Levels of enzymes of the pentose phosphate pathway in *Pachysolen tannophilus* Y-2460 and selected mutants

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The compositions of intracellular pentose phosphate pathway enzymes have been examined in mutants of Pachysolen tannophilus NRRL Y-2460 which possessed enhanced D-xylose fermentation rates. The levels of oxidoreductive enzymes involved in converting D-xylose to D-xylulose via xylitol were 1.5–14.7-fold higher in mutants than in the parent. These enzymes were still under inductive control by D-xylose in the mutants. The D-xylose reductase activity (EC 1.1.1.21) which catalyses the conversion of D-xylose to xylitol was supported with either NADPH or NADH as coenzyme in all the mutant strains. Other enzyme specific activities that generally increased were: xylitol dehydrogenase (EC 1.1.1.9), 1.2–1.6-fold; glucose-6-phosphate dehydrogenase (EC 1.1.1.49), 1.9–2.6-fold; D-xylulose-5-phosphate phosphoketolase (EC 4.1.2.9), 1.2-2.61fold; and alcohol dehydrogenase (EC 1.1.1.1). 1.5–2.7-fold. The increase of enzymatic activities, 5.3–10.3-fold, occurring in D-xylulokinase (EC 2.7.1.17), suggested a pivotal role for this enzyme in utilization of D-xylose by these mutants. The best ethanol-producing mutant showed the highest ratio of NADH- to NADPH-linked D-xyloSe reductase activity and high levels of all other pentose phosphate pathway enzymes assayed.

Keywords: Pentose phosphate pathway; *Pachysolen tannophilus;* D-xylose metabolism

Introduction

Xylans, the principal constituents of hemicelluloses, can be obtained from woody angiosperms (hardwoods) and agricultural residues. Sugars derived from xylans offer potential feedstock for generating food and fuel. The hemicellulosic sugars can be recovered from lignocellulose more readily than glucose but are fermented with greater difficulty than glucose or pure sugars.[1]

In recent years, numerous yeasts, particularly *Pachysolen tannophilus,*[2] *Candida shehatae,*[3] and *Pichia stipitis*[4] have been found to produce ethanol from D-xylose. Of these, *P. tannophilus* has been studied most extensively,[5] and a number of mutants exhibiting improved rates of D-xylose fermentation have been obtained from the type culture, *P. tannophilus* NRRL Y-2460.[6] This research sought to characterize the parent strain and the mutants biochemically in order to determine if altered levels of key enzymes could account for the improved performance of the mutants.

In yeasts and moulds, D-xylose is converted to D-xylulose in two enzymatic steps.[7] In the oxidoreductive pathway, D-xylose is first reduced by an NADPH-linked D-xylose reductase (alditol:$NADP^+$/NAD^+ 1 -oxidoreductase, EC 1.1.1.21) to form xylitol. The reoxidation of xylitol to D-xylulose is then effected by another NAD-linked dehydrogenase (xylitol:NAD^+ 2-oxidoreductase, D-xylulose reductase, EC 1.1.19). D-Xylulose thus formed is then phosphorylated to form a key intermediate, D-xylulose-5-phosphate. Beyond this point, much of the pathway is assumed from biochemical studies with other organisms. In one route, phosphate acetylating enzyme, phosphoketolase (D-xylulose-5-phosphate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.9) converts D-xylulose-5-phosphate to glyceraldehyde-3-phosphate and acetyl phosphate in the presence of cocarboxylase and Mg^{2+}. In another route, D-xylulose-5-phosphate proceeds by way of epimerase, isomerase, transketolase and transaldolase to form glyceraldehyde-3-phosphate and D-fructose-6-phosphate. Glyceraldehyde-3-phosphate is an intermediate in both the pentose phosphate pathway (PPP) and Embden–Meyerhof–Parnas (EMP) pathway.[8-10] The net result of a homoethanolic fermentation through this latter route involving transketolase is that 3 mol of xylose yield 5 mol

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of ethanol and 5 mol of carbon dioxide, i.e. 1.67 mol C_2 per mol C_5 utilized. Both the ATP and the ethanol yields of this homoethanolic route are identical to those obtained with glucose via the Embden–Meyerhofpathway.

The pathway for fermenting D-xylose by *P. tannophilus* is poorly characterized. *P. tannophilus* unambiguously exhibits an anaerobic fermentation of D-xylose; however it forms significant quantities of acetic acid under anaerobic conditions or when cells are grown aerobically on nitrate.[11] It is possible that this product could arise from phosphoketolase activity. We decided therefore to determine the enzymic activities of various PPP enzymes in parent and previously obtained mutant yeast strains[6] in order to better characterize the pathway and elucidate the rate-limiting step(s) in the overall process.

The mutant strains examined here were enriched and selected on the basis of their abilities to grow preferentially in broth and to form large colonies on agar with either nitrate or ammonia as nitrogen sources and with xylitol as a carbon source. Nitrate was chosen as the sole nitrogen source for enrichment and selection because of its recognized ability to induce higher levels of pentose phosphate pathway enzymes in yeasts, fungi and plant cells.[12,13] Xylitol was chosen as the sole carbon source because it was restrictive for these cells, and it could be used to distinguish between fast- and slow-growing colonies on a plate. The mutant strains examined have produced ethanol approximately twice as fast and in nearly 30% better yield than the parent strain under the conditions employed?

The objective of the present work was to determine intracellular levels of some of the enzymes of the PPP in the parent and isolated mutant strains, when grown under welldefined aerobic conditions. The results of this research should be useful in future comparative biochemical studies with *C. shehatae, P. stipitis* and other yeasts.

Materials and methods

Chemicals

D-Xylose, xylitol, D-xylulose; NAD; NADH; NADP; NADPH, type 1; lactic dehydrogenase, type 1 (LDH); adenosine 5'-triphosphate, phosphoenol pyruvate, D-xylulose, thiamin pyrophosphate, phosphoriboisomerase, pyruvate kinase, D-ribulose-5-phosphate 3-epimerase, and D-ribose-5-phosphate were obtained from sigma Chemical Company. Bacto Yeast Nitrogen Base was obtained from Difco. Other chemicals used were of reagent grade.

Microorganisms

Pachysolen tannophilus Boidin et Adzet NRRL Y-2460 (ATCC 32691) was obtained from the yeast collection at the USDA Northern Regional Research Center, Peoria, IL, and maintained on yeast malt agar (YMA, Difco). Mutagenesis, enrichment, isolation and selection of mutant strains of *P. tannophilus* Y-2460, viz. $NO_3 \rightarrow NO_3$-7,$NO_3 \rightarrow$ U-4, $NO_3 \rightarrow NO_3$-4, U→U-27 and U→NO_3-6, have been described previously.[6] Of these strains, $NO_3 \rightarrow$NO3-7 was the fastest grower on xylitol; $NO_3 \rightarrow$U-4 and $NO_3 \rightarrow NO_3$-4 showed enhanced rates of aerobic and specific anaerobic fermentation, and U→U-27 and U→NO_3-6 showed enhanced growth on xylitol, but no increase in specific anaerobic fermentation of D-xylose.

Growth medium and culture conditions

All experiments were carried out in a synthetic medium containing 0.67% Bacto Yeast Nitrogen Base (YNB, Difco) filter sterilized in 10 × strength and either 4.5 or 6.0% D-xylose or D-glucose as sole carbon sources. An inoculum was grown by transferring each strain from an agar slant into a 125 ml Erlenmeyer flask containing 50 ml of the medium and agitating at 100 rev min^{-1}, 32°C, for 48 h. Inoculum cells were washed with sterile water and diluted so that cells grown under different conditions would all give the same initial absorbance at 525 nm.[14] Five ml of each cell suspension containing on average 45 mg (±2) dry weight (0.8g wet wt) cells was used as inoculum. The strains were grown on a rotatory shaker (100 rev min^{-1}) at 32°C in 125ml Erlenmeyer flasks containing 50ml of medium. The cells were harvested by centrifugation (7000 rev min^{-1} , 20 min, 44°C) after 48 h and washed with potassium phosphate buffer (0.1 M, pH 7.1) containing 10 mM cysteine-HCl, 1 mM dithiothreitol, 1 mM EDTA, and 5 mM $MgCl_2$.

Preparation of cell homogenates

The harvested yeast cells (4–6g), usually from six Erlenmeyer flasks, were suspended in 15 ml 100 mM potassium phosphate buffer, pH 7.1, and placed in precooled thick-walled bottles containing 35 g (HCl-washed and rinsed in water) glass beads (0.5 mm diameter). This mixture was broken in a Braun model cell homogenizer by agitating for 3 min while cooling to 1–5°Cwith liquid CO_2. The supernatant solution was separated from glass beads and cell debris by centrifugation (12 000 rev min^{-1} , 30 min, 4-6°C) and used immediately for enzyme assays.

Enzyme assays

NAD(H) and NADP(H)-dependent reduction or oxidation of the polyol, ketose or aldose substrates by cell-free extracts were followed on a Gilford model 250 spectrophotometer at 340 nm using 1 cm light path quartz cuvettes thermostatically controlled at 30°C. The enzyme sample in the assay was diluted appropriately to give a linear reaction velocity for at least 5 min. In every assay, control recordings were made without added cell homogenate, coenzyme or substrate, respectively. ε_{340} nm values were converted to NAD(P)H concentrations using ε (mM) = 6.22.

Activities of all enzymes are expressed as international units (U), where one unit is the amount of enzyme necessary to catalyse the oxidation or reduction of one micromole of coenzyme (NAD/NADP) per minute at 30°C under the given experimental conditions. Specific activities are expressed as units mg $protein^{-1}$. The reaction mixtures and other conditions for the individual enzymes are as follows.

D-Xylose reductase (EC 1.1.1.21) (alditol:$NADP^+$/NAD^+ 1-axidoreductase). The activity of D-xylose reductase was determined using NADPH and NADH as coenzyme according to the method described by Chiang and Knight.[7] The reaction mixture contained 0.1 M Tris–HCl buffer, pH 7.8, 0.6 ml; 0.2 ml diluted enzyme sample; 1.15 × 10^{-3} M NADPH, 0.1 ml; and at zero time was initiated by the addition of 0.1 ml 0.1 M D-xylose.

NAD-xylitol dehydrogenase (EC 1.1.1.9) (D-xylulose reductase, xylitol:NAD^+ 2-oxidoreductase). The reaction mixture contained 0.1 M glycine–NaOH buffer, pH 8.6, 0.8 ml; 0.1 ml diluted enzyme sample; 0.034 M NAD^+, 0.01 ml; and was initiated by the addition of 0.1 ml 0.1 M xylitol[15].

D-Xylulokinase (ATP:D-xylulose 5-phosphotransferase, EC 2.7.1.17). D-Xylulokinase assay was carried out according to the method described by Simpson.[16] The reaction mixture contained 0.5 M Tris–glycine–KCbuffer (with 0.01 M EDTA), pH 7.8, 0.1 ml; 0.25 ml freshly prepared mixture of 0.01 M phosphoenolpyruvate (1 part); 0.05 M $MgCl_2$ (1 part) and 0.01 M ATP (0.5 part); 0.03 M NADH, 0.03 ml; 0.01 M D-Xylulose, 0.1 ml; 0.03 ml L-lactic dehydrogenase (containing about 10 units), and the diluted enzyme sample to make the volume 1 ml.

D-Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:NAD(P)$^+$ 1-oxidoreductase, EC 1.1.1.49). The reaction mixture contained 0.055 M Tris–HCl buffer, pH 7.8, containing 0.0033 M $MgCl_2$, 2.7 ml; 0.006 M NADP, 0.1 ml; 0.1 M glucose-6-phosphate, 0.1 ml; and was initiated by the addition of the diluted enzyme, 0.1 ml.[17]

D-Xylulose-5-phosphate phosphoketolase (D-xylylose-5-phosphate D-glyceraldehyde-3-phosphate-lyase, EC4.1.2.9). The spectrophotometric measurement of triose phosphate with NADH and α-glycerophosphate dehydrogenase was monitored according to Horecker.[18] The assay mixture was as follows: 0.5 M potassium phosphate buffer, pH 6.0, 0.22 ml; 0.1 M ribose-5-phosphate, 0.03 ml; 0.1 M $MgCl_2$, 0.01 ml; 0.3 M glutathione, 0.01 ml; 0.005 M thiamin pyrophosphate, 0.01 ml; 0.005 ml isomerase-epimerase mixture (phosphoriboisomerase and D-xyldose-5-phosphate 3-epimerase). The mixture was incubated for 5 min at 38°C, then the diluted enzyme preparation (0.01 ml) was introduced into the assay mixture, and incubation was continued for another 15 min. The reaction was stopped by immersing the tube for 1 min in a boiling water bath. The assay of fructose-1,6-diphosphate was carried out using 0.05 ml of the incubation mixture in a 1 cm path quartz cuvette as follows: 0.02 M phosphate buffer, pH 7.5,l ml; 0.01 ml NADH (0.001 M in 0.01 M NaOH). The absorbance was measured before and after the addition of α-glycerophosphate dehydrogenase.

Alcohol dehydrogenase (alcohol:NAD oxidoreductase EC 1.1.1.1). The activity of yeast alcohol dehydrogenase was determined by the method of Vallee and Hoch.[19] In this method the rate of increase in absorbance at 340 nm due to reduction of NAD is measured. The reaction mixture contained 0.032 M pyrophosphate buffer, pH 8.8, 1.5 ml; 2 M absolute ethanol, 0.5 ml; 25 × 10^{-3} M NAD, 1.0 ml; and was initiated by the addition of the diluted enzyme, 0.2 ml.

Analytical procedures

The samples (1 ml) were removed periodically and assayed for cell growth, sugar utilization and product formation as previously described.[6,14] Protein determination in cell-free extracts was carried out with Folin–Ciocalteu reagent as described by Lowry *et al.*[20] Crystalline bovine serum albumin was used as the standard.

Results

Induction of D-xylose reductase and NAD$^+$-xylitol dehydrogenase

D-Xylose reductase and xylitol dehydrogenase activities were observed in the cell-free extracts of the strains which were grown on D-xylose as sole carbon source (when as little as 5 - 10 μg protein mixture/assay was employed). None of the strains exhibited D-xylose reductase or xylitol dehydrogenase activity when grown on D-glucose as sole carbon source, even when much higher concentrations of protein (250 μg) were employed. The results indicated that the enzymes are inducible in both the parent and the mutants.

D-Xylose reductase

Specific activities of NADPH- and NADH-linked D-xylose reductase activity in cell-free extracts of xylose-grown *P. tannophilus* and five of its mutants are presented in Table 1. As compared to the parent, a 1.5–5.2-fold increase in the specific activity of NADPH-linked xylose reductase was observed in the mutants. Even more striking increases in specific activities of NADH-linked D-xylose reductase activities were observed. NADH-linked activities increased up to 14.7-fold as compared to the parent strain. The mutant strain previously shown to exhibit the highest specific ethanol fermentation rate among these various strains was $NO_3 \rightarrow NO_3$-4.[6] This strain, while not exhibiting the highest specific activities, showed the highest ratio of NADH- to NADPH-linked activity. This ratio was 0.26 in the parent and 1.0 in the $NO_3 \rightarrow NO_3$-4 mutant.

Xylitol dehydrogenase

NAD-linked xylitol dehydrogenase activity showed an enhancement of 1.1 –2.6-foldover the parent strain (Table 2). An average increase in activity of 1.4-fold was observed. Again, the $NO_3 \rightarrow NO_3$-4 mutant exhibited a substantial increase in activity, 2.4-fold as compared to the parent. NADP-linked xylitol dehydrogenase activity was not detected in any of the strains when examined with con-

Table 1 NADPH-and NADH-linked D-xylose reductase activity in NRRL Y-2460 and its mutants

Strain	Ethanol production (mM)	Enzyme activities: NADPH-dependent (U mg^{-1})	Fold[a] specific activity	NADH-dependent (U mg^{-1})	Fold[a] specific activity	NADH/NADPH linked xylose reductase	Fold rise in the ratio[b]
NRRL Y-2460	79[c]	0.033	1.0	0.008	1.0	0.26	1.0
$NO_3 \rightarrow NO_3$-7	99[d]	0.051	1.54	0.024	3.0	0.49	1.8
$NO_3 \rightarrow$U-4	140[c], 133[d]	0.076	2.3	0.044	5.5	0.57	2.2
$NO_3 \rightarrow NO_3$-4	155[c], 134[d]	0.078	2.36	0.080	10.0	1.00	3.8
U→U-27	80[d]	0.173	5.24	0.118	14.7	0.68	2.6
U→NO_3-6	98[c], 65[d]	0.121	3.67	0.074	9.25	0.61	2.3

[a] All as compared to parent strain
[b] Of NADH/NADPH-linked activity
[c] 48 h experiment
[d] 44 h experiment

Table 2 Specific activities of NAD-linked xylitol dehydrogenase in NRRL Y-2460 and isolated mutants

Strain	Specific activity (U mg^{-1})	Fold specific activity
Y-2460	0.049	1.0
$NO_3 \rightarrow NO_3$-7	0.083	1.7
$NO_3 \rightarrow$U-4	0.055	1.1
$NO_3 \rightarrow NO_3$-4	0.12	2.4
U→U-27	0.16	2.6
U→NO_3-6	0.07	1.2

Table 3 Specific activities of D-xylulokinase in NRRL Y-2460 and isolated mutants

Strain	Specific activity (U mg^{-1})	Fold specific activity
Y-2460	0.057	1.0
$NO_3 \rightarrow NO_3$-7	0.62	10.3
$NO_3 \rightarrow$U-4	0.51	8.5
$NO_3 \rightarrow NO_3$-4	0.34	5.8
U→U-27	0.27	4.5
U→NO_3-6	0.33	5.5

centrations of either NADP from 0.34 to 1.0 mM or D-xylitol from 100 to 200 mM.

D-Xylulokinase

Production of D-xylulokinase was enhanced by 4.5–103 times in mutants as compared to the parent strain (Table 3). The greatest specific activity of D-xylulokinase found was 0.62 in the strain $NO_3 \rightarrow NO_3$-7, which is 10.3 times greater than the parent strain NRRL Y-2460. This particular strain, $NO_3 \rightarrow NO_3$-7 exhibited the most rapid growth observed on xylitol plates.[6]

Glucose-6-phosphate dehydrogenase

In contrast to other enzymes examined, glucose-6-phosphate dehydrogenase did not increase significantly in most of the mutants tested. A small increase (<50%) was observed in the fermentative strain, $NO_3 \rightarrow NO_3$-4 *(Table 4)*. No attempt was made to determine the NAD^+-linked activity, if any, of this enzyme.

D-Xylulose-5-phosphate phosphoketolase

Phosphoketolase activity was detected in all the strains examined in the present study with specific activities in the range 0.06–0.16 U mg^{-1} of total cell protein. A rise of 1.2–2.6-foldwas generally observed in the mutant strains as compared to the parent strain. However, contrary to the other increases in activity, in $NO_3 \rightarrow$U-4 strain, the phosphoketolase activity was lower by 34% than the parent strain. The greatest increase in activity was observed with the most fermentative mutant, $NO_3 \rightarrow NO_3$-4 (Table 5).

Alcohol dehydrogenase

The specific activities of alcohol dehydrogenase also indicated elevated levels in mutants between 1.5-fold and 2.7-fold over the parent strain. NAD was the most active coenzyme for the ADH *(Table 6)*. However, with some mutants, slight activity was also recorded with NADP as the coenzyme (data not shown). Again, the greatest increase in activity was observed with the most active fermentative strain, $NO_3 \rightarrow NO_3$-4.

Summary of results

Three observations may be summarized from the results. (1) All mutants exhibited increases in the NADH- and NADPH-dependent D-xylose reductase activity with the NADH-dependent activity increasing more. The consequent increase in the NADH:NADPH activity ratio was greatest in the best fermenter and least in the strain exhibiting fastest growth on xylitol. (2) This fast-growing stain also exhibited the highest levels of D-Xylulokinase. (3) The best fermenter exhibited the greatest increases observed for glucose-6-phosphate dehydrogenase, phosphoketolase and alcohol dehydrogenase.

Discussion

The results reported here indicate that the mutants obtained earlier are regulatory in nature. Broad changes were observed in the levels of many enzymes simultaneously. The strains, however, differed significantly from one another and from the parent in the balance of constituent enzymes. It seems probable that the balance of activities is important in determining the overall direction of the pathways involved. Expression of the enzymes is influenced by various metabolic factors, and levels of individual enzymatic activities attained under the conditions employed are the result of complex regulatory functions.

The apparent levels of enzymes observed here in crude preparations are assumed to be indicative of effective intracellular activities. The D-xylose reductase and the

Table 4 Glucose-6-phosphate dehydrogenase levels in *P. tannophilus* and its mutants

Strains	Specific activity (U mg^{-1})	Fold specific activity
Y-2460	1.9	1.0
$NO_3 \rightarrow NO_3$-7	2.0	1.05
$NO_3 \rightarrow$U-4	1.9	1.00
$NO_3 \rightarrow NO_3$-4	2.6	1.40
U→U-27	2.3	1.20
U→NO_3-6	2.1	1.10

Table 5 Specific activities of D-xylulose-5phosphate D-glyceraldehyde-3-phosphate lyase in NRRL Y-2460 and isolated mutants

Strain	Specific activity (U mg^{-1})	Fold specific activity
Y-2460	0.0092	1.0
$NO_3 \rightarrow NO_3$-7	0.014	1.5
$NO_3 \rightarrow$U-4	0.006	(0.65)[a]
$NO_3 \rightarrow NO_3$-4	0.024	2.6
U→U-27	0.016	1.8
U→NO_3-6	0.0113	1.2

[a] Decreased

Table 6 Specific activities of alcohol dehydrogenase in NRRL Y-2460 and isolated mutants

Strain	Specific activity (U mg^{-1})	Fold specific activity
Y-2460	0.09	1.0
$NO_3 \rightarrow NO_3$-7	0.09	1.0
$NO_3 \rightarrow$U-4	0.16	1.8
$NO_3 \rightarrow NO_3$-4	0.25	2.7
U→U-27	0.21	2.3
U→NO_3-6	0.23	2.5

xylitol dehydrogenase activities in the mutant strains are still under the regulation of xylose induction. This inducibility had been shown with *P. tannophilus* Y-2460 in earlier work by Smiley and Bolen[21] and by Bolen and Detroy.[22]

The ratio of NADH/NADPH-linked D-xylose reductase activities observed in crude preparations is probably a factor that can be altered by metabolic regulation, and the relative activities might be a function of regulatory effectors in the cytoplasm or reaction mixture. Several different ratios of relative cofactor activity were obtained in different strains when they were grown under identical conditions, suggesting that overall regulatory patterns differ in these strains. This conclusion is further supported by the observation that many different enzymes involved in xylose metabolism were affected by the mutation and selection process.

An additional possibility which might account for the observed increases in enzymatic activities is that some of these strains are simply diploid or polyploid in nature. However, this explanation is unlikely because the ratios of the enzymes did not vary in a consistent manner from one mutant strain to another. For example, $NO_3 \rightarrow NO_3$-7, the fastest growing strain on xylitol, had the highest xylulokinase activity, but a relatively low level of NADH-linked xylose reductase; the best fermenting strain, $NO_3 \rightarrow NO_3$-4, showed the greatest rise in relative NADH-linked xylose reductase and also the greatest rise in alcohol dehydrogenase. For these reasons, we have concluded that these mutants have amplified expressions of certain enzymes, but do not appear to have amplified their genome as a whole.

The intracellular balances of NAD(P)/NAD(P)H appear to be very important in regulating the activities of xylose reductase[23] and xylitol dehydrogenase[24] in *P. tannophilus.* The NADP/NADPH ratio regulates the activity of glucose-6-phosphate dehydrogenase from rat liver.[25,26] Other studies have also shown the importance of NAD(P)/NAD(P)H ratios in regulating the metabolic activity of yeasts.[27] In *Candida utilis,* glucose-6-phosphate dehydrogenase is further regulated by the levels of erythrose-4-phosphate and glyceraldehyde-3-phosphate.[28] Regulation of this enzyme is important because its activity largely determines intracellular concentrations of NADPH.

Given that ethanol dehydrogenase must compete with xylose reductase for reductant, the relative K_m values of these two enzymes for NADH could be critical in determining xylitol and ethanol product ratios. Under the conditions employed, respiration also enters into consideration[29,30] Intracellular NADH levels should increase as cells are shifted to anaerobic conditions. In *P. tannophilus,* such a shift is associated with an increase in xylitol production. Ditzelmüller et al.[24] have shown that purified xylitol dehydrogenase from *P. tannophilus* is strongly influenced by the 'catabolic reduction charge' (NADH/NAD + NADH). An increase in the charge favours the accumulation of xylitol and NAD^+. The 'anabolic reduction charge' (NADPH/NADP + NADPH) did not influence the NAD-linked oxidation of xylitol.

In the following, the activities of each of the enzymes observed here are discussed with regard to previous findings and current knowledge of the D-xylose fermentation.

D-Xylose reductase

Either NADH or NADPH will serve as a coenzyme for the reduction of xylose to xylitol in *P. tannophilus.* The ratio of NADH/NADPH coupled activity observed here was about 0.26 in the parent and as high as 1.0 in the best fermenting mutant. Earlier studies by Bruinenberg *et al.*[31] have indicated a ratio of 0.04 for *P. tannophilus* CBS 4044 (=NRRL Y-2460 = ATCC 32691). Some of the difference between our results and those of Bruinenberg *et al.*[31] might be attributed to the manners in which the cultures were grown or maintained. In contrast to Bruinenberg *et al*.,[31] we did not observe significant NADP-coupled xylitol dehydrogenase activity in the parent or in mutant strains.

Changes in xylose reductase levels in yeast mutants have been previously demonstrated. The faster fermenting mutant, XF217, showed less (0.24 U mg protein^{-1}) while the parent, Candida sp. C2, showed more (0.41 U mg protein^{-1}) D-xylose reductase activity.[32] It should be noted, however, that neither of these strains showed D-xylose reductase activity with NADH as the coenzyme, and in our laboratory neither of these strains was capable of fermenting D-xylose under anaerobic conditions.[33]

Bruinenberg *et al.* [31] showed a correlation between the fermentative ability of a yeast and its NADH-linked D-xylose reductase activity. They found that NADH-linked D-xylose reductase activity was 34 times higher in *P. stipitis* than in *P. tannophilus,* and the *P. stipitis* had 8 times the xylose fermentation rate of *P. tannophilus.* This correlation has been supported by the fact that the highest ratio of NADH- to NADPH-linked D-xylose reductase activity was observed in the best fermenting mutant.

Because both NADH and NADPH-linked D-xylose reductase activities are present in *P. tannophilus,* it is appropriate to ask whether one or two enzymes are involved. Ditzelmüller *et al.* [23] recently purified and characterized D-xylose reductase from *P. tannophilus.* The purified enzyme showed specificity for NADPH. Less than 0.5% of the NADPH-linked V_{max} was observed with NADH. In contrast to the NADPH specificity shown by the purified *P. tannophilus* enzyme, a homogeneous preparation of *P. stipitis* xylose reductase obtained by Verduyn *et al.*[34] showed 70% as much activity with NADH as with NADPH. Moreover, the ratio of NADH/NADPH-linked activities remained practically constant between 0.72 and 0.78 during purification stages. However, earlier findings and the results reported here suggest that two separate D-xylose reductases are present in *P. tannophilus.* This conclusion is supported by the various ratios of NADH/NADPH-linked activities observed in our different mutants and, in fact, Ditzelmüller *et al.*[35] have recently reported separate NADH- and NADPH-linked D-xylose reductase activities in cell-free preparations derived from *P. tannophilus.*

Xylitol dehydrogenase

Our studies showed modest increases in NAD-linked xylitol dehydrogenase activity in the selected mutants. Although the best fermenter showed one of the better increases in xylitol dehydrogenase, a strain that showed no significant increase in specific ethanol fermentation rate (U→U-27) showed the highest increase in xylitol dehydrogenase. Therefore, no direct independent correlation between the enzymatic and fermentative activities can be made from these data. McCracken and Gong[32] reported a moderate increase in the level of this enzyme in Candida sp. mutant XF217.

Ditzelmüller *et al.*[24] partially purified xylitol dehydrogenase from *P. tannophilus.* With purified enzyme, they observed specificity for NAD(H), but 6–11% of the NAD(H)-linked V_{max} was obtained when NADP(H) was

substituted at the same levels. The findings of Ditzelmüller *et al.*[24] agree well with the ratios of crude activities observed by Bruinenberg *et al.*[31] We did not observe significant NAD-linked xylitol dehydrogenase activities in our studies.

D-Xylulokinase

D-Xylulokinase catalyses the principal driving reaction in the nonoxidative phase of the pentose phosphate pathway. Mutants in the present study showed 5.5–10.3-fold increases in D-xylulokinase levels. Although increases in D-Xylulokinase were recorded for all of the strains reported here, the greatest increase was observed in the strain which grew the fastest on xylitol agar. McCracken and Gong[32] reported a sixfold increase in the specific activity of this enzyme in their XF217 mutant as compared to the parent Candida strain. Their mutant had also been selected for rapid growth on xylitol.

Phosphoketolase

Phosphoketolase carries out the cleavage of either D-xylulose-5-phosphate or D-fructosed-6-phosphate to form glyceraldehyde-3-phosphate or erythrose-4-phosphate and acetyl phosphate. The overall free energy change for this reaction has been estimated to be -10.5 kcal mol^{-1}. If the acetyl phosphate is in turn linked to the production of ATP and acetate the free energy change is an additional -3.1 kcal mol^{-1}. In either case, the reaction represents a substantial driving force in the overall metabolic pathway. For this reason, it is probably under strict metabolic regulation. Evans and Ratledge[10] have stressed the role of phosphoketolase in xylose utilization by yeasts. These investigators have found xylulose-5-phosphate phosphoketolase in 20 out of 25 yeasts examined. It seems likely that phosphoketolase is responsible for the production of acetic acid under anaerobic conditions.

It is useful to compare the relative activities of D-xylulokinase and phosphoketolase. In the wild-type cells, the measured phosphoketolase activity constituted about 16% of the level of the D-xylulokinase activity observed. In the mutants, phosphoketolase activity was at most 7% of the D-xylulokinase activity and in one instance (the fastest grower) was as little as 1%. One might speculate that phosphoketolase could contribute glyceraldehyde-3-phosphate to an otherwise depleted metabolic pool especially when it is depleted by high fermentative activity. This action would thereby facilitate turnover of the reaction mediated by transketolase. Phosphoketolase probably does not contribute directly to uptake or assimilation of xylose.

Glucose-6-phosphate dehydrogenase

The primary function of glucose-6-phosphate dehydrogenase is to supply NADPH for anabolic processes. In this instance, NADPH is available both for growth and xylose assimilation. The slight elevation in levels observed with this enzyme was surprising given the fact that the $NO_3 \rightarrow NO_3$-4 strain was obtained by selecting for rapid growth on nitrate as a sole nitrogen source. Utilization of nitrate places a high demand on the ability of the cell to produce NADPH. For example, Hankinson and Cove[36] found roughly a twofold increase in glucose-6-phosphate dehydrogenase activity in wild-type strains of *Aspergillus nidulans* when it was grown in the presence of nitrate, and Osmond and Ap Rees[13] found a 2.5-fold increase in activity of this enzyme when *C. utilis* was grown on nitrate.

Alcohol dehydrogenase

In these experiments, the levels of alcohol dehydrogenase increased from 1.8 to 2.7-fold over the parent strain. While there was no simple correlation between alcohol dehydrogenase levels and an ability to rapidly ferment D-xylose, the best fermenting mutant, $NO_3 \rightarrow NO_3$-4, showed the highest fold increase, and the fastest grower did not exhibit an increase.

Conclusions

The mutants obtained by selecting for rapid growth on xylitol agar, either with or without nitrate as a nitrogen source, appear to be regulatory in nature because many separate enzymatic activities were affected. Faster growth on xylitol resulted in higher enzyme levels. All of the mutant strains examined here showed increased levels of pentose phosphate pathway enzymes. The fastest grower was not the best alcohol producer, but it had the highest activity of D-xylulokinase observed. The best anaerobic ethanol producer also showed the highest ratio of NADH/NADPH-linked xylose reductase, the highest levels of glucose-6-phosphate dehydrogenase, phosphoketolase and alcohol dehydrogenase, and the second highest level of xylitol dehydrogenase. These studies do not, therefore, point to a single rate-limiting step in the utilization of xylose. Rather, D-xylulokinase appears to be critical for aerobic assimilation and growth, and a consortium of enzymes is important for fermentation. Intracellular competition for reductant and consequent regulatory processes appear to be crucial in this latter process.

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