0273-2289 /97/63-65-0109 $10.00

Diminished Respirative Growth and Enhanced Assimilative Sugar Uptake Result in Higher Specific Fermentation Rates by the Mutant *Pichia stipitis* FPL-061

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ABSTRACT

A mutant strain of *Pichia stipitis*, FPL-061, was obtained by selecting for growth on L-xylose in the presence of respiratory inhibitors. The specific fermentation rate of FPL-061, was higher than that of the parent, *Pichia stipitis* CBS 6054, because of its lower cell yield and growth rate and higher specific substrate uptake rate. With a mixture of glucose and xylose, the mutant strain FPL-061 produced 29.4 g ethanol/L with a yield of 0.42 g ethanol/g sugar consumed. By comparison, CBS 6054 produced 25.7 g ethanol/L with a yield of 0.35 g/g. The fermentation was most efficient at an aeration rate of 9.2 mmoles $O_2 L^{-1} h^{-1}$. At high aeration rates (22 mmoles $O_2 L^{-1} h^{-1}$), the mutant cell yield was less than that of the parent. At low aeration rates, (1.1 to 2.5 $O_2 L^{-1} h^{-1}$), cell yields were similar, the ethanol formation rates were low, and xylitol accumulation was observed in both the strains. Both strains respired the ethanol once sugar was exhausted. We infer from the results that the mutant, *P. stipitis* FPL-061, diverts a larger fraction of its metabolic energy from cell growth into ethanol production.

Index Entries: *Pichia stipitis;* respiration; ethanol production; specific productivity; mutation; salicyl hydroxamate; antimycin A.

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INTRODUCTION

Pichia stipitis can convert xylose, glucose, mannose and galactose into ethanol *(1)*. However, glucose slows down its rate of xylose assimilation and ultimately results in lower productivity and yield *(2)*. This could be a result of catabolite repression or differential transport *(3–5)*. *P. stipitis* also has a lower ethanol yield at high aeration rates *(6-8)*. This appears to be caused by the respirative consumption of ethanol. Unfortunately, the sugar uptake and fermentation rates decrease as the aeration rate decreases*(9)*.

It is difficult to obtain improved fermentative mutants of *P. stipitis* because cell growth depends on aeration and fermentative activity occurs under oxygen-limiting conditions *(10,11)*. Strategies for obtaining improved yeast mutants with enhanced fermentation on mixed sugars suggest that derepression of enzymes for xylose metabolism enhance growth and fermentation rates *(12-15)*. Selection for mutants that can grow on carbon sources that do not induce pentose phosphate pathway enzymes, but which can be assimilated by that route can result in higher rates of xylose uptake. Moreover, by selecting for mutants that resist respiratory inhibitors, one can obtain lower respiratory activities *(16)*.

The objective of the present work was to characterize a mutant *P. stipitis* that had been selected for rapid growth on noninductive carbon sources in the presence of respiratory inhibitors. We have compared the *P. stipitis* parental strain with FPL-061 during fermentation of pentose and hexose sugar mixtures at various aeration rates.

MATERIALS AND METHODS

Yeast Strains and Inoculum Preparation

P. stipitis CBS 6054 was mutagenized with nitrosoguanidine (NG), and the cells were first screened by selecting for growth on L-xylose in the presence of salicylhydroxamic acid (SHAM, Sigma, St. Louis, MO) and antimycin A (AA, Sigma). FPL-061 was selected following screens of respiratory resistant mutants in microfuge tubes *(16)*. *P. stipitis* CBS 6054 and *P. stipitis* FPL-061 were cultivated on fresh plates of yeast extract peptone xylose (YEPX) agar at 31 to 32°C. Cells from a 48-h-old YEPX plate were washed with sterile water and used as the inoculum. The optical densities of cell suspensions were measured at 525 nm and adjusted to equivalent values by dilution with water.

Shake Flask Fermentation

Fermentation media contatied 1.7 g/L filter sterilized yeast nitrogen base (YNB) without ammonium sulfate or amino acids (Difco). Urea, 2.27 g/L, and peptone, 6.56 g/L, were used as nitrogen sources. Fifty mL of medium containing xylose or sugar mixture in a 125-mL Erlenmeyer flask

was shaken at 100 rpm, at 25 to 30°C. The fermentation was monitored in triplicate flasks for 3 to 10 d by removing 1.3 mL samples for sugar, ethanol, and cell analyses. The final pH was 4.0 to 4.5.

Fermentations used glucose, xylose, or arabinose or a 1:1 mixture of glucose and xylose. Final sugar concentrations ranged from 60 to 80 g/L. Individual sugars and sugar mixtures were autoclave separately and added to media after they had cooled to room temperature. For aeration optimization, the dissolved oxygen transfer rate was estimated by the sulfite oxidation method *(17)*. For medium containing 70 g/ L-xylose in various volumes of media 12.5 to 87.5 mL, oxygen transfer rates corresponding to 1.1 to 22.6 mmol of $O_2 L^{-1} h^{-1}$ were used.

Analytical Methods

Cell densities were measured at 525 nm. An OD of 1.0 was equivalent to 0.21 mg dry weight of cells/mL. Sugars and fermentation products were determined by high performance liquid chromatography (HPLC) (Hewlett Packard series 1050) with an RI detector. Sugars and products were separated on an Aminex Carbohydrate HPX 87C, column (300 × 7.8 cm) maintained at 85°C *(18)*. The mobile phase was degassed distilled water at a flow rate of 0.5 mL/min at a pressure of 50 to 55 bar. Filtered clear samples (980 µL) were mixed with 20 µL of sucrose (500 g/L) as internal standard before injection.

RESULTS AND DISCUSSION

Selection of the FPL-061 Mutant

D-Xylose is the form found in nature; L-xylose is uncommon. Both, however, can be reduced to xylitol, which is neither D nor L. L-Xylose is not metabolized by *P. stipitis* CBS 6054. Presumably, this sugar does not induce assimilative enzymes, but this has not been confirmed. L-Xylose is, however, a (poor) substrate for aldose reductase *(18)*, and once reduced to xylitol, L-xylose can be metabolized. Growth on L-xylose, therefore, should select for strains constitutively derepressed for aldose reductase. It is not sufficient to identify rapid growers because such are usually poor fermenters. Hence, mutants were selected for growth in the presence of respiratory inhibitors. *P. stipitis* possesses both AA-sensitive and SHAM-sensitive respiratory pathways *(19)*. Blockage of either pathway alone is insufficient to inhibit growth, so both inhibitors were used. Mutants growing under these conditions were screened for fermentative activities in microfuge tubes *(20)*. Initial concentrations of cells from various strains were adjusted to a constant value so that strains with higher specific fermentation rates (rather than higher growth rates) could be identified. The properties of FPL-061 were then confirmed by trials in replicate shake flasks. Methods used to identify FPL-061 have been patented (16).

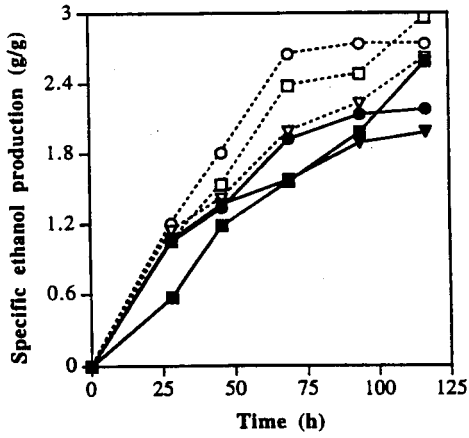

Fig. 1. Effect of xylose, glucose, and mixture of xylose and glucose on specific production in *Pichia stipitis* CBS 6054 (solid line) and *Pichia stipitis* FPL-061 (broken line). Symbols: (■), CBS 6054 with 74 g/L xylose; (▼), CBS 6054 with 70 g/L glucose; (●), CBS 6054 with 36 g/L xylose and 36 g/L glucose; (●), FPL 061 with 74 g/L xylose; (▼), FPL 061 with 70 g/L glucose; (○), FPL 061 with 36 g/L xylose and 36 g/L glucose.

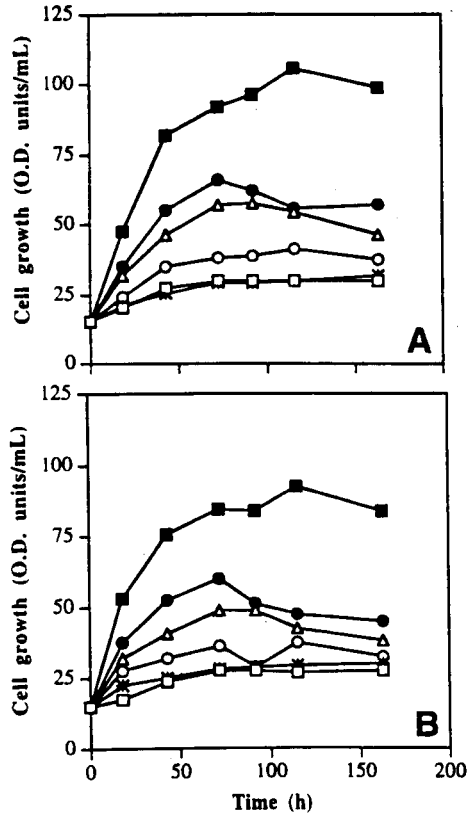

Fig. 2. Effect of aeration rates on cell growth during xylose fermentation in **(A)** *Pichia stipitis* CBS 6054 and **(B)** *Pichia stipitis* FPL-061. Symbols: aeration rate in mmol O_2 L^{-1} h^{-1}: (■), 22.5; (●), 11.8; (▽), 9.2; (○), 5.4; (□), 2.5; (X), 1.1.

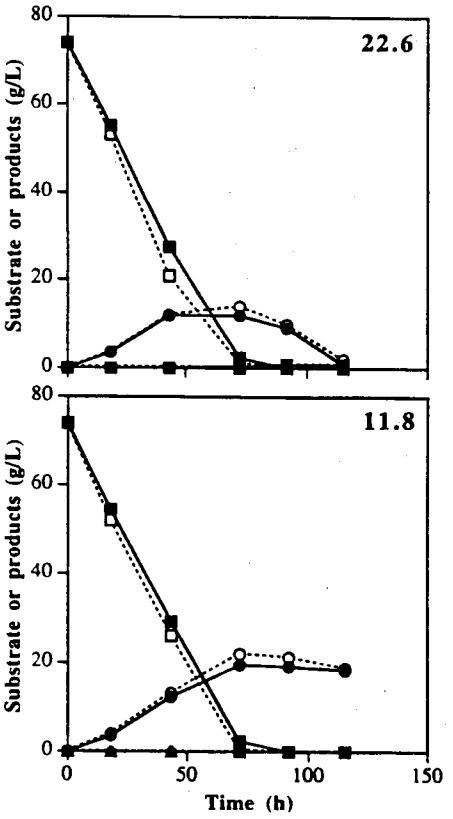

Fig. 3. Effect of aeration at 22.6 and 11.3 mmoles O_2 L^{-1} h^{-1} on ethanol production during xylose fermentation in *Pichia stipitis* CBS 6054 (solid line) and *Pichia stipitis* FPL-061 (broken line). Symbols: (■), CBS 6054 xylose; (♦), CBS 6054 ribitol; (▲), CBS 6054 xylitol; (●), CBS 6054 ethanol; (□), FPL 061 xylose; (○), FPL 061 ribitol; (△), FPL 061 xylitol; (○), FPL 061 ethanol.

Growth and Fermentation of Various Sugars and Sugar Mixtures

CBS 6054 grew faster than FPL-061 on a mixture of glucose and xylose, but on a specific cell basis, FPL-061 produced more ethanol in glucose, xylose, and sugar mixtures (Fig. 1). When grown on a 1:1 glucose: xylose mixture, FPL-061 assimilated xylose slightly faster than CBS 6054 (data not shown). FP?L-061 produced a maximum ethanol concentration of 29.4 g/L with a yield of 0.42 g/g sugar consumed. By comparison, under the same conditions, CBS 6054 produced 25.7 g/L of ethanol with a yield of 0.35 g/g. Neither FPL-061 or CBS 6054, fermented L--arabinose, but both strains grew slowly on this sugar and converted it to arabitol.

Effect of Various Aeration Rates on Xylose Fermentation

The parental strain grew better than the mutant, particularly with high aeration. Growth rates of both strains were severely retarded with low aeration (Fig. 2). The mutant strain *P. stipitis* FPL-061 produced ethanol

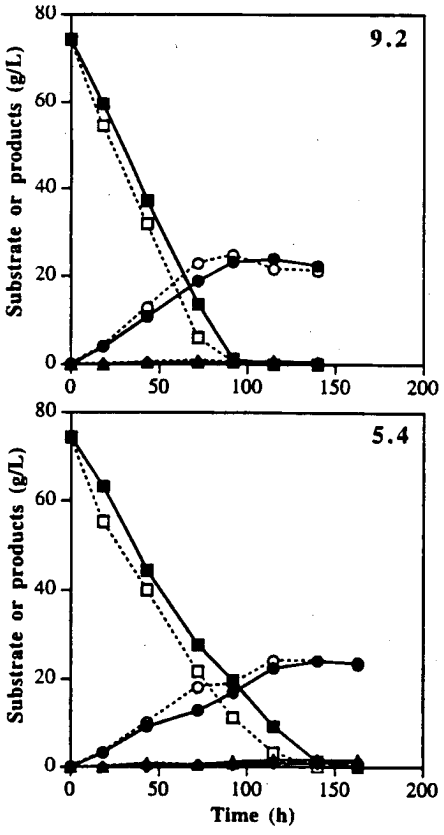

Fig. 4. Effect of aeration at 9.2 and 5.4 mmoles O_2 L^{-1} h^{-1} on ethanol production during xylose fermentation in *Pichia stipitis* CBS 6054 (solid line) and *Pichia stipitis* FPL-061 (broken line). Symbols same as Fig. 3.

from xylose faster than *P. stipitis* CBS 6054 when optimally aerated (Figs. 3 and 4). At the highest aeration rate, ethanol respiration was noted with both strains (Fig. 3). Reassimilation of ethanol at high aeration is well-recognized *(21).* In contrast, at low aeration rates, we observed a low ethanol formation rate and xylitol accumulation by both strains (Fig. 5). FPL-061 and CBS 6054 both fermented xylose maximally at an aeration rate of 9.2 mmoles O_2L^{-1} h^{-1}, but at that aeration rate, FPL-061 fermented faster (Fig. 4).

The mutant growth rates and cell yields were lower (Fig. 2), but its specific fermentation rates (Fig. 1) were higher than the parent, suggesting that FPL-061 has a deficient respirative capacity. This might have arisen when it gained resistance to AA or SHAM. For aeration rates of 5.4 to 9.2 mmol $O_2L^{-1}h^{-1}$, substrate uptake by FPL-061 was more rapid than by CBS-6054, even though growth was less (cf. Fig. 1 and Fig. 3). However, for higher and lower aeration rates, no appreciable difference was observed. This suggests that substrate uptake is derepressed in the mutant. This conclusion was supported by the observation that FPL-061 ferments a mixture of glucose and xylose more rapidly than CBS 6054. Another possibility is

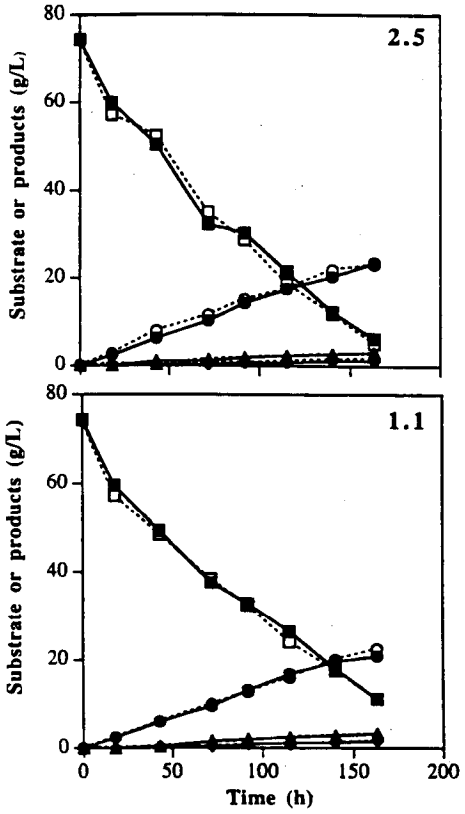

Fig. 5. Effect of aeration at 2.5 and 1.1 mmoles O_2 L^{-1} h^{-1} on ethanol production during xylose fermentation in *Pichia stipitis* CBS 6054 (solid line) and *Pichia stipitis* FPL-061 (broken line). Symbols same as Fig. 3.

that the mutant diverts a larger portion of metabolic energy into sugar uptake. In either case, of the sugar taken up, more is shunted into ethanol at intermediate aeration levels. At the lowest aeration rate, there was no discernible difference between the sugar uptake rates for the mutant and the parent (Fig. 5), and the cell growth rates were also similar (and very low). This indicates that the high affinity oxygen uptake systems in these two yeasts are similar.

Conclusions

The mutant strain *P. stipitis* FPL-061 produced ethanol at a higher yield as compared to its parental strain, *P. stipitis* CBS 6054. Elevated sugar uptake without concomitant growth suggests that the assimilative pathway is derepressed in the mutant. During xylose fermentation, an aeration rate of 9.2 mmol $O_2L^{-1}h^{-1}$ for ethanol production. Because the growth rate of the mutant was reduced at high aeration rates, it is probably defective in a low affinity oxygen uptake system that is responsible for energy production.

ACKNOWLEDGMENT

Hassan K. Sreenath is supported in part by the USDA, Forest Service International Forest Products Program. Additional support is provided by USAID Office of Research grant 10.299.

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