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Comparative metabolic network analysis of two xylose fermenting recombinant *Saccharomyces cerevisiae* strains

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Abstract

The recombinant xylose fermenting strain *Saccharomyces cerevisiae* TMB3001 can grow on xylose, but the xylose utilisation rate is low. One important reason for the inefficient fermentation of xylose to ethanol is believed to be the imbalance of redox co-factors. In the present study, a metabolic flux model was constructed for two recombinant *S. cerevisiae* strains: TMB3001 and CPB.CR4 which in addition to xylose metabolism have a modulated redox metabolism, i.e. ammonia assimilation was shifted from being NADPH to NADH dependent by deletion of *gdh1* and over-expression of *GDH2*. The intracellular fluxes were estimated for both strains in anaerobic continuous cultivations when the growth limiting feed consisted of glucose (2.5 g L^{-1}) and xylose (13 g L^{-1}). The metabolic network analysis with ^{13}C labelled glucose showed that there was a shift in the specific xylose reductase activity towards use of NADH as co-factor rather than NADPH. This shift is beneficial for solving the redox imbalance and it can therefore partly explain the 25% increase in the ethanol yield observed for CPB.CR4. Furthermore, the analysis indicated that the glyoxylate cycle was activated in CPB.CR4.

Keywords: *Saccharomyces cerevisiae*; Yeast; Glucose; Xylose; Ethanol; NADH; NADPH; Carbon labelling; Metabolic network analysis; Metabolic engineering

1. Introduction

Lignocellulosic materials are an abundant and inexpensive resource that could support the sustainable production of liquid transportation fuels (Hayn et al., 1993). Xylose is the most abundant pentose sugar in the hemicellulose (around one-fourth of dry weight) of hardwoods and crop residues, and it is second only to glucose in natural abundance of all monosaccharides. Thus, the efficient utilisation of the xylose component of hemicellulose in addition to hexoses offers the opportunity to significantly reduce the production costs of bioethanol (Aristidou and Penttilä, 2000; Sreenath and Jeffries, 2000). *Saccharomyces cerevisiae*, which is one of the most prominent ethanol-producing microorganisms using hexose sugars, has the drawback that it is unable to utilise xylose. The first successful approach to construct xylose-utilising recombinant *S. cerevisiae* strains has been to express the xylose reductase (XR)- and xylitol dehydrogenase (XDH)-encoding genes *XYL1* and *XYL2* from *Pichia stipitis* in *S. cerevisiae* (Kötter and Ciriacy, 1993). However, over-expression of the endogenous xylulokinase (XK) significantly improved xylose utilisation (Ho et al., 1998). Despite the successful expression of the three-enzyme set, XR/XDH/XK, the xylose utilisation rate is still significantly lower than the glucose utilisation rate, and the ethanol yield on xylose is lower than on glucose due to undesired xylitol excretion (Eliasson et al., 2000). One important reason for the inefficient fermentation of xylose to

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ethanol can be explained by the imbalance of redox co-factors (as reviewed by Hahn-Hägerdal et al. (2001) and Zaldivar et al. (2001)). Xylose metabolism involves reduction to xylitol by the NADH/NADPH-dependent XR (Hahn-Hägerdal et al., 2001), followed by oxidation of xylitol to xylulose by the exclusively NAD^+-dependent enzyme XDH. The ratio of the specific XR activity utilising different co-factors has been determined to be NADH/NADPH approximately 0.65 in *P. stipitis* (Skoog and Hahn-Hägerdahl, 1990) though the co-factor preference for XR is not fully established (Wahlbom et al., 2001). In *S. cerevisiae* NADPH is mainly regenerated in the oxidative part of the pentose phosphate (PP) pathway, where reduction of $NADP^+$ is coupled to conversion of glucose-6-phosphate to xylose-5-phosphate and generation of CO_2. When less NADH is consumed in the XR reaction, then less NAD^+ is available for the XDH reaction. Consequently, excess NADH must be removed by undesired xylitol excretion or aeration, and in both cases the excess of NADH results in reduced ethanol yield. To improve the ethanol yield, it would be desirable to regenerate NADPH in a way, which is not directly coupled to CO_2 production and which simultaneously eliminates the production of excess NADH (Verho et al., 2003). By deletion of *ZWF1* (encoding glucose-6-phosphate dehydrogenase), in combination with over-expression of *GDP1,* which allows regeneration of NADPH not linked to CO_2 production, it has been shown that recombinant xylose utilisation could be improved with respect to the xylose utilisation rate and ethanol yield on xylose (Verho et al., 2003). In an alternative approach, the expression level of *ZWF1* was modulated, and it was shown that a low level of glucose-6-phosphate dehydrogenase resulted in improved ethanol yield on xylose with concomitant reduction in xylitol yield. However, the improved ethanol yield was at the expense of a reduced xylose consumption rate (Jeppsson et al., 2003).

Metabolic engineering of the ammonium assimilation in xylose fermenting *S. cerevisiae* has been used as a strategy to improve ethanol yield by modulating the redox metabolism (Roca et al., 2003). The reference strain TMB3001, carrying XR and XDH from *P. stiptis* and over-expressed endogenous XK (Eliasson et al., 2000), was modified by deleting the *GDH1* gene encoding an NADPH-dependent glutamate dehydrogenase, and by over-expressing the *GDH2* gene encoding an NADH-dependent glutamate dehydrogenase. These modifications in the ammonia assimilation pathway resulted in beneficial alterations of the redox metabolism and hereby of the xylose metabolism. The resulting strain CPB.CR4 had a 16% higher ethanol yield, mainly due to a 44% reduction of xylitol excretion (Roca et al., 2003).

The significant improvement of ethanol yield was suspected to be a result of successful redirection of intracellular fluxes in the central carbon metabolism, but metabolic flux analysis is insufficient for estimation of the entire set of intracellular metabolic fluxes (Christensen and Nielsen, 2000). Metabolic network analysis with a ^{13}C labelled tracer gives a more detailed picture of the fluxes in the central carbon metabolism (Szyperski, 1995; Christensen and Nielsen, 1999; Wiechert, 2001). We report here a comparative analysis of the central carbon metabolism for *S. cerevisiae* CPB.CR4 and the reference strain TMB3001. The use of labelled [1-^{13}C] glucose allowed a good estimation of the active metabolic pathways in the two strains and the fluxes through the different branches of the central carbon metabolism.

2. Materials and methods

2.1. Strains

The *S. cerevisiae* strains used in this study were derived from the CEN.PK113-7D wild type strain (van Dijken et al., 2000). The gene encoding XR and XDH from *P. stipitis* and the endogenous gene for XK have already been integrated into the chromosome of CEN.PK113-7D, using the integrative plasmid YipXR/XDH/XK leading to the stable construct TMB3001 (Eliasson et al., 2000). The *GDH1* gene has been deleted using the *loxP-kanMX-loxP* disruption cassette (Güldener et al., 1996). *GDH2* has been put under a *PGK* constitutive promoter and the CEN.MSI (*Δgdh1GDH2*) strain was obtained. Transformation of CEN.MSI with the plasmid YipXR/XDH/XK was performed using the lithium acetate method as described by Gietz et al. (1992), leading to the xylose fermenting strain CPB.CR4 (Roca et al., 2003). The strains were stored at 4 °C on YPD agar plate.

2.2. Cultivation

The cultivation was carried out in a 400 ml bioreactor specially designed for labelling experiments with a working volume of 200 ml. Initial batch cultivation with 2.5 g L^{-1} glucose and 13 g L^{-1} xylose was first performed, and then a medium with the same composition as the batch cultivation medium was continuously fed to the bioreactor at a dilution rate of 0.058 h^{-1}. When steady state was achieved after three residence times, the feeding medium was switched to a medium containing 2.5 g glucose (a mixture of 1.65 g naturally labelled glucose and 0.85 g [1-^{13}C] glucose) and 13 g xylose per litre until a new isotopic steady state was achieved after another three residence times (Grotkjær et al., 2004). The bioreactor was continuously sparged with nitrogen (0.5 vvm) with a maximum content of 0.2 ppm oxygen. The medium had the following composition per litre: 5 g