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EXPERIMENTAL ARTICLES

Metabolic Properties of *Pachysolen tannophilus* Mutants Producing Xylitol and Ethanol from D-Xylose

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Abstract—Activity of the major enzymes of D-xylose metabolism in the mutants of xylose-utilizing yeasts *Pachysolen tannophilus* selectively producing xylitol or ethanol was studied. The xylitol-producing strain exhibited low activities of xylitol dehydrogenase, xylose reductase with preferential affinity to NADPH, NAD^+-dependent malate dehydrogenase, and cytochrome *c* oxidase (4.40, 4.80, 1.87, and 0.28 μmol mg^{-1} min^{-1}, respectively). The cells of the ethanol-producing mutants exhibited elevated activity of NADH/NADPH-xylose reductase, xylitol dehydrogenase, 1-glycerophosphate dehydrogenase, and lactate dehydrogenase to 6.80, 8.60, 4.68, and 16.48 μmol mg^{-1} min^{-1}, respectively. Effect of the NADPH/NADH imbalance on ethanol production accumulation and xylitol accumulation is discussed.

Keywords: D-xylose, xylitol, ethanol, *Pachysolen tannophilus*

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It is known that xylose-utilizing yeasts usually convert D-xylose either to xylitol or to ethanol. Only wild-type *Pachysolen tannophilus* strains can produce these two compounds simultaneously in comparable amounts [1]. Xylitol accumulation is presumed to induce an imbalance between NADPH/NADH coenzymes [2, 3] responsible for the initial stages of D-xylose conversion into D-xylulose [4, 5]. Further D-xylulose degradation is mediated by enzymes of the pentose phosphate cycle (Warburg–Dickens–Horecker), glycolysis (Embden–Meyerhof–Parnas), and the Krebs cycle. Therefore, the stages limiting and enhancing ethanol production involve different stages of general carbohydrate metabolism pathways taking place both in the cytosol and in mitochondria.

The specific features of D-xylose catabolism responsible for selective production of xylitol or ethanol by yeast cells remain unknown. In our opinion, mutant *Pa. tannophilus* strains, which, in contrast to wild-type strains, selectively accumulate one of these products, are a convenient model for such investigations [6]. Their unique traits are probably related to some changes involving not only the initial stages of D-xylose degradation, but also the general pathways of carbohydrate catabolism.

The goal of the present work was therefore to determine the activity of xylose reductase (EC 1.1.1.21) and xylitol dehydrogenase (EC 1.1.1.9), which catalyze D-xylulose synthesis, as well as of cytosolic 1-glycerophosphate dehydrogenase (EC 1.1.1.8), lactate dehydrogenase (EC 1.1.1.27), mitochondrial cytochrome *c* oxidase (EC 1.9.3.1) and total NAD^+-dependent malate dehydrogenase (EC 1.1.1.37) in mutant *Pa. tannophilus* strains that produce either xylitol or ethanol.

MATERIALS AND METHODS

Subjects of research. The study was performed with mutant *Pa. tannophilus* 22-Y-1532 strains no. 390, no. 442, and no. 664, which exhibited altered growth on D-xylose, xylitol, or ethanol as sole carbon sources ($Glu^+Xyl^+XylOH^{\pm}EtOH^-$, $Glu^+Xyl^+XylOH^-EtOH^+$, and $Glu^+Xyl^{\pm}XylOH^{\pm}EtOH^{\pm}$ phenotypes, respectively) and produced either ethanol or xylitol from D-xylose [6]. The parent strain *Pa. tannophilus* 22-Y-1532, which accumulates both compounds in comparable amounts, was used as the control.

To obtain inocula for fermentation, yeasts were grown in 500-mL flasks containing 100 mL liquid YEPD medium with 2% D-glucose as the sole carbon source [7] at 30 ± 2°C under shaking (230 rpm). After 18–24 h, 10 mL of yeast suspension was transferred into new 500 mL-flasks containing 100 mL liquid YEPD medium with 2% D-xylose as the sole carbon source [7] and incubated under the same conditions. The obtained biomass was used for microaerobic fermentation experiments in the concentration of 6.0 g/L (absolutely dry matter).

Microaerobic fermentation was performed for 24 h in 250-mL flasks containing 100 mL liquid YEPD medium with 2% D-xylose at 30 ± 2°C with shaking

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Table 1. Assimilation of D-xylose by *Pa. tannophilus* mutants

Mutant strain	Xylitol, g/g*	Ethanol, g/g*	D-xylose consumption		Biomass, g/g*
			Rate, g L^{-1} h^{-1}	Extent, %	
664	**0.25 ± 0.01**	0.05 ± 0.01	0.51 ± 0.09	56.0 ± 1.0	0.05 ± 0.01
390	≤0.01	**0.24 ± 0.01**	0.73 ± 0.07	86.0 ± 1.0	0.09 ± 0.01
442		**0.26 ± 0.01**		78.6 ± 1.0	
C	0.11 ± 0.01	0.21 ± 0.01	0.62 ± 0.09	68.8 ± 1.0	0.12 ± 0.01

C is the parent haploid strain *Pa. tannophilus* 22-Y-1532.

* The yield of microaerobic fermentation products was calculated per 1 g of D-xylose consumed. The highest xylitol and ethanol production rates and the corresponding enzyme activities are shown in bold.

(100 rpm). Biomass concentration was determined at the end of fermentation on an SF-26 spectrophotometer (Poland) as described in detail in [6]. The concentrations of reducing agents were determined using the Fehling's test. Ethanol and xylitol were determined by gas chromatography on a Vista 600 chromatograph (Varian, United States) as described in [1].

Cell-free extracts were obtained following the protocol described in [6]. To disintegrate the mitochondrial membranes, the cells were ground with fused silica powder in 0.01 M Tris-HCl buffer (pH 7.5) with 0.1% Triton X-100, and the mixture was centrifuged at 20000 rpm at 0°C for 30 min [8]. Protein concentrations in the obtained extracts were determined using the Lowry assay [9], with bovine serum albumin as the standard.

Total and specific enzyme activity of xylose reductase and xylitol dehydrogenase were assessed by changes in NAD(P)H/$NAD(P)^+$ concentrations; the optical density of the reaction mixture was measured at 340 nm in specimens with equal protein concentrations on an SF-26 spectrophotometer at 20°C. The correction for endogenous activity was introduced using an internal control that contained all components of the reaction mixture except the substrate (D-xylose or xylitol). For each enzyme, the amount necessary to reduce (oxidize) 1 μmol NAD(P)H/$NAD(P)^+$ in 1 min was taken for an activity unit [8].

The total activity of lactate dehydrogenase, NAD^+-dependent malate dehydrogenase, and 1-glycerophosphate dehydrogenase in cell-free extracts was determined by spectrophotometry based on changes in NADH concentrations [10]. Cytochrome *c* oxidase activity was accessed using the Smith's method [11] after cytochrome *c* was reduced with twofold amount of ascorbate (by weight) in 0.02 M phosphate buffer (pH 7.0) for 2 h and purified from the excess of ascorbate on a Sephadex G-25 column.

Statistical analysis of experimental data was performed using the Student's *t*-test [12] with the significance threshold of 0.05 by means of MS Excel.

RESULTS AND DISCUSSION

Microaerobic transformation of D-xylose. Table 1 summarizes the characteristics of microaerobic D-xylose fermentation by mutant *Pa. tannophilus* 22-Y-1532 strains accumulating either xylitol or ethanol. In strain 664, enhanced xylitol production (nearly 2.3-fold) was accompanied by slower culture growth, lower extent of D-xylose consumption, and suppressed growth of yeast biomass. These facts indirectly suggest that in strain no. 664, which showed weak growth on media containing xylose, xylitol, or ethanol as sole carbon sources ($Glu^+Xyl^{\pm}XylOH^{\pm}EtOH^{\pm}$ phenotype), reactions introducing D-xylose into general carbohydrate catabolism pathways were inhibited.

Higher D-xylose consumption rates and consumption extent in strains nos. 390 and 442 in comparison to the *Pa. tannophilus* 22-Y-1532 control strain were associated with more efficient ethanol production (by 19 and 27%, respectively; Table 1). Taking into account that these mutants exhibited lower biomass growth than the control strain (0.09 vs .0.12 g per 1 g D-xylose consumed), we assumed that genetic modifications in these strains with the phenotypes $Glu^+Xyl^+XylOH^{\pm}EtOH^-$ (no. 390; weak growth on xylitol and inability to utilize ethanol as the sole carbon source) and $Glu^+Xyl^+XylOH^-EtOH^+$ (no. 442; lack of growth on xylitol as the sole carbon source) affected some steps of the general carbohydrate catabolism.

Activity of the key enzymes of D-xylose catabolism. Previous studies involving a broad variety of wild-type yeast strains confirmed that xylose reductase and xylitol dehydrogenase not only play a central role in D-xylose catabolism [2, 3, 13], but also affect strongly the production of xylitol and ethanol [4, 5]. Analysis of their activity in *Pa. tannophilus* 22-Y-1532 mutants revealed the following (Table 2).

Strain no. 664 ($Glu^+Xyl^{\pm}XylOH^{\pm}EtOH^{\pm}$) exhibited the minimal activity of the enzymes regulating D-xylulose formation. Interestingly, in this strain, 67% of the total xylose reductase activity was NADPH-dependent, whereas 95% of xylitol dehydrogenase activity was NAD^+-dependent. This fact further emphasizes the importance of the initial stages of

Table 2. Initial stages of D-xylose catabolism in *Pa. tannophilus* mutants

Principal product	Mutant	Activity, μmol/mg min					
		Xylose reductase			Xylitol dehydrogenase		
		NADPH	NADH	total	NAD^+	$NADP^+$	total
Xylitol	664	3.00 ± 0.08	1.40 ± 0.08	4.40 ± 0.08	4.55 ± 0.10	0.25 ± 0.10	4.80 ± 0.10
Ethanol	390	2.80 ± 0.10	3.20 ± 0.10	6.00 ± 0.10	8.00 ± 0.10	0.40 ± 0.10	**8.40 ± 0.10**
	442	3.60 ± 0.10	3.20 ± 0.10	**6.80 ± 0.10**	6.45 ± 0.09	0.35 ± 0.09	6.80 ± 0.09
C		5.00 ± 0.09	5.40 ± 0.09	10.40 ± 0.09	7.70 ± 0.09	0.30 ± 0.09	8.00 ± 0.09

D-xylose catabolism in the development of the NADPH/NADH imbalance promoting xylitol accumulation (figure).

Ethanol-producing *Pa. tannophilus* 22-Y-1532 mutants exhibited fairly high total xylose reductase and xylitol dehydrogenase activity. In strains no. 390 ($Glu^+Xyl^+XylOH^{\pm}EtOH^-$) and no. 442 ($Glu^+Xyl^+XylOH^-EtOH^+$)), the share of NADH-dependent xylose reductase activity was also higher (53%), while xylitol dehydrogenase retained high affinity to NAD^+. In mutant no. 390, its total activity was even higher than in the control (Table 2). The observed characteristics of these enzymes explained well the efficiency of D-xylose utilization by ethanol-producing *Pa. tannophilus* 22-Y-1532 mutants; however, the reason for selective ethanol accumulation remained insufficiently clear. In contrast to xylitol, ethanol is the end product of microaerobic D-xylose catabolism (figure). Therefore, we also investigated the activities of several enzymes of the Warburg–Dickens–Horecker and Embden–Meyerhof–Parnas pathways, as well as of the Krebs cycle in mutant *Pa. tannophilus* 22-Y-1532 strains with altered ethanol and xylitol production.

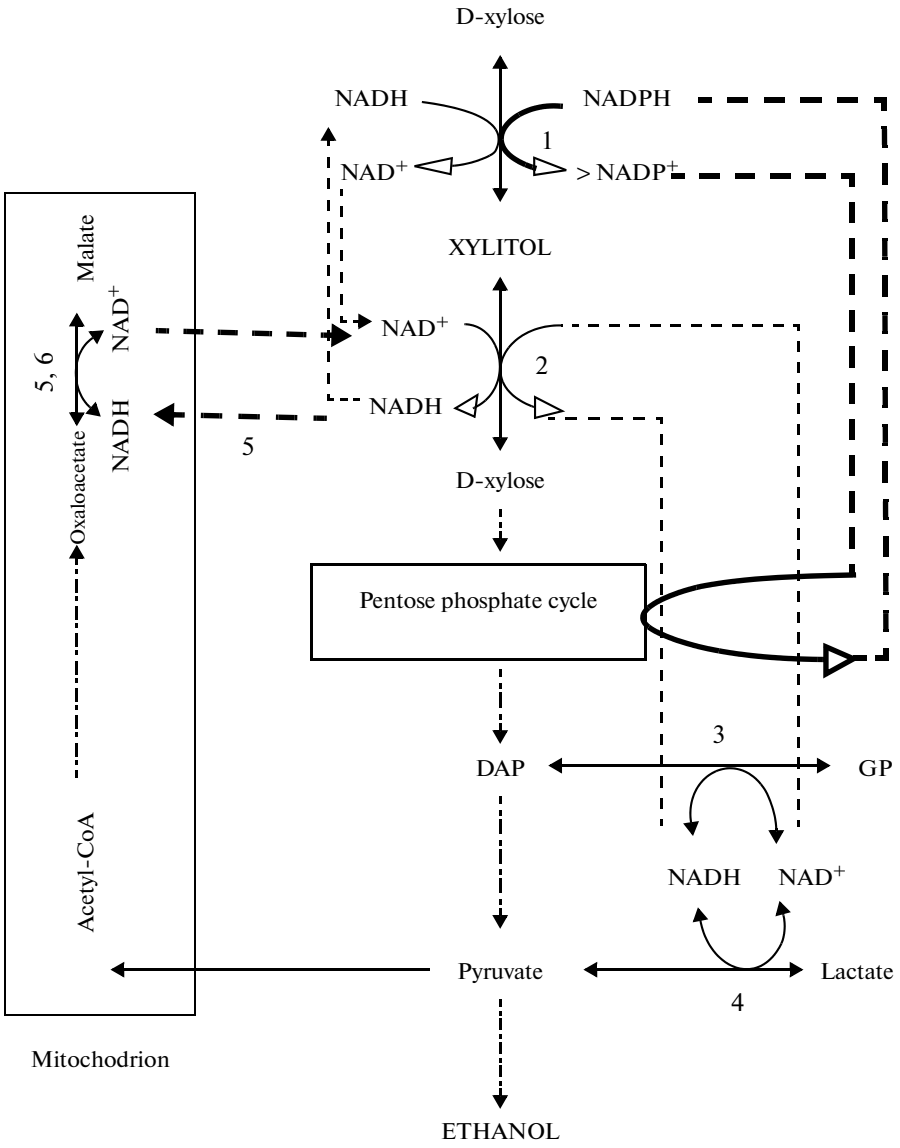
Activity of enzymes of the general carbohydrate catabolism pathways. It is known that the transformation of D-xylulose 5-phosphate into fructose-6-phosphate and glyceraldehyde-3-phosphate, intermediate products of glycolysis, occurs by means of non-oxidative reactions of the pentose phosphate cycle. Their subsequent fate depends on the aeration of the fermentation medium. However, even under microaerobic conditions, the amount of pyruvate that xylose-consuming yeasts utilize for ethanol production is lower than the amount entering the Krebs cycle [14]. This results in differences in the accumulation of xylitol and ethanol, as well as in the yeast biomass growth (Table 1). The metabolic properties of a yeast cell that underlie this phenomenon are poorly understood. Presumably, they are closely related to the crucial role that oxygen plays in the maintenance of the NADPH/NADH balance [2, 15]. To evaluate the intensity of transmembrane transfer of reduced NADH equivalents into mitochondria under conditions of oxygen limitation, we assessed the activity of 1-glycerophosphate dehydrogenase and NAD^+-dependent malate dehydrogenase, components of the glycerol phosphate and malate shuttle systems (Table 3).

In the xylitol-accumulating mutant strain no. 664 ($Glu^+Xyl^{\pm}XylOH^{\pm}EtOH^{\pm}$), their activity was the lowest, indirectly suggesting that the electron and proton transport from NADH onto the respiratory chain was nearly completely blocked.

The levels of 1-glycerophosphate dehydrogenase and NAD^+-dependent malate dehydrogenase activity differed among ethanol-producing mutants of *Pa. tannophilus* 22-Y-1532. In strain no. 390 ($Glu^+Xyl^+XylOH^{\pm}EtOH^-$), they were by 46 and 43% lower than in the control, respectively. In strain no. 442 ($Glu^+Xyl^+XylOH^-EtOH^+$), the total 1-glycerophosphate dehydrogenase activity differed little from the control value, while the activity of NAD^+-dependent malate dehydrogenase activity was only 79% of the control. Thus, D-xylose conversion into ethanol does not rule out the possibility of NAD^+ regeneration due to the transportation of reduced equivalents into mitochondria, although its intensity may vary (figure).

It was previously shown that inhibition of malate dehydrogenase, α-ketoglutarate dehydrogenase, or fumarase in the Krebs cycle also stimulated ethanol production in *Pa. tannophilus* [14]. However, little is known about the functional changes in the respiratory chains that accompany such metabolic reorganization in xylose-utilizing yeasts. Cytochrome *c* oxidase is one of the most important components of the mitochondrial energy system. Therefore, we assumed it would be informative to compare the activity of NAD^+-dependent malate dehydrogenase and cytochrome *c* oxidase among *Pa. tannophilus* 22-Y-1532 mutants (Table 3).

In the ethanol-producing strain no. 442 ($Glu^+Xyl^+XylOH^-EtOH^+$), cytochrome *c* oxidase activity was not changed much, while the inhibition of NAD^+-dependent malate dehydrogenase was more pronounced. These data confirm that oxygen might be involved in the regulation of the NADH/NAD^+ equilibrium under microaerobic conditions.



NAD(P)H regeneration in mutant strains selectively producing xylitol or ethanol from D-xylose. DAP, dioxyacetone phosphate; GP, glycerol-3-phosphate. Enzymes: xylose reductase (1); xylitol dehydrogenase (2); 1-glycerophosphate dehydrogenase (3); lactate dehydrogenase (4); NAD^+-dependent malate dehydrogenase (5); and cytochrome c oxidase (6). Pathways of NAD(P)H regeneration: principal (standard dashed lines) and alternative (fine dashed lines).

In strain no. 390 ($Glu^+Xyl^+XylOH^{\pm}EtOH^-$), another ethanol-producing mutant, the activity levels of both these enzymes were lower than in *Pa. tannophilus* 22-Y-1532 by 43 and 60%, respectively. Finally, in the xylitol-accumulating strain no. 664 ($Glu^+Xyl^{\pm}XylOH^{\pm}EtOH^{\pm}$), cytochrome *c* oxidase was nearly completely inhibited, while the activity of NAD^+-dependent malate dehydrogenase was retained on a low level (Table 3). Therefore, the NADH/NAD^+ ratio in *Pa. tannophilus* 22-Y-153 mutants that selectively produce ethanol or xylitol may be regulated by organic electron and proton acceptors other than oxygen.

Possible candidates for this role are dioxyacetone phosphate (an intermediate of the Embden–Meyerhof–Parnas pathway) and oxaloacetate released in response to elevated cytosolic citrate concentrations accompanying repression of the Krebs cycle [16]. Their reduced forms can be used for lipid production, as well as for NADPH generation in the presence of the malic enzyme found in some yeast species [17, 18]. However, this hypothesis is appropriate only if the levels of 1-glycerophosphate dehydrogenase and NAD^+-dependent malate dehydrogenase activity are relatively high.

The reaction of pyruvate reduction typical for facultatively anaerobic bacteria is another means to

Table 3. Enzyme activity of general carbohydrate catabolism pathways in *Pa. tannophilus* mutants

Principal product	Mutant	Activity, μmol/mg min			
		1-glycerophosphate dehydrogenase	NAD^+-dependent malate dehydrogenase	Cytochrome *c* oxidase	Lactate dehydrogenase
Xylitol	664	1.10 ± 0.11	1.87 ± 0.09	0.28 ± 0.09	5.34 ± 0.10
Ethanol	390	2.61 ± 0.09	3.04 ± 0.09	0.56 ± 0.09	**16.48 ± 0.11**
	442	**4.68 ± 0.10**	**4.26 ± 0.09**	**1.29 ± 0.09**	7.14 ± 0.09
C		4.86 ± 0.09	5.37 ± 0.10	1.39 ± 0.10	7.72 ± 0.10

maintain the NADH/NAD^+ balance under conditions of oxygen limitation [19]. Its importance for the regulation of D-xylose catabolism in yeast has long been ignored. Therefore, we determined the total lactate dehydrogenase activity in mutant *Pa. tannophilus* 22-Y-1532 strains accumulating different products of microaerobic D-xylose fermentation (Table 3).

It was found that in the xylitol-accumulating strain no. 664 ($Glu^+Xyl^{\pm}XylOH^{\pm}EtOH^{\pm}$) the level of lactate dehydrogenase activity was 69% of the control level. Ethanol-producing strains differed considerably from each other: in strain no. 442 ($Glu^+Xyl^+XylOH^-EtOH^+$), lactate dehydrogenase activity was nearly the same as in *Pa. tannophilus* 22-Y-1532, while in no. 390 ($Glu^+Xyl^+XylOH^{\pm}EtOH^-$), it was significantly higher. Therefore, pyruvate may act as an electron and proton acceptor for NADH under conditions of microaerobiosis, although the role of lactate remains unclear (figure).

Thus, the phenomenon of selective xylitol or ethanol production by yeast cells should be considered as a coordinated reorganization of their carbohydrate metabolism as a whole, rather than as a simple consequence of altered enzyme activity at the initial stages of D-xylose catabolism. It can be blocked at the xylitol stage because a NADPH/NADH imbalance develops, for a variety of reasons. In particular, the contribution determined by the differences in xylose reductase and xylitol dehydrogenase specificity is significant [2, 4–6], but it is also important to take into account the inhibition of NADH/NAD^+-dependent dehydrogenases of the general carbohydrate degradation pathways. Thus, xylitol accumulation may be considered as a particular manifestation of the Pasteur's effect involving D-xylose as a reserve carbon source in yeasts.

Selective ethanol production is rather infrequent among xylose-utilizing yeast strains [1]. It is based on flexible regulation of the NADPH/NADH balance under microaerobic conditions. Apart from the respiratory chain, there are at least three or four other sites of NADH regeneration involving different stages of the general carbohydrate catabolism pathways (figure). Their functioning consistently produces ATP, NADPH, or the substrates for different processes of biosynthesis. Under such conditions, ethanol production can be activated by inhibitors that, similarly to sodium azide, would suppress the inclusion of Embden–Meyerhof–Parnas pathway intermediates in other metabolic cycles of the cell [20].

It is known that the growth of xylose-utilizing yeast strains is strongly limited by furfural, oxymethylfurfural, volatile organic acids, and lignofurane compounds commonly present in industrial sources of D-xylose [21]. Therefore, the possibility to employ the toxic components of acidic lignocellulose hydrolysates and sulfite waste liquor for the regulation of ethanol production by xylose-utilizing yeasts may be of a considerable theoretical and practical interest [22].

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