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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

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Xylitol production by a *Pichia stipitis* D-xylulokinase mutant

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Abstract Xylitol production by *Pichia stipitis* FPL-YS30, a *xyl3-Δ1* mutant that metabolizes xylose using an alternative metabolic pathway, was investigated under aerobic and oxygen-limited culture conditions. Under both culture conditions, FPL-YS30 (*xyl3-Δ1*) produced a negligible amount of ethanol and converted xylose mainly into xylitol with comparable yields (0.30 and 0.27 g xylitol/g xylose). However, xylose consumption increased five-fold under aerobic compared to oxygen-limited conditions. This suggests that the efficiency of the alternative route of xylose assimilation is affected by respiration. As a result, the FPL-YS30 strain produced 26 g/l of xylitol, and exhibited a higher volumetric productivity (0.22 g xylitol l^{-1} h^{-1}) under aerobic conditions.

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Introduction

Xylose is the second most abundant sugar in nature (Pettersen 1984). Therefore, its degradation into CO_2 is important for carbon cycling. Recently, the conversion of xylose into value-added chemicals, such as xylitol, ethanol, and lactic acid, has become attractive to the fermentation industry (Lynd et al. 1999). In particular, the bioconversion process for xylitol production has been intensively studied during the last decade because xylitol can be used as a functional sweetener (Emidi 1978). So far three types of yeasts are known to produce xylitol. First, there are wild type xylose assimilating yeasts, such as *Candida boindii* (Vandeska et al. 1995), *C. guilliermondii* (Meyrial et al. 1991), *C. tropicalis* (Kim et al. 1999), *C. parapsilosis* (Oh et al. 1998), and *Debaryomyces hansenii* (Cruz et al. 2002), Second, recombinant *Saccharomyces cerevisiae* (Hallborn et al. 1991; Lee et al. 2003; Meinander and Hahn-Hagerdal 1997) containing *XYL1* from *Pichia stipitis* is known to convert xylose into xylitol. Third, mutant strains of *Pichia stipitis* also produce xylitol from xylose. Cho and Jeffries reported the accumulation of xylitol by *P. stipitis* alcohol dehydrogenase (*ADH*) disrupted mutants (Cho and Jeffries 1998) and (Kim et al. 2001) reported xylitol production with a xylitol dehydrogenase defective mutant of *P. stiptis*.

We disrupted the *XYL3* gene coding for D-xylulokinase in *P. stipitis*. The resulting strain, FPL-YS30 (*xyl3-Δ1*) utilized xylose slowly and accumulated a significant amount of xylitol in the medium (Jin et al. 2002). A metabolic pathway via arabinitol and ribulose-5-phosphate, bypassing the xylulokinase step, was proposed as an alternative pathway mediating xylose assimilation in the xylulokinase mutant. This alternative pathway consists of xylose reductase, xylitol dehydrogenase, arabinitol dehydrogenase, and ribulokinase reactions. This pathway is also involved in L-arabinose assimilation in *P. stipitis* (Fig. 1). In the present study, we investigated the capability of the alternative pathway for xylitol production by culturing the FPL-YS30 strain under aerobic and oxygen-limited conditions.

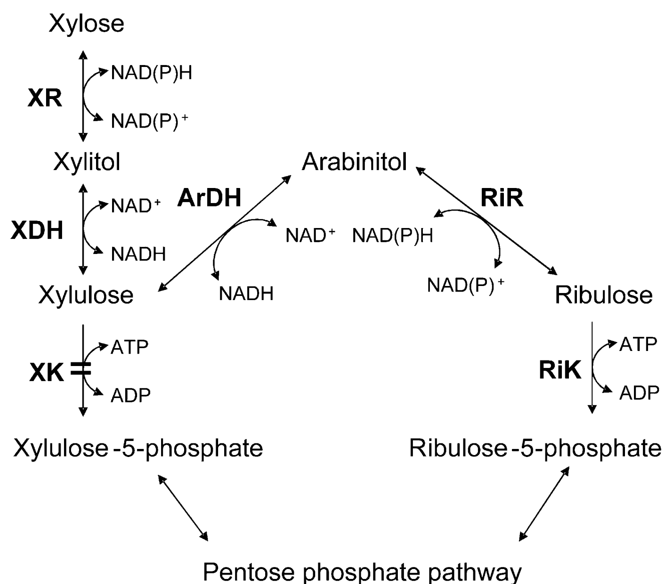

Fig. 1 Alternative route of xylose assimilation in *Pichia stipitis* FPL-YS30 (Δ*xyl3*). XR Xylose reductase; XDH xylitol dehydrogenase; XK xylulokinase; ArDH arabinitol dehydrogenase; RiR ribulose reductase; RiK ribulokinase

Materials and methods

Microorganisms and fermentation conditions

P. stipitis strains FPL-UC7 (*ura3-2*) (= NRRL Y-21448) (Lu et al. 1998) and *P. stipitis* FPL-YS30 (*xyl3-Δ1*) (*xyl3::URA3*) (=NRRL Y-30785) (Jin et al. 2002) were used in this study. Yeast strains were maintained at 4°C on agar plates of YPD (10 g/l of yeast extract, 20 g/l of Bactopeptone, and 20 g/l of glucose) medium. YPD medium was used for inoculum preparation. YP medium (10 g/l of yeast extract, 20 g/l of Bactopeptone) with 100 g/l of xylose was used for xylose fermentation. Fermentation experiments were carried out at 30°C in 50 ml of medium in a 125 ml Erlenmeyer flask. Cells were grown in two different culture conditions, i.e. oxygen-limited and aerobic conditions by agitating at 100 and 200 rpm, respectively.

Analytical methods

Xylose, xylitol, and ethanol concentrations were determined by HPLC (HP, Wilmington, Del.) using an ION 300 column (Interaction Chromatography, San Jose, Calif.) with 5 mM sulfuric acid solution as a mobile phase at a flow rate of 0.6 ml/min. Cell growth was monitored by optical density at 600 nm (OD600). One OD600 at 600 nm was equivalent to 0.21 g cell/l for *P. stipitis*.

Results

To investigate characteristics of the alternative pathway for xylose assimilation that bypasses xylulokinase, we cultured FPL-YS30 (*xyl3-Δ1*), and its parental strain, *P. stipitis* UC7 (*ura3*), in YP medium with 100 g/l of xylose under two different conditions (100 and 200 rpm). Overall, FPL-YS30 consumed xylose very slowly compared to *P. stipitis* UC7 at 100 rpm (Fig. 2). During the first 40 h FPL-YS30 consumed xylose faster than *P. stipitis* UC7 (Fig. 3), but the maximum specific xylose consumption rates of FPL-YS30 were only 17% and 41% of *P. stipitis* UC7 cultured with aeration at 100 and 200 rpm, respectively. FPL-YS30 consumed only 18 g/l of xylose with aeration at 100 rpm whereas it consumed 99 g/l of xylose with aeration at 200 rpm within the 120 h of the fermentation experiment. *P. stipitis* UC7 mainly produced ethanol from xylose with yields of 0.40 and 0.32 g ethanol/g xylose with aeration at 100 and 200 rpm, respectively. In contrast, ethanol production from xylose by FPL-YS30 was negligible (less than 2 g/l) under both conditions. FPL-YS30 produced mainly xylitol from xylose with a yield of 0.30 and 0.27 g xylitol/g xylose at aeration at aeration rates of 100 and 200 rpm whereas xylitol production by *P. stipitis* UC7 under the same conditions was almost negligible (less than 3 g/l). Xylitol accumulation by FPL-YS30 began as soon as 20 h into the fermentation experiments, which can be regarded as fully aerobic conditions (Figs. 2b, 3b). In contrast, ethanol was not produced by *P. stipitis* UC7 within that same time period under the same conditions (Figs. 2a, 3a).

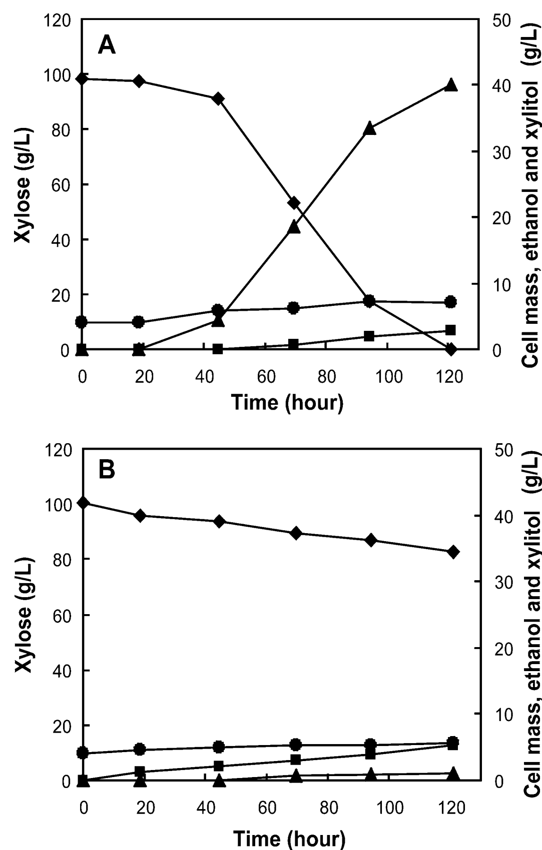

Fig. 2 Fermentation profile of *P. stipitis* UC7 (*ura3*) (**a**) and FPL-YS30 (*xyl3-Δ1*) (**b**) under oxygen-limited-conditions (100 rpm). Symbols: ◆ xylose; ■ xylitol; ▲ ethanol; ● biomass

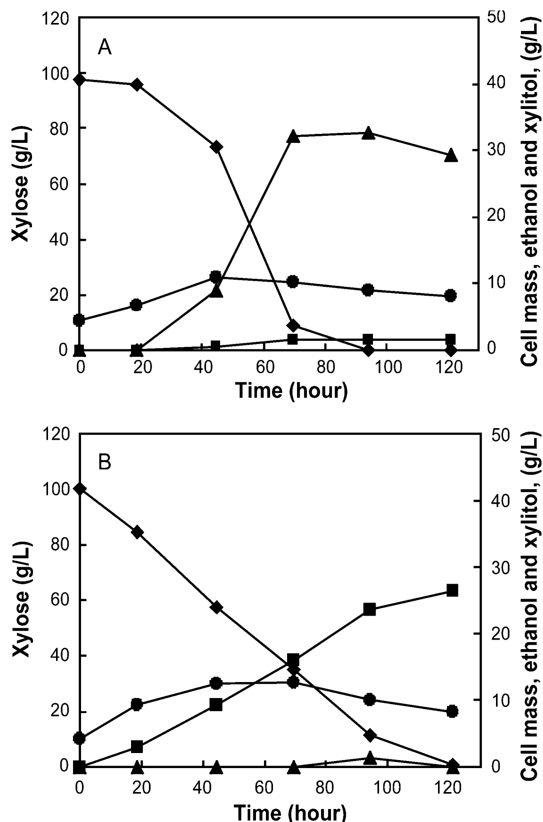

Fig. 3 Fermentation profile of *P. stipitis* UC7 (**a**) and FPL-YS30 (*xyl3*-Δ1) (**b**) under aerobic conditions (200 rpm). Symbols: ◆ xylose; ■ xylitol; ▲ ethanol; ● biomass

Discussion

We suggested that FPL-YS30 (*xyl3-Δ1*) uses an alternative pathway to metabolize xylose because it did not show any detectable D-xyluokinase activity yet it was still able to grow on xylose (Jin et al. 2002). Based on xylose consumption profiles, the metabolic capacity of the alternative pathway was inferior to the main pathway. This suggests that the xylulokinase pathway is mainly responsible for xylose consumption in wild type *P. stipitis*. This is also supported by previous results that D-xyulokinase activity is three fold higher than D-ribulokinase activity in *P. stipitis* UC7 grown on xylose (Jin et al. 2002). Although xylose metabolism via either pathway is aeration-dependent, the alternative pathway required a higher aeration rate than the main pathway. The mutant did not produce any ethanol even though it could grow using the alternative pathway. This was similar to our earlier observation that *P. stipitis* produced very little ethanol from L-arabinose (Shi et al. 2000) even though it could grow slowly on this sugar.

Kim et al. (2001) reported an increase in xylitol production by a *P. stipitis* xylitol dehydrogenase mutant. Cho and Jeffries (Cho and Jeffries 1998) also reported that *P. stipitisPsADH* disruptants produce xylitol. Although these mutants exhibit similar phenotypes in terms of xylitol production, metabolic mechanisms underlying these similar phenotypes are distinct. First, the xylitol dehydrogenase mutant requires co-substrates, which support growth and regeneration of cofactor, to produce xylitol from xylose since its xylose metabolic pathway blocked. By comparison, *adhΔ* and *xyl3Δ* mutants can produce xylitol from xylose as a sole carbon source. Second, the xylitol dehydrogenase mutant and the *xyl3-Δ1* mutant accumulate xylitol because of direct disruption in xylose metabolic pathway, but *adhΔ* mutants are thought to produce xylitol because of indirect metabolite interactions through cytosolic NADH. Third, *adhΔ* mutants prefer oxygen-limited conditions for xylitol production, like most xylitol producing yeast strains, but the *xyl3-Δ1* mutant exhibited the same xylitol yield independent of aeration. This suggests that xylitol accumulation by *adhΔ* mutants is mainly caused by redox imbalance in the cytosol under oxygen-limited conditions. In contrast, the YS30 (*xyl3-Δ1*) mutant produced xylitol under the same conditions that its parent produced ethanol. These results suggest that FPL-YS30 (*xyl3-Δ1*) produces xylitol mainly because of insufficient glycolytic capacity of the alternative pathway rather than because of a redox imbalance. This also indicates that the oxidative phase of the PPP, which produces NADPH for xylitol accumulation, is sufficient even without activity coded for by *XYL3*. This is a new class of *P. stipitis* mutants that accumulate xylitol during xylose fermentation by a different metabolic mechanism compared to mutants reported previously.

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