SHORT COMMUNICATION

Isobutanol production from D-xylose by recombinant *Saccharomyces cerevisiae*

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Abstract

Simultaneous overexpression of an optimized, cytosolically localized valine biosynthesis pathway together with overexpression of xylose isomerase XylA from *Clostridium phytofermentans*, transaldolase Tal1 and xylulokinase Xks1 enabled recombinant *Saccharomyces cerevisiae* cells to complement the valine auxotrophy of *ilv2,3,5* triple deletion mutants for growth on D-xylose as the sole carbon source. Moreover, after additional overexpression of ketoacid decarboxylase Aro10 and alcohol dehydrogenase Adh2, the cells were able to ferment D-xylose directly to isobutanol.

Isobutanol is a branched-chain alcohol and is naturally produced in low amounts by the yeast *Saccharomyces cerevisiae* as a degradation product of valine. Compared to bioethanol isobutanol would be a superior biofuel because of its physico-chemical properties (Weber *et al.*, 2010). To increase isobutanol production from D-glucose with recombinant yeasts different strategies have been performed like overexpression of genes of valine biosynthesis or its degradation (Chen *et al.*, 2011; Lee *et al.*, 2012). In our own work (Brat *et al.*, 2012), we could increase isobutanol production of *S. cerevisiae* strains by re-locating the mitochondrial valine biosynthesis enzymes Ilv2, Ilv5 and Ilv3 into the cytosol via truncation of their mitochondrial targeting sequences. Highest isobutanol production titers from D-glucose were obtained by overexpression of codon-optimized truncated Ilv2, 5 and 3 genes in the absence of valine in a strain deleted for the mitochondrial valine biosynthesis pathway. Additionally, enzymes involved in valine degradation, Aro10 alpha-keto acid decarboxylase and Adh2 alcohol dehydrogenase were overproduced.

In contrast to D-glucose, pentose sugars like D-xylose cannot be naturally utilized by wild-type *S. cerevisiae*. However, as D-xylose can make up more than 30% of plant biomass (Weber *et al.*, 2010), the ability to ferment this sugar is necessary to enhance the cost-effectiveness of fermentation processes from lignocellulosic hydrolysates. The inability of *S. cerevisiae* to ferment D-xylose to ethanol could be overcome by either expression of xylose isomerases or of fungal xylose reductases together with xylitol dehydrogenases, and further optimization of xylulose phosphorylation and of the flux through the non-oxidative part of the pentose phosphate pathway (Weber *et al.*, 2010).

The aim of this work was to demonstrate for the first time direct fermentation of D-xylose to isobutanol by recombinant yeast cells. Therefore, we overexpressed the xylose isomerase of *Clostridium phytofermentans* (Brat *et al.*, 2009) together with two crucial steps in further xylose metabolism, xylulokinase and transaldolase (Ni *et al.*, 2007) and combined this with overexpression of the optimized cytosolic isobutanol production pathway (Fig. 1).

Yeast strains used in this work were Isoy16 (Δ*ilv2* Δ*ilv5* Δ*ilv3)* and Isoy17 (Δ*ilv2* Δ*ilv5* Δ*ilv3* with unknown beneficial mutations for growth on media lacking valine)

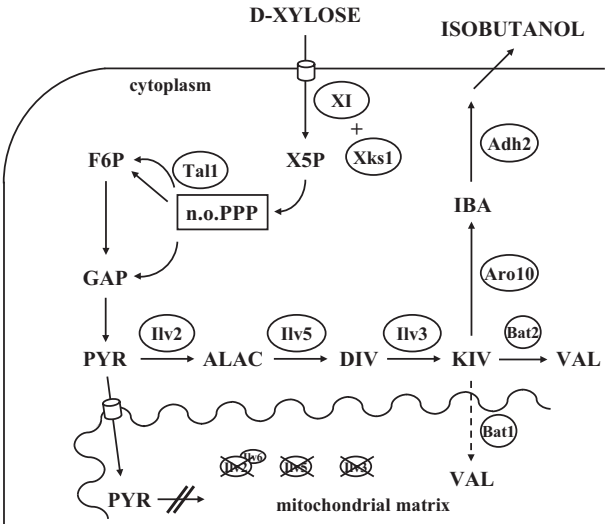

Fig. 1. Schematic illustration of isobutanol production from D-xylose. The pentose sugar D-xylose is converted by xylose isomerase (XI) and xylulokinase (Xks1) to xylulose-5-phosphate (X5P). X5P can be further converted via the non-oxidative pentose phosphate pathway (n.o.PPP) including transaldolase (Tal1) to intermediates of glycolysis to provide pyruvate (PYR). PYR is metabolized to 2-ketoisovalerate (KIV) via the cytosolic ILV pathway, which consists of N-terminally truncated enzymes Ilv2, Ilv5 and Ilv3. Finally, KIV can be either transaminated by branched-chain amino acid aminotransferase Bat1 or Bat2 to valine (VAL) or KIV can be decarboxylated to isobutyraldehyde (IBA) by Aro10 and finally reduced to isobutanol by Adh2. F6P = fructose -6-phosphate; GAP = glyceraldehyde-3-phosphate; ALAC = 2-acetol actate; DIV = 2,3-dihydroxyisovalerate.

(Brat *et al.*, 2012), both derived from CEN.PK2-1C. Plasmids expressing wild-type Ilv2, Ilv5, Ilv3, Aro10 and Adh2 were p426H7-ILV2wt, p423H7-ILV5wt, pRS42KH7-ILV3wt and pRS42HH7-Aro10-Adh2, respectively (Brat *et al.*, 2012). Empty control vectors were p424H7, p426H7, pRS42K, pRS42N, pRS42HH7 (Becker & Boles, 2003; Taxis & Knop, 2006; Brat *et al.*, 2012). The plasmid expressing truncated codon-optimized ORFs *ILV2ΔN54*, *ILV5ΔN48* and *ILV3ΔN19* was p425-synthILV235 (Brat *et al.*, 2012). *Saccharomyces cerevisiae* was grown in selective medium (1.7 g L^{-1} Difco yeast nitrogen base without amino acids and with 5 g L^{-1} ammoniumsulfate), supplemented with amino acids but omitting the selective plasmid marker nutrients (SC) (Zimmermann, 1975). Compared to SC media, SM media contained only the auxotrophic requirements. As carbon sources, media contained either 20 g L^{-1} D-glucose (SCD, SMD) or 20 g L^{-1} D-xylose (SCX, SMX). In the growth assays with Isoy16 transformants, 5 g L^{-1} leucine + 5 g L^{-1} isoleucine + 5 g L^{-1} valine were used as nitrogen source and to complement the branched-chain amino acids requirement. To test the performance of the cytosolic valine/isobutanol pathway on D-xylose valine was omitted. For maintenance of plasmids with resistance markers, media containing antibiotics were used (geneticin 200 mg L^{-1}, hygromycin B 200 mg L^{-1}, nourseothricin 100 mg L^{-1}). "Selective medium" means medium without auxotrophic requirements or with antibiotics for plasmid selection.

The codon-optimized *xylA*-open reading frame (ORF) from *C. phytofermentans* (Brat *et al.*, 2009) was cloned via homologous recombination into the multicopy vector p424H7 (*TRP1* marker), resulting in plasmid p424H7-synthXI, and the *TAL1*-ORF together with the *XKS1*-ORF into the multicopy vector pRS42NH7 (Brat *et al.*, 2012) (*natNT2* nourseothricin resistance marker), resulting in plasmid pRS42NH7-Tal1-Xks1. *XylA* and *TAL1* were cloned between the strong and constitutive *HXT7* promoter fragment and the *CYC1* terminator, whereas the *XKS1*-ORF was placed between the *PFK1* promoter and *FBA1* terminator. The *ARO10*-ORF and *ADH2*-ORF were cloned together into the multicopy plasmid pRS42HH7 (Brat *et al.*, 2012) containing the *hphNT1* hygromycin B resistance marker, resulting in pRS42HH7-Aro10-Adh2. *ARO10* was placed between the *FBA1* promoter and *PGK1* terminator, *ADH2* between the constitutive *HXT7* promoter fragment and *FBA1* terminator. Plasmid construction, re-isolation, amplification in *Escherichia coli* and verification were carried out essentially as described before (Brat *et al.*, 2012). Metabolites produced during fermentations were analyzed by HPLC and static headspace GC-MS (Brat *et al.*, 2012).

To test whether D-xylose could be utilized as the sole carbon and energy source and could also be used by the cytosolically located valine pathway, the triple *ilv2,3,5* deletion mutant Isoy16 was transformed with the plasmids p424H7-synthXI, pRS42NH7-Tal1-Xks1 and p425-synthILV235 overexpressing *xylA*, *TAL1*, *XKS1* and *ILV2ΔN54*, *ILV5ΔN48* and *ILV3ΔN19*. As control, plasmids expressing wild-type Ilv enzymes as well as empty vectors were transformed instead of p425-synthILV235. Transformants were selected on selective SC agar plates containing 20 g L^{-1} D-glucose. To test whether D-xylose could be used as the sole carbon source and could also complement the valine auxotrophy via the cytosolic valine pathway, growth assays were performed (Fig. 2). Transformants were collected from SMX + leu + ile agar plates and resuspended in sterile water to an $OD_{600\ nm}$ of 1. Cell suspensions were serially diluted in 10-fold steps, and 7 μL of each dilution was spotted on selective SMD + leu + ile + val and on selective SMX + leu + ile agar plates, either with or without valine, and incubated for 7 days at 30 °C (Fig. 2). The results show that the yeast cells could utilize D-xylose as the sole carbon source and could metabolize it into valine via the mitochondrial (Ilv2wt + Ilv5wt + Ilv3wt) as well as the cytosolic (p425-synthILV235) valine pathway.

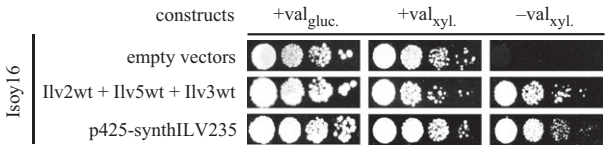

Fig. 2. Growth complementation test of valine auxotrophic mutants on D-xylose medium. The different Isoy16 (*Δilv2, Δilv3, Δilv5*) transformants, all additionally overexpressing *XKS1*, *TAL1* and codon-optimized *xylA* from *C. phytofermentans*, were pre-grown on selective SMX + leu + ile -val medium (empty vector control with valine), washed with sterile water and spotted in serial dilutions on selective SM + leu + ile agar plates with either 20 g L^{-1} D-glucose (gluc.) or D-xylose (xyl.), with or without valine, and incubated for 7 days at 30 °C.

To investigate isobutanol production from D-xylose, strain Isoy17 was transformed with the plasmids expressing the xylose and valine pathways, and additionally with the plasmid pRS42HH7-Aro10-Adh2 expressing Aro10 keto-acid decarboxylase and Adh2 alcohol dehydrogenase (Brat *et al.*, 2012). Aerobic fermentation experiments were performed in 500-mL shake flasks. Transformants were pre-grown in selective SCX medium without valine (empty vector control with valine), harvested and used to inoculate SCX medium without valine in shake flasks at an $OD_{600\ nm}$ of 1 (Fig 3a). During the fermentations, after taking samples for metabolite analyses equivalent volumes of fresh medium were added to cultures to compensate for volume losses. Fermentations were performed up to 146 h in triplicate using different precultures (Fig. 3). Metabolite analysis revealed that D-xylose was used as a carbon source but was not completely consumed (Fig. 3b). The cells expressing the codon-optimized cytosolic isobutanol pathway (p425-synthILV235) consumed about 12 g D-xylose and produced up to 1.36 ± 0.11 mg L^{-1} isobutanol with a yield of 0.16 ± 0.04 mg isobutanol per g D-xylose (Fig. 3c). Additionally, ethanol was produced but only in low amounts (0.415 ± 0.065 g L^{-1} with a yield of 0.040 ± 0.014 g ethanol per g D-xylose) due to the low D-xylose utilization rate and the aerobic conditions (Fig. 3d). Accordingly, most of the consumed D-xylose was probably respired. In comparison, cells overexpressing wild-type Ilv2, Ilv5 and Ilv3 enzymes and those with the empty vector did not produce neither any isobutanol nor ethanol.

Our results show that yeast cells expressing a cytosolically located isobutanol pathway together with a D-xylose degradation pathway are able to convert D-xylose into valine and isobutanol. As the yeast cells have not yet been optimized by further genetic or evolutionary engineering which is crucial for efficient D-xylose utilization (Kuyper *et al.*, 2005a,b), the isobutanol production rates and titers from D-xylose are still low. Nevertheless, the results dem-

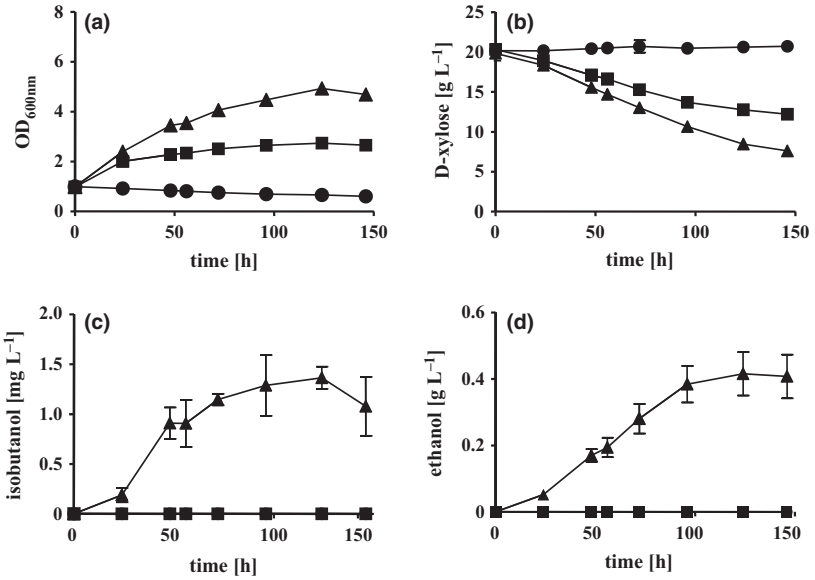

Fig. 3. Isobutanol production from D-xylose with recombinant *S. cerevisiae* cells. Fermentation experiments were performed aerobically at 30 °C in shake flasks in selective SC-val medium containing 20 g L^{-1} D-xylose. All transformants contained plasmids overexpressing the codon-optimized *xylA* gene from *C. phytofermentans*, *XKS1*, *TAL1*, *ARO10* and *ADH2*. Additionally, transformants contained plasmids overexpressing wild-type *ILV2*, *ILV5* and *ILV3* (closed squares), codon-optimized truncated *ILV2ΔN54*, *ILV5ΔN48* and *ILV3ΔN19* (closed triangles) or the corresponding empty vectors (closed circles) (in c and d closed circles overlap with closed squares). Experiments were performed in triplicate with given standard deviations (in a and b standard deviations were within the area of the symbols). a–d: $OD_{600\ nm}$ (a), D-xylose consumption (b), isobutanol production (c) and ethanol production (d).

onstrate the feasibility of isobutanol production from lignocellulosic sugars. Further optimization of the pentose phosphate pathway together with elimination of competing pathways (e.g. pyruvate decarboxylase) should greatly enhance isobutanol production from pentoses.

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