J Ind Microbiol Biotechnol (2013) 40:1383–1392
DOI 10.1007/s10295-013-1344-9

BIOENERGY/BIOFUELS/BIOCHEMICALS

SIMB
Society for Industrial Microbiology and Biotechnology

Overexpression of NADH-dependent fumarate reductase improves D-xylose fermentation in recombinant *Saccharomyces cerevisiae*

Laura Salusjärvi · Sanna Kaunisto · Sami Holmström · Maija-Leena Vehkomäki · Kari Koivuranta · Juha-Pekka Pitkänen · Laura Ruohonen

Received: 2 July 2013 / Accepted: 9 September 2013 / Published online: 10 October 2013

Abstract Deviation from optimal levels and ratios of redox cofactors NAD(H) and NADP(H) is common when microbes are metabolically engineered. The resulting redox imbalance often reduces the rate of substrate utilization as well as biomass and product formation. An example is the metabolism of D-xylose by recombinant *Saccharomyces cerevisiae* strains expressing xylose reductase and xylitol dehydrogenase encoding genes from *Scheffersomyces stipitis*. This pathway requires both NADPH and NAD^+. The effect of overexpressing the glycosomal NADH-dependent fumarate reductase (FRD) of *Trypanosoma brucei* in D-xylose-utilizing *S. cerevisiae* alone and together with an endogenous, cytosol directed NADH-kinase (POS5Δ17) was studied as one possible solution to overcome this imbalance. Expression of FRD and FRD + POS5Δ17 resulted in 60 and 23 % increase in ethanol yield, respectively, on D-xylose under anaerobic conditions. At the same time, xylitol yield decreased in the FRD strain suggesting an improvement in redox balance. We show that fumarate reductase of *T. brucei* can provide an important source of NAD^+ in yeast under anaerobic conditions, and can be useful for metabolic engineering strategies where the redox cofactors need to be balanced. The effects of FRD and NADH-kinase on aerobic and anaerobic D-xylose and D-glucose metabolism are discussed.

Keywords Fumarate reductase · NADH-kinase · *Saccharomyces cerevisiae* · NADH · NADPH · D-Xylose · Metabolic engineering · D-Xylose fermentation

Introduction

Lignocellulosic feedstocks from agriculture and forestry provide cheap and sustainable resources for production of transportation biofuels and biochemicals. A significant proportion of the hemicellulose fraction of lignocellulose is pentose sugars and therefore the ability to metabolize them along hexoses is essential for the production hosts to be used in plant material-based processes. The most abundant pentose sugar is D-xylose that is the primary constituent of xylans making up the bulk of hemicellulose in plant cell walls [55].

S. cerevisiae is one of the most prominent organisms for the industrial bioethanol production due to its very efficient hexose fermentation and good inhibitor tolerance [55]. Consequently, the extension of substrate range for fermentation of D-xylose is one of the most active fields in metabolic engineering of *S. cerevisiae* [20, 27]. D-Xylose-fermenting *S. cerevisiae* strains have been constructed by over-expression of the genes of *Scheffersomyces stipitis* (former *Pichia stipitis*) encoding NAD(P)H-dependent xylose reductase (XR) and NAD^+-dependent xylitol dehydrogenase (XDH) [21, 22]. D-Xylose utilization and ethanol production was further improved by over-expression of the endogenous gene encoding xylulokinase [9, 14, 47]. The xylose pathway with the XR and XDH from *S. stipitis*,

Electronic supplementary material The online version of this article (doi:10.1007/s10295-013-1344-9) contains supplementary material, which is available to authorized users.

L. Salusjärvi · S. Kaunisto · S. Holmström · M.-L. Vehkomäki · K. Koivuranta · J.-P. Pitkänen · L. Ruohonen (✉)
VTT, Technical Research Centre of Finland, PO Box 1000, 02044 VTT, Finland
e-mail: laura.ruohonen@vtt.fi

L. Salusjärvi
e-mail: laura.salusjarvi@vtt.fi

however, results in redox cofactor imbalance due to different cofactor requirements of XR and XDH enzymes [8]. In recombinant *S. cerevisiae*, this has been attributed to one of the major reasons for low ethanol production rates from D-xylose, substantial production of xylitol as a side product, and dependence of oxygen for growth on D-xylose [8, 9, 16]. D-Xylose fermentation by *S. cerevisiae* has also been enabled by expression of xylose isomerase (XI) encoding gene from bacteria or fungi that directly isomerizes D-xylose to D-xylulose with no involvement of redox cofactors [6, 13]. Expression of the XI pathway in *S. cerevisiae* has resulted in higher ethanol yields but lower growth rates and productivities on D-xylose compared with the strains with the XR-XDH pathway [6, 19, 24, 25, 34].

One of the first approaches to relieve the redox imbalance of recombinant D-xylose-fermenting *S. cerevisiae* was the expression of the bacterial transhydrogenase that catalyses the conversion of NADH and $NADP^+$ to NAD^+ and NADPH, however, when expressed in *S. cerevisiae* it operated in the reverse direction converting NADPH to NADH [1]. The other strategies for relieving the redox imbalance include among others expression of a $NADP^+$-dependent D-glyceraldehyde 3-phosphate dehydrogenase [7, 51], modification of the cofactor preference of the ammonium assimilation pathway from NADPH to NADH [40], alteration of the cofactor affinity of XR and XDH [18, 35, 41, 53, 54], or disruption of the oxidative pentose phosphate pathway [17]. Typically, these approaches resulted in a 15–50 % increase in ethanol yields on D-xylose with concomitant decrease in xylitol production but the fermentation rates were still lower than that of D-glucose.

Recently, the endogenous NADH-kinase Pos5p of *S. cerevisiae* was overexpressed in mitochondria and cytosol of *S. cerevisiae* with the oxidoreductive D-xylose pathway aiming at reduced NADH levels and increased NADPH availability for the XR reaction and subsequent enhancement in D-xylose fermentation [15]. The mitochondrial NADH-kinase Pos5p phosphorylates NADH to NADPH using ATP as a phosphate donor. Pos5p has kinase activity also towards NAD^+, but with an affinity 50-fold lower compared with NADH [5, 31, 44]. Pos5p is the major source of mitochondrial NADPH and important for example iron-sulphur cluster biogenesis and for full respiratory activity [32, 44, 45]. The first 17 amino acids of the Pos5p constitute the signal peptide, which targets the enzyme into mitochondria and removal of these amino acids led to cytosolic expression of the enzyme [46]. When *S. cerevisiae* expressing the oxidoreductive D-xylose pathway and POS5Δ17 was grown anaerobically on a mixture of D-glucose and D-xylose, increased xylitol and decreased ethanol yields from D-xylose were observed, contrary to the set hypothesis [15]. The result suggested increased NADPH availability for the XR reaction but insufficient NAD^+ supply for efficient conversion of xylitol to D-xylulose. Under anaerobic conditions, NADH produced in glycolysis and in the synthesis of biomass can be oxidized to NAD^+ only by the reactions catalyzed by alcohol dehydrogenase and glycerol-3-phosphate dehydrogenase producing ethanol and glycerol, respectively [39, 48]. Thus, as increasing the glycerol production would reduce the carbon flux to ethanol, the results suggested that yet another reaction producing NAD^+ elsewhere than the central carbon pathways would be required to increase the carbon flux from xylitol onwards (Fig. 1).

Fumarate reductases catalyze the reduction of fumarate to succinate in the citric acid cycle. Most of the FRDs belong to a multimeric complex associated with the respiratory chain and transfer electrons from quinol to fumarate [49]. However, also soluble monomeric FRDs, which transfer electrons from non-covalently bound $FADH_2$/$FMNH_2$ or NADH to fumarate, have been identified from some species. These include FRDs from several *Shewanella* species that use quinol as electron donor and are involved in fumarate respiration [11]. *S. cerevisiae* has two soluble FRDs (cytosolic and mitochondrial) being unique using $FADH_2$/$FMNH_2$ to reduce fumarate, and they are neither linked to the electron transfer chain nor involved in the oxidative phosphorylation [2]. Most interestingly, the African trypanosome *Trypanosoma brucei* expresses a soluble NADH-dependent FRD located in the peroxisome-like organelle glycosome. This 120-kDa enzyme plays an important role in maintaining the glycosomal NAD^+/NADH balance in *T. brucei* [4]. In addition to fumarate reductase activity, it has domains with homology to AbpE enzymes involved in thiamine biosynthesis and cytochrome *b*5 reductases/nitrate reductases involved in biosynthesis of unsaturated fatty acids and nitrate metabolism, suggesting that the enzyme may be multifunctional, possessing several different metabolic roles [4].

The aim of the present study was to increase both cytosolic NADPH and NAD^+ by combining in cells the cytosolic NADH–kinase and fumarate reductase reactions that specifically produce NADPH and NAD^+ cofactors. Fumarate has been shown to accumulate during D-xylose fermentation with the XR-XDH strain [3], suggesting that this metabolite is abundant and present for the reduction. NADH-dependent fumarate reductase of *T. brucei* was expressed in *S. cerevisiae* with the oxidoreductive D-xylose pathway either alone or together with the truncated cytosol-directed form of the endogenous NADH-kinase. The strains generated were studied in aerobic and anaerobic batch fermentations using D-glucose and D-xylose as carbon sources.

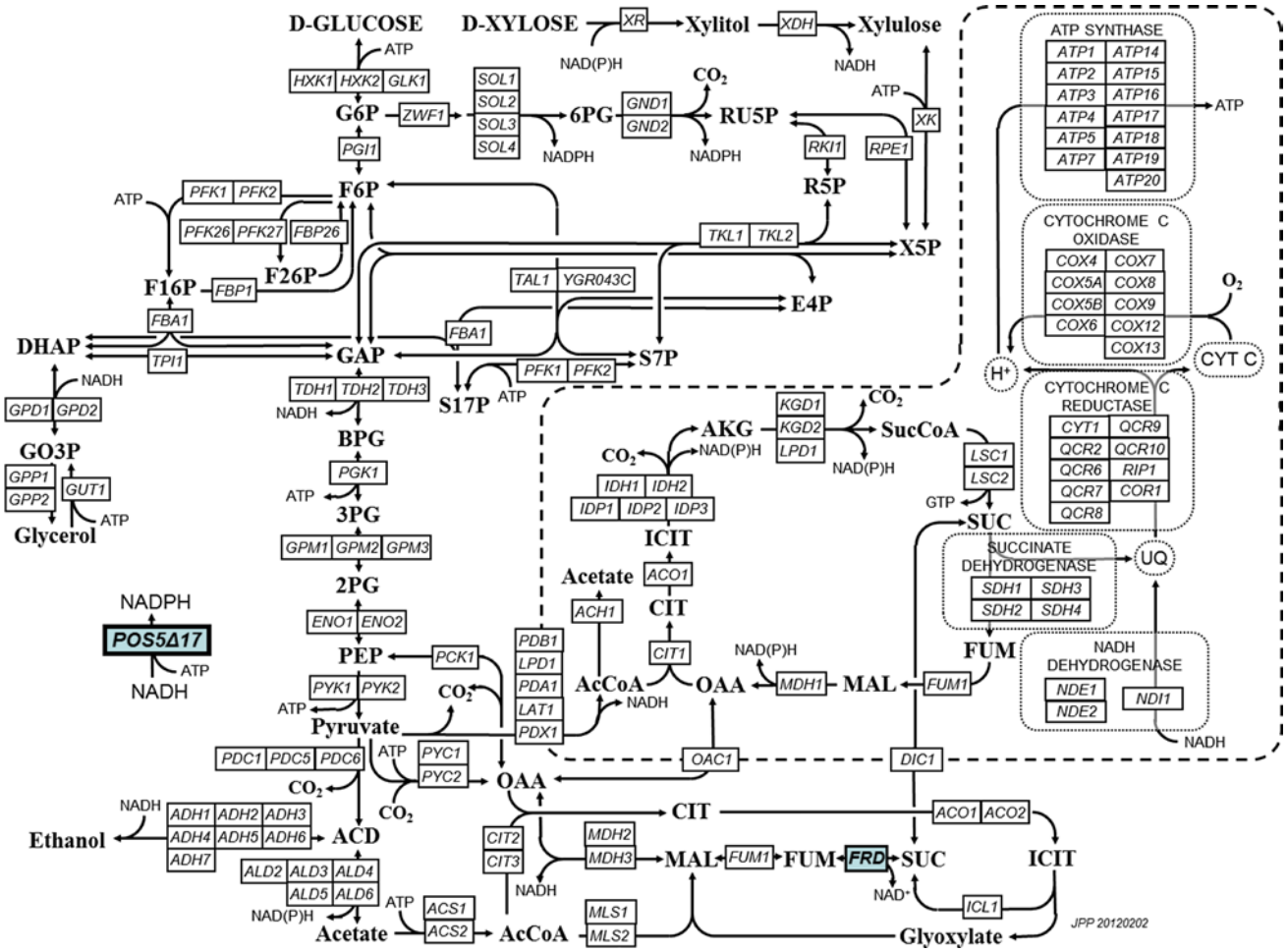

Fig. 1 Schematic picture of D-glucose and D-xylose metabolic pathways in recombinant *S. cerevisiae*, also showing the relevant cofactors and overexpressed fumarate reductase (FRD) and NADH–kinase reactions

Materials and methods

Plasmid construction

The plasmids and yeast strains used in this study are listed in Table 1. The *FRD* from *T. brucei* (GenBank: AF457132) was codon optimized for *S. cerevisiae* and synthesized by GENEART (Regensburg, Germany). The synthesized gene was ligated into *Bgl*II site between the *PGK1* promoter and terminator of vector B1181, which was constructed from YEplac195 [10] as described earlier [42]. The POS5Δ17 lacking the first 17 amino acids (mitochondrial targeting sequence) was amplified by PCR from the chromosomal DNA of *S. cerevisiae* using the following primers: 5′CGGGATCCCGAAGCTTAAAA**ATG**AGTACGTTGGATTCACATTCC3′ and 5′CGGGATCCCGAAGCTTTTAATCATTATCAGTCTGTCTCTTG3′, and cloned between the *TPI1* promoter and *ADH1* terminator of plasmid pMLV12 (BluescribeM13 with *TPI1*-promoter and *ADH1*-terminator). The p*TPI1*-POS5Δ17-t*ADH1* cassette was released by *Bam*HI digestion and cloned into *Bam*HI site of the vector pKK27-1.

PKK27-1 was constructed by releasing the KanMX4 cassette from the plasmid pFA6a-KanMX4 [52] by *BamH*I + *Eco*RI digestion. The KanMX4 cassette was further ligated into YEplac195 [10] digested with *Bam*HI + *Eco*RI resulting in pKK27-1.

Strain construction

Yeast strain used in the study was VTT C-10880, i.e., [CEN.PK113-1A (*URA3. HIS3. LEU2. TRP1. MAL2-8*^{c}*. SUC2*), *ura3*::*XYL1 XYL2, xks1*::*XKS1*]. *XYL1* and *XYL2* genes of *S. stipitis* were chromosomally integrated into *URA3* locus under *PGK1* and *ADH1* promoters, respectively. The integration cassette was constructed as

Table 1 Strains and plasmids used in the study

Strain/plasmid	Relevant genotype	References
Plasmid		
B1181	YEplac195 with *PGK1* promoter and terminator, *URA3* marker	[40, 41]
pKK27-1	YEplac195 with KanMX marker	[40] this study
pPGK1-FRD	FRD in B1181 under *PGK1* promoter	This study
pTPI1-POS5Δ17	POS5Δ17 in pKK27-1 under *TPI1* promoter	This study
Strain		
VTT C-10880	CEN.PK113-1A (*URA3. HIS3. LEU2. TRP1. MAL2-8^c. SUC2*) *ura3*::*XYL1 XYL2, xks1*::*XKS1*	This study
Control (Ctrl)	VTT C-10880/B1181 + pKK27-1	This study
FRD	VTT C-10880/pPGK1-FRD + pKK27-1	This study
FRD + POS5Δ17	VTT C-10880/pPGK1-FRD + pTPI1-POS5Δ17	This study

described earlier [47]. *XKS1* of *S. cerevisiae* was integrated into *XKS1* locus as described below.

The *XKS1* expression cassette p*ADH1* m- *XKS1*- t*ADH1* was released as a *Bam*HI-fragment from plasmid pMLV14 (BluescribeM13) and ligated into the bacterial plasmid pMLV24. The plasmid pMLV24 contains *loxP-S. cerevisiae MEL5* (encoding α-galactosidase)-*loxP* marker cassette with 60-bp flanking regions for targeting to the *XKS1* locus. The *XKS1* flanking regions were from nucleotides-250 to -192 and from nucleotides 1,801–1,861, where numbers are relative to the nucleotide A in the *XKS1* ATG start codon. The *Bam*HI cloning site was included in the *XKS1* 5′ flanking sequence. The resulting plasmid, containing *XKS1*, was named pMLV46. The expression cassette for *XKS1*, together with the *loxP-MEL5-loxP* marker, was released from pMLV46 with *Pst*I and *Spe*I and introduced into yeast cells by transformation. Blue colored, *MEL5* (α-galactosidase) expressing yeast colonies were collected from agar-solidified YP containing 2 % w/v D-galactose, supplemented with 5-Bromo-4-chloro-3-indolyl-α-D-galactopyranoside (*X-α-Gal*, 40 μg/ml). To remove the *MEL5* marker cassette from the yeast chromosome, the transformant was retransformed with a plasmid pSH47 [12], expressing the Cre recombinase. Integration of the expression cassette p*ADH1* m-*XKS1*-t*ADH1* into the *XKS1* locus in the genome was verified with PCR. The yeast strain generated was named VTT C-10880.

Control plasmids (B1181 and pKK27-1) and plasmids expressing FRD (pPGK1-FRD) and POS5Δ17 (pTPI1-POS5Δ17) were transformed into VTT C-10880 strain resulting in control (Ctrl), FRD and FRD + POS5Δ17 strains, respectively.

Batch bioreactor cultivations

FRD and FRD + POS5Δ17 and their control strain were cultivated in the 15-vessel parallel bioreactor prototype system Medicel Explorer Cultivation Unit (Medicel, Espoo, Finland) [36]. Cultivation conditions were: temperature 30 °C, 800 rpm stirrer speed with a 26-mm-diameter Rushton-type impeller, cultivation volume of 200 ml, and pH 5, maintained by adding 1 M NaOH. For aerobic conditions, 100 ml/min (0.5 vvm) air was sparged into the reactors. For anaerobic conditions, the head space of the reactors was flushed with 50 ml/min nitrogen (99.999 %). Cultivation medium contained 1.7 g/l yeast nitrogen base without amino acids and ammonium sulphate (Difco), 1 g/l glutamate (Fluka), 0.0135 g/l adenine, 0.348 g/l arginine, 0.266 g/l aspartic acid, 0.058 g/l histidine, 0.036 g/l myo-inositol, 0.525 g/l isoleucine, 0.262 g/l leucine, 0.091 g/l lysine, 0.149 g/l methionine, 0.083 g/l phenylalanine, 0.105 g/l serine, 0.119 g/l threonine, 0.082 g/l tryptophan, 0.03 g/l tyrosine, 0.117 valine, 1.6 mg/l Geneticin sulphate G-418 (Gibco) and either 20 g/l D-glucose (AnalaR NORMAPUR) or 50 g/l D-xylose (Sigma-Aldrich, St. Louis, MO, USA).

Inocula were cultivated in two phases on the above medium with 10 g/l D-glucose and 20 g/l D-xylose in shake flasks at 30 °C with 150 rpm shaking. For the first phase, 25-ml cultivations in 100-ml Erlenmeyer flasks were inoculated from single colonies on plates and incubated overnight. For the second phase, 75-ml cultivations in 250-ml Erlenmeyer flasks were inoculated by adding the total contents of the first phase and incubated for 5 h. An adequate amount of the cell suspension from the second phase was centrifuged and re-suspended in the cultivation medium so that the initial optical density (A600 nm) in the bioreactor cultivations was 1 in aerobic and 3 in anaerobic conditions. Three replicate cultivations were carried out with each of the strains.

Prior to the bioreactor cultivations, FRD and FRD + POS5Δ17 and their control strain were also cultivated in aerobic and anaerobic shake cultures as described in Supplemental Material Figs. 8 and 9.

Extracellular metabolite analysis

Samples were taken with the automated sampling device of the reactor system to empty tubes in a cold bath at −30 °C and let to freeze. Samples of 4 ml for extracellular metabolite and cell-density analyses were taken every 3 h from the glucose cultivations and every 12 h from the xylose cultivations. Samples of 10 ml for enzyme analyses were taken after 24 h cultivation. Concentrations of D-xylose, D-glucose, glycerol, xylitol, ethanol, and acetate were measured with HPLC by using a Waters 2690 Separation Module and Waters System Interphase Module liquid chromatography coupled with a Waters 2414 differential refractometer and a Waters 2487 dual λ absorbance detector (Waters Co., Milford, MA, USA). A fast Acid Analysis Column (100 × 7.8 mm, Bio-Rad, Hercules, CA, USA) and an Aminex HPX-87H Organic Acid Analysis Column (300 × 7.8 mm, Bio-Rad) were equilibrated with 5 mM H_2SO_4 (Titrisol, Merck, Germany) in water at 55 °C and samples were eluted with 5 mM H_2SO_4 in water at a 0.6 ml/min flow rate. Data were acquired with Waters Empower software. Cell dry weight was measured gravimetrically by drying the washed cells at 105 °C in Eppendorf tubes. Optical density was measured with spectrophotometer at 600-nm wavelength. In addition, CO_2 and O_2 levels in the exhaust gas were measured from the exhaust gas at 1.25-h intervals with BlueSens probes (BlueSens, Herten, Germany).

Enzyme activity measurement

Samples for the enzyme activity assays were harvested from the batch cultures from the time point 24 h. Cells were broken with glass beads in 100 mM Tris–HCl, pH 8, supplemented with the Complete Mini, EDTA-free Protease Inhibitor Cocktail (Roche) using a Precellys homogenizator. Protein concentrations were measured by Bradford reagent (Bio-Rad). NAD- and NADH-kinase activities were determined according to the previously described methods [44]. One unit (U) of enzyme activity was defined as 1 μmol of NADH or NADPH produced in 1 min at 30 °C in 1 ml of assay mixture. Specific activity was expressed in units per mg of total soluble proteins. Fumarate reductase activity assay mixture consisted of 100 mM fumarate and 0.4 mM NADH in 100 mM Tris–HCl, pH 8, in a total volume of 220 μl. The reaction was started by addition of 10 μl of the cell lysate prepared as described above and the oxidation of NADH was followed at 340 nm. One unit (U) of enzyme activity was defined as 1 μmol of NADH oxidized in 1 min at 30 °C in an assay mixture. Both assays were performed using a Konelab Arena photometric analyzer (Thermo Electron Oy, Vantaa, Finland).

Results

The NADH-dependent fumarate reductase FRD of *T. brucei* was overexpressed alone and together with the endogenous cytosol-directed NADH-kinase (POS5Δ17) in D-xylose-utilizing *S. cerevisiae* to study their effect on the aerobic and anaerobic metabolism of D-glucose and fermentation of D-xylose.

Enzyme activities

The functional overexpression of POS5Δ17 and FRD was confirmed by in vitro enzymatic assays. Since Pos5p functions both as NADH and NAD^+ kinase the activity was measured with both of these cofactors. The NADH-FRD and Pos5p activities were measured from the 24-h time point of three replicate aerobic and anaerobic D-glucose and D-xylose cultures. NADH-FRD activity was not detected in the control strain not expressing FRD. In FRD strain fumarate reductase activity was 53 ± 10 mU/mg protein in the D-glucose cultivations and 38 ± 12 mU/mg protein in the D-xylose cultivations. This was substantially more than 7 mU/mg protein measured from *T. brucei* cell extracts [30] that confirms the functional overexpression of FRD in *S. cerevisiae*. Fumarate reductase activity was not significantly different between the aerobic and anaerobic conditions (data not shown). NADH-kinase activity of the control strain with the empty B1181 and pKK27-1 plasmids was 0.2 ± 0.1 U/mg protein while its cytosolic overexpression increased the activity to 1.1 ± 0.3 U/mg protein. NAD^+-kinase activity was 0.05 ± 0.03 U/mg protein in the control strain and 0.08 ± 0.02 U/mg protein in the strains with *POS5Δ17* overexpression. There were no significant differences in the NAD^+ and NADH kinase activities between the aerobic and anaerobic D-xylose and D-glucose cultures (data not shown).

Aerobic and anaerobic cultivations on D-glucose

The effect of overexpression of FRD alone or combination of FRD and POS5Δ17 on D-glucose metabolism of VTT C-10880 was studied in aerobic and anaerobic batch fermentations on 20 g/l D-glucose. The product yields on carbon utilized and the specific rates of D-glucose utilization and metabolite production are presented in Fig. 2. The g/l titers over the time course of the fermentations are shown the Fig. 1 and 2 in Supplemental Material. Overall, the differences between the strains on D-glucose were rather small. The expression of FRD and FRD + POS5Δ17 slightly increased the D-glucose consumption rate but decreased the ethanol yield both under aerobic and anaerobic conditions. The CO_2 yield of FRD + POS5Δ17 was lower compared with the control under anaerobic conditions.

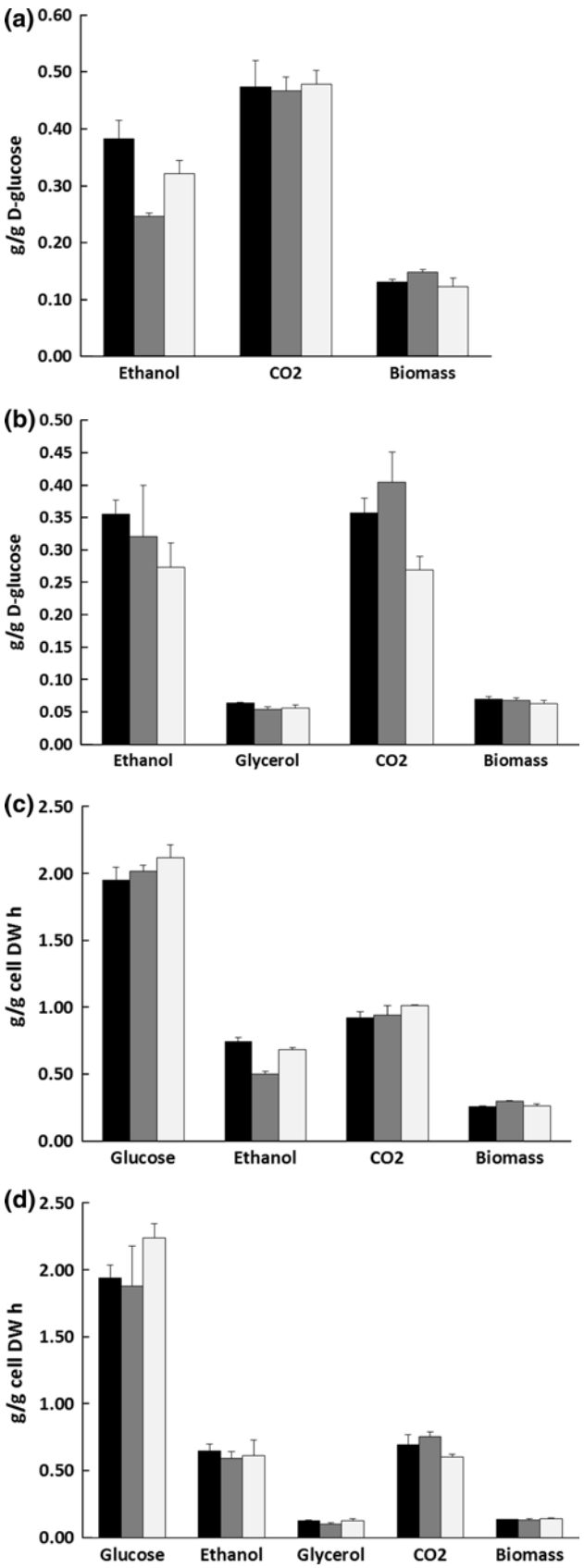

Fig. 2 The yields (g/g) of ethanol, glycerol, carbon dioxide (CO_2) and biomass over total D-glucose consumed in **a** aerobic and **b** anaerobic batch cultures on 20 g/l D-glucose. The specific consumption and production rates (g g^{-1} h^{-1}) of D-glucose, ethanol, glycerol, carbon dioxide (CO_2) and biomass in **c** aerobic and **d** anaerobic batch cultures on 20 g/l D-glucose. The yields and rates were calculated from the logarithmic phase (4–6 h) and (0–6 h) of the aerobic and anaerobic cultivations, respectively. (Ctrl *black bars*, FRD *grey bars*, and FRD + POS5Δ17 *light grey bars*)

Aerobic and anaerobic cultivations on D-xylose

The effect of overexpression of FRD alone or FRD + POS5Δ17 on xylose fermentation was studied in aerobic and anaerobic batch fermentations on 50 g/l D-xylose. The product yields on D-xylose utilized and the specific rates of D-xylose utilization and metabolite production are presented in Fig. 3. The g/l titers over the time course of the fermentations are shown the Figs. 2–7 in Supplemental Material. The cultivations were also carried out in shake flasks on 20 g/l D-xylose. Aerobic growth of strains measured as OD600 and fermentation measured as weight loss of glycerol-lock shake flasks due to CO_2 escape are shown in Supplemental Material Figs. 8 and 9.

Under aerobic conditions, FRD expression decreased the D-xylose consumption rate (31 %) and also xylitol, glycerol, and CO_2 production rates and yields were decreased while acetate yield was increased. FRD expression decreased the D-xylose consumption rate also under anaerobic conditions. However, ethanol yield increased 60 % and its production rate 40 %. At the same time, xylitol yield and production rate decreased but the specific glycerol production rate increased.

Under aerobic conditions, the expression of FRD + POS5Δ17 had no effect on the D-xylose consumption rate but the specific xylitol production rate was the lowest of the strains studied (73 % lower compared with the control). On the other hand, there was an increase in glycerol production while acetate production was decreased. Under anaerobic conditions, the expression of FRD + POS5Δ17 decreased the D-xylose consumption rate significantly (73 %). Ethanol yield was slightly higher compared with the control (23 %) but its production rate was lower as well as the rates of xylitol, glycerol, acetate, and CO_2 production.

Discussion

In the present study, fumarate reductase (FRD) and NADH-kinase (POS5Δ17) reactions were introduced into the cytosol of recombinant *S. cerevisiae* with the oxidoreductive

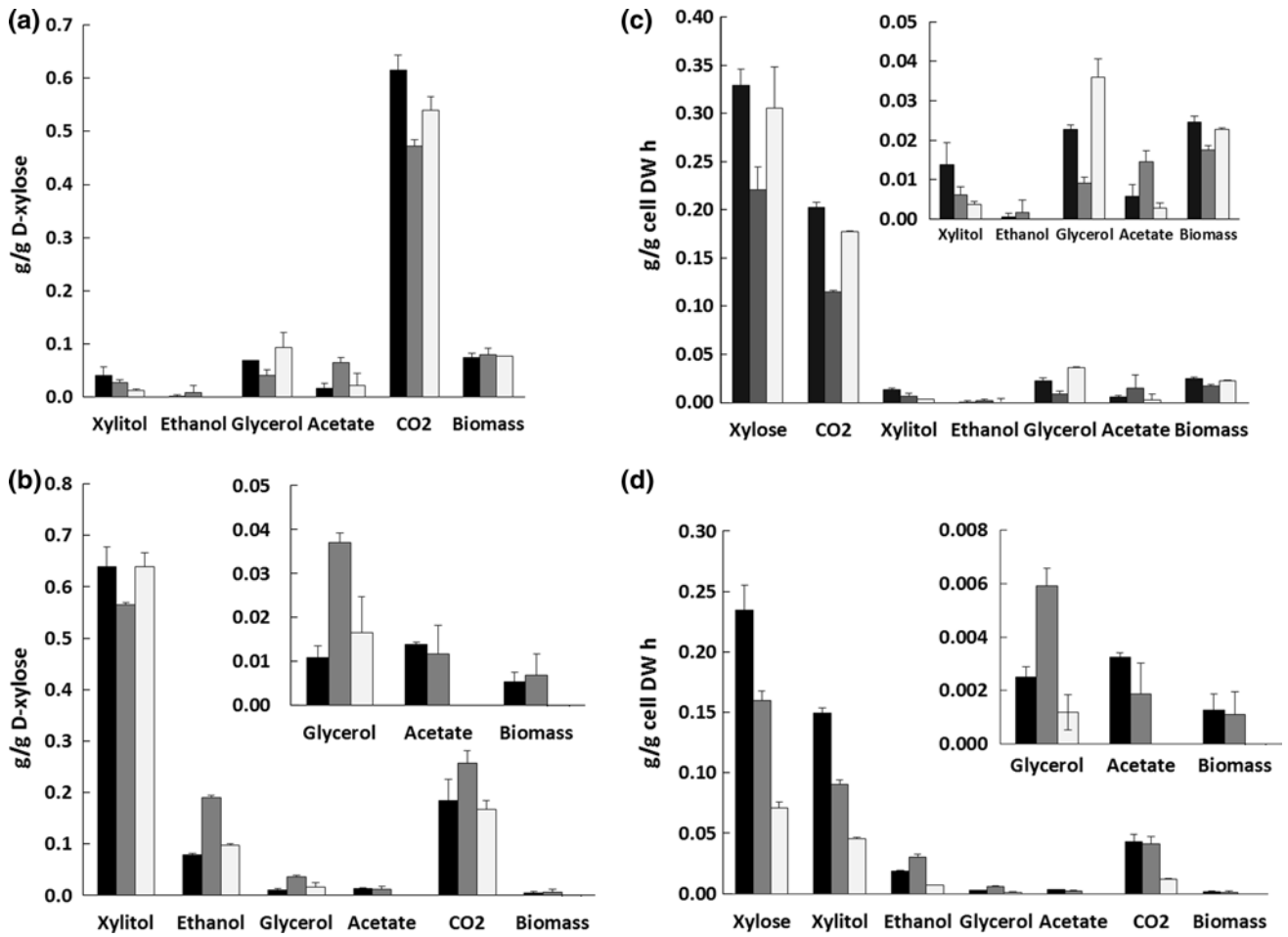

Fig. 3 The yields (g/g) of xylitol, ethanol, glycerol, acetate, carbon dioxide (CO_2) and biomass over total D-xylose consumed in **a** aerobic and **b** anaerobic batch cultures on 50 g/l D-xylose. The specific consumption and production rates (g g^{-1} h^{-1}) of D-xylose, ethanol, glycerol, acetate, carbon dioxide (CO_2), and biomass in **c** aerobic and **d** anaerobic batch cultures on 50 g/l D-xylose. The yields and rates were calculated from the D-xylose consumption phase (0–90 h) of the aerobic and anaerobic cultivations. (Ctrl *black bars*, FRD *grey bars*, and FRD + POS5Δ17 *light grey bars*)

XR-XDH D-xylose pathway. The aim was to increase the cytosolic NAD^+ and NADPH pools in order to alleviate the redox imbalance caused by the oxidoreductive D-xylose utilization pathway, and thus enhance D-xylose fermentation. Alternative solutions to redox imbalance have been previously provided for instance by the expression of the D-xylose isomerase pathway in *S. cerevisiae* that has resulted in ethanol yields ranging from 0.13 to 0.48 g/g of D-xylose consumed depending on the strain background and fermentation conditions [26]. The other interesting option is alteration of the cofactor affinity of XR or XDH that has resulted in yeast strains able to produce 0.12–0.37 g ethanol/g consumed D-xylose as summarized by Krahulec et al. [23].

Expression of FRD and FRD + POS5Δ17 resulted in 60 and 23 % increase in ethanol yield on D-xylose under anaerobic conditions. The maximum ethanol yield was 0.19 g/g with the strain expressing FRD, which was lower compared with the best ethanol yields reported in the literature [26]. However, as the aim of the present study was to demonstrate the effect of FRD and FRD + POS5Δ17 expression on D-xylose fermentation, we used a very basic laboratory yeast strain with the XR-XDH pathway and small replicate cultures in a 15-vessel parallel bioreactor system that were not expected to result in the highest possible ethanol yields. The results suggest that in a strain with the XR-XDH pathway provision of NAD^+ may be at least as important as the provision of NADPH, as the expression of FRD alone was adequate to increase the ethanol yield and to decrease the xylitol yield from D-xylose. Similar results, supporting the present data, were recently obtained by overexpression of the water-forming NADH oxidase from *Lactococcus lactis* in *S. cerevisiae* with the XR-XDH pathway [56]. The reason may be that under anaerobic conditions there are only

two major ways to oxidize NADH and close the redox balance; alcoholic fermentation and glycerol production [38, 50]. Indeed, FRD expression resulted also in increased yield and production rate of glycerol suggesting that the NADH produced in glycolysis and oxidation of xylitol was not completely oxidized by production of ethanol.

On the other hand, for the regeneration of NADPH more options exist. The cytosolic NADPH can be regenerated via the reactions catalyzed by glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in the oxidative pentose phosphate pathway, and by isocitrate dehydrogenase or acetaldehyde dehydrogenase [29]. Our previous results from proteome and metabolic flux analyses of D-xylose-fermenting *S. cerevisiae* suggest that on D-xylose NADPH regeneration mainly takes place via the reaction catalyzed by acetaldehyde dehydrogenase, and via the reactions in the oxidative pentose phosphate pathway that, however, simultaneously result in loss of carbon as CO_2 [37, 43].

Compared with the expression of FRD only, the expression of FRD + POS5Δ17 significantly decreased the D-xylose consumption and ethanol, xylitol, glycerol and acetate production rates, suggesting that the phosphorylation of NADH to NADPH by NADH-kinase was energetically too costly under anaerobic conditions on D-xylose. This was observed also in the previous study where expression of POS5Δ17 in xylose-utilizing *S. cerevisiae* resulted in decreased rates of D-glucose and D-xylose consumption and growth, and biomass yield compared with the control strain when cells were grown on D-glucose or on a mixture of D-glucose and D-xylose under both aerobic and anaerobic conditions [15]. However, in the present study FRD + POS5Δ17 rather increased the D-glucose consumption rate and had no effect on biomass yield or production rate in both the aerobic and anaerobic D-glucose cultures. These results are more in line with earlier results from overexpression of the endogenous NADH-kinase in *A. nidulans* that resulted in increased specific growth rate and biomass yield, suggesting that the overexpression increased NADPH availability for biomass formation [33]. The present results additionally indicate that the extra NAD^+ provided by FRD may have a positive effect on the D-glucose consumption rate counteracting the negative effect of NADH-kinase expression reported before. The FRD alone slightly increased the aerobic and anaerobic D-glucose consumption rates and aerobic biomass production. The yield and fermentation rate of ethanol were decreased under both aerobic and anaerobic conditions proposing that the FRD reaction served as an alternative redox sink replacing the need for regeneration of NAD^+ by fermentation.

FRD reduced the D-xylose uptake rate under aerobic conditions on xylose, resulting in decreased xylitol, glycerol and CO_2 production rates while the acetate production rate was increased. This suggests that FRD expression resulted in surplus of oxidized cofactors which were reduced to NADH e.g. by acetaldehyde dehydrogenase under these conditions when also the oxidative phosphorylation was active. On the other hand, FRD + POS5Δ17 restored the D-xylose uptake rate to the level of the control strain and the yield and production rate of xylitol were further decreased as compared to the FRD strain. This in turn indicates that under aerobic conditions the ATP supply was adequate and the redox cycle created by FRD and NADH-kinase worked most optimally, providing NADPH and NAD^+ for xylose reductase and xylitol dehydrogenase reactions. However, the carbon was directed rather to glycerol than to acetate, as was the case when FRD was expressed alone. Apparently there was no need to oxidize a surplus of reduced cofactors, which could have been formed by the xylitol dehydrogenase reaction if the NADH-kinase and FRD reactions were not completely in balance. Moreover, hardly any ethanol was observed in aerobic xylose cultures, indicating that the ethanol produced was consumed as a co-substrate of D-xylose [28, 43] that may have further increased the NADH pool to be oxidized via the glycerol synthesis.

Overall, the results show in particular, the beneficial effect of provision of NAD^+ on D-xylose fermentation by the oxidoreductive xylose pathway. Expression of the NADH-using FRD provides a simple and still a novel way to enhance D-xylose fermentation of *S. cerevisiae* strains with XR-XDH pathway without extensive metabolic engineering. Moreover, its expression takes advantage of the higher concentration of fumarate observed in D-xylose fermenting *S. cerevisiae* with XR-XDH pathway [3]. Simultaneously increasing the anaerobic pool of NADPH by overexpression of NADH-kinase turned out to be more challenging, but appeared beneficial under aerobic conditions, as seen by decreased xylitol production. Combination of FRD with an ATP independent production of NADPH, such as the expression of $NADP^+$-dependent glyceraldehyde 3-phosphate dehydrogenase [51] would be an interesting option to test.

Acknowledgments This study was financially supported by the Academy of Finland being part of the KETJU Research Programme (2006–2010). Technical assistance of Pirjo Tähtinen is gratefully acknowledged.

References

1. Anderlund M, Nissen TL, Nielsen J, Villadsen J, Rydstrom J, Hahn-Hägerdal B, Kielland-Brandt MC (1999) Expression of the *Escherichia coli* pntA and pntB genes, encoding nicotinamide nucleotide transhydrogenase, in *Saccharomyces cerevisiae* and its effect on product formation during anaerobic glucose fermentation. Appl Environ Microbiol 65:2333–2340

2. Arikawa Y, Enomoto K, Muratsubaki H, Okazaki M (1998) Soluble fumarate reductase isoenzymes from *Saccharomyces cerevisiae* are required for anaerobic growth. FEMS Microbiol Lett 165:111–116
3. Bergdahl B, Heer D, Sauer U, Hahn-Hägerdal B, van Niel EW (2012) Dynamic metabolomics differentiates between carbon and energy starvation in recombinant *Saccharomyces cerevisiae* fermenting xylose. Biotechnol Biofuels 5:34
4. Besteiro S, Biran M, Biteau N, Coustou V, Baltz T, Canioni P, Bringaud F (2002) Succinate secreted by *Trypanosoma brucei* is produced by a novel and unique glycosomal enzyme, NADH-dependent fumarate reductase. J Biol Chem 277:38001–38012
5. Bieganowski P, Seidle HF, Wojcik M, Brenner C (2006) Synthetic lethal and biochemical analyses of NAD and NADH kinases in *Saccharomyces cerevisiae* establish separation of cellular functions. J Biol Chem 281:22439–22445
6. Brat D, Boles E, Wiedemann B (2009) Functional expression of a bacterial xylose isomerase in *Saccharomyces cerevisiae*. Appl Environ Microbiol 75:2304–2311
7. Bro C, Regenberg B, Forster J, Nielsen J (2006) In silico aided metabolic engineering of *Saccharomyces cerevisiae* for improved bioethanol production. Metab Eng 8:102–111
8. Bruinenberg PM, de Bot PHM, van Dijken JP, Scheffers WA (1983) The role of redox balances in the anaerobic fermentation of xylose by yeasts. Eur J Appl Microb Biotechnol 18:287–292
9. Eliasson A, Christensson C, Wahlbom CF, Hahn-Hägerdal B (2000) Anaerobic xylose fermentation by recombinant *Saccharomyces cerevisiae* carrying *XYL*, *XYL2*, and *XKS1* in mineral medium chemostat cultures. Appl Environ Microbiol 66:3381–3386
10. Gietz RD, Sugino A (1988) New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. Gene 74:527–534
11. Gordon EH, Pealing SL, Chapman SK, Ward FB, Reid GA (1998) Physiological function and regulation of flavocytochrome c3, the soluble fumarate reductase from *Shewanella putrefaciens* NCIMB 400. Microbiology 144(Pt 4):937–945
12. Guldener U, Heck S, Fielder T, Beinhauer J, Hegemann JH (1996) A new efficient gene disruption cassette for repeated use in budding yeast. Nucleic Acids Res 24:2519–2524
13. Harhangi HR, Akhmanova AS, Emmens R, van der Drift C, de Laat WT, van Dijken JP, Jetten MS, Pronk JT, Op den Camp HJ (2003) Xylose metabolism in the anaerobic fungus *Piromyces* sp. strain E2 follows the bacterial pathway. Arch Microbiol 180:134–141
14. Ho NW, Chen Z, Brainard AP (1998) Genetically engineered *Saccharomyces* yeast capable of effective cofermentation of glucose and xylose. Appl Environ Microbiol 64:1852–1859
15. Hou J, Vemuri GN, Bao X, Olsson L (2009) Impact of overexpressing NADH kinase on glucose and xylose metabolism in recombinant xylose-utilizing *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 82:909–919
16. Jeffries TW (2006) Engineering yeasts for xylose metabolism. Curr Opin Biotechnol 17:320–326
17. Jeppsson M, Johansson B, Hahn-Hägerdal B, Gorwa-Grauslund MF (2002) Reduced oxidative pentose phosphate pathway flux in recombinant xylose-utilizing *Saccharomyces cerevisiae* strains improves the ethanol yield from xylose. Appl Environ Microbiol 68:1604–1609
18. Jeppsson M, Bengtsson O, Franke K, Lee H, Hahn-Hägerdal B, Gorwa-Grauslund MF (2006) The expression of a *Pichia stipitis* xylose reductase mutant with higher K(M) for NADPH increases ethanol production from xylose in recombinant *Saccharomyces cerevisiae*. Biotechnol Bioeng 93:665–673
19. Karhumaa K, Sanchez RG, Hahn-Hägerdal B, Gorwa-Grauslund MF (2007) Comparison of the xylose reductase-xylitol dehydrogenase and the xylose isomerase pathways for xylose fermentation by recombinant *Saccharomyces cerevisiae*. Microb Cell Fact 6:5
20. Kim SR, Park YC, Jin YS, Seo JH (2013) Strain engineering of *Saccharomyces cerevisiae* for enhanced xylose metabolism. Biotechnol Adv. doi:10.1016/j.biotechadv.2013.03.004
21. Kötter P, Amore R, Hollenberg CP, Ciriacy M (1990) Isolation and characterization of the *Pichia stipitis* xylitol dehydrogenase gene, *XYL2*, and construction of a xylose-utilizing *Saccharomyces cerevisiae* transformant. Curr Genet 18:493–500
22. Kötter P, Ciriacy M (1993) Xylose fermentation by *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 38:776–783
23. Krahulec S, Klimacek M, Nidetzky B (2012) Analysis and prediction of the physiological effects of altered coenzyme specificity in xylose reductase and xylitol dehydrogenase during xylose fermentation by *Saccharomyces cerevisiae*. J Biotechnol 158:192–202
24. Kuyper M, Harhangi HR, Stave AK, Winkler AA, Jetten MS, de Laat WT, den Ridder JJ, Op den Camp HJ, van Dijken JP, Pronk JT (2003) High-level functional expression of a fungal xylose isomerase: the key to efficient ethanolic fermentation of xylose by *Saccharomyces cerevisiae*? FEMS Yeast Res 4:69–78
25. Madhavan A, Tamalampudi S, Ushida K, Kanai D, Katahira S, Srivastava A, Fukuda H, Bisaria VS, Kondo A (2009) Xylose isomerase from polycentric fungus *Orpinomyces*: gene sequencing, cloning, and expression in *Saccharomyces cerevisiae* for bioconversion of xylose to ethanol. Appl Microbiol Biotechnol 82:1067–1078
26. Madhavan A, Srivastava A, Kondo A, Bisaria VS (2012) Bioconversion of lignocellulose-derived sugars to ethanol by engineered *Saccharomyces cerevisiae*. Crit Rev Biotechnol 32:22–48
27. Matsushika A, Inoue H, Kodaki T, Sawayama S (2009) Ethanol production from xylose in engineered *Saccharomyces cerevisiae* strains: current state and perspectives. Appl Microbiol Biotechnol 84:37–53
28. Meinander NQ, Hahn-Hägerdal B (1997) Influence of cosubstrate concentration on xylose conversion by recombinant, *XYL1*-expressing *Saccharomyces cerevisiae*: a comparison of different sugars and ethanol as cosubstrates. Appl Environ Microbiol 63:1959–1964
29. Minard KI, Jennings GT, Loftus TM, Xuan D, McAlister-Henn L (1998) Sources of NADPH and expression of mammalian NADP+-specific isocitrate dehydrogenases in *Saccharomyces cerevisiae*. J Biol Chem 273:31486–31493
30. Mracek J, Snyder SJ, Chavez UB, Turrens JF (1991) A soluble fumarate reductase in *Trypanosoma brucei* procyclic trypomastigotes. J Protozool 38:554–558
31. Outten CE, Culotta VC (2003) A novel NADH kinase is the mitochondrial source of NADPH in *Saccharomyces cerevisiae*. EMBO J 22:2015–2024
32. Pain J, Balamurali MM, Dancis A, Pain D (2010) Mitochondrial NADH kinase, Pos5p, is required for efficient iron-sulfur cluster biogenesis in *Saccharomyces cerevisiae*. J Biol Chem 285:39409–39424
33. Panagiotou G, Grotkjaer T, Hofmann G, Bapat PM, Olsson L (2009) Overexpression of a novel endogenous NADH kinase in *Aspergillus nidulans* enhances growth. Metab Eng 11:31–39
34. Parachin NS, Gorwa-Grauslund MF (2011) Isolation of xylose isomerases by sequence- and function-based screening from a soil metagenomic library. Biotechnol Biofuels 4:9
35. Petschacher B, Nidetzky B (2008) Altering the coenzyme preference of xylose reductase to favor utilization of NADH enhances ethanol yield from xylose in a metabolically engineered strain of *Saccharomyces cerevisiae*. Microb Cell Fact 7:9

36. Pitkänen J-P, Vuolanto A (2004) Reactor device. Patent FI118192
37. Pitkänen J-P, Aristidou A, Salusjärvi L, Ruohonen L, Penttilä M (2003) Metabolic flux analysis of xylose metabolism in recombinant *Saccharomyces cerevisiae* using continuous culture. Metab Eng 5:16–31
38. Postma E, Verduyn C, Scheffers WA, Van Dijken JP (1989) Enzymatic analysis of the Crabtree effect in glucose-limited chemostat cultures of *Saccharomyces cerevisiae*. Appl Environ Microbiol 55:468–477
39. Pronk JT, Yde Steensma H, Van Dijken JP (1996) Pyruvate metabolism in *Saccharomyces cerevisiae*. Yeast 12:1607–1633
40. Roca C, Nielsen J, Olsson L (2003) Metabolic engineering of ammonium assimilation in xylose-fermenting *Saccharomyces cerevisiae* improves ethanol production. Appl Environ Microbiol 69:4732–4736
41. Runquist D, Hahn-Hägerdal B, Bettiga M (2010) Increased ethanol productivity in xylose-utilizing *Saccharomyces cerevisiae* via a randomly mutagenized xylose reductase. Appl Environ Microbiol 76:7796–7802
42. Ruohonen L, Aristidou A, Frey AD, Penttilä M, Kallio PT (2006) Expression of *Vitreoscilla* hemoglobin improves the metabolism of xylose in recombinant yeast *Saccharomyces cerevisiae* under low oxygen conditions. Enzyme Microb Technol 39:6–14
43. Salusjärvi L, Poutanen M, Pitkänen J-, Koivistoinen H, Aristidou A, Kalkkinen N, Ruohonen L, Penttilä M (2003) Proteome analysis of recombinant xylose-fermenting *Saccharomyces cerevisiae*. Yeast 20:295–314
44. Shi F, Kawai S, Mori S, Kono E, Murata K (2005) Identification of ATP-NADH kinase isozymes and their contribution to supply of NADP(H) in *Saccharomyces cerevisiae*. FEBS J 272:3337–3349
45. Shi F, Li Z, Sun M, Li Y (2011) Role of mitochondrial NADH kinase and NADPH supply in the respiratory chain activity of *Saccharomyces cerevisiae*. Acta Biochim Biophys Sin (Shanghai) 43:989–995
46. Strand MK, Stuart GR, Longley MJ, Graziewicz MA, Dominick OC, Copeland WC (2003) POS5 gene of *Saccharomyces cerevisiae* encodes a mitochondrial NADH kinase required for stability of mitochondrial DNA. Eukaryot Cell 2:809–820
47. Toivari MH, Aristidou A, Ruohonen L, Penttilä M (2001) Conversion of xylose to ethanol by recombinant *Saccharomyces cerevisiae*: importance of xylulokinase (*XKS1*) and oxygen availability. Metab Eng 3:236–249
48. van Dijken JP, Scheffers WA (1986) Redox balances in the metabolism of sugars by yeast. FEMS Microbiol Rev 32:199–224
49. Van Hellemond JJ, Tielens AG (1994) Expression and functional properties of fumarate reductase. Biochem J 304(Pt 2):321–331
50. Verduyn C, Zomerdijk TPL, van Dijken JP, Scheffers WA (1984) Continuous measurement of ethanol production by aerobic yeast suspensions with an enzyme electrode. Appl Microbiol Biotechnol 19:181–185
51. Verho R, Londesborough J, Penttilä M, Richard P (2003) Engineering redox cofactor regeneration for improved pentose fermentation in *Saccharomyces cerevisiae*. Appl Environ Microbiol 69:5892–5897
52. Wach A, Brachat A, Pohlmann R, Philippsen P (1994) New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. Yeast 10:1793–1808
53. Watanabe S, Pack SP, Saleh AA, Annaluru N, Kodaki T, Makino K (2007) The positive effect of the decreased NADPH-preferring activity of xylose reductase from *Pichia stipitis* on ethanol production using xylose-fermenting recombinant *Saccharomyces cerevisiae*. Biosci Biotechnol Biochem 71:1365–1369
54. Watanabe S, Saleh AA, Pack SP, Annaluru N, Kodaki T, Makino K (2007) Ethanol production from xylose by recombinant *Saccharomyces cerevisiae*-expressing protein engineered NADP(+)-dependent xylitol dehydrogenase. J Biotechnol 130:316–319
55. Zaldivar J, Nielsen J, Olsson L (2001) Fuel ethanol production from lignocellulose: a challenge for metabolic engineering and process integration. Appl Microbiol Biotechnol 56:17–34
56. Zhang GC, Liu JJ, Ding WT (2011) Overexpression of water-forming NADH oxidase decreases xylitol formation during xylose fermentation of *Saccharomyces cerevisiae*. Appl Environ Microbiol 78:1081–1086