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EXPERIMENTAL ARTICLES

Production of Xylitol and Ethanol and Activity of the Key Enzymes of D-Xylose Consumption in *Pachysolen tannophilus* Mutant Strains

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Abstract—Production of xylitol and ethanol, as well as activities of the key enzymes of D-xylose consumption, were studied in *Pachysolen tannophilus* mutants with altered growth on D-xylose, xylitol, ethanol, or D-glucose as sole carbon sources. Suppressed activity of xylose reductase with preferential affinity for NADPH and of xylitol dehydrogenase to 4.40 and 4.80 μmol mg^{-1} min^{-1}, respectively, resulted in accumulation of xylitol (0.25 g per 1 g D-xylose consumed). The highest levels of NADH/NADPH-xylose reductase and xylitol dehydrogenase (6.00–6.80 and 6.80–8.40 μmol mg^{-1} min^{-1}, respectively) were found in the strains producing 0.24–0.26 g ethanol per 1 g D-xylose. Application of *Pa. tannophilus* mutants for analysis of the regulation of D-xylose catabolism in yeasts is discussed.

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

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The direction of D-xylose degradation by xylose-utilizing yeasts is known to depend on the aeration intensity. Under conditions of oxygen deficit (so-called microaerobiosis), yeasts start producing xylitol and ethanol [1] in the proportion that correlates strongly to the activities of the key enzymes involved, xylose reductase and xylitol dehydrogenase [2]. However, it is still not quite clear what the metabolic shifts that result in selective accumulation of particular target products of microaerobic D-xylose fermentation are. Insufficient understanding of the life cycles of *Candida intermedia, C. parapsilosis*, and *C. silvanorum*, which produce primarily xylitol [1], as well as of ethanol-producing species *Pichia stipitis* and *C. shehatae* [1], encumbers experimental research aimed at answering this question.

Pachysolen tannophilus strains, for which the conditions required for stabilization of vegetative growth or for induction of the sex process have been determined [3], may be a convenient model to address this problem. However, the collection variants of this species accumulate both target products of microaerobic D-xylose fermentation in comparable amounts [1]. For this reason, the metabolic regulation of xylitol and ethanol production should be studied in mutant *Pa. tannophilus* strains selected for impaired D-xylose catabolism. Such strains characterized with altered xylitol and ethanol production have been obtained previously using specialized techniques [4–7]. In a similar manner, we obtained mutant strains based on the selected stably haploid *Pa. tannophilus* strain 22-Y-1532 [8]. Some of them were presumably able to synthesize individual products of microaerobic D-xylose fermentation.

The goal of the present work was therefore to analyze xylitol and ethanol accumulation by mutant *Pa. tannophilus* 22-Y-1532 strains, and to assess the correlation between the product levels and the xylose reductase and xylitol dehydrogenase activity.

MATERIALS AND METHODS

Subjects. The study was performed with nine mutant *Pa. tannophilus* 22-Y-1532 strains characterized by altered growth on media containing D-xylose, xylitol, ethanol, or D-glucose as sole carbon sources (Table 1) and isolated as described in [8]. The parent haploid strain served as the control. Its ploidy was verified previously using a range of criteria [3, 9] and was confirmed by genetic testing [8].

Yeasts were grown on solid JEPD medium containing 2% D-xylose as the sole carbon source, 2% peptone, 0.5% yeast extract, and 2% agar-agar [9] at 30°C for 2–14 days. Microbiological analysis of the mutant strains was performed under a Jenamed Variant microscope (Germany) using ocular and objective magnification of ×18 and ×40, respectively; both macromorphological (colony diameter, color, shape, surface, and texture) and micromorphological traits (cell size, budding type, and the character of sporulation) were evaluated [3].

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Table 1. Growth of *Pa. tannophilus* mutants on different carbon sources

Strain no.	Carbon source				Phenotype
	glucose*	xylose*	xylitol	ethanol	
4	±	±	±	±	$Glu^{\pm}Xyl^{\pm}XylOH^{\pm}EtOH^{\pm}$
13	+	+	±	–	$Glu^{+}Xyl^{+}XylOH^{\pm}EtOH^{-}$
228	+	+	+	+	$Glu^{+}Xyl^{+}XylOH^{+}EtOH^{+}$
390	+	+	±	–	$Glu^{+}Xyl^{+}XylOH^{\pm}EtOH^{-}$
442	+	+	–	+	$Glu^{+}Xyl^{+}XylOH^{-}EtOH^{+}$
497	+	+	–	+	$Glu^{+}Xyl^{+}XylOH^{-}EtOH^{+}$
517	+	+	+	±	$Glu^{+}Xyl^{+}XylOH^{+}EtOH^{\pm}$
664	+	±	±	±	$Glu^{+}Xyl^{\pm}XylOH^{\pm}EtOH^{\pm}$
686	+	+	+	±	$Glu^{+}Xyl^{+}XylOH^{+}EtOH^{\pm}$
C	+	+	+	+	$Glu^{+}Xyl^{+}XylOH^{+}EtOH^{+}$

Here and in Tables 2 and 3, data for the parent strain *Pa. tannophilus* 22-Y-1532 (C) were used as the control. Notation: +, strong growth; +, normal growth; ±, weak growth on selective medium; –, complete lack of growth on the selective medium.

* D isomers.

Microaerobic fermentation was performed in 250-mL round-bottomed flasks containing 100 mL liquid JEPD medium on a shaker at 100 rpm, at 30 ± 2°C for 24 h; yeast were inoculated using 6.0 g dry biomass per 1 L medium [1].

Biochemical analysis. The concentrations of reducing carbohydrates were determined using the Fehling's reagent. Biomass amounts were quantified using an SF-26 spectrophotometer (LOMO, USSR). Colorimetric measurements of culture samples taken at the end of fermentation were performed against liquid JEPD medium at 620 nm. Ethanol and xylitol were determined by gas chromatography as described in [1].

Cell-free extracts. To obtain cell-free extracts, yeast cells were resuspended in 10 mM potassium phosphate buffer (pH 6.5) containing 1 mM 2-mercaptoethanol and ground with 0.4–0.5 mm glass beads on a Vortex homogenizer (Germany) in five 1-min sessions with cooling pauses. Cell debris was precipitated by centrifugation (45000 rpm at 0°C for 20 min). Protein concentrations in the obtained extracts were determined using the Lowry assay [10], with bovine serum albumin as a standard.

Proteins of the cell-free extracts were separated by PAGE in a denaturing 15% gel. Xylose reductase and xylitol dehydrogenase activity in the presence of different coenzymes was determined directly in the gel using the tetrazolium violet test. Following electrophoresis, the gels were incubated at 30°C in the staining solution of the following composition: 50 mM Tris-HCl (pH 8.0); 0.2 mM $NADP^+$ or NAD^+; 1 mM xylitol; 0.3 mM tetrazolium violet; and 0.01 mM phenazine methosulfate. The analysis was performed as proposed by Zverlov et al. [11].

Statistical analysis of the data was performed using the conventional Student's test with the significance threshold of 0.05 [12]. For each strain studied, as well as for the positive and negative controls, the sample size was at least five values. Data deviations from the means did not exceed 5%.

RESULTS AND DISCUSSION

General characterization of *Pa. tannophilus* mutants. According to the classification proposed by Kreger-van Rij, the life cycle of the studied yeast species is diplo-haplontic, with haploid phase predominance [9]. Experimental analysis of *Pa. tannophilus* strains is often complicated due to their pronounced homothallism (the lack of a particular mating type and the difficulty in discriminating between the vegetative and the generative phases of the life cycle) [13]. Therefore, the mutant strains exhibiting altered growth on D-xylose, xylitol, ethanol, or D-glucose as sole carbon sources were isolated based on the selected stably haploid strain *Pa. tannophilus* 22-Y-1532. However, we were aware that mutagen-induced changes could have affected not only the genes participating in D-xylose catabolism, but also those responsible for morphogenesis of the yeast. To rule out the possibility of intrapopulational instability (simultaneous presence of the cells in the haploid and diploid phases), we evaluated the morphological traits of *Pa. tannophilus* mutants.

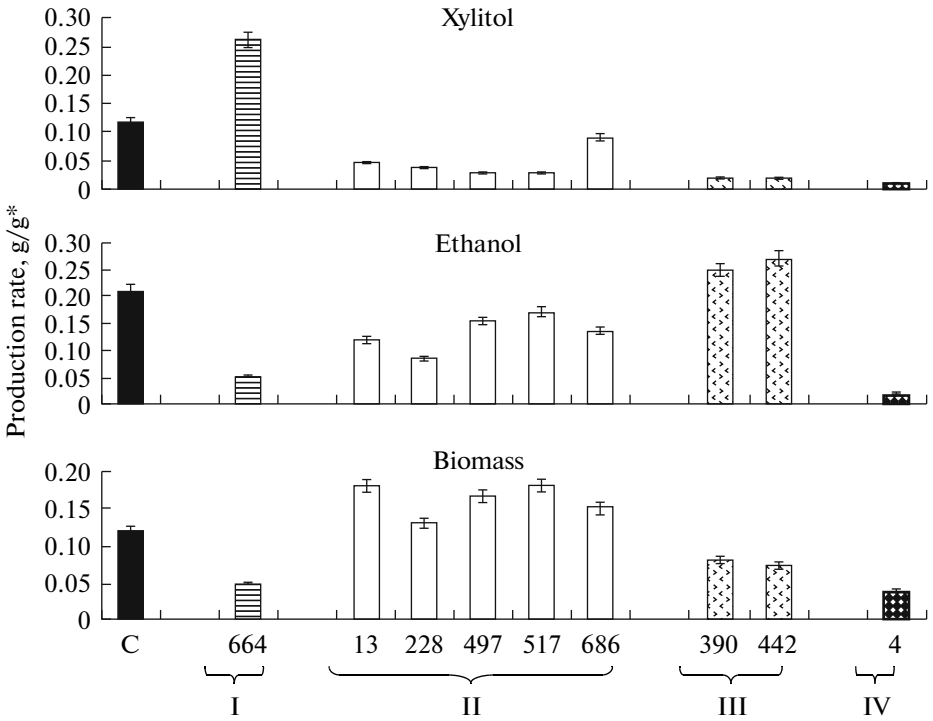
In most cases, their macromorphology agreed well with the characteristics of the control haploid strain: white rounded colonies 1.0 mm in diameter with a glistening convex surface. The colonies retained their mucoid texture for the first two days of growth on JEPD; then they became butter-like. These traits were slightly modified in the strains with altered growth on D-xylose, xylitol, ethanol, or D-glucose as sole carbon sources, as summarized in Table 2.

Table 2. Macro- and micromorphological characteristics of mutant *Pa. tannophilus* strains

Strain no.	Colony				Cell			Sporulation, days
	d, mm	color	shape	texture	shape	size, μm	budding	
4	0.3	Matte white	Round convex	Butter-like	Rounded and oval	(3.0–6.0) × (3.5–8.0)	Bipolar	4–5
13	1.2	Glossy white		Mucoid		(1.5–2.5) × (2.0–3.5)		5–6
228	1.0			Butter-like	Irregular	(1.5–4.0) × (2.0–6.0)		
390					Rounded and ellipsoid	(1.5–4.5) × (1.5–6.0)		
442	0.9				Rounded and oval	(1.5–6.5) × (2.0–7.5)	Multilateral	6–8*
497	1.0					(1.5–4.0) × (2.0–4.5)	Bipolar	5–6
517	1.5			Mucoid		(1.5–3.5) × (2.0–5.0)		6–7
664	1.0			Butter-like	Rounded and ellipsoid	(1.5–3.0) × (2.0–5.0)		5–6
686	0.8					(1.5–4.5) × (1.5–6.0)		7–8
C	1.0					(1.5–4.5) × (2.0–6.5)		5–6

Characteristics of the parent strain *Pa. tannophilus* 22-Y-1532 (C) were used as the control.

* Formation of individual ascophores.



Xylitol, ethanol, and biomass production by mutant *Pa. tannophilus* strains. C is the parent haploid strain *Pa. tannophilus* 22-Y-1532; mutant strain numbers are given in Arabic figures, while Roman numerals and bar hatching show the groups of mutants.

*Production rates were calculated as amounts of xylitol, ethanol, or biomass (in g) produced per 1 g of D-xylose consumed. The relative error of each experimental point did not exceed 5%.

At the same time, micromorphological traits of the mutant strains varied much more. For instance, cells of the parent haploid strain were 1.5–4.5 × 2.0–6.5 μm in size, exhibited bipolar budding, and sporulated on day 5–6 of growing on JEPD. Colonies of strains nos. 13, 497, 517, and 664 were composed of smaller cells, whereas the cells of strain no. 4 were considerably larger. In mutants nos. 4, 517, and 686, sporulation was shifted by 24–48 h. Finally, strain no. 442, incapable of utilizing xylitol, exhibited multilateral budding, which is uncharacteristic of *Pa. tannophilus* [9] (Table 1). However, the type of colony growth, the cell size and shape, and ascophore morphology indicated that it was a haploid strain [3]. The above variations were probably due to pleiotropic effects of mutations that impaired the strains' ability to assimilate D-xylose, xylitol, ethanol, or D-glucose as sole carbon sources, or to aneuploidy of mutant cells.

Thus, intrapopulational stability was experimentally confirmed for all studied mutant strains of *Pa. tannophilus* 22 22-Y-1532. Their haploid nature enabled the phenotypic expression of not only dominant but also recessive mutations, which was an important prerequisite for our study of xylitol and ethanol production by these strains.

Microaerobic fermentation of D-xylose. It is known that, during the first 24 h, D-xylose concentration does not considerably affect the functioning of transport systems in a yeast cell [14]. Therefore, the direction of its catabolism can be expected to largely reflect the enzymatic activity typical for a given strain. For this reason, it was within this period of time that we analyzed the characteristics of microaerobic D-xylose fermentation by *Pa. tannophilus* 22-Y-1532 mutants. Based on the experimental data, the mutant strains were divided in four groups (figure). Group I included strain no. 664, which accumulated xylitol 2.3 times more efficiently than the parent haploid strain (Tables 1 and 2), exhibiting the extent of D-xylose utilization of 56.0% and its consumption rate of 0.5 g L^{-1} h^{-1}.

Group II was the most numerous one and included five strains with different phenotypes: nos. 13, 228, 497, 517, and 686 (Tables 1 and 2). During microaerobic fermentation, the growth of their biomass was comparable to that of the control, or was even higher (figure), while the extent of D-xylose consumption ranged from 54.7 to 64.6%, and its assimilation rate was 0.5 g L^{-1} h^{-1} for all strains.

Mutants of group III, nos. 390 and 442, exhibited the most efficient D-xylose assimilation (Tables 1 and 2). The consumption rates were equal (0.7 g L^{-1} h^{-1}), while the utilization extent was 86.0% in strain no. 390 and 78.6% in strain no. 442. These strains accumulated hardly any xylitol, while producing by 19.0 and

Table 3. Xylose reductase and xylitol dehydrogenase activity in *Pa. tannophilus* mutants under microaerobic conditions

Group	Strain no.	Specific activity, μmol mg^{-1} min^{-1}*					
		xylose reductase			xylitol dehydrogenase		
		NADH	NADPH	total	NAD^+	$NADP^+$	total
I	664	*1.40*	3.00	*4.40*	4.55	0.25	4.80
II	13	**3.40**	3.40	**6.80**	5.80	0.20	6.00
	228	2.80	3.20	6.00	5.40	*0.03*	5.43
	497	*1.60*	**3.80**	5.40	4.80	**0.40**	5.90
	517	2.00	*2.40*	*4.40*	6.00	0.40	6.40
	686	2.40	3.00	5.40	*4.20*	0.20	*4.40*
III	390	**3.20**	2.80	6.00	**8.00**	**0.40**	**8.40**
	442	**3.20**	3.60	**6.80**	6.45	0.35	6.80
IV	4	2.00	*2.40*	*4.40*	6.35	0.25	6.60
C	22-Y-1532	5.40	5.00	10.40	7.70	0.30	8.00

* A unit of activity was assumed to be equal to the enzyme amount capable of reducing or oxidizing 1 μmol NAD(P)(H) in 1 min. The minimal and the maximal values of xylose reductase and xylitol dehydrogenase activity are shown in italics and in bold, respectively. The relative error of each experimental point did not exceed 5%.

27.0% more ethanol than the parent strain; at the same time, their biomass growth was slower.

Finally, group IV included strain no. 4, which exhibited weak growth on all selective media (Table 1) and had a number of distinguishing morphological traits (Table 2). This mutant showed the lowest D-xylose consumption rate (0.5 g L^{-1} h^{-1}) and the lowest utilization extent (53.6%), as well as the lowest production of xylitol, ethanol, and biomass. We assume that these traits were a direct consequence of its impaired ability to assimilate D-glucose, which is the principal carbon source for a yeast cell.

Thus, mutant *Pa. tannophilus* 22-Y-1532 strains varied in their ability to produce xylitol and ethanol. Strains of groups I–III were good models for the production rates previously determined for xylose-utilizing yeast of the genera *Candida, Pachysolen*, and *Pichia* [1], indirectly indicating that the genetic changes induced in the source haploid cells differed in their nature. To arrive at the final conclusion as to whether *Pa. tannophilus* mutants were appropriate subjects for analysis of xylitol and ethanol production, we investigated the characteristics of the key enzymes of D-xylose catabolism, xylose reductase and xylitol dehydrogenase, in these strains.

Total and specific activity of the key enzymes of D-xylose degradation. In most mutants, the total activity of xylose reductase and xylitol dehydrogenase was lower by 1.5–2.4 times and 1.2–1.8 times, respectively, than in the haploid strain 22-Y-1532 (Table 3). Nevertheless, these levels correlated with the ethanol and xylitol production rates. In particular, the lowest activity of both enzymes corresponded to xylitol production from D-xylose. Interestingly, in strain no. 664, xylose reductase exhibited predominant affinity to NADPH, while over 95% of xylitol dehydrogenase activity were associated with NAD^+ (Tables 1 and 2). Similar properties of xylose reductase and xylitol dehydrogenase were previously described in *Candida intermedia, C. parapsilosis*, and *C. silvanorum* yeasts, which accumulate xylitol in the course of microaerobic fermentation of D-xylose [2].

The mutants with enhanced ethanol production exhibited the highest activity of the enzymes in question. In strains nos. 390 and 442, xylose reductase had a strongly pronounced double NADH/NADPH specificity, while xylitol dehydrogenase was strictly NAD^+-specific (Tables 1 and 2). Such properties of the key enzymes of D-xylose degradation are also characteristic of ethanol-producing *C. shehatae* and *P. stipitis* strains [2].

The reported correlation between the activities of xylose reductase and xylitol dehydrogenase on the one hand and accumulation of the target products on the

other hand indicates that the mechanisms responsible for the regulation of D-xylose catabolism are basically similar in collection strains of xylose-utilizing yeasts and in *Pa. tannophilus* mutants. Thus, it should be essentially possible to identify the limiting stages in xylitol and ethanol production and to find approaches to producer strain development using *Pa. tannophilus* 22-Y-1532 strains with altered growth on D-xylose, xylitol, ethanol, or D-glucose as models. From this point of view, strains nos. 664, 390, and 442 are worth special consideration, since they selectively synthesize the products of microaerobic D-xylose fermentation.

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