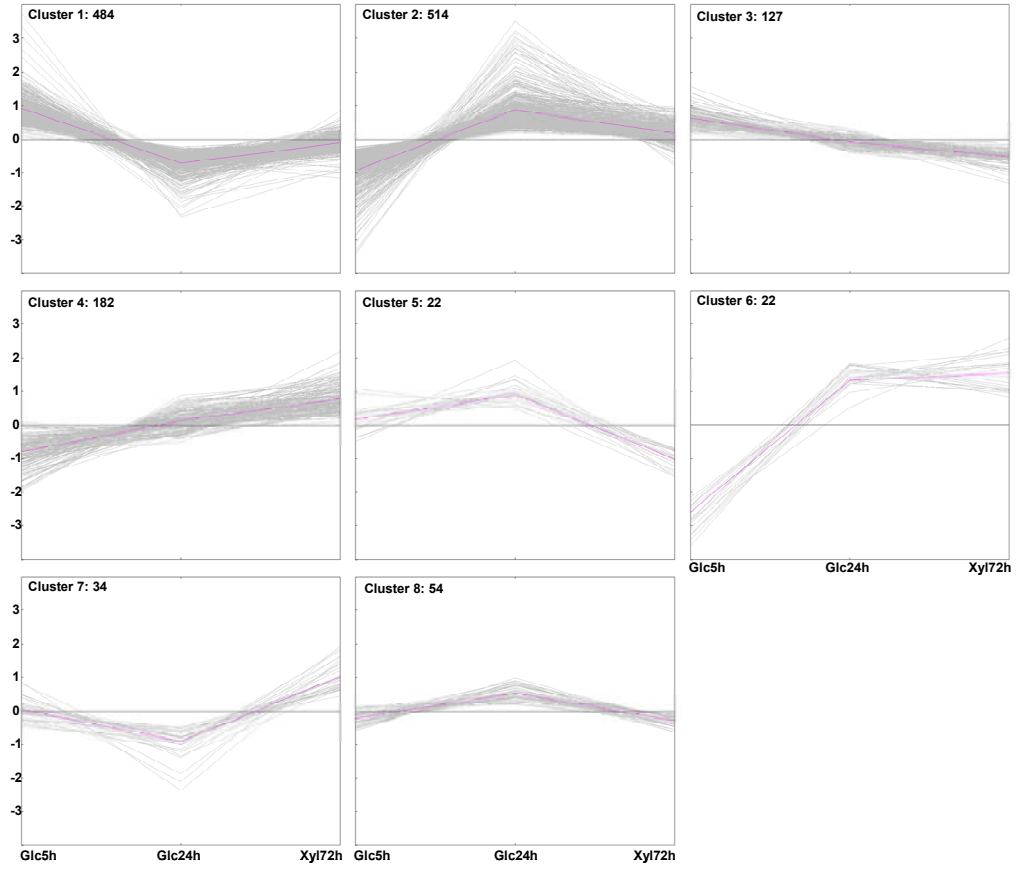

Figure 9. Transcription analysis of the aerobic batch cultures of H2217 on 333 mM xylose and 278 mM glucose. The eight clusters of the 1439 differentially expressed genes in glucose repressed (Glc5h), glucose derepressed (Glc24h), and cells grown on xylose for 72 h (Xyl72h) were determined by K-means with Euclidean distance.

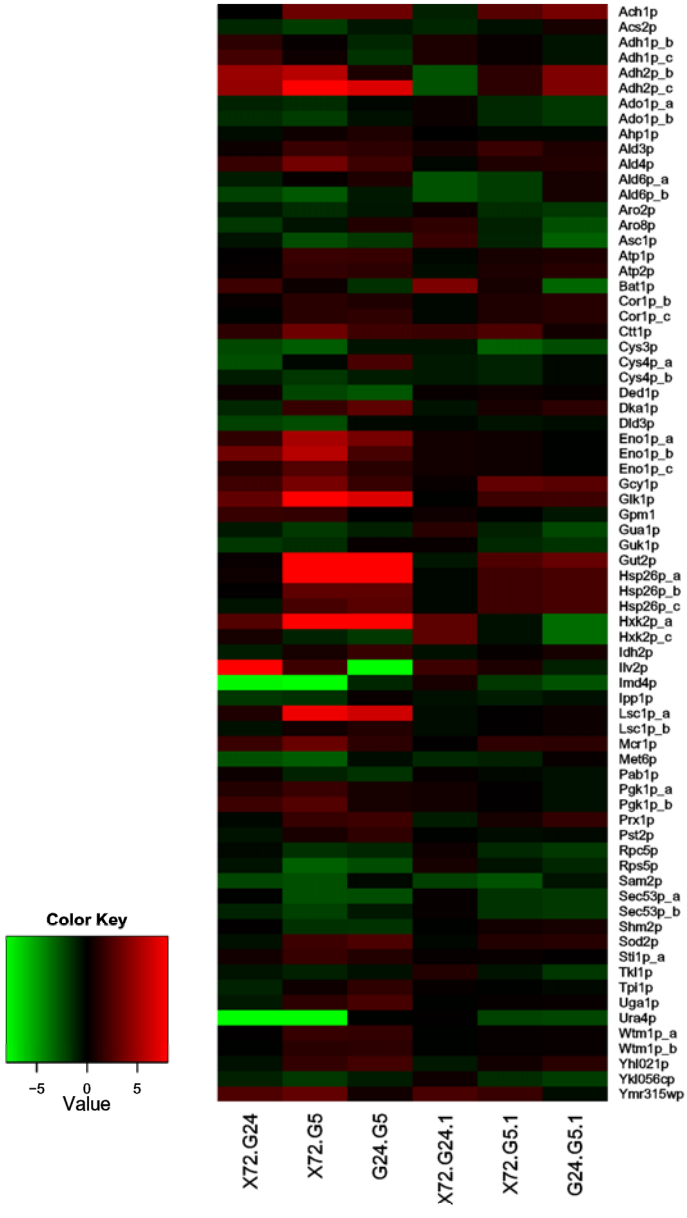

Figure 10. Heat map showing the abundance ratios of the protein spots, which had different abundances in cells growing on glucose or xylose in aerobic batch fermentations. X72.G24; xylose 72h vs. glucose 24h, X72.G5; xylose 72h vs. glucose 5h, and G24.G5; glucose 24h vs. glucose 5h (glucose derepressed vs. repressed cells). a to c refer to different isoforms of the proteins. The corresponding gene expression ratios are shown in the three right-hand columns of the picture.

Whereas xylose seemed partially repress expression of the respiratory, TCA cycle and gluconeogenic genes, *HXK1*, *HXT16* and *SUC2* had their highest expression levels on xylose in batch cultures (Fig. 9, cluster 4) (IV). Alike the genes encoding proteins of respiration, the TCA cycle and gluconeogenesis, these genes are regulated via the Snf1p/Mig1p-pathway, and are normally expressed on non-fermentable carbon sources (Klein *et al.*, 1998; Lutfiyya and Johnston, 1996; Rodriguez *et al.*, 2001; Özcan and Johnston, 1999). Thus, these genes were rather induced than repressed by xylose. In the cells grown in the chemostat culture on xylose + glucose, *HXK1* was downregulated compared with the cells from the corresponding glucose culture, indicating that in this case the response to xylose was also dependent on the culture conditions (Fig. 8) (III). In other transcription analysis studies of xylose-fermenting *S. cerevisiae* strains, *HXK1* had increased expression on xylose compared with glucose repressed cells both under aerobic and oxygen-restricted conditions (Jin *et al.*, 2004), as observed in study IV. However, it was downregulated in a chemically mutagenised TMB400 strain grown on xylose compared with the glucose-grown cells (Wahlbom *et al.*, 2003b). *HXT16*, in turn, had increased expression in evolved C1 strain compared with its TMB3001 parent strain (Sonderegger *et al.*, 2004b), as in study IV.

The data from studies III and IV showed that xylose does not cause similar carbon catabolite repression of genes as glucose and also that it fails to repress some genes normally repressed by glucose. The only partial repression of gluconeogenic genes and induction of some genes normally repressed by glucose may lead to simultaneous operation of glycolytic and gluconeogenic reactions during growth on xylose. This may affect negatively the fermentation of xylose and increase the ATP consumption. Glucose-repressible genes respond to the repression signal in a hierarchical manner depending on the actual mechanism and signalling cascades leading to repression (Verma *et al.*, 2005), and different genes also respond to different concentrations of the repressing carbon source (Yin *et al.*, 2003). The level of transcription of genes may also depend on both a repressing signal and on an inducing signal that may be specific for different genes (Belinchón and Gancedo, 2007). For example, the gene encoding isocitrate lyase requires the presence of a C2 carbon source to be fully induced (Fernández *et al.*, 1993). Thus, the regulatory network for glucose repression and derepression is complex and strictly fine-tuned. Therefore it is not surprising that during growth on xylose, which is not normally utilised as a carbon source by *S. cerevisiae*, its function is altered.

3.3 Effect of xylose on the major carbon fluxes

A metabolic flux analysis of the chemostat cultures on 56 mM glucose or on 180 mM xylose with 17 mM glucose indicated that on xylose + glucose the total glycolytic flux to pyruvate was only about 60% of that of glucose-grown cells (I, Fig. 1). The same analysis showed that on xylose as the main carbon source the flux from glucose 6-phosphate to ribulose 5-phosphate was almost tenfold higher compared with the glucose culture. This suggests cycling of the carbon through the upper glycolysis back to the PPP, where NADPH is produced in the reactions catalysed by glucose 6-phosphate dehydrogenase (Zwf1p) and 6-phosphogluconate dehydrogenase (Gnd1p) (Fig. 8). In the latter reaction, one carbon is lost as carbon dioxide. Consequently, the relative carbon flux through the TCA cycle was decreased (I). The decreased protein amounts and transcript levels of enzymes and genes related to the TCA cycle (II, III) discussed in section 3.2 are consistent with this result. However, of the genes encoding the enzymes of the PPP, only *GND1* had increased expression in the aerobic and anaerobic chemostat cultures on xylose + glucose compared with the corresponding glucose-limited cultures (Fig. 8) (III, and unpublished results Appendix V).

The role of the PPP in *S. cerevisiae* is to produce NADPH and precursors for nucleotide and amino acid biosyntheses. However, relatively little is known about the regulation of reactions in this pathway in *S. cerevisiae*. As NADPH is required in various enzymatic reactions involved in protection against oxidative stress, many genes of the PPP are regulated by the transcription factors controlling the expression of genes responding to oxidative stress (*e.g.* Yap1p or Stb5p) (Lee *et al*., 1999; Larochelle *et al*., 2006). Moreover, the genes *GND2*, *TKL2*, *SOL4* and *NQM1* encoding the second isoenzymes of the reactions of the PPP are induced after diauxic shift (DeRisi, 1997), and their expression also appears to respond similarly under other conditions such as heat shock and nitrogen depletion (Gasch *et al*., 2000). The flux through the PPP is controlled by the rate of the reaction carried out by Zwf1p that is regulated by the balance of NADPH and NADP+ and by the level of ATP (Voet and Voet, 1995; Vaseghi *et al.*, 1999).

In the aerobic batch cultures *GND2* had the highest expression on xylose (Fig. 9, cluster 4) (IV). In addition, *ZWF1*, *TKL2* and *NQM1* had a similar trend in their expression although the difference was not statistically significant based on ANOVA (IV, Fig. 3). However, this supports the previously reported coregulation

of these genes under different conditions, as discussed above. *GND1*, *RPE1* and *TKL1* in turn had lower expression in cells grown on xylose compared with the glucose repressed cells, but the expression was higher than in glucose derepressed cells (Fig. 9, cluster 1) (IV). On the other hand, the abundance of Tkl1p was lowest in xylose-grown cells (Fig. 10). Thus, in contrast to the genes of the second isoenzymes of PPP that are repressed by glucose, these genes appeared to be induced by glucose and in study IV interestingly, to a lesser extent also by xylose. In other studies, the mutants with improved xylose metabolism compared with their parent strains have been shown to have increased expression of *ZWF1* (Sonderegger *et al.*, 2004b), *SOL3*, *GND1*, *TAL1* and *TKL1* (Wahlbom *et al.*, 2003b) and increased activity of transketolase, transaldolase and glucose 6-phosphate dehydrogenase (Pitkänen *et al.*, 2005). In agreement with these results, the overexpression of genes encoding the enzymes of the non-oxidative PPP has improved xylose utilisation and thus, it seems likely that flux through this pathway limits the xylose catabolism at least in strains with a high level of expression of genes encoding XR and XDH or in strains with xylose isomerase (Johansson and Hahn-Hägerdal, 2002; Karhumaa *et al.*, 2005; Ni *et al.*, 2007).

Moreover, an improved uptake of xylose was measured from chemostat isolates with enhanced growth on xylose (Pitkänen *et al.*, 2005). When sugar transport and consequently glycolytic flux was enhanced by overexpression of permeases, *Kluyveromyces lactis* acquired the ability to grow on galactose and raffinose non-respiratively (Goffrini *et al.*, 2002). Thus, one potential reason for the partial repression of respiratory genes and possibly inadequate regulatory network for efficient xylose fermentation is the lower glycolytic flux compared with growth on glucose. In addition to low flux through the oxidative part of PPP, this may also be due to lack of a specific transporter for xylose in *S. cerevisiae* (Hamacher *et al.*, 2002; Lee *et al.*, 2002; Saloheimo *et al.*, 2007).

Several studies have suggested that the expression level of the glycolytic genes would be connected with the rate of glycolytic flux that is in turn related to the availability of carbon and the efficiency of its uptake. In a study of yeast strains with different hexose uptake capacities, the expression of *TPI1*, *PGK1*, *PDC1* and *ADH1* was shown to correlate with the glycolytic rate, whereas the expression levels of gluconeogenic genes had an inverse correlation (Elbing *et al.*, 2004). Additionally, Mig1p remained dephosphorylated (and so repressed its target genes) only at high glycolytic rates (Elbing *et al.*, 2004). Blank and Sauer,

(2004) showed that repression of the genes of the TCA cycle was regulated by the growth rate, and the activity of the TCA cycle increased with decreasing rates of glucose uptake. In study IV of aerobic batch fermentations, *PGI1* and *ENO2* had higher expression in cells grown on xylose compared with the glucose derepressed cells but lower expression compared with the repressed cells (Fig. 9, cluster 1). This correlates with the specific growth rate of the cells on glucose and xylose (repressed > xylose > derepressed cells) (IV). However, the abundance of Adh1p, Pgk1p and Gpm1p was higher in cells grown on xylose in comparison with glucose repressed and derepressed cells (Fig. 10). These results are in contrast to those of Jin and co-workers, who did not observe changes in the expression levels of the genes encoding fermentative enzymes in their transcription analysis of cells grown in shake flask cultures on xylose and glucose (Jin *et al.*, 2004).

On the other hand, several studies suggest that regulation of the glycolytic flux takes place mainly at the post-transcriptional level (Daran-Lapujade *et al*., 2004; Wiebe *et al.*, 2007; de Groot *et al*., 2007; Daran-Lapujade *et al*., 2007). Only a small transient increase in the expression of glycolytic genes was observed along an increase in glycolytic flux after a shift from aerobic to anaerobic conditions in glucose-limited chemostat cultures (Wiebe *et al.*, 2007). In study of aerobic and anaerobic chemostat cultures by de Groot and co-workers (2007), most of the glycolytic proteins had increased abundance under anaerobic compared with aerobic conditions, whereas the corresponding transcript levels remained constant. In a transcriptome comparison of cells from a glucose-limited chemostat culture and from chemostat cultures on ethanol or acetate, decreased expression of only *HXK1* and *TDH1* was observed during periods of decreased glycolytic flux on the latter two carbon sources (Daran-Lapujade *et al.*, 2004).

In study IV, 2-DE gel comparison of glucose repressed and derepressed cells and cells grown on xylose showed that Hxk2p had two isoforms, one of which had its highest abundance in cells grown on xylose whereas the other was most abundant in the glucose repressed cells (Fig. 10). Further staining of 2-DE gels with a phosphoprotein-specific Pro-Q Diamond fluorescent dye suggested that Hxk2p actually had three isoforms with different phosphorylation patterns in cells grown on glucose and xylose (Fig. 11). Two out of three of these phosphorylated isoforms were not present in the glucose repressed cells and the level of phosphorylation of all three isoforms was higher in the glucose derepressed cells and in the cells grown on xylose compared with the glucose

repressed cells (Fig. 11). It has been shown that Hxk2p is dephosphorylated on fermentable carbon sources and that on poorly fermentable carbon sources both phosphorylated and dephosphorylated forms are present (Randez-Gil *et al.*, 1998). Thus, based on the phosphorylation pattern of Hxk2p, xylose appears to be detected rather like a non-fermentable than a fermentable carbon source by *S. cerevisiae*.

The phosphorylation was not limited to Hxk2p but Glk1p, Eno1p and Eno2p also had several phosphorylated pI forms that were present in different quantities in glucose repressed, derepressed and in xylose-grown cells (Fig. 11). Like Hxk2p, Glk1p catalyses the phosphorylation of glucose in the first reaction of glycolysis, but *GLK1* is expressed during growth on non-fermentable carbon sources whereas Hxk2p is predominant during growth on glucose (Herrero *et al.*, 1995). Enolases catalyse the conversion of 2-phosphoglycerate to phosphoenolpyruvate in glycolysis. The *ENO1* is repressed by glucose, whereas on non-fermentable carbon sources both *ENO1* and *ENO2* are expressed (Entian *et al.*, 1987).

The role of phosphorylation in the regulation of the activity and function of these enzymes under the conditions studied remains unclear. However, data is emerging for several glycolytic proteins, besides Hxk2p (Ahuatzi *et al.*, 2006), that have other functions in the cell in addition to their roles in glycolysis that may necessitate complex regulation mechanisms. For example, several post-translationally processed forms of Eno2p were identified in 2-DE gel analysis of osmotically stressed yeast cells (Larsen et al., 2001). More recent studies demonstrated that Eno1/2p participate in the targeting of nuclear encoded tRNA to mitochondrial import (Entelis *et al.*, 2006), and that enolases are also involved in vacuole fusion and protein transport to the vacuole in yeast (Decker and Wickner, 2006; Sirover, 2005). Moreover, pyruvate decarboxylase isoenzymes Pdc1p and Pdc5p of *S. cerevisiae* appear to be involved in regulation of thiamine metabolism and also possess nuclear localisation (Mojzita and Hohmann, 2006). Acetyl-CoA synthetases (Acs1/2p) in turn function not only in the synthesis of mitochondrial and cytosolic acetyl-CoA for the assimilatory reactions of carbon metabolism, but also in nuclear provision of acetyl-CoA for histone deacetylation (Takahashi *et al.*, 2006). The post-translational modification of proteins may also be a way to modulate the flux through a pathway. Shenton and Grant (2003) demonstrated that several glycolytic proteins (*e.g.* Tdh3p, Eno2p and Adh1p) were S-thiolated in response to oxidative stress. This decreased their activity, which was proposed to lead to increased flux through the PPP at the expense of the glycolytic flux under oxidative stress conditions.

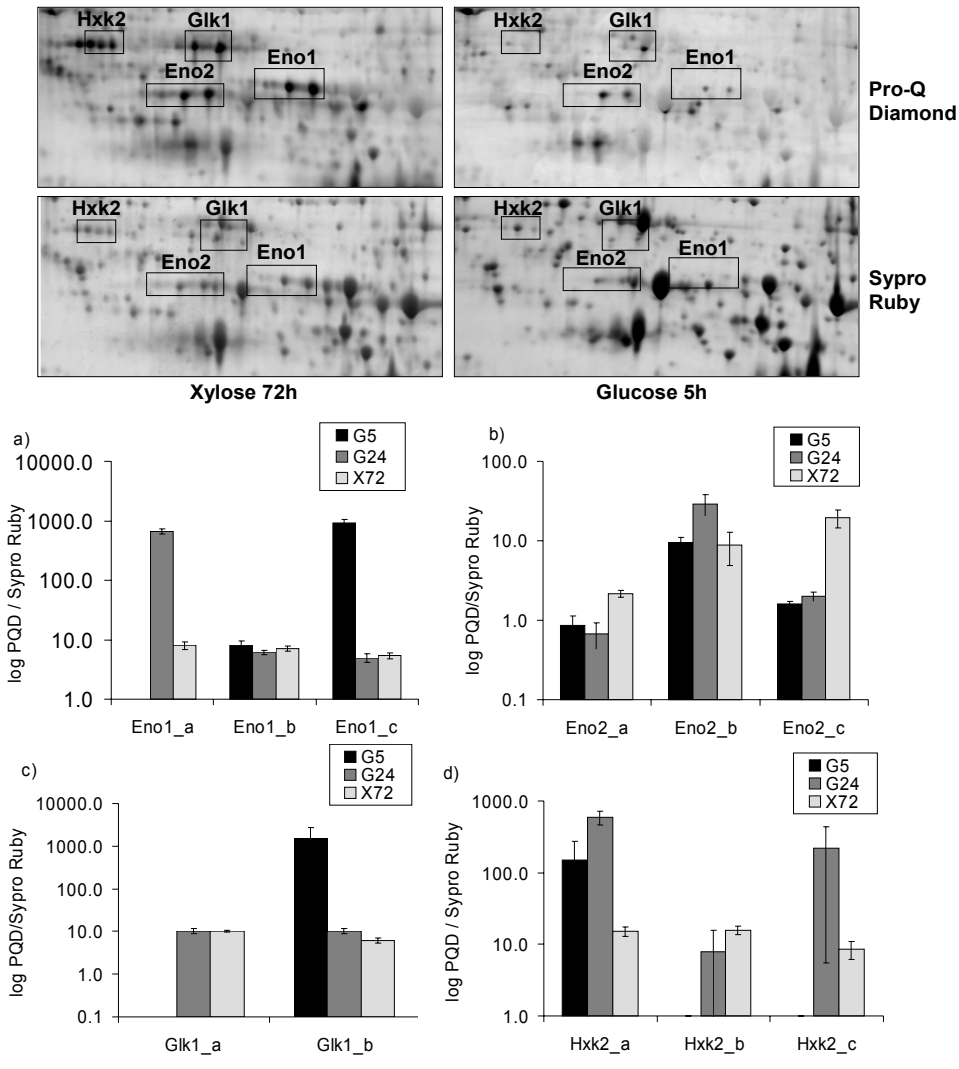

Figure 11. Images of 2-DE gels showing the locations of hexokinase 2 (Hxk2p), glucokinase (Glk1p), enolase 2 (Eno2p) and enolase 1 (Eno1p) in samples from cells grown for 72 h on xylose and for 5 h on glucose and stained either with phosphoprotein specific Pro-Q Diamond (upper) or Sypro Ruby (lower). The ratio of Pro-Q Diamond phosphoprotein stain intensity to Sypro Ruby signal intensity (total protein amount) of phosphorylated protein isoforms is presented below. a to c refer to different isoforms on gels. G5 = cells after 5 h on glucose; G24 = cells after 24 h on glucose; X72 = cells after 72 h on xylose.

3.4 Expression of genes for sugar transport

In the yeast *S. cerevisiae* xylose uptake takes place by facilitated diffusion by members of the hexose transporter family that transport glucose, fructose and mannose (Bisson *et al.*, 1993; Hamacher *et al.*, 2002; Özcan and Johnston, 1999; Saloheimo *et al.*, 2007). At least Hxt1p, Hxt4p, Hxt5p, Hxt7p and Gal2p are reported to transport xylose but with an affinity for xylose that is one to two orders of magnitude lower than for glucose (Hamacher *et al.*, 2002; Sedlak and Ho, 2004; Saloheimo *et al.*, 2007). In study I, low concentration of glucose was shown to increase the specific xylose uptake rate. This may be due to its positive effect on induction of *HXT* genes. In the aerobic chemostat culture on xylose + glucose (III) *HXT2* had 5-fold higher expression compared with the glucose-limited chemostat culture (III, Fig. 2), but anaerobically it was downregulated on xylose + glucose under the otherwise identical culture conditions (unpublished results, Appendix V). In study IV of aerobic batch cultures, *HXT16* (Fig. 9, cluster 4) and *HXT4* (Fig. 9, cluster 7) had higher expression in cells grown on xylose compared with the glucose repressed and derepressed cells.

Previous transcript analyses of recombinant xylose-utilising *S. cerevisiae* have also shown increased expression of *HXT2* on xylose, but in contrast to study III, compared with glucose repressed cells (Jin *et al.*, 2004). *HXT5*, *HXT16* and *GAL2* had increased expression in chemostat cultures on xylose in mutant strains with improved xylose fermentation capacity compared with their parent strains (Sonderegger *et al.*, 2004b; Wahlbom *et al.*, 2003b). Interestingly, all the *HXT*s upregulated on xylose in these and studies III and IV are normally expressed at low concentrations of glucose (Özcan and Johnston, 1996; Özcan and Johnston, 1999).

The genes encoding maltose permeases *MAL11* and *MAL31* and additionally *MAL12* and *MAL32* encoding α-glucosidases had higher expression in cells grown on xylose + glucose in the anaerobic chemostat culture compared with the anaerobic glucose-limited chemostat culture (unpublished results, Appendix V). Further, in aerobic batch cultures *MAL11* and *MAL31* had about the same expression levels in xylose-grown cells as in glucose derepressed cells (Fig. 9, cluster 6) (IV). However, in the aerobic chemostat culture the presence of xylose was able to repress the expression of *MAL11*, *MAL12* and *MAL32* compared with the glucose derepressed cells in the glucose culture (III, Fig. 2, Table 2).

Several *MAL* genes had increased expression on xylose also in transcription analysis of xylose-fermenting *S. cerevisiae* carried out by Jin and co-workers (Jin *et al.*, 2004). In two separate chemostat culture studies of transcriptional responses of *S. cerevisiae* to different nutrient limitations, the induced expression of *MAL11* and *MAL32* was shown to be specific for carbon limitation (Boer *et al.*, 2003; Tai *et al.*, 2005). This suggests that the induction of these genes in anaerobic chemostat culture on xylose may be indicative of growth on a poorly fermentative carbon source.

3.5 Carbon recognition and regulation of metabolism during growth on xylose

In the aerobic batch cultures, *RGT2* and *MTH1* had the highest levels of expression in cells grown on xylose (Fig. 9, cluster 4) (IV). In addition, *SNF3* and *RGT1* had higher expression in xylose-grown cells compared with the glucose repressed cells, but the expression was lower compared with the derepressed cells (Fig. 9, cluster 2) (IV). Previously, in a transcriptional comparison of a mutated recombinant strain with enhanced performance on xylose compared with its parent strain, *MTH1* expression was increased in the mutant strain (Sonderegger *et al.*, 2004b). Additionally, Jin and co-workers also detected increased *MTH1* expression in cells grown on xylose compared with the glucose repressed cells (Jin *et al.*, 2004). *SNF3* and *RGT2* encode glucose sensors that are involved in induction of the transcription of genes for hexose transporters through the signal transduction pathway that releases the transcriptional repressor Rgt1p and its co-repressors Mth1p and Std1p from the upstream binding sites of *HXT*s (Kim and Johnston, 2006). *RGT2* is normally expressed at high concentrations of glucose and in study IV it appeared also to respond to high extracellular xylose, whereas *SNF3* that is repressed by glucose via Snf1p/Mig1p and is thus normally expressed in low levels of glucose, was only partially repressed by xylose. Furthermore, *MTH1* expression is repressed by glucose via the Snf1p/Mig1p-pathway (Kim *et al.*, 2006). Thus, it appears that xylose was not able to repress *MTH1* and *SNF3* via the Snf1p/Mig1p-pathway in a similar manner to glucose.

During growth on xylose carbon flows through the PPP and phosphorylation of glucose by Hxk2p does not take place. Despite this, *HXK2* had higher expression

in aerobic batch cultures in cells grown on xylose compared with the glucose derepressed cells, but the expression was lower than in the repressed cells (Fig. 9, cluster 1) (IV). This is interesting as Hxk2p also plays a regulatory role and together with Mig1p is involved in the repression of genes not needed during growth on glucose (Moreno *et al.*, 2005; Palomino *et al.*, 2005; Ahuatzi *et al.*, 2006). In addition to regulating the expression of hexose transporters, Rgt1p is also involved in repression of *HXK2* at low glucose concentrations (Palomino *et al.*, 2005; Palomino *et al.*, 2006). Consistently, in aerobic batch cultures in which *RGT1* was expressed at its highest level in glucose derepressed cells, *HXK2* had its lowest expression (Fig. 9, cluster 2 and cluster 1, respectively) (IV).

In the comparison of chemostat cultures on 56 mM glucose and 180 mM xylose + 17 mM glucose, *TYE7* encoding an E-box DNA-binding protein was induced in both aerobic and anaerobic cultures with xylose + glucose (III, Fig. 2, Appendix V). Tye7p has been shown to be a multicopy suppressor of *gcr2* mutants with a defect in expression of the glycolytic genes (Sato *et al.*, 1999). Moreover, Tye7p was able to complement a *sck1* null mutation in *K. lactis* exhibiting a reduced flux of glycolysis (Lemaire *et al.*, 2002). However, in the present study overexpression of *TYE7* in the strain H2217 with the xylose pathway did not result in enhanced growth on xylose or utilisation of xylose, reduced xylitol production or an improved ethanol yield compared with the control strain in the shake flask cultures on xylose with and without antimycin A (unpublished results). Moreover, the recent transcription data from multiple nutrient limitations suggest that Tye7p rather functions together with Cbf1p in the regulation of genes encoding the upper part of the sulphur assimilation pathway (Knijnenburg *et al.*, 2007).

Several other genes encoding transcription factors also had higher expression in the aerobic chemostat culture on xylose + glucose compared with the glucose-limited culture (III, Table 1). These included *SKN7*, *BUR6*, *MED1*, *MED8*, *ZAP1* and *NRG2*. The functions regulated by the transcription factors encoded by these genes are related to osmo- and oxidative stress (Skn7p), stress response (Bur6p, Nrg2p), zinc metabolism (Zap1p) and regulation of transcription related to growth on different carbon sources (Med1p, Med8p and Nrg2p) (Balciunas *et al.*, 1999; Geisberg *et al.*, 2001; Janiak-Spens *et al.*, 2005; Lyons *et al.*, 2000; Palomino *et al.*, 2006; Raitt *et al.*, 2000; Vyas *et al.*, 2005). *NRG2* and its

homologue *NRG1* encode transcriptional repressors that participate in the regulation of glucose repression, haploid invasive growth and in the control of expression of a set of stress response genes (Vyas *et al.*, 2005). Med1p and Med8p are part of a mediator complex that regulates RNA-polymerase III -dependent transcription. Deletion of *MED1* caused reduced expression from *GAL1* and to a lesser extent also from *FBP1*, *MIG1* and *CAT8* promoters. Moreover, *med1* disruption suppressed some phenotypes associated with deletion of *snf1* (Balciunas *et al.*, 1999). Med8p, in turn, is involved together with Rgt1p in repression of *HXK2* in conditions of low glucose (Palomino *et al.*, 2006).

In the aerobic batch cultures *NRG2* and *GAL83* had their lowest expression in cells grown on xylose (Fig. 9, cluster 3) (IV). *GAL83* encodes one of the β-subunits of the Snf1-kinase complex and allows nuclear localisation of the Snf1-kinase in the presence of a non-fermentable carbon source (McCartney *et al.*, 2005). Unexpectedly, *GAL83* had its highest expression in glucose repressed cells and the expression was also higher in glucose derepressed cells compared with cells grown on xylose. This suggests differences in regulation of Snf1p functions between glucose- and xylose-grown cells. Overall, expression levels of several genes encoding transcription factors involved in regulation of carbon metabolism and stress responses were altered during growth on xylose. However, the interpretation of these mixed responses is difficult. The expression changes of these regulators were not clearly connected to changes in the expression of any group of genes that they regulate. Additionally, expression of transcription factors involved in regulation of the genes of enzymes in both fermentative and non-fermentative pathways was altered in xylose-grown cells.

3.6 Expression of genes and abundance of proteins for fermentation, ethanol utilisation and acetyl-CoA synthesis

In the aerobic chemostat cultures, the abundance of alcohol dehydrogenase Adh2p and the acetaldehyde dehydrogenases Ald6p (cytosolic) and Ald4p (mitochondrial) was increased in cells grown on xylose + glucose compared with the cells from glucose culture (II, Fig. 2), as was *ALD6* and *ALD4* expression (Fig. 8) (III). In addition, *ACS1* encoding acetyl-CoA synthase had increased and *ACH1* encoding acetyl-CoA hydrolase decreased expression in cells from the

chemostat culture on xylose + glucose (Fig. 8) (III). The metabolic flux analysis showed consistently increased flux to cytosolic acetyl-CoA in cells from xylose + glucose compared with the glucose-grown cells (I, Fig. 1). The increased abundance of these transcripts and proteins in xylose-grown cells suggests that under these conditions ethanol and acetate were utilised as co-substrates for growth with xylose. Oxidation of acetaldehyde to acetate by either Ald6p or mitochondrial Ald4p also serves as a supply for NADPH that is in particular needed during growth on xylose for the reaction catalysed by XR in the xylose pathway. These reactions may also contribute to ATP production, as cytosolic acetaldehyde can enter mitochondria where it can be oxidised to acetate by Ald4p with the formation of NADH. Moreover, ethanol produced in the cytosol can enter mitochondria and be oxidised to acetaldehyde and further acetate by mitochondrial alcohol dehydrogenase and acetaldehyde dehydrogenases, respectively, to produce NADH for the energy metabolism (Bakker *et al.*, 2001; Boubekeur *et al.*, 2001). Increased *ACS1* expression suggests that acetate may also have been converted to acetyl-CoA that serves as a precursor in the TCA or glyoxylate cycles and in amino acid and lipid metabolism. Whereas genes encoding the enzymes of TCA and glyoxylate cycles were downregulated in the aerobic chemostat culture on xylose + glucose (Fig. 8) (III), several genes encoding enzymes on the ergosterol biosynthetic pathway had increased expression, suggesting the channelling of acetyl-CoA into this pathway (III, Supplemental Fig. 1).

Adh2p and Ald4p had increased abundance in cells grown on xylose also in aerobic batch cultures (IV), when compared with both the glucose repressed and derepressed cells, but in contrast to chemostat cultures, Ald6p (a and b isoforms) had lower abundance on xylose compared with the glucose repressed and derepressed cells (Fig. 10). This was also seen at the transcript level (Fig. 9, cluster 5). By contrast, *ALD5* encoding another mitochondrial NADPH-dependent isoform of the acetaldehyde dehydrogenases had the highest expression in cells grown on xylose (Fig. 9, cluster 7), and Ald3p was the most abundant in xylose-grown cells at the protein level (Fig. 10). Ald5p takes part in acetate production during fermentation (Saint-Prix *et al.*, 2004), but has also been proposed to have a role in the biosynthesis of electron transport chain components (Kurita and Nishida, 1999). As a summary, in chemostat culture on xylose + glucose both the mitochondrial and cytosolic pyruvate dehydrogenase bypasses leading to synthesis of acetyl-CoA appeared to be active, whereas in cells grown in batch

cultures on xylose, low abundance of Ald6p and increased expression of *ALD5* suggest that the mitochondrial bypass was more active. This difference may be due to the different physiological state of the cells in chemostat and batch cultures.

3.7 Starvation response and expression of genes for amino acid catabolism and biosynthesis

In the transcription analysis of cells grown in chemostat cultures on glucose and xylose + glucose the expression changes of ~ 15% of the responding genes correlated with the gene expression changes observed in a study of acid-to-alkali transition phase of yeast colonies during growth on an agar plate (Palková *et al.*, 2002) (III, Tables 1 and 2). This transition was accompanied by the secretion of ammonia with concomitant induction of *ATO1-3* encoding transporters of the YaaH-family, which were shown to be involved in ammonia secretion. Ammonia acts as a starvation signal related to adaptation and survival under starvation conditions (Palková *et al.*, 2002). A more recent study showed that *ATO1* (*ADY2*) also encodes an acetate transporter (Paiva *et al.*, 2004). All three of these transporters also had increased expression in cells from the aerobic chemostat on xylose + glucose compared with cells from the glucose-limited culture (III, Fig. 2). Other common features with study of Palková and co-workers included the gene expression changes suggesting the activation of pathways for acetyl-CoA production (*e.g.* induction of *ADH2, ALD4*, *ALD6*, *ACS1* and *FOX2*), for amino acid catabolism (*e.g.* induction of *UGA2*, *ICL2*, *ARO10* and *AAD14*), for uptake of carboxylic acids (*e.g.* induction of *JEN1*) and for transport of phosphate and zinc (*e.g*. induction of *PHO84*, *PHO89* and *ZRT1*) (III, Tables 1 and 2). Moreover, several genes encoding enzymes of the TCA cycle and many genes related to general stress response were downregulated in cells grown on xylose + glucose and under the acid-to-alkali transition of yeast colonies (III, Tables 1 and 2) (Palková *et al.*, 2002).

Interestingly, relatively many of the induced and downregulated genes during the acid-to-alkali transition of yeast colonies (Palková *et al.*, 2002), and also those responding to xylose as the carbon source (*e.g. JEN1*, *ACS1*, *ADH2*, *ALD4*, *ARO10*, *ICL2*, *CTA1*, *ATO2*, *ATO3*), also appeared to have correspondingly higher or lower expression specifically under carbon limited conditions (Boer *et*

al., 2003), proposing that the expression of these genes would generally be related to carbon limitation. This suggests that during growth on xylose + glucose in the chemostat *S. cerevisiae* sensed a more severe carbon limitation than in the glucose-limited chemostat culture. However, similar "starvation" related transcriptional responses were not observed in transcription analysis of anaerobic chemostat cultures on xylose + glucose and on glucose (unpublished results).

In the aerobic batch cultures on xylose and glucose, expression of *ATO1* and *2* was increased in glucose derepressed cells and was either approximately at the same level or higher on xylose, whereas *ATO3* had its highest expression in cells grown on xylose (Fig. 9, clusters 2, 6 and 4, respectively) (IV). Most of the other genes mentioned above and related to "starvation response" did not specifically have higher expression in cells grown on xylose in aerobic batch cultures but also responded to glucose deprivation and/or growth on ethanol (glucose derepressed cells).

The higher expression of *ICL2*, *ARO10*, *AAD14* and *GRE2* in cells grown on xylose + glucose in chemostat cultures suggested activation of pathways for degradation of carbon skeletons from some amino acids and consequent production of fusel alcohols (III, Table 1) (Dickinson *et al.*, 2003; Hauser *et al.*, 2006; Luttik *et al.*, 2000; Vuralhan *et al.*, 2005). The transcript and proteome analysis of aerobic batch cultures (IV) give further support for the activation of amino acid catabolic pathways during growth on xylose. Fusel alcohols and acids are produced by the "Ehrlich pathway" that is coupled to phenylalanine, leucine or methionine degradation pathways (Schoondermark-Stolk *et al.*, 2006; Vuralhan *et al.*, 2005). *ARO10* encoding the decarboxylase activity required in the first step of the "Ehrlich pathway" (Vuralhan *et al.*, 2005) had a higher expression in xylose-grown cells, although only compared with glucose repressed cells (Fig. 9, cluster 2) (IV). Moreover, *PDR12* encoding a multidrug resistance transporter that functions in the export of fusel acids (Hazelwood *et al.*, 2006) had its highest expression on xylose (Fig. 9, cluster 4) (IV). *BAT1* encoding a mitochondrial branched-chain amino acid aminotransferase that catalyses the first step in leucine catabolism had the highest expression (Fig. 9, cluster 7) and protein abundance in cells grown on xylose (Fig. 10). Additionally, several other genes (*ILV2*, *LEU9*, *LEU1*, *LEU2*) encoding enzymes for leucine biosynthesis, as well as *BAP2* encoding a high affinity leucine

permease had their highest expression in cells grown on xylose (Fig. 9, clusters 7 and 4, respectively) (IV). In case the reactions carried out by Ilv2p and Leu2p would take place in the direction of leucine catabolism, NAD^+ and NADPH cofactors required in xylose pathway, would be produced. Consequently, catabolism of amino acids may have been used as a way to balance redox cofactors in xylose-grown cells.

On the other hand, several genes and proteins involved in methionine uptake and biosynthesis had their lowest expression and protein abundance on xylose (Fig. 9, clusters 3 and 5 and Fig. 10) (IV). Methionine biosynthesis requires ATP and is highly NADPH-consuming (Stephanopoulos *et al.*, 1998). Hypothetically, increased demand and possibly limited availability of this co-factor and also of ATP during xylose metabolism could lead to downregulation of the expression of genes on this pathway. Of *ZWF1*, *GND1*,*2*, *IDP2* and *ALD6*, which in *S. cerevisiae* encode the NADPH-producing reactions in the cytoplasm (Grabowska and Chelstowska, 2003), only *GND2* had increased expression in cells grown on xylose in the aerobic batch cultures (Fig. 9, cluster 4) (IV).

3.8 Stress and redox responses

In the aerobic chemostat culture on xylose + glucose, 45% of the promoters of the downregulated genes had at least one binding site for Msn2p/Msn4p transcription factors that are activated under a number of stress conditions (Martinez-Pastor *et al.*, 1996), whereas only 14% of the genes with higher expression on xylose + glucose had binding sites for these transcription factors in their promoter regions (III). Thus, the general stress responsive genes were rather downregulated than induced during growth on xylose in chemostat culture. However, genes encoding the transcription factors Skn7p, Bur6p and Nrg2p had increased expression in the xylose culture (III, Table 1). Skn7p is part of the phosphorelay system, through which cells can respond to osmotic and other environmental stresses. Skn7p is also activated by oxidative stress but independently from the phosphorelay system (Ikner and Shiozaki, 2005; Janiak-Spens *et al.*, 2005). Both Bur6p and Nrg2p participate in regulation of the genes related to general environmental stress response and moreover Nrg2p contributes to repression of some of the glucose repressible genes (Geisberg *et al.*, 2001; Vyas *et al.*, 2005). In addition *GRE2*, which has been shown to be induced under

both osmotic and oxidative stress conditions, had increased expression in the cells from aerobic chemostat culture on xylose + glucose (III, Table 1) (Garay-Arroyo and Covarrubias, 1999). This gene encodes a broad-specificity reductase possessing both methylglyoxal and isovaleraldehyde reductase activities and it also plays a role in ergosterol metabolism (Chen *et al.*, 2003; Hauser *et al.*, 2006; Warringer and Blomberg, 2006).

Similar kinds of changes were observed in transcription analysis of aerobic batch cultures on xylose and glucose (IV). In aerobic batch cultures most of the stress responsive genes, including *MSN4*, had their highest expression in glucose derepressed cells and lowest in glucose repressed cells (Fig. 9, cluster 2) (IV). Only *GCY1*, *CTT1*, *ALD3, SSK22* and *AHA1* encoding a co-chaperone of Hsp82p, were induced on xylose (Fig. 9, cluster 4) (IV). In addition, *GND1*, *YPD1*, *RHR2* and *DOG2* had higher expression in xylose-grown cells compared with the glucose derepressed cells (Fig. 9, cluster 1) (IV). Of these genes *GCY1*, *RHR2, SSK22, YPD1* and *ALD3* are expressed in particular in response to osmotic stress, whereas *GND1* and *CTT1* respond to oxidative stress (Bro *et al.*, 2004; Izawa *et al.*, 1998; Janiak-Spens *et al.*, 2005; Navarro-Avino *et al.*, 1999; Norbeck and Blomberg, 1997). *DOG2* is induced by both oxidative and osmotic stresses and in addition by glucose starvation (Tsujimoto *et al.*, 2000). Thus, although growth on xylose seemed not to provoke a general stress response, it appears that cells were experiencing some stress during growth on xylose, perhaps due to redox imbalance as a result of xylose utilisation.

In aerobic batch cultures several genes that had their highest expression in cells grown on xylose encoded functions involved in cell wall organisation and biogenesis, mating and regulation of the cell cycle and pseudohyphal growth. In addition, some genes related to post-translational modification of proteins and catabolism of proteins by ubiquitinylation had increased expression in cells grown on xylose (Fig. 9, cluster 4) (IV). Pseudohyphal growth is hypothesised to be an adaptation that allows *S. cerevisiae* to search for more optimal growth substrates and it is also induced by fusel alcohols that are formed by catabolism of amino acids as discussed in section 3.7 (Dickinson, 1996; Gagiano *et al.*, 2002).

3.9 Transcription analysis of anaerobic chemostat cultures (unpublished)

The transcription changes in the comparison of the cells from the anaerobic chemostat culture on 56 mM glucose with the anaerobic 180 mM xylose + 17 mM glucose chemostat culture (Appendix V) were for the most part different from the responses observed in the comparison of the corresponding aerobic chemostat cultures (III). Only six genes had higher expression in cells grown in aerobic and anaerobic chemostat cultures on xylose + glucose compared with the glucose limited chemostat cultures under aerobic and anaerobic conditions. These included *GND1* encoding 6-phosphogluconate dehydrogenase, catalysing the latter of the two NADPH producing steps in the oxidative PPP and *TYE7* discussed in section 3.5 (Table 3).

The genes with lower expression in cells grown on xylose + glucose under both aerobic and anaerobic conditions included genes encoding three of the four proteins of the trehalose synthase complex (Table 3). Transcription of these genes is activated under glucose-limitation and thus, the residual xylose present in the cultivation medium appeared to repress the expression of these genes. Further, the genes for trehalose synthesis are activated via the stress responsive Msn2p/Msn4p-transcription factors (Winderickx *et al.*, 1996). Under aerobic conditions, the majority of genes with one or more binding sites for Msn2p/Msn4p had lower expression in cells growing on xylose + glucose, as discussed in section 3.8 (III).

In addition, several genes encoding proteins involved in iron uptake and homeostasis had lower expression in cells grown on xylose + glucose both in aerobic and anaerobic chemostat cultures (Table 3). The TCA cycle and the respiratory chain harbour iron- and copper-containing proteins (De Freitas *et al*., 2003). Speculatively, decreased expression of genes and abundance of enzymes of the TCA cycle as seen in the aerobic chemostat culture on xylose (I, III) could decrease the intracellular iron demand, which may further affect the expression of genes related to regulation of iron homeostasis. This does not, however, explain the lower anaerobic expression of these genes in cells grown on xylose + glucose. On the other hand, *FET3*, *FIT2* (Table 3) and *FTR1* (downregulated on xylose + glucose under aerobic conditions, III) are induced during the diauxic shift (Haurie *et al.*,

2003) and consequently decreased expression of these genes was perhaps due to the repressive effect of the residual xylose in the growth medium.

Table 3. The genes with increased and decreased expression in cells grown on 180 mM xylose + 17 mM glucose compared with the cells from the glucose-limited chemostat culture with 56 mM glucose under both aerobic and anaerobic conditions.

ORF Id	Gene	Description
Genes with higher expression on xylose + glucose		
YHR183w	*GND1*	6-phosphogluconate dehydrogenase
YOR344c	*TYE7*	basic helix-loop-helix transcription factor
YBR067c	*TIP1*	major cell wall mannoprotein with possible lipase activity
YNR030w	*ALG12*	alpha-1,6-mannosyltransferase localized to the ER
YOL121c	*RPS19A*	40S small subunit ribosomal protein S19.e
YJL190c	*RPS22A*	ribosomal protein S15a.e.c10
Genes with lower expression on xylose + glucose		
YML100w	*TSL1*	large subunit of trehalose 6-phosphate synthase
YBR126c	*TPS1*	synthase subunit of trehalose 6-phosphate synthase/phosphatase complex
YDR074w	*TPS2*	phosphatase subunit of the trehalose 6-phosphate synthase/phosphatase complex
YHR008c	*SOD2*	superoxide dismutase (Mn) precursor, mitochondrial
YMR058w	*FET3*	cell surface ferroxidase, high affinity
YOL158c	*ENB1*	endosomal ferric enterobactin transporter, expressed under conditions of iron deprivation
YLR205c	*HMX1*	ER localized, heme-binding peroxidase involved in the degradation of heme
YOR382w	*FIT2*	cell wall mannoprotein, involved in the retention of siderophore-iron in the cell wall
YOR383c	*FIT3*	cell wall mannoprotein, involved in the retention of siderophore-iron in the cell wall
YGR023w	*MTL1*	potential cell wall stres sensor
YGR032w	*GSC2*	1,3-beta-D-glucan synthase subunit
YDR388w	*RVS167*	involved in regulation of actin cytoskeleton and viability following starvation or osmotic str
YHL021c		weak similarity to *Pseudomonas* gamma-butyrobetaine hydroxylase
YFR039c		similarity to hypothetical protein YGL228w
YBL085w	*BOI1*	BEM1 protein-binding protein
YIL120w	*QDR1*	multidrug transporter

The notable difference between the anaerobic xylose + glucose and glucose chemostat cultures was the reduced expression levels of *MET3*, *MET14*, *MET10* and *CYS4* encoding catalytic enzymes in the methionine biosynthesis pathway, reduced expression of several genes encoding sulphate and methionine permeases, and in addition, lower expression of *MET32* and *MET30*, encoding the transcriptional regulators of the genes of sulphur amino acid metabolism in the cells grown on xylose + glucose in anaerobic chemostat culture (Appendix V) (Blaiseau *et al.*, 1997; Rouillon *et al.*, 2000; Thomas and Surdin-Kerjan, 1997). The reduced expression of several genes on this pathway was also seen in aerobic batch

cultures in cells grown on xylose (see, section 3.7) (IV). Sulphur amino acid biosynthesis requires both ATP and NADPH, which may hypothetically be a reason for the lower expression of these genes on xylose as already discussed in section 3.7.

However, *LYS9* and *LYS1* encoding the saccharopine dehydrogenases of the two last steps of the lysine biosynthesis had higher expression in the cells grown anaerobically on xylose + glucose compared with the glucose-limited chemostat culture (Appendix V). Lys9p and Lys1p utilise the co-factors NADPH and NAD^+, respectively. Consequently, in case the reactions carried out by these enzymes would take place in the direction of lysine catabolism, NADPH and NAD^+ would be produced to balance the redox co-factors during metabolism of xylose. In addition, *GCY1* encoding the NADPH -dependent aldoketoreductase was induced in the cells growing in the anaerobic chemostat on xylose + glucose, similar to the cells from the aerobic batch cultures on xylose (see section 3.8). Previously, the expression of *GCY1* has been connected to osmotic stress and to glycerol catabolism (Nevitt *et al.*, 2004; Norbeck and Blomberg, 1997). Gcy1p shows some homology to glycerol dehydrogenases (Norbeck and Blomberg, 1997), but when overexpressed in the present study in a yeast multicopy vector in H2217, no activity towards glycerol was detected. By contrast, NADPH-dependent activity of 12.6 nkat/mg of total protein towards methylglyoxal was measured from the crude cell extracts, whereas the activity in a control strain with the empty vector was 8 nkat/mg protein. Methylglyoxal, although toxic, is formed during normal yeast glucose metabolism and it functions as a signal initiator in the HOG-MAPK cascade (Maeta *et al.*, 2005). Methylglyoxal is degraded to lactic acid by glyoxalase or by methylglyoxal reductase and lactaldehyde dehydrogenase. Gre2p, which had higher expression in cells grown in the aerobic chemostat culture on xylose + glucose compared with the glucose culture (III), possesses NADPH-dependent methylglyoxal reductase activity but the deletion of *GRE2* was not shown to cause methylglyoxal hypersensitivity as did the deletion of *GLO1* encoding glyoxalase (Maeta *et al.*, 2005 and references therein). As *GLO1* did not respond within the glucose and xylose samples analysed it is likely that although possessing some activity towards methylglyoxal, Gcy1p does not play a major role in methylglyoxal metabolism, but has some other function in the cell. Overexpression of *GCY1* in a strain with xylose pathway did not result in improved xylose metabolism as studied in shake flask cultures on xylose with and without antimycin A.

Growth on xylose + glucose in the anaerobic chemostat culture affected the expression of only a few genes in the central carbon metabolism. The transcripts for the glyoxylate cycle encoded by *CIT2* and *MDH2* and *PCK1* encoding phosphoenolpyruvate carboxykinase, the key enzyme in gluconeogenesis, had lower expression in the cells grown on xylose + glucose compared with the cells from the glucose culture (Fig. 12). As discussed in section 3.2, under aerobic conditions *ICL1* encoding isocitrate lyase of the glyoxylate cycle had correspondingly lower expression in the cells grown on xylose + glucose (Fig. 9). Thus, it appears that residual xylose in the growth medium was able to repress the expression of genes of the glyoxylate cycle and gluconeogenesis in the chemostat cultures also under anaerobic conditions. However, whereas under aerobic conditions the genes for ethanol utilisation and acetyl-CoA synthesis pathways had higher expression in the cells grown on xylose + glucose compared with cells from the glucose-limited culture (Fig. 8), under anaerobic conditions *ALD6*, *ALD4*, *ACS1* and *ACS2* had lower expression in the cells grown on xylose + glucose compared with the glucose derepressed cells in the glucose-limited chemostat culture (Fig. 12). Consistently, flux to acetate was also lower under anaerobic conditions compared with the aerobic chemostat culture (I). Thus, it seems that xylose repressed these genes differently depending on the presence or absence of oxygen.

There were no changes in the expression of genes of the TCA cycle between cells grown on xylose + glucose and glucose under anaerobic conditions. However, several other glucose-repressible genes had decreased expression levels in cells grown on xylose + glucose, suggesting again a repressive effect of residual xylose. These included several genes encoding enzymes for oxidation of fatty acids and leading to formation of acetyl-CoA and NADH (Appendix V), and genes for acetyl-CoA transport across the mitochondrial membrane, encoded by *CAT2*, *YAT1* and *YAT2*. In addition, the transcript levels of genes *GPD1* and *GUT2* for glycerol 3-phosphate shuttle and *NDE1* and *NDE2* for NADH dehydrogenases were reduced in the cells growing on xylose + glucose (Fig. 12) (Bakker *et al.*, 2001). The lower expression of *GPD1* was also seen as a reduced amount of Gpd1p, but Gut2p abundance was similar in the cells from anaerobic xylose + glucose and glucose chemostat cultures (II). Instead of decreasing the expression of these genes, anaerobic xylose metabolism would rather benefit from activation of NAD^+ -regenerating systems (Kötter and Ciriacy, 1993), and the decreased expression of the genes mentioned above is also in contrast to the results of Jin and

co-workers, who observed the increased transcript levels of these genes in shake flask cultures on xylose. The conditions were, however, oxygen-limited that probably explains the difference in responses observed (Jin *et al.*, 2004). Overall, it is possible that the transcriptional responses of cells grown on xylose under anaerobic conditions in present study were at least partly owing to extreme difficulty to metabolise xylose under these conditions.

The genes encoding transport proteins of oxaloacetate and citrate, *OAC1* and *CTP1* had, on the other hand, increased expression in cells grown on xylose + glucose (Fig. 12). The physiological role of Oac1p is to import oxaloacetate into the mitochondria for anaplerotic reactions of the TCA cycle (Palmieri *et al.*, 1999), whereas Ctp1 transports citrate from mitochondria to the cytosol (Kaplan *et al.*, 1995). This allows citrate produced in mitochondria to be utilised for fatty acid and sterol biosynthesis, and for gluconeogenesis once it is broken down to acetyl-CoA and oxaloacetate (Young *et al.*, 2003). The increased expression of Ctp1p in cells grown on xylose + glucose may thus be indicative of shortage of cytosolic acetyl-CoA in cells grown on xylose + glucose. This is supported by the decreased expression of *ALD6* and *ACS1/2* (Fig. 12). In addition, flux leading to its synthesis in cytosol was lower in cells grown on xylose + glucose compared with glucose-grown cells (I, Fig. 2).

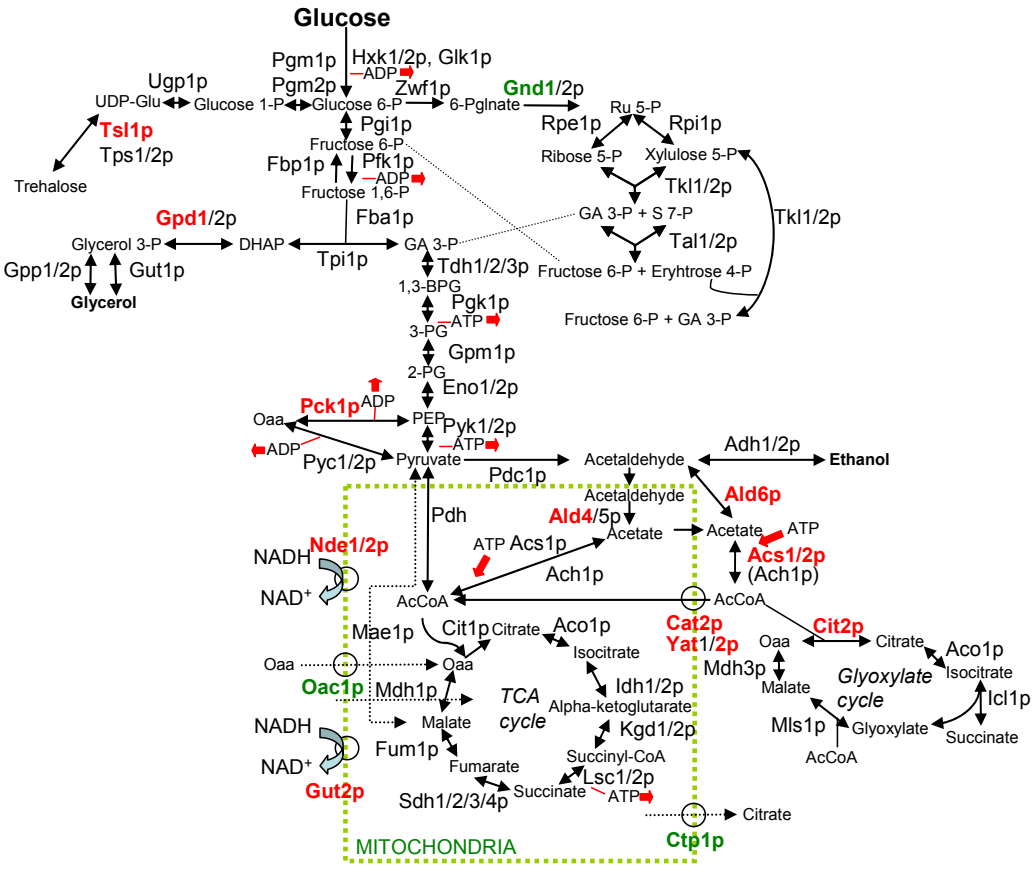

Figure 12. Central carbon metabolism of H2490 grown in anaerobic chemostat cultures on 180 mM xylose + 17 mM glucose or on 56 mM glucose. The proteins encoded by genes with higher and lower expression in cells grown on xylose + glucose compared with the glucose-limited culture are coloured green and red, respectively.

4. Conclusions

The transcription and proteome analyses of xylose-metabolising recombinant *S. cerevisiae* allowed the identification of numerous changes in the gene expression, protein abundance and post-translational modification of proteins in cells metabolising xylose compared with cells growing on glucose. One of the major observations was that xylose appears not to be recognised as a fully fermentable carbon source but causes only partial repression of *e.g.* genes encoding enzymes of the TCA and glyoxylate cycles and gluconeogenesis. However, some of the genes encoding gluconeogenic enzymes and genes involved in the utilisation of alternative carbon sources had increased expression in the cells growing on xylose. This suggests either that xylose imposes a different repression effect on the expression of these genes depending on the glucose repression pathway and mechanisms that control their expression *i.e.* xylose fails to repress the genes, or that these genes were induced by xylose. The other evident observation was the increased expression of genes and abundance of proteins of both cytosolic and mitochondrial acetyl-CoA-producing pathways in cells growing on xylose. These responses may be due to utilisation of ethanol as a cosubstrate with xylose under aerobic conditions, but may also be indicative of shortage of acetyl-CoA during growth on xylose. On the other hand, a cytosolic acetaldehyde dehydrogenase reaction also provides NADPH needed in the xylose pathway.

Not only did metabolism on xylose affect the main carbon dissimilation pathways, but differences were also seen in the expression of genes and abundance of proteins of the amino acid synthetic and catabolic pathways. Enzymatic reactions on these pathways may also serve as a supply for redox cofactors. Increased expression of genes encoding enzymes of amino acid catabolism in cells growing on xylose may also be related to growth on a poor carbon source and thus induction of pathways for utilisation of carbon skeletons derived from amino acids for growth. On the other hand, genes for the methionine biosynthetic pathway had attenuated expression and respective proteins were less abundant in cells grown on xylose, especially under anaerobic conditions. This may be related to limited availability of NADPH and ATP during growth on xylose. Apart from some enzymatic reactions in amino acid metabolism, of the cytosolic NADPH-producing reactions only genes encoding

the cytosolic acetaldehyde dehydrogenase (Ald6p) and 6-phosphogluconate dehydrogenase of the PPP had increased expression in cells grown on xylose. Metabolic flux analysis suggested correspondingly increased flux from glucose 6-phosphate to ribulose 5-phosphate. In addition to *GND2*, also other genes encoding the second isoenzymes of the reactions of the PPP appeared to have increased expression on xylose whereas *GND1*, *RPE1*, *TKL1* and *TAL1* had their highest expression in glucose repressed cells.

The proteome analysis revealed differences in abundances of post-translationally modified forms of some enzymes and in particular, differences in phosphorylation of the glycolytic enzymes Hxk2p, Glk1p, Eno1p and Eno2p in cells grown on xylose and glucose. Phosphorylation of proteins is usually linked to regulation of activity of regulatory proteins in cellular signalling cascades, the glucose repression pathway being one example. The present results suggest that phosphorylation may play an unforeseen role in regulation of the activities of metabolic enzymes during growth on different carbon sources and/or under different stress conditions. This is supported also by other studies, however, the regulation mechanisms of glycolytic enzymes at the post-translational level are still only moderately known. Post-translational modifications also bring another level of complexity to proteome data analysis. The interpretation and integration of data from different system-wide analyses remains a challenging task that will require still further progress in the field of bioinformatics.

The results suggest interesting differences in nutrient sensing and signalling, regulation of carbon dissimilatory pathways and balance of glycolysis and gluconeogenesis between cells grown on glucose and xylose. Whereas the redox cofactor imbalance in xylose fermentation by *S. cerevisiae* has been overcome by the heterologous expression of xylose isomerase, an interesting question remains: would xylose be more efficiently fermented if the cells were engineered to react as if they were fermenting a fully repressive carbon source and would therefore find themselves in a state similar to full glucose repression? The more efficient utilisation of xylose will most probably require complex and global changes in cellular metabolism. This provides a significant challenge to the further engineering of *S. cerevisiae* strains capable of utilisation of xylose-rich lignocellulosic substrates.

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Appendix B: Publications I–IV of this publication is not included in the PDF version. Please order the printed version to get the complete publication (http://www.vtt.fi/publications/index.jsp)

Appendix A: Transcription analysis data of the anaerobic chemostat cultures with H2490

Table A1. The genes of strain H2490 with increased expression in the anaerobic chemostat culture on 180 mM xylose + 17 mM glucose compared with the anaerobic glucose-limited chemostat culture with 56 mM glucose.

ORF Id	Gene	Fold change	Description
			Amino acid metabolism
YHR208w	*BAT1*	2,4	branched chain amino acid aminotransferase, mitochondrial
YMR062c	*ECM40*	6,4	acetylornithine acetyltransferase
YCL030c	*HIS4*	3,8	phosphoribosyl-AMP cyclohydrolase/phosphoribosyl-ATP pyrophosphatase/histidinol dehydrogenase
YGL009c	*LEU1*	2,6	3-isopropylmalate dehydratase
YIR034c	*LYS1*	5,2	saccharopine dehydrogenase
YNR050c	*LYS9*	2,5	saccharopine dehydrogenase ($NADP^+$, L-glutamate forming)
YML096w		7,6	similarity to asparagine synthases
			Carbon utilization
YOR120w	*GCY1*	5,4	galactose-induced protein of the aldo/keto reductase family
YHR183w	*GND1*	2,3	6-phosphogluconate dehydrogenase
YGR292w	*MAL12*	15,3	alpha-glucosidase of the *MAL1* locus
YBR299w	*MAL32*	14,2	alpha-glucosidase
YGR287c		10,3	strong similarity to maltase
YOL157c		14,5	strong similarity to alpha-glucosidases
YIL172c		11,1	identical to FSP2P and similarity to other alpha-glucosidases
			Cell cycle, differentiation and growth
YMR094w	*CTF13*	4,1	kinetochore protein complex, CBF3, 58 KD subunit
YNR030w	*ECM39*	3,7	involved in cell wall biogenesis and architecture
YCL024w	*KCC4*	12,2	kinase coordinate cell cycle progression with the organization of the peripheral cytoskeleton
YPL187w	*MFALPHA1*	2,6	mating pheromone alpha-1 precursor
YNL180c	*RHO5*	2,6	similarity to *S.pombe* CDC42P and other GTP-binding proteins
YEL040w	*UTR2*	2,4	cell wall protein
			DNA synthesis, recombination and repair
YBR088c	*POL30*	3,1	Proliferating Cell Nuclear Antigen (PCNA)
YKL045w	*PRI2*	7,8	DNA-directed DNA polymerase alpha , 58 KD subunit (DNA primase)
YDR030c	*RAD28*	15,2	protein involved in the same pathway as RAD26P, has beta-transducin (WD-40) repeats
			Mitochondrion
YPL271w	*ATP15*	11,7	F1F0-ATPase complex, F1 epsilon subunit
YHR208w	*BAT1*	2,4	branched chain amino acid aminotransferase, mitochondrial
YPL172c	*COX10*	4,7	farnesyl transferase
YML129c	*COX14*	3,0	cytochrome-c oxidase assembly protein
YBR291c	*CTP1*	7,9	citrate transport protein, mitochondrial (MCF)
YMR062c	*ECM40*	6,4	acetylornithine acetyltransferase, catalyzes the fifth step in arginine biosynthesis
YBR282w	*MRPL27*	7,1	ribosomal protein YmL27 precursor, mitochondrial
YNL137c	*NAM9*	3,4	ribosomal protein, mitochondrial
YKL120w	*OAC1*	3,2	mitochondrial inner membrane transporter, transports oxaloacetate, sulfate, and thiosulfate (MCF)
YNL131w	*TOM22*	2,4	mitochondrial outer membrane import receptor complex subunit
			Protein synthesis
YNL048w	*ALG11*	2,7	required for asparagine-linked glycosylation
YOR302w	*CPA1*	2,5	leader peptide
YNL255c	*GIS2*	5,6	strong similarity to nucleic acid-binding proteins, similarity to *Tetrahymena thermophila* cnjB protein

YBR282w	*MRPL27*	7,1	ribosomal protein YmL27 precursor, mitochondrial
YNL137c	*NAM9*	3,4	ribosomal protein, mitochondrial
YHL001w	*RPL14B*	2,2	ribosomal protein
YNL162w	*RPL42A*	2,6	ribosomal protein L36a.e
YOL121c	*RPS19A*	2,3	40S small subunit ribosomal protein S19.e
YJL190c	*RPS22A*	2,9	ribosomal protein S15a.e.c10
YNR037c	*RSM19*	5,6	strong similarity to *Mycoplasma* ribosomal protein S19
YNL007c	*SIS1*	8,3	heat shock protein
YMR260c	*TIF11*	3,9	translation initiation factor eIF1a
			Stress, protein folding and destination
YNL048w	*ALG11*	2,7	required for asparagine-linked glycosylation
YPL172c	*COX10*	4,7	farnesyl transferase
YJR032w	*CPR7*	4,0	member of the cyclophilin family
YJR032w	*CPR7*	4,0	member of the cyclophilin family
YNR028w	*CPR8*	6,7	cyclophilin (peptidylprolyl cis-trans isomerase or PPIase)
YJR036c	*HUL4*	2,6	hect domain E3 ubiquitin-protein ligase
YNL007c	*SIS1*	8,3	heat shock protein
YMR095c	*SNO1*	2,5	similarity to *M.leprae* hisH protein
YOR247w	*SRL1*	2,0	similarity to vanadate sensitive suppressor SVS1P
YDR293c	*SSD1*	0,4	involved in the tolerance to high concentration of $Ca2^{+}$
YBR067c	*TIP1*	2,1	esterase
YNL131w	*TOM22*	2,4	mitochondrial outer membrane import receptor complex subunit
YNL064c	*YDJ1*	2,7	protein chaperone involved in regulation of the HSP90 and HSP70 functions; involved in protein translocation across membranes
			Transcription
YNL255c	*GIS2*	5,6	strong similarity to nucleic acid-binding proteins, similarity to *Tetrahymena thermophila* cnjB protein
YBR009c	*HHF1*	4,7	histone H4
YNL031c	*HHT2*	2,1	histone H3
YOR344c	*TYE7*	5,2	basic helix-loop-helix transcription factor
YDR259c	*YAP6*	3,1	transcription factor of a fungal-specific family of bzip proteins
			Transport
YPL271w	*ATP15*	11,7	F1F0-ATPase complex, F1 epsilon subunit
YBR291c	*CTP1*	7,9	citrate transport protein, mitochondrial (MCF)
YGR289c	*MAL11*	6,9	general alpha-glucoside permease
YBR298c	*MAL31*	7,9	maltose permease
YKL120w	*OAC1*	3,2	oxaloacetate transport protein, mitochondrial
YGL008c	*PMA1*	2,6	H^{+}-transporting P-type ATPase, major isoform, plasma membrane
YNL131w	*TOM22*	2,4	mitochondrial outer membrane import receptor complex subunit
YNL064c	*YDJ1*	2,7	mitochondrial and ER import protein
YFR045w		13,5	similarity to mitochondrial citrate transport proteins
YIL088c		3,3	weak similarity to *A.thaliana* aminoacid permease AAP4
			Unclassified
YOR248w	*TOS11*	2,0	hypothetical protein
YDR133c		2,2	questionable ORF
YOL109w	*ZEO1*	2,2	weak similarity to *G.hirsutum* embryonic abundant protein D-7
YNL190w		2,3	weak similarity to MUCIN 1 PRECURSOR *Mesocricetus auratus* (Golden hamster)
YOL155c		2,6	similarity to glucan 1,4-alpha-glucosidase Sta1p and YAR066w
YIL169c		2,7	similarity to glucan 1,4-alpha-glucosidase and YAR066w
YAR068w		2,9	strong similarity to hypothetical protein YHR214w-a
YNL166c	*BNI5*	2,9	similarity to *S. pombe* SPBC1711.05 serine-rich repeat protein of unknown function
YER186c		3,3	weak similarity to hypothetical protein YMR316w
YPL144w		3,3	hypothetical protein

YOL003c		3,4	similarity to *C.elegans* hypothetical protein, YDR126w, YNL326c and YLR246w
YOL070c		3,9	hypothetical protein
YGL098w		4,2	hypothetical protein
YKL037w		4,4	weak similarity to *C.elegans* ubc-2 protein
YDR533c		4,8	strong similarity to hypothetical proteins YPL280w, YOR391c and YMR322c
YHR214w-a		5,0	strong similarity to hypothetical protein YAR068w
YNL047c		5,8	similarity to probable transcription factor ASK10P and hypothetical protein YPR115w, and strong similarity to hypothetical protein YIL105c
YBL049w		6,2	strong similarity to hypothetical protein - human
YDR528w	*HLR1*	7,5	similarity to LRE1P
YDR401w		7,7	questionable ORF
YBR089w		8,6	questionable ORF
YFL064c		9,9	strong similarity to subtelomeric encoded proteins
YNR022c		13,7	weak similarity to protein phosphatases
YHR134w	*WSS1*	28,9	similarity to *S. pombe* SPCC1442.07c putative Zn-protease
YBL059w		44,8	weak similarity to hypothetical protein YER093c-a
YKR077w		67,5	hypothetical protein
YDR221w		196,3	weak similarity to the beta subunit of an ER luminal alpha-glucosidase from mouse

Table A2. The genes of strain H2490 with decreased expression in anaerobic chemostat culture on 180 mM xylose + 17 mM glucose compared with the anaerobic glucose-limited chemostat with 56 mM glucose.

ORF Id	Gene	Fold change	Description
			Amino acid metabolism
YBL098w	*BNA4*	4,1	kynurenine 3-mono oxygenase, required for biosynthesis of nicotinic acid from tryptophan via the kynurenine pathway
YGR155w	*CYS4*	3,9	cystathionine beta-synthase
YKR069w	*MET1*	5,4	siroheme synthase
YFR030w	*MET10*	7,1	sulphite reductase flavin-binding subunit
YKL001c	*MET14*	4,5	ATP adenosine-5'-phosphosulphate 3'-phosphotransferase
YJR010w	*MET3*	9,9	sulphate adenylyltransferase
YIL046w	*MET30*	3,7	involved in regulation of sulfur assimilation genes and cell cycle progression
YDR253c	*MET32*	2,9	transcriptional regulator of sulfur amino acid metabolism
YBR213w	*MET8*	18,4	siroheme synthase
YKR080w	*MTD1*	2,4	methylene tetrahydrofolate dehydrogenase (NAD^+)
YLR142w	*PUT1*	29,1	proline oxidase
YLR058c	*SHM2*	2,0	serine hydroxymethyltransferase, cytoplasmic
YBR006w	*UGA2*	2,3	succinate semialdehyde dehydrogenase
			Carbon utilization
YAL054c	*ACS1*	27,1	acetyl-coenzyme A synthetase 1
YLR153c	*ACS2*	2,2	acetyl-coenzyme A synthetase 2
YOR374w	*ALD4*	4,9	aldehyde dehydrogenase, mitochondrial
YPL061w	*ALD6*	5,9	aldehyde dehydrogenase, cytosolic
YGR177c	*ATF2*	9,0	alcohol O-acetyltransferase
YBR023c	*CHS3*	4,2	chitin synthase III
YCR005c	*CIT2*	4,4	citrate (si)-synthase, peroxisomal
YML054c	*CYB2*	16,5	lactate dehydrogenase cytochrome b2
YDL174c	*DLD1*	2,1	D-lactate ferricytochrome C oxidoreductase

YBR020w	*GAL1*	4,8	galactokinase
YER020w	*GPA2*	16,8	guanine nucleotide-binding regulatory protein
YDL022w	*GPD1*	6,2	glycerol-3-phosphate dehydrogenase (NAD^+), cytoplasmic
YGR032w	*GSC2*	2,3	1,3-beta-D-glucan synthase subunit
YIL155c	*GUT2*	3,0	glycerol 3-phosphate dehydrogenase, mitochondrial
YJL153c	*INO1*	7,8	myo-inositol 1-phosphate synthase
YOL126c	*MDH2*	7,5	malate dehydrogenase, cytoplasmic
YKR097w	*PCK1*	18,5	phosphoenolpyruvate carboxykinase
YBR050c	*REG2*	5,4	regulatory subunit of the Glc7p type-1 protein phosphatase
YGL179c	*TOS3*	26,0	protein kinase, related to and functionally redundant with Elm1p and Sak1p for the phosphorylation and activation of Snf1p
YDR074w	*TPS2*	2,2	alpha,alpha-trehalose-phosphate synthase, 102 KD subunit
YML100w	*TSL1*	3,5	alpha,alpha-trehalose-phosphate synthase, 123 KD subunit
			Cell cycle, differentation and growth
YBL085w	*BOI1*	4,1	BEM1 protein-binding protein
YOL016c	*CMK2*	12,2	Ca2+/calmodulin-dependent ser/thr protein kinase, type II
YER020w	*GPA2*	16,8	guanine nucleotide-binding regulatory protein
YDR072c	*IPT1*	2,2	mannosyl diphosphorylinositol ceramide synthase
YIL046w	*MET30*	3,6	involved in regulation of sulphur assimilation genes and cell cycle progression
YGR023w	*MTL1*	2,0	potential cell wall stress sensor
YKR048c	*NAP1*	3,4	nucleosome assembly protein I
YDR388w	*RVS167*	3,0	involved in regulation of actin cytoskeleton and viability following starvation or osmotic stress
YHR172w	*SPC97*	14,5	spindle pole body component
YDR293c	*SSD1*	2,5	involved in tolerance to high concentration of $Ca2^+$
YPL057c	*SUR1*	2,9	required for mannosylation of sphingolipids
YDR457w	*TOM1*	2,2	E3 ubiqitin ligase required for G2/M transition
			DNA recombination and repair
YJR035w	*RAD26*	2,6	DNA repair and recombination protein
YDL059c	*RAD59*	3,2	DNA repair and recombination protein
			Homeostasis of ions
YDR270w	*CCC2*	4,1	probable copper-transporting ATPase
YDR040c	*ENA1*	27,2	P-type ATPase involved in Na^+ and Li^+ efflux
YDR038c	*ENA5*	2,8	P-type ATPase involved in Na^+ efflux
YMR058w	*FET3*	1,9	cell surface ferroxidase, high affinity
YOR383c	*FIT3*	4,8	mannoprotein involved in the retention of siderophore-iron in the cell wall
YLR214w	*FRE1*	4,8	ferric (and cupric) reductase
YLR205c	*HMX1*	18,6	ER localized, heme-binding peroxidase involved in the degradation of heme
YJL094c	*KHA1*	2,9	K^+/H^+ exchanger
YBR296c	*PHO89*	79,9	Na^+-coupled phosphate transport protein, high affinity
YJL198w	*PHO90*	4,7	low affinity phosphate transporter
YBR294w	*SUL1*	3,6	high-affinity sulphate transport protein
YLR092w	*SUL2*	3,1	sulphate transporter
YGR065c	*VHT1*	2,3	plasma membrane H^+-biotin symporter
			Lipid and isoprenoid biosynthesis
YPL069c	*BTS1*	20,5	geranylgeranyl diphosphate synthase
YML042w	*CAT2*	6,1	carnitine O-acetyltransferase
YBR177c	*EHT1*	2,2	alcohol acyl transferase
YML075c	*HMG1*	2,5	3-hydroxy-3-methylglutaryl-coenzyme A reductase 1
YPL057c	*SUR1*	2,9	required for mannosylation of sphingolipids
			Mitochondrion
YAL054c	*ACS1*	27,1	acetyl-CoA synthetase
YML042w	*CAT2*	6,1	carnitine O-acetyltransferase
YGR174c	*CBP4*	6,11	ubiquinol--cytochrome-c reductase assembly factor

YML054c	*CYB2*	16,6	lactate dehydrogenase cytochrome b2
YDL174c	*DLD1*	2,2	D-lactate ferricytochrome C oxidoreductase (D-LCR)
YIL155c	*GUT2*	3,0	glycerol 3-phosphate dehydrogenase, mitochondrial
YMR145c	*NDH1*	3,9	mitochondrial cytosolically directed NADH dehydrogenase
YDL085w	*NDH2*	15,9	mitochondrial cytosolically directed NADH dehydrogenase
YLR142w	*PUT1*	5,4	proline oxidase
YHR008c	*SOD2*	2,9	superoxide dismutase (Mn) precursor, mitochondrial
			Oxidation of fatty acids
YOR317w	*FAA1*	2,1	long-chain-fatty-acid--CoA ligase
YER015w	*FAA2*	5,1	long-chain-fatty-acid--CoA ligase
YKR009c	*FOX2*	4,1	hydratase-dehydrogenase-epimerase, peroxisomal
YIL160c	*POT1*	4,5	acetyl-CoA C-acyltransferase, peroxisomal
YJR019c	*TES1*	5,4	peroxisomal acyl-CoA thioesterase
			Stress and protein fate, folding, destination
YGR174c	*CBP4*	6,0	ubiquinol--cytochrome-c reductase assembly factor
YDR256c	*CTA1*	9,0	catalase A, peroxisomal
YAL042w	*ERV46*	3,2	component of copii vesicles involved in transport between the ER and golgi complex
YDL022w	*GPD1*	6,2	glycerol 3-phosphate dehydrogenase (NAD^+), cytoplasmic
YDL079c	*MRK1*	3,0	glycogen synthase kinase 3 (GSK-3) homologue; activates Msn2p-dependent transcription of stress responsive genes
YDR404c	*RPB7*	5,4	DNA-directed RNA polymerase II, 19 KD subunit
YHR008c	*SOD2*	2,9	superoxide dismutase (Mn) precursor, mitochondrial
YDR293c	*SSD1*	2,6	involved in tolerance to high concentration of $Ca2^+$
YOR010c	*TIR2*	5,4	cold shock induced protein
YOR009w	*TIR4*	8,9	cell wall mannoprotein
YLR327c	*TMA10*	2,5	strong similarity to STF2P
YGR241c	*YAP1802*	3,5	protein involved in clathrin cage assembly
			Transcription
YBR020w	*GAL1*	4,8	galactokinase
YGL133w	*ITC1*	2,7	subunit of ISW2 chromatin remodeling complex
YIL046w	*MET30*	3,7	involved in regulation of sulphur assimilation genes and cell cycle progression
YDR253c	*MET32*	2,9	transcriptional regulator of sulphur amino acid metabolism
YKR048c	*NAP1*	3,4	nucleosome assembly protein I
YDR432w	*NPL3*	5,7	nucleolar protein
YDR404c	*RPB7*	5,4	DNA-directed RNA polymerase II, 19 KD subunit
YIL101c	*XBP1*	3,3	stress-induced transcriptional repressor
			Transport
YBR132c	*AGP2*	2,4	amino-acid permease
YNR002c	*ATO2*	7,8	ammonia transporter
YNR056c	*BIO5*	2,8	similarity to choline transport protein HNM1P
YML042w	*CAT2*	6,2	carnitine O-acetyltransferase
YDR270w	*CCC2*	4,2	Cu(+2)-transporting P-type ATPase
YPL265w	*DIP5*	2,9	dicarboxylic amino acid permease
YDR040c	*ENA1*	27,3	P-type ATPase involved in Na^+ and Li^+ efflux
YDR038c	*ENA5*	2,9	P-type ATPase involved in Na^+ efflux
YOL158c	*ENB1*	2,6	endosomal ferric enterobactin transporter, expressed under conditions of iron deprivation
YMR058w	*FET3*	1,9	cell surface ferroxidase, high affinity
YKR039w	*GAP1*	4,9	general amino acid permease
YDL234c	*GYP7*	3,1	GTPase-activating protein for YPT7P
YMR011w	*HXT2*	7,8	high-affinity hexose transporter
YDR497c	*ITR1*	2,5	myo-inositol permease, major
YKL217w	*JEN1*	40,3	carboxylic acid transporter protein
YJL094c	*KHA1*	2,9	K^+/H^+ exchanger

YLL061w	*MMP1*	5,4	high affinity S-methylmethionine permease
YGR055w	*MUP1*	2,6	high affinity methionine permease
YHL036w	*MUP3*	2,6	very low affinity methionine permease
YDR432w	*NPL3*	5,7	nucleolar protein
YBR296c	*PHO89*	79,9	Na^+-coupled phosphate transport protein, high affinity
YJL198w	*PHO90*	4,7	low affinity phosphate transporter
YKL188c	*PXA2*	3,9	ABC transporter, peroxisomal
YIL120w	*QDR1*	13,4	similarity to antibiotic resistance proteins
YPL274w	*SAM3*	11,7	high affinity S-adenosylmethionine permease
YBR294w	*SUL1*	3,6	high-affinity sulphate transport protein
YLR092w	*SUL2*	3,1	sulphate transporter
YGR065c	*VHT1*	2,4	plasma membrane H^+-biotin symporter
YER024w	*YAT2*	4,9	carnitine O-acetyltransferase
YLL055w		4,0	similarity to DAL5P
			Unclassified
YGL245w		34,7	strong similarity to glutamine--tRNA ligase
YDL110c		29,1	hypothetical protein
YKL187c		14,9	strong similarity to hypothetical protein YLR413w
YGR161c		13,0	hypothetical protein
YPL004c		12,9	strong similarity to YGR086c
YGR243w		12,4	strong similarity to hypothetical protein YHR162w
YMR195w	*ICY1*	11,8	similarity to ICY2
YDR413c		11,4	weak similarity to NADH dehydrogenase
YDR509w		7,2	questionable ORF
YCL061c	*MRC1*	6,6	similarity to URK1
YDL039c	*PRM7*	5,6	hypothetical protein
YFR008w		5,3	weak similarity to human centromere protein E
YLR414c		5,1	weak similarity to YLR413w
YBR032w		5,0	hypothetical protein
YDL038c		5,0	similarity to mucin proteins
YLR194c		4,6	hypothetical protein
YLR099c	*ICT1*	4,1	similarity to YDR125c
YER064c		3,9	similarity to hypothetical protein YIL056w
YBR016w		3,7	strong similarity to hypothetical proteins YDL012c and YDR210w
YHL021c		3,6	weak similarity to *Pseudomonas* gamma-butyrobetaine hydroxylase
YLR101c		3,4	questionable ORF
YLR462w		3,4	strong similarity to subtelomeric encoded proteins
YAL053w		3,2	strong similarity to hypothetical proteins YOR365c,YGL139w,YPL221w
YGR266w		3,2	hypothetical protein
YBR287w		3,0	similarity to hypothetical *S. pombe* protein
YPL054w	*LEE1*	3,0	zinc-finger protein of unknown function
YNL191w		2,9	similarity to *Synechocystis* hypothetical protein
YMR082c		2,8	hypothetical protein
YGL080w		2,8	strong similarity to *C.elegans* R07E5.13 protein
YGR149w		2,8	similarity to PIR:T40675 hypothetical protein SPBC776.05 *S. pombe*
YHR097c		2,7	strong similarity to hypothetical protein YDR348c
YKR075c		2,6	weak similarity to negative regulator REG1P
YOR382w	*FIT2*	2,5	hypothetical protein
YKL056c		2,3	strong similarity to human IgE-dependent histamine-releasing factor
YGR160w	*FYV13*	2,1	weak similarity to *C. elegans* Y113G7B.12 protein of unknown function
YFR039c		1,9	similarity to hypothetical protein YGL228w

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Transcriptome and proteome analysis of xylose-metabolising *Saccharomyces cerevisiae*

Abstract

Increasing concern about global climate warming has accelerated research into renewable energy sources that could replace fossil petroleum-based fuels and materials. Bioethanol production from cellulosic biomass by fermentation with baker's yeast *Saccharomyces cerevisiae* is one of the most studied areas in this field. The focus has been on metabolic engineering of *S. cerevisiae* for utilisation of the pentose sugars, in particular D-xylose that is abundant in the hemicellulose fraction of biomass. Introduction of a heterologous xylose-utilisation pathway into *S. cerevisiae* enables xylose fermentation, but ethanol yield and productivity do not reach the theoretical level.

In the present study, transcription, proteome and metabolic flux analyses of recombinant xylose-utilising *S. cerevisiae* expressing the genes encoding xylose reductase (XR) and xylitol dehydrogenase (XDH) from *Pichia stipitis* and the endogenous xylulokinase were carried out to characterise the global cellular responses to metabolism of xylose. The aim of these studies was to find novel ways to engineer cells for improved xylose fermentation. The analyses were carried out from cells grown on xylose and glucose both in batch and chemostat cultures. A particularly interesting observation was that several proteins had post-translationally modified forms with different abundance in cells grown on xylose and glucose. Hexokinase 2, glucokinase and both enolase isoenzymes 1 and 2 were phosphorylated differently on the two different carbon sources studied. This suggests that phosphorylation of glycolytic enzymes may be a yet poorly understood means to modulate their activity or function.

The results also showed that metabolism of xylose affected the gene expression and abundance of proteins in pathways leading to acetyl-CoA synthesis and altered the metabolic fluxes in these pathways. Additionally, the analyses showed increased expression and abundance of several other genes and proteins involved in cellular redox reactions (*e.g.* aldo-ketoreductase Gcy1p and 6-phosphogluconate dehydrogenase) in cells grown on xylose. Metabolic flux analysis indicated increased NADPH-generating flux through the oxidative part of the pentose phosphate pathway in cells grown on xylose.

The most importantly, results indicated that xylose was not able to repress to the same extent as glucose the genes of the tricarboxylic acid and glyoxylate cycles, gluconeogenesis and some other genes involved in the metabolism of respiratory carbon sources. This suggests that xylose is not recognised as a fully fermentative carbon source by the recombinant *S. cerevisiae* that may be one of the major reasons for the suboptimal fermentation of xylose. The regulatory network for carbon source recognition and catabolite repression is complex and its functions are only partly known. Consequently, multiple genetic modifications and also random approaches would probably be required if these pathways were to be modified for further improvement of xylose fermentation by recombinant *S. cerevisiae* strains.

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