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Physiological Properties of a Mutant of *Pachysolen tannophilus* Deficient in NADPH-Dependent D-Xylose Reductase†

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A D-xylose reductase mutant of *Pachysolen tannophilus* was isolated on the basis of its poor growth on D-xylose but normal growth on xylitol and D-glucose. Fractionation of cell extracts indicated that the mutant was deficient in D-xylose reductase activity that used NADPH exclusively as a cofactor, but not in activity that used both NADH and NADPH. Mutant cultures grown on D-xylose as the sole carbon source exhibited some properties that would be desired in improved strains. Growth rate, growth yield, and D-xylose consumption rate of the mutant were less sensitive than those of the wild type to changes in aeration rate. D-Xylose was utilized more efficiently in that less of a by-product, xylitol, was produced. In addition, under low aeration conditions, more ethanol was produced. A disadvantage was a relatively slow rate of D-xylose utilization.

Properties of yeast cultures that produce ethanol from D-xylose depend on the extent of oxygen limitation. As oxygen supply increases, D-xylose is used more rapidly, and the growth rate and biomass yield increase (6, 10, 22, 27). In addition, the rate of ethanol production and the yield or concentration of ethanol accumulated are affected (2, 6, 9, 10, 19, 22, 27).

The biochemical basis for the effects of oxygen limitation is not known. Elucidation of the factors involved is of interest in improving D-xylose fermentation, because higher rates of D-xylose utilization and ethanol production, as well as greater ethanol yields, can be obtained aerobically than anaerobically (1, 9, 15, 25, 28; J. P. van Dijken and W. A. Scheffers, U.S. Patent 4,701,414, 20 October 1987). Knowledge of the effects of oxygen limitation can also be useful in devising strategies to obtain mutants or recombinants with improved properties.

The present study investigated a number of properties of a mutant of *Pachysolen tannophilus* deficient in NADPH-dependent D-xylose reductase activity. D-Xylose reductase catalyzes the first step of D-xylose metabolism in yeasts. In *P. tannophilus*, this enzyme has been reported to use either NADH or NADPH as a cofactor (13, 17, 26). Comparison between the wild type and mutant under changing aeration conditions suggests that the NADPH-dependent D-xylose reductase activity is linked to oxygen utilization and that it plays an important role in growth under aerobic conditions.

MATERIALS AND METHODS

Organisms. The wild-type strain was *P. tannophilus* NRRL Y2460. The type strain carrying the *xyl13* mutant allele is a methionine auxotroph of the wild type (12). For the purposes of the present study, the auxotrophic requirement was eliminated by appropriate crosses (11). This strain is on deposit with the culture collection of the National Research Council of Canada as no. 5410.

Temperature. All liquid-phase experiments were conducted at 30°C.

Growth rate determination. Inocula for the wild-type or mutant were grown by placing a loopful of cells from a plate containing 1% (wt/vol) yeast extract (Difco), 1% (wt/vol) Bacto-Peptone (Difco), and 2% (wt/vol) D-glucose (YEPD) into 6 ml of medium containing 0.67% (wt/vol) yeast nitrogen base (YNB) without amino acids (Difco), 54 mM glycerol, and 67 mM D-xylose and incubating in rotated tubes (23) for 30 h. One milliliter of inoculum was then transferred to the test medium, which consisted of 100 ml of 0.67% (wt/vol) YNB, without amino acids, and either 267 mM D-xylose, 267 mM L-arabinose, 395 mM xylitol, 232 mM D-glucose, or 434 mM glycerol. Because of the small quantities available, growth rate on D-xylulose was determined using 50 ml of 0.67% (wt/vol) YNB without amino acids and 67 mM of the ketopentose. Xylitol was tested at the 395 mM level because of poor growth of *P. tannophilus* at lower concentrations (20). The culture, placed in a 250-ml Erlenmeyer flask fitted with a 15-mm-internal-diameter sidearm, was incubated at 200 rpm in a gyratory shaker. A_{600} was measured at intervals through the sidearm. Calculations employed absorbance values of less than 0.3 in order to avoid questions arising from the lack of linearity in the relation between absorbance and cell density at higher absorbancies (24).

Enzyme assays and cell extracts. D-Xylose reductase and xylitol dehydrogenase activities were assayed in glycerol-grown cells which were induced for D-xylose-metabolizing enzymes by the addition of D-xylose (17). Inocula were prepared by growing a loopful of cells from a YEPD plate in 6 ml of medium containing 0.67% YNB without amino acids and 54 mM glycerol for 30 h in rotated tubes (23). One milliliter of inoculum was transferred to 100 ml of medium containing 0.67% (wt/vol) YNB without amino acids and 434 mM glycerol in a 250-ml loosely capped Erlenmeyer flask, which was incubated at 200 rpm in a gyratory shaker. After 16 h, 11 ml of 2.67 M D-xylose was added and shaking was

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continued. At 6 and 24 h after D-xylose addition, cells were harvested by centrifugation and cell extracts were obtained by sonication (17). Enzyme activities were determined as described previously (14).

Fractionation of D-xylose reductase activities. Cell extracts were fractionated by ion-exchange fast-protein liquid chromatography (8) with minor modifications. Extracts from 24-h D-xylose-induced cells grown as described above were desalted and membrane filtered. A 3-ml sample of extract containing 5 to 8 mg of protein per ml was applied to a Mono Q anion exchanger previously equilibrated with 20 mM imidazole buffer (pH 7.0), containing 2 mM 2-mercaptoethanol. After the column was washed with 10 ml of buffer, linear gradients of 0 to 0.25 M NaCl and 0.25 to 0.50 M NaCl in the same buffer were applied. Elution volumes were 30 and 10 ml for the first and second gradient, respectively. Fractions of 2 ml were collected and assayed for NADPH- and NADH-dependent D-xylose reductase activity. Fractionation was carried out in a Pharmacia fast-protein liquid chromatography apparatus equipped with an automatic gradient controller, automatic fraction collector, and UV monitor.

Aeration rate dependencies of mutant and wild type. Aeration rates were altered by varying the ratio of flask to medium volume. The capacity of the Erlenmeyer flask used was 500 ml, while medium volumes were 50 and 450 ml (ratios of 10 and 1.11, respectively). The flasks bore a 15-mm-inner-diameter sidearm through which culture absorbance was measured. Inocula consisted of 48-h cultures grown in 0.67% (wt/vol) YNB without amino acids and containing 217 mM glycerol and 133 mM D-xylose. One milliliter of inoculum was transferred to the test medium, which contained 0.67% (wt/vol) YNB without amino acids and 267 mM D-xylose. The flasks were capped loosely and incubated at 200 rpm on a gyratory shaker. A_{600} was measured periodically, and at each time a sample was also removed for residual sugar and metabolite determinations. Growth rates were determined in separate experiments using the same physical set-up, but absorbance was measured more frequently during early log phase and samples were not removed.

Anaerobic cultures. Model KLF 2000 Fermenters (Bioengineering AG, Wald, Switzerland) with 2 liter capacities were employed. The inocula used were grown aerobically. A loopful of cells from a YEPD plate was transferred to 100 ml of 0.67% (wt/vol) YNB without amino acids and 56 mM D-glucose in a 250-ml flask, which was incubated as described above. After 24 h, the culture was divided into three portions, and each portion was used to inoculate one 2-liter flask that contained 500 ml of 0.67% (wt/vol) YNB without amino acids, 55 mM glycerol, and 67 mM D-xylose. After incubation as described for 24 h, the cells were harvested by centrifugation, washed once with distilled water, and suspended in 150 ml of water. The wild-type and mutant cultures were adjusted to about the same A_{600} (14-mm-internal-diameter cuvette), and the cell suspensions were added to separate fermentors each containing 1,100 ml of medium made up so that the concentrations were 0.67% (wt/vol) for YNB without amino acids and 267 mM for D-xylose after the inoculum was added. The cultures were maintained anaerobic by sparging at 34 ml/min with high-purity-grade nitrogen gas (containing 20 ppm [μl/liter] oxygen). The rate of stirring was 1,500 rpm. Loss of volatile materials in the off-gas was minimized with a condensor at 4°C. Samples were removed for analyses at intervals.

Analytical methods. Protein was measured by the method of Lowry et al., using bovine serum albumin as a standard (16). Ethanol and acetic acid concentrations were determined by gas chromatography (14). D-Xylose and xylitol concentrations were measured by high-pressure liquid chromatography using an Aminex HPX-87P column (Bio-Rad Laboratories) with water as the eluant. Absorbance of cultures was measured with a Coleman model 30 spectrophotometer.

TABLE 1. Growth rate of wild type and mutant on different carbon sources

Carbon source	Growth rate (h^{-1})	
	Wild type	Mutant
D-Xylose	0.25	0.05
L-Arabinose	0.24	0.04
Xylitol	0.17	0.18
D-Xylulose	0.36	0.28
D-Glucose	0.37	0.37
Glycerol	0.33	0.32

RESULTS

Identification of metabolic defect in the mutant. Growth rates on different carbon sources (Table 1) suggested that the mutant was deficient in D-xylose reductase activity. The growth rate of the mutant on D-xylose was one-fifth that of the wild-type value. The growth rate on L-arabinose, the catabolism of which is also thought to require D-xylose reductase (3, 4), was similarly depressed. However, growth rates on xylitol or D-xylulose, both of which enter the D-xylose catabolic pathway subsequent to D-xylose reductase, were comparable to those of the wild type. In addition, growth rates on D-glucose and glycerol, which do not use the D-xylose catabolic pathway, were similar to those of the wild type.

The mutant was deficient in NADPH-dependent, but not NADH-dependent, D-xylose reductase activity. After 24 h of induction by D-xylose, specific activity for NADPH-dependent D-xylose reductase with the mutant was 40 μmol/min per μg of protein, which was about one-quarter the wild-type value of 170 μmol/min per μg of protein. In contrast, the specific activities for the NADH-dependent D-xylose reductase were similar for the wild type and mutant: 76 and 86 μmol/min per μg of protein , respectively. Specific activities for NAD-dependent xylitol dehydrogenase were also similar for the wild type and mutant: 1.18 and 1.16 μmol/min per μg of protein, respectively. The level of each enzyme measured was about one-quarter as high when the induction period was 6 instead of 24 h.

The lower NADPH-dependent D-xylose reductase activity in mutant cell extracts was due to absence of the activity that specifically requires NADPH. Fast-protein liquid chromatography factionation of wild-type cell extracts resulted in two D-xylose reductase activity peaks (Fig. 1). One peak used only NADPH as a cofactor (designated as enzyme A in reference 26), and one used either NADPH or NADH (enzyme B, reference 26). However, only a single peak that used either cofactor was found in the mutant cell extract (enzyme B).

Effects of aeration rate. Changes in aeration rate had greater effects on growth and D-xylose utilization of the wild type (Fig. 2). When aeration rate was decreased, growth rate of the wild type decreased by a factor of about one-third, while that for the mutant changed relatively little (Table 2).

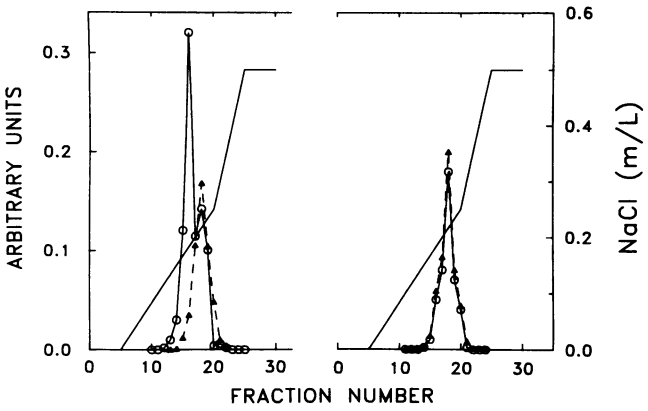

FIG. 1. Fractionation of D-xylose reductase activities in cell extracts of the wild type (left) and mutant (right). Symbols: ○, NADPH-dependent activity: ▲, NADH-dependent activity. The concentration of NaCl in the eluting gradient is shown by the solid line without symbols.

In addition, the volumetric rate of D-xylose consumption at times when the rate of ethanol production was close to maximal decreased by a factor of about one-third with the wild type while that for the mutant remained largely unchanged. Cell yields, as estimated by absorbance values, were similar for the two strains at the higher aeration rate, but the decrease was greater with the wild type at the lower aeration rate (Fig. 2). The wild type produced more xylitol, and the amount produced was more sensitive to a change in aeration rate.

In contrast to aspects of growth, D-xylose consumption rate, and xylitol production, the maximum concentration of ethanol accumulated by the mutant was more sensitive to changes in aeration rate (Fig. 2). Another difference in

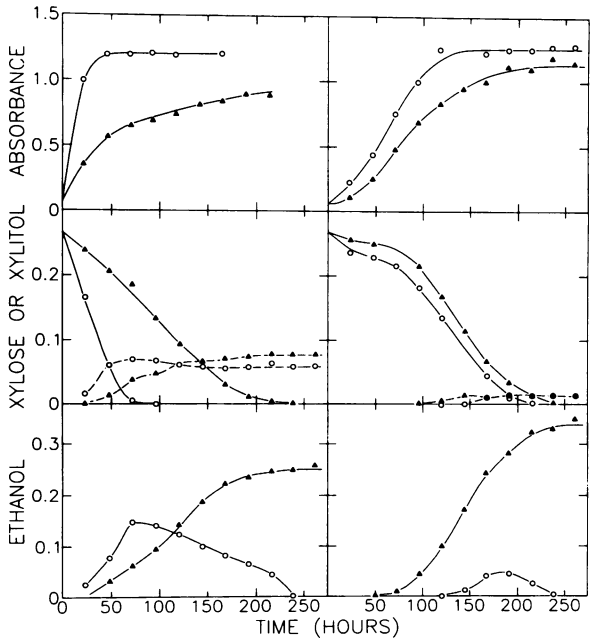

FIG. 2. Effect of aeration rate on cultures of the wild type (left) and mutant (right). Xylitol data are represented by the dashed lines. All concentrations are in moles per liter. The flask capacity was 500 ml, and the medium volumes were 50 (○) and 450 (▲) ml.

TABLE 2. Some effects of changes in aeration rate on cultures of wild type and mutant inoculated into 267 mM D-xylose

Aeration rate (flask/medium volume)	Growth rate (h^{-1})		D-Xylose use rate[a] (mmol/liter/h)		Ethanol production rate[a] (mmol/liter/h)	
	Wild type	Mutant	Wild type (50 h)	Mutant (150 h)	Wild type (50 h)	Mutant (150 h)
High (10)	0.46	0.05	4.6	1.9	2.0	0.65
Low (1.1)	0.15	0.04	1.5	2.1	1.3	3.0

[a] Rates were measured at times close to maximal for ethanol production rate.

ethanol production was that as aeration rate decreased, the volumetric rate of production by the wild type decreased while that for the mutant increased (Fig. 2, Table 2). Experiments at aeration rates intermediate to those in Fig. 2 yielded results for growth rate, D-xylose use rate, and product formation consistent with those described above (data not shown).

In general, the performance of the wild type and mutant on D-xylose became similar as aeration rate decreased in that rates for growth, D-xylose consumption, and ethanol production tended to converge towards each other (Table 2 and Fig. 2). Under anaerobic conditions the rate of D-xylose consumption by the two strains was similar (Fig. 3). This was consistent with the general trend for the two strains to behave more alike as aeration rate decreased. Other effects under anaerobic conditions included production of more ethanol but less xylitol by the mutant.

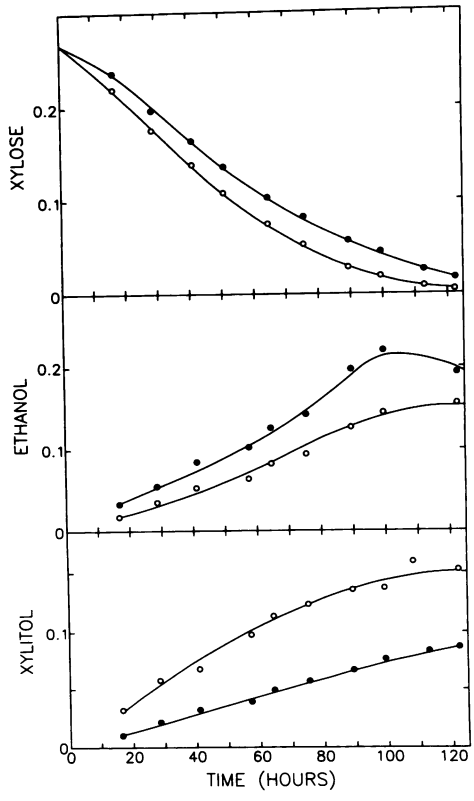

FIG. 3. Performance of wild type and mutant under anaerobic conditions. All concentrations are in moles per liter. Initial absorbances were 13.0 and 13.1 for the wild type and mutant, respectively. Symbols: ○, wild type; ●, mutant.

DISCUSSION

The ability of D-xylose reductase in pentose-fermenting yeasts to use either NADH or NADPH as a cofactor is ascribed to the presence of several enzymes that differ in cofactor specificity. However, the number and specificity of these enzymes in *P. tannophilus* are unclear. Enzymes have been reported that are active with only NADPH (7), with only NADH (8), or with both NADPH and NADH (5, 18, 26). The isolation of a mutant deficient in NADPH- but not NADH-dependent activity demonstrated that *P. tannophilus* possesses at least one enzyme which is highly specific for NADPH.

The greater sensitivity to aeration rate of the rates of growth and D-xylose consumption by the wild type suggested that NADPH-dependent D-xylose reductase plays an important role in growth under aerobic conditions. This greater sensitivity suggested also that the NADPH-dependent component of D-xylose reductase was linked to oxygen utilization. Such involvement may have arisen through the cofactor regeneration mechanisms thought to be operative under aerobic conditions for the two initial steps of D-xylose catabolism (21). These mechanisms consist of regeneration of the NADPH required for D-xylose reductase via the pentose phosphate shunt, while the NAD^+ required for xylitol dehydrogenase is regenerated by oxidation of NADH via the respiratory chain. The alternate mechanism, use of NADH by D-xylose reductase with subsequent regeneration of this cofactor by xylitol dehydrogenase, may not function to an appreciable extent under relatively aerobic conditions because the NADH produced by xylitol dehydrogenase would tend to be oxidized by the respiratory chain.

The lower amount of ethanol produced by the mutant at higher aeration rate was consistent with a difference, relative to the wild type, in the partitioning of oxygen among alternate pathways (21). Oxygen is proposed to be utilized concurrently in D-xylose media via three routes: (i) oxidation of NADH produced by xylitol dehydrogenase via the respiratory chain, (ii) oxidation of pyruvate via the tricarboxylic acid cycle, and (iii) oxidative utilization of ethanol even when D-xylose is present in the medium. In the wild type, ethanol accumulation is suggested to decrease as more oxygen becomes available to the culture, in part because more oxygen is channeled through the second and third routes listed above, which has the effect of decreasing ethanol accumulation. However, in the mutant, because of the lower rate of D-xylose use, the requirement of oxygen for oxidation of NADH produced by xylitol dehydrogenase was lower than with the wild type. As a result, more oxygen was available for oxidation of pyruvate or for oxidative utilization of ethanol, and consequently, less ethanol was accumulated.

The greater volumetric rate of ethanol production by the wild type at the higher aeration rate may have resulted from the associated greater rate of D-xylose consumption. An implication of the greater rate of ethanol production resulting from the increased rate of D-xylose use by the wild type was that ethanol accumulation occurred while oxygen was being consumed through the respiratory chain-linked regeneration of the NAD^+ required by xylitol dehydrogenase.

The D-xylose reductase mutant displayed several properties which are desirable in improved D-xylose-fermenting strains. These properties included lowered sensitivity of growth and D-xylose consumption rates to aeration rate, less xylitol production, and greater ethanol accumulation. However, an undesirable effect was a decrease in the rate of D-xylose utilization.

The manifestation of several useful properties through the presence of a single mutant allele indicated that genetic manipulation is a useful approach with which to obtain improved D-xylose-fermenting yeasts. Other studies support the same conclusion (12, 14).

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