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Increased Xylose Reductase Activity in the Xylose-Fermenting Yeast *Pichia stipitis* by Overexpression of *XYL1*

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ABSTRACT

The *Pichia stipitis* xylose reductase gene *(XYL1)* was inserted into an autonomous plasmid that *P. stipitis* maintains in multicopy. The plasmid pXOR with the *XYL1* insert or a control plasmid pJM6 without *XYL1* was introduced into *P. stipitis*. When grown on xylose under aerobic conditions, the strain with pXOR had up to 1.8-fold higher xylose reductase (XOR) activity than the control strain. Oxygen limitation led to higher XOR activity in both experimental and control strains grown on xylose. However, the XOR activities of the two strains grown on xylose were similar under oxygen limitation. When grown on glucose under aerobic or oxygen-limited conditions, the experimental strain had XOR activity up to 10 times higher than that of the control strain. Ethanol production was not improved, but rather it decreased with the introduction of pXOR compared to the control, and this was attributed to nonspecific effects of the plasmid.

Index Entries: *Pichia stipitis*; xylose reductase; gene expression; fermentation; metabolism.

INTRODUCTION

Over the past decade, considerable research has focused on the metabolism of xylose, and strains of microorganisms, which can ferment xylose, have been identified or developed *(1–13)*. D-Xylose is a primary component of biomass hemicellulosic materials, and it is an excellent substrate for fuel production because it can be

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fermented to ethanol. In the long-term view, environmentally friendly renewable fuels could displace fossil fuels used in transportation.

Pichia stipitis, Candida shehatae, and *Pachysolen tannophilus* are three yeast species generally accepted as the best wild-type xylose fermenters *(1)*. In *P. stipitis*, oxygen levels greatly influence the fate of xylose. Under fully aerobic culture conditions, where oxygen is in excess, no fermentation occurs *(14)*. Ethanol is produced in culture only during oxygen limitation *(15)*. During oxygen limitation, yeast cells grow more slowly and use less xylose for respiration *(16)*. Skoog and Hahn-Hägerdal *(17)* demonstrated that pyruvate decarboxylase (PDC) activity in *P. stipitis* increases with declining oxygen uptake. Alexander et al. *(18)* showed that in a similar yeast, *C. shehatae*, alcohol dehydrogenase (ADH) activity is elevated when oxygen availability declines. Other enzymes are probably also regulated during the transition from aerobic conditions to oxygen limitation.

Efforts have been made to broaden the substrate range of *Saccharomyces cerevisiae*, a high-productivity glucose fermenter that is incapable of xylose utilization. The *Escherichia coli* gene for xylose isomerase (XOI), an enzyme that directly converts xylose into xylulose, was integrated into *S. cerevisiae*, but the protein was inactive in *S. cerevisiae (4)*. When the *E. coli* gene for XOI was incorporated into *Schizosaccharomyces pombe*, active protein was produced, but xylitol formation and low ethanol productivity were problems *(2)*.

The major enzymes involved in xylose assimilation (xylose reductase [XOR], xylitol dehydrogenase [XDH], and xylulose kinase [XUK]) have been investigated *(19)*. XOR and XDH activities are induced in *P. stipitis, P. tannophilus,* and *C. shehatae* when these yeast are grown on xylose *(18,20)*. The gene for XUK from *S. cerevisiae* has been cloned and introduced back into *S. cerevisiae* on a high-copy-number plasmid, resulting in an increased level of XUK activity *(5)*. The gene for XOR was cloned from *P. stipitis* and introduced into *S. cerevisiae*, resulting in XOR activity *(6,8,10)*. This recombinant *S. cerevisiae* strain could neither grow on nor ferment xylose, but it did produce xylitol. Recently, *P. stipitis* genes for both XOR and XDH were introduced into *S. cerevisiae*, resulting in the expression of active protein products and production of ethanol in low titers *(11,12)*.

P. stipitis does not produce ethanol under fully aerobic conditions, but its ethanol yield improves and approaches theoretical as conditions become oxygen limited *(21)*. However, the growth rate of this yeast is significantly reduced when the culture is oxygen limited *(22)*. To increase ethanol production, it would be useful to develop a *P. stipitis* strain in which relevant fermentative enzymes are expressed on induction by xylose, rather than being oxygen regulated. We have begun to investigate the regulation of the *XYL1* promoter in *P. stipitis*, and we have overexpressed *XYL1* in *P. stipitis*. This strain has elevated levels of XOR activity, and it was analyzed for its ability to ferment xylose and glucose separately.

MATERIALS AND METHODS

Microbial Strains

E. coli DH1 (*recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17, *supE*44, *relA*1) or *E. coli* DH5α (*recA*1 *endA*1 *hsdR*17 *supE*44 *thi*-1 *gyrA*96 *relA*1 Δ*lacU*169[Ø80*lacZ*ΔM15]) were used as bacterial hosts for plasmids. *P. stipitis ura3* strain TJ26 *(23)* was used as the recipient strain in yeast transformations.

Media

The minimal medium used in yeast fermentation analysis contained 6.5 g/L urea, 10% glucose or xylose, and 1.7 g/L yeast nitrogen base without amino acids or ammonium sulfate (Difco, Detroit, MI) at pH 6.6. *E. coli* was cultivated in Luria-Bertani medium. When selection was required for *E. coli*-harboring plasmids, 50 μg/mL ampicillin was added to the medium.

DNA Manipulations and Molecular Biological Enzymes

Yeast genomic DNA was isolated and purified as described by Rose et al. *(24)*, except Novozyme 234 was used instead of Zymolyase 100,000. Bacterial plasmids were purified according to Sambrook et al. *(25)* or by the Wizard DNA purification systems from Promega (Madison, WI). Restriction enzymes were obtained from Promega or New England Biolabs (Beverly, MA), and digestions were performed as recommended by the suppliers. Restriction fragments were purified from low melting agarose by phenol extraction.

Cell Growth

Inoculum (100 mL) was grown in a 250-mL baffled flask at 30°C for 72 h shaken at 150 rpm. The cells were harvested by centrifugation, resuspended in fresh medium, and used to inoculate shake flasks. To obtain oxygen-limited conditions, 100 mL of medium was put in a foam-plugged 125-mL flask. For the aerobic cultures, 75 mL of medium was placed in a 250-mL baffled flasks. Four flasks were used for each condition, so that cells could be harvested at four different time points. Glucose and xylose fermentations of both strains under the different aeration conditions were done side by side in a single incubator/shaker. The fermentations were done at 30°C with continuous shaking at 180 rpm.

Fermentation Analysis

Cells from each condition and sugar source were harvested every day for 4 d at indicated times. A small amount of culture was taken immediately before harvest, and the optical density of the sample was determined using a Beckman DU spectrophotometer at 600 nm.

The cells were collected by centrifugation. The supernatant was passed through a 0.2- or 0.45-μm pore Nalgene filter, and stored at –20°C for later gas chromatographic and high-performance liquid chromatography (HPLC) analysis. The cells were then washed with water and the volume was split in two. One-half was washed with 1*M* sorbitol for later DNA analysis. The other half was washed with 0.1*M* MOPS (pH 6.8) for later enzyme activity analysis. The cells were then quick-frozen in liquid nitrogen and stored at –80°C.

Preparation of Cell Extracts and XOR Activity Assays

Cell pellet (0.5 mL) slurry in 0.1*M* MOPS (pH 6.8) buffer was added to 0.5 g of 425–600 μ glass beads (Sigma, St. Louis, MO) in a borosilicate glass culture tube. Cells were vortexed vigorously for five 1-min bursts, with cooling in crushed ice for 1–2-min in between bursts. Homogenates were centrifuged at 13,800*g* for 20 min at 4°C, and the supernatant solution was transferred to a new tube. Within 4 h of cell breakage, the xylose-dependent oxidation of reduced nicotinamide adenine

dinucleotide phosphate (NADPH) was followed at 340 nm, 30°C, according to a modification of the protocol by Chiang and Knight *(26)*. The assay mixture contained 0.1*M* Tris-HCl (pH 7.2), 5–50 µL cell extract, and 100 µL $1.5 \times 10^{-3}M$ NADPH in a total volume of 900 µL. The reaction was started with the addition of 100 µL 0.1*M* xylose.

Protein Assays

Total protein was measured by the bicinchoninic acid assay (Pierce, Rockford, IL) using bovine serum albumin as standard.

Southern Blot Analysis

Southern transfer was performed by capillary blotting *(27)*. DNA hybridizations were done with a Genius 1 kit (Boehringer Mannheim, Indianapolis, IN) as described by the manufacturer. Zeta-Probe GT membrane (Bio-Rad, Hercules, CA) was used to crosslink the nucleic acids. Hybridizations were typically done in 25% formamide at 37°C. The 65°C washing times were shortened from 15 to 5 min.

Transformation

Electrotransformation of *P. stipitis* was performed as previously described *(23)*. Transformation of *E. coli* was done by electroporation with a Gene Pulser together with a Gene Controller apparatus and cuvets from Bio-Rad according to the manufacturer's protocol.

Densitometry

Densitometry was done using a Hewlett-Packard Desk Scanner with the Quantitative Images program (National Institutes of Health, Washington, DC) on a Macintosh II computer.

HPLC

The sugars, ethanol, and byproducts of fermentation were separated by HPLC (Hewlett Packard series 1050, Wilmington, DE) using a Bio-Rad carbohydrate column (HPX 87C, 300 × 7.8 cm) maintained at 85°C. The mobile phase was degassed distilled water at a flow rate of 0.5 mL/min at a pressure of 50–55 bar. The clear filtered sample (980 µL) was mixed with 20 µL of sucrose (500 g/L) as an internal standard before injection. The sugars and ethanol were quantitated by a refractive index (RI) detector.

Gas Chromatography (GC)

Ethanol was separated by GC (Hewlett Packard series 2, model 5890) using Porapack Q column from Alltech (Deerfield, IL) with the initial and final oven temperature at 180°C. The detector temperature was 220°C, and the injector temperature was 200°C. The carrier gas was helium, and a flame ionization detector was used.

RESULTS

pTN131 *(10)* was cleaved with *Bam*HI restriction enzyme, and the approx 5.5-kb DNA fragment containing the *P. stipitis XYL1* gene was isolated and purified. This

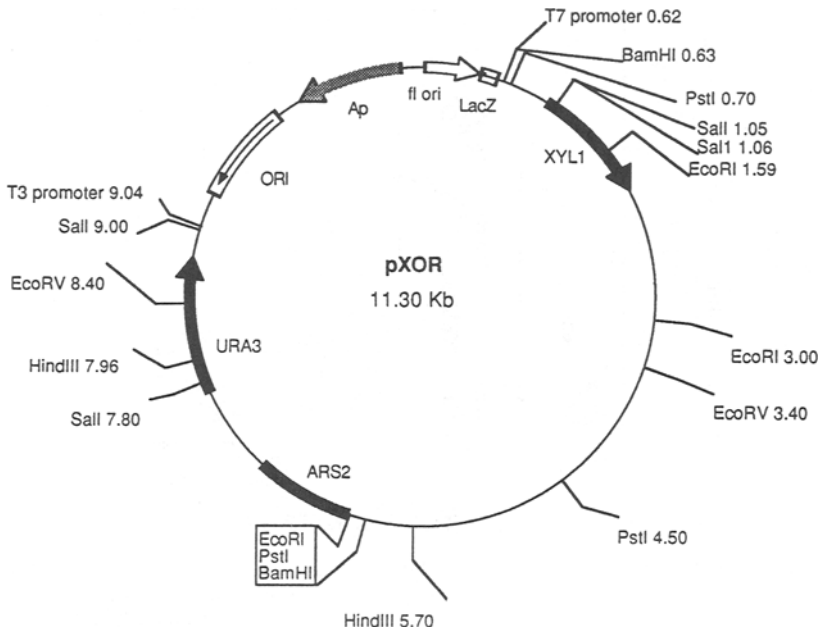

Fig. 1. Physical map of plasmid pXOR containing the *P. stipitis* xylose reductase gene *(XYL1)*.

fragment was inserted at the unique *Bam*HI site in pJM6 *(23)*, creating pXOR (Fig. 1). Uncut pXOR was introduced into *P. stipitis* TJ26 *(ura3-1)*, and a Ura$^+$ colony harboring pXOR as an autonomous plasmid was isolated (TJ26 [pXOR]). To obtain a control strain, a TJ26 Ura$^+$ colony harboring pJM6 as an autonomous plasmid was isolated (TJ26 [pJM6]).

TJ26 (pXOR) had about 1.8-fold higher XOR activity than TJ26 (pJM6) after 1 d of growth in xylose medium under aerobic conditions (Fig. 2). At the same time point under oxygen-limited conditions, the XOR activities in both the pXOR strain and the control strain were higher, and the activities of the two strains were similar. After 2 d of xylose fermentation (Fig. 3), the XOR activities of both strains at both levels of aeration decreased and were similar.

Introduction of the pXOR plasmid increased XOR activity levels even when glucose was the sole carbon source. After 1 d of growth in glucose medium with either high or low levels of aeration, TJ26 (pXOR) showed up to 10 times higher XOR activity than the control (Fig. 2). The pXOR strain continued to show higher XOR activity on glucose after 2 d of fermentation (Fig. 3).

Following 4 d of fermentation, ethanol production by the pXOR strain was compared to that of the control (Fig. 4). Surprisingly, the ethanol production of the pXOR strain was lower than the pJM6 strain under the conditions tested. In fact, the pJM6 strain consistently produced more ethanol than the pXOR strain on each of the 4 d of fermentation (data not shown). The shake-flask fermentations were repeated, producing similar trends in XOR activity and ethanol production (data not shown).

The cell growth of TJ26 (pJM6) and TJ26 (pXOR) during the shake-flask fermentation was determined spectrophotometrically. Optical density at 600 nm was

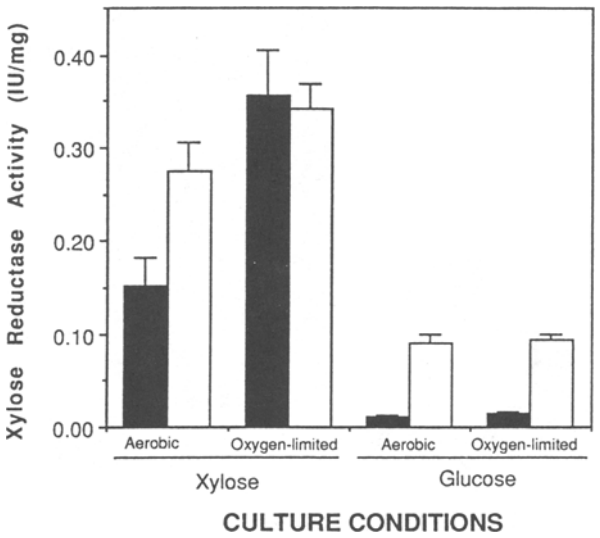

Fig. 2. Xylose reductase activity in the TJ26::pJM6 control strain (■) and in the *P. stipitis* TJ26::pXOR strain (□) after 1 d of growth in minimal medium. The aerobic cultures were harvested at 24 h postinoculation, and the oxygen-limited cultures were harvested after 27 h postinoculation. Error bars show the standard deviation of at least three replicate assays from the same sample, taken from a representative shake-flask experiment. Values are given in IU/mg of total protein in the cell extract.

determined from the shake flasks at the time of harvest, one flask for each condition at each time point (data not shown). Optical density was also monitored daily from the shake flask harvested at the final time point. The growth patterns of the pJM6 strain and the pXOR strain were compared and found to be similar, except the pJM6 strain grew to a higher cell density when grown on xylose under oxygen-limited conditions. In other repetitions, the pJM6 strain grew to a higher cell density under all conditions tested.

We compared carbohydrate utilization by the two strains under aerobic and oxygen-limited conditions (data not shown). Under both conditions, TJ26 (pXOR) used less sugar than TJ26 (pJM6) when grown on xylose. The glucose utilization patterns of the two transformants varied with the plasmid and the aeration conditions employed. No direct correlation between xylose or glucose consumption and ethanol production was seen.

In order to determine the copy number of plasmids pJM6 and pXOR, we harvested cells from one experiment (the same experiment from which all the other results shown were obtained) daily. Total DNA was extracted from the cells grown under the high aeration condition and digested to completion with *Bam*HI restriction enzyme. The DNA fragments were separated by agarose gel electrophoresis and analyzed by Southern blotting. Probing with *P. stipitis URA3* yielded two bands of different sizes, one band from the native genomic copy of the gene and the other band from the plasmid-borne copy of the gene. Copy number was calculated by comparing the intensity of the two bands using densitometry. Between one and eight copies of the plasmid (either pJM6 or pXOR) were found per cell under the conditions used in this experiment.

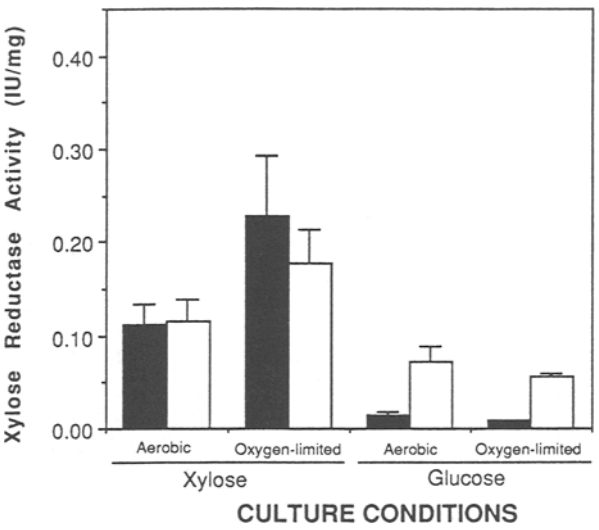

Fig. 3. Xylose reductase activity in the TJ26::pJM6 control strain (■) and in the *P. stipitis* TJ26::pXOR strain (□) after 2 d of growth in minimal medium. The aerobic cultures were harvested at 46.5 h postinoculation, and the oxygen-limited cultures were harvested after 42.5 h postinoculation. Error bars show the standard deviation of at least three replicate assays from the same sample, taken from a representative shake-flask experiment. Values are given in IU/mg of total protein in the cell extract.

DISCUSSION

When the xylose reductase gene *(XYL1)* from *P. stipitis* was introduced into *P. stipitis* TJ26 *(ura3)* on a multicopy plasmid, XOR activity increased when the transformant was grown on glucose as a sole carbon source. Introduction of pXOR also increased XOR activity in aerobically grown cells with xylose as a sole carbon source. These increases can be explained by an increase in the copy number of *XYL1*. Oxygen levels did not affect the XOR activities of either the pXOR or pJM6 strains when grown on glucose. When grown on xylose, both strains had higher XOR activity when limited for oxygen. However, under oxygen limitation, the XOR activities of the two strains grown on xylose were similar. Under oxygen limitation, there are likely other levels of regulation in addition to gene dosage for expression of *XYL1*. It is possible that the XOR activity of *P. stipitis* is regulated not only at the level of transcription, but also at the level of translation and post-translationally by such factors as other gene products and metal ions.

Ethanol production was not increased by overexpression of *XYL1*, suggesting that either XOR is not rate limiting under the conditions employed for the strain tested or that any benefit resulting from overexpression XOR was countered by negative effects owing to the multicopy plasmid. Introduction of pXOR and the resulting increase in XOR activity could alter the metabolism of the *P. stipitis* in an unanticipated manner, resulting in lower ethanol production, slower growth, and decreased carbohydrate utilization. For example, in some *E. coli* strains bearing multicopy plasmids, changes in cell growth were seen and postulated to occur owing to interaction of the host cell machinery with the plasmid and plasmid

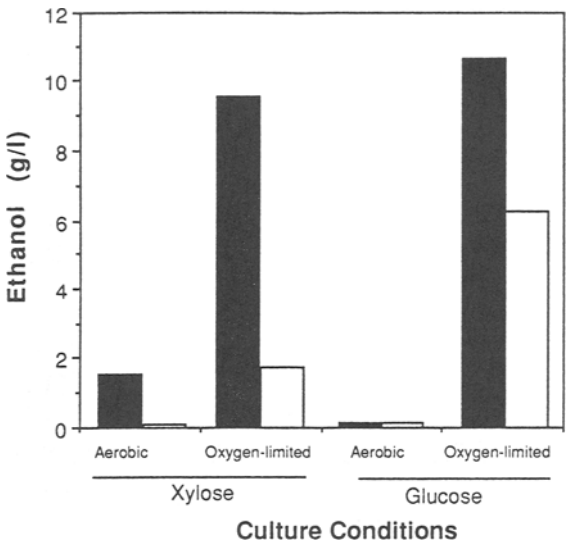

Fig. 4. Quantification of ethanol in culture supernatant after 4 d of a representative fermentation by *P. stipitis* TJ26 strains harboring plasmid pJM6 (■) or pXOR (□). The aerobic culture samples were taken at 92 h postinoculation. The oxygen-limited samples were taken at 95.75 h postinoculation. The ethanol concentrations shown were determined from the shake flask supernatant samples at the time of harvest, one flask for each condition at each time point.

gene products *(28,29)*. pXOR is about twice the size of pJM6, this difference might account in part for the poor fermentative performance of the TJ26 (pXOR) strain.

The *XYL1* DNA fragment used to construct pXOR is large, and different results might be obtained if less of the *XYL1* 3'-flanking region were used. The genetic background of the host strain also determines how gene overexpression affects metabolism. We know from other studies that *P. stipitis* TJ26 is a poor fermentative strain (data not shown). If this deficiency results from decreased fermentative or increased respirative enzyme activity, overexpressing genes for xylose assimilation, such as *XYL1*, can be expected to have little effect.

The copy number of the autonomous plasmid varies from one time point to the next. We assume that the copy number may vary also from one fermentation experiment to the next. The basis for variability is not fully understood, but copy number appears to increase with frequent subculturing (data not shown). Therefore, subculturing may be useful to increase the gene dosage further beyond the relatively low copy numbers (between one and eight) that were achieved in the experiments described here. Alternatively, expression vectors may be incorporated into the host genome for prolonged stability, eliminating the requirement of maintaining selective pressure.

Regulation of *XYL1* expression by glucose was altered in TJ26 (pXOR), resulting in higher XOR activity under conditions when transcription is normally not induced owing to the presence of glucose. This suggests that the plasmid-borne *XYL1* 5'-flanking sequence does not have all the necessary regulatory and control regions for full and regulated expression. Alternatively, the increase in gene dosage may be titrating negative transcriptional regulators of *XYL1*. However, the 5'-flanking

sequence could be used in developing strains to carry out simultaneous fermentation of glucose and xylose. In a commercial process, fermentation of both sugars would be desirable.

Oxygen appears to affect XOR activity when TJ26 (pXOR) was grown on xylose, resulting in higher activity than the control strain under relatively aerobic conditions. No such effect was seen when glucose was the carbon source. Normally, ethanol production by *P. stipitis* is low under aerobic conditions, and this is an area for improvement. Therefore, the *XYL1* 5'-flanking sequence may be useful when higher levels of desired enzyme activity are required in the presence of high levels of oxygen.

Other fermentative enzymes need to be studied. Pyruvate is a key fermentative intermediate, and two enzymes involved in the conversion of pyruvate to ethanol are PDC and ADH. In *S. cerevisiae*, increasing PDC and ADH activity does not lead to an increased rate of ethanol production *(30)*. However, ethanol production by *E. coli* is increased when PDC and ADH activities are increased *(31)*. *P. stipitis* could be transformed with these genes under the regulation of the *XYL1* 5'-flanking sequence, thus allowing for increased ethanol production in the presence of xylose. Strains that overexpress two or more genes simultaneously could be developed in an effort to produce a strain that has higher ethanol production.

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