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EXPERIMENTAL ARTICLES

The Activity of Key Enzymes in Xylose-Assimilating Yeasts at Different Rates of Oxygen Transfer to the Fermentation Medium

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Abstract—The activities of xylitol dehydrogenase and xylose reductase in the yeasts *Candida shehatae, C. didensiae, C. intermediae, C. tropicalis, Kluyveromyces marxianus, Pichia stipitis, P. guillermondii, Pachysolen tannophilus*, and *Torulopsis molishiama* were studied at different oxygen transfer rates (OTRs) to the fermentation medium (0, 5, and 140 mmol O_2/(l h)). The activities of these enzymes were maximum in the yeasts *P. stipitis* and *C. shehatae*. The xylitol dehydrogenase of all the yeasts was NAD^+-dependent, irrespective of the intensity of aeration. The xylose reductase of the yeasts *C. didensiae, C. intermediae, C. tropicalis, Kl. marxianus, P. guillermondii*, and *T. molishiama* was NADPH-dependent, whereas the xylose reductase of *P. stipitis, C. shehatae*, and *Pa. tannophilus* was specific for both NADPH and NADH. The effect of OTR on the activities of the different forms of xylitol dehydrogenase and xylose reductase in xylose-assimilating yeasts is discussed.

Key words: yeasts, oxygen transfer rate (OTR), xylose reductase, xylitol dehydrogenase.

The ability of yeasts to utilize xylose-containing substrates depends on whether xylose reductase and xylitol dehydrogenase are present in these yeasts or not. The enzymes convert D-xylose into D-xylulose, which then becomes involved into the main metabolic pathways (glycolysis and the pentose phosphate and tricarboxylic acid cycles) [1, 2]. The incomplete oxidation of pentoses (which takes place under the condition of limited yeast respiration) gives rise either to ethanol (in certain yeasts) or to xylitol (in the other yeasts) [3–5]. There is evidence that oxygen deficiency in the fermentation medium may influence the activities of xylitol dehydrogenase and xylose reductase in yeast cells [3, 6–8]. In this case, the metabolism of D-xylose is blocked at the stage of xylitol formation [1, 3, 6, 7]. The mechanism of oxygen action on the activity of different forms of xylitol dehydrogenase and xylose reductase is as yet unknown.

The aim of this work was to study the effect of the oxygen transfer rate to the fermentation medium on the functioning of xylitol dehydrogenase and xylose reductase in xylose-assimilating yeasts.

MATERIALS AND METHODS

Experiments were carried out with the xylose-assimilating yeasts *Candida shehatae* Y-1632, *Candida didensiae* F-3, *Candida intermediae* TabII (85), *Candida tropicalis* W-1, *Pichia stipitis* Y-2160, *Pichia guillermondii* Y-1017, *Kluyveromyces marxianus* Y-488, *Torulopsis molishiama* 55, and *Pachysolen tannophilus* Y-1532, Y-1533, and Y-1634 [9]. By ability to form different products of D-xylose metabolism, the yeasts were divided into three groups. Group I included yeasts that produce xylitol; group II included yeasts that produce xylitol and ethanol in comparable amounts; and group III included yeasts that produce ethanol [9]. The yeasts were grown in standard nutrient media containing 2% D-xylose [10].

Cultivation was carried out at 30 ± 2°C for 24 h in three modes: (1) in 100-ml round-bottom flasks containing 100 ml of the fermentation medium without shaking (inoculum size 10.0 ± 0.5 g dry wt); (2) in 250-ml Erlenmeyer flasks with 100 ml of the fermentation medium with shaking at 100 rpm (inoculum size 6.0 ± 0.5 g dry wt); and (3) in 1000-ml Erlenmeyer flasks with 100 ml of the fermentation medium with shaking at 200 rpm (inoculum size 1.0 ± 0.5 g dry wt). The oxygen transfer rate (OTR) was evaluated by the colorimetric method with sulfite [11] and expressed in mmol O_2/(l h).

The activity and the specificity of xylose reductase (EC 1.1.1.21) and xylitol dehydrogenase (EC 1.1.1.9) for the pyridine nucleotide coenzymes were determined in cell-free extracts [12].

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Table 1. The production of biomass, xylitol, and ethanol by the yeast *Pa. tannophilus* Y-1532 at different oxygen transfer rates to the fermentation medium

Parameter	Oxygen transfer rate, mmol/(l h)		
	0.0	5.0	140.0
Consumption of D-xylose, %	<5.0	**95.0**	**95.0**
D-Xylose utilization rate, g/(l h)	<0.01	0.46	**0.91**
Yield (g/g) of			
xylitol	<0.01	**0.16**	<0.01
ethanol	<0.01	**0.25**	<0.01
biomass	<0.01	0.08	**0.41**
Production rate (g/(l h)) of			
xylitol	<0.01	**0.08**	<0.01
ethanol	<0.01	**0.13**	<0.01
biomass	<0.01	0.05	**0.53**
Specific growth rate (μ), h^{-1}	<0.01	0.05	**0.23**

Note: The maximum values of particular parameters are shown in bold. Experimental errors did not exceed 5%.

RESULTS

The effect of OTR on the production of xylitol and ethanol. It is known that the oxygen transfer rate to the fermentation medium, which is mainly determined by the level of mechanical aeration [3, 8], governs the conversion of D-xylose to particular products. In this work, relevant experiments were carried out with the yeast *Pa. tannophilus*, which ferments D-xylose with the formation of xylitol and ethanol in comparable amounts [7, 10]. Experimental data on the consumption of D-xylose and the production of biomass, ethanol, and xylitol as functions of OTR are summarized in Table 1.

In cultivation mode 1, when mechanical aeration was absent (some researchers call such conditions anaerobic [3, 8]), the consumption of D-xylose and the production of biomass and the products of the incomplete oxidation of D-xylose (ethanol and xylitol) were insignificant (Table 1).

In cultivation mode 2 (OTR = 5 mmol O_2/(l h)), D-xylose was consumed at a significant rate (0.46 g/(l h)), but the specific growth rate did not exceed 0.05 h^{-1}. The oxygen supply rate in cultivation mode 2, which is usually referred to as microaerobic [4, 7], promoted the production of ethanol and xylitol (Table 1).

In cultivation mode 3 (OTR = 140 mmol O_2/(l h)), the consumption rate of D-xylose was two times as high as in cultivation mode 2, and the specific growth rate reached 0.23 h^{-1}. The yield and the production rate of biomass were at maxima (Table 1). Ethanol and xylitol were not detected in the culture liquid. The oxygen supply rate in cultivation mode 3, which can be called aerobic [6], stimulated biosynthetic processes in yeast cells and their growth.

Thus, different aeration levels can be used to regulate the production of biomass, ethanol, and xylitol and to study the effect of oxygen supply on the activity of the key enzymes of xylose metabolism, xylose reductase and xylitol dehydrogenase.

The effect of aeration on the activity of different forms of xylose reductase and xylitol dehydrogenase. It is known that xylose reductase and xylitol dehydrogenase possess different specificities for the pyridine nucleotide coenzymes, which are involved in many biochemical redox processes [1, 13, 14]. Different aeration levels may differently influence the activities of xylose reductase and xylitol dehydrogenase that are specific for particular pyridine nucleotides.

The data presented in Table 2 show that the yeasts of group I predominantly possess NADPH-specific xylose reductase activity (97–98% of the total xylose reductase activity), irrespective of the oxygen transfer rate to the fermentation medium. This implies that xylitol-producing yeasts contain only NADPH-linked xylose reductase.

In the yeast *Pa. tannophilus* (group II) cultivated in mode 2, the activities of NADPH- and NADH-linked xylose reductases comprised 2.0–2.1 and 1.39–1.56 μmol/(mg min), respectively. Under anaerobic conditions (cultivation mode 1), both activities were greatly suppressed. At the same time, the aerobic conditions typical of cultivation mode 3 (OTR = 140 mmol O_2/(l h)) considerably suppressed only NADH-linked xylose reductase. In general, irrespective of aeration level, the yeast *Pa. tannophilus* contained both forms of xylose reductase.

In the yeasts of group III cultivated in the absence of aeration (cultivation mode 1), the activity of NADPH-linked xylose reductase ranged from 5.02 (*C. shehatae*) to 10.6 μmol/(mg min) (*P. stipitis*). The activity of NADH-linked xylose reductase was high only under microaerobic conditions, ranging from 4.93 (*C. shehatae*) to 10.37 μmol/(mg min) (*P. stipitis*).

Thus, NADPH-linked xylose reductase was prevalent in the yeasts that were cultivated either under anaerobic or under aerobic conditions, i.e., at an OTR equal either to 0 or to 140 mmol O_2/(l h). The ethanol-producing yeasts that were cultivated under microaerobic conditions were characterized by considerable NADH-specific xylose reductase activity, which comprised, respectively, 41–43, 59, and 68% of the total xylose reductase activity of the yeasts *Pa. tannophilus, C. shehatae*, and *P. stipitis.*

The specific activities of NAD^+- and $NADP^+$-linked xylitol dehydrogenases are shown in Table 2 in bold. Irrespective of aeration level, the NAD^+-specific xylitol dehydrogenase activity of the yeasts comprised 97–99% of the total xylitol dehydrogenase activity. Under aerobic conditions, the NAD^+-specific xylitol dehydrogenase activity of all the yeasts was at a maximum and

Table 2. The specific activities of xylose reductase and xylitol dehydrogenase in the yeasts cultivated at different aeration rates

Group	Yeast species and strains	Activities of xylose reductase and xylitol dehydrogenase, μmol/(mg min)*					
		0.0 mmol O_2/(l h)**		5.0 mmol O_2/(l h)**		140.0 mmol O_2/(l h)**	
		NADPH/$NADP^+$	NADH/NAD^+	NADPH/$NADP^+$	NADH/NAD^+	NADPH/$NADP^+$	NADH/NAD^+
I	*C. didensiae* F-3	0.03/**<0.01**	<0.01/**<0.01**	0.63/**<0.01**	0.03/**<0.01**	1.66/**0.09**	0.03/**1.11**
	C. intermediae TabII (85)	1.39/**0.06**	0.08/**0.35**	4.74/**0.21**	0.16/**3.62**	6.53/**0.23**	0.11/**2.68**
	C. tropicalis W-1	0.80/**0.04**	0.04/**0.48**	6.43/**0.06**	0.14/**1.10**	4.27/**0.08**	0.15/**6.25**
	Kl. marxianus Y-488	0.34/**<0.01**	<0.01/**<0.01**	0.16/**<0.01**	<0.01/**0.04**	0.04/**0.04**	<0.01/**0.47**
	P. guillermondii Y-1017	0.40/**0.04**	0.01/**0.50**	0.61/**<0.01**	0.02/**0.18**	0.76/**<0.01**	<0.01/**3.49**
	T. molishiama 55	<0.01/**<0.01**	<0.01/**<0.01**	0.11/**0.01**	<0.01/**0.01**	0.02/**<0.01**	<0.01/**0.47**
II	*Pa. tannophilus* Y-1532, Y-1533, Y-1634	0.07–0.14/**<0.01–0.04**	0.04–0.05/**0.43–1.00**	2.00–2.10/**<0.01–0.12**	1.39–1.56/**4.04–5.26**	2.10–2.34/**<0.01–0.15**	0.12–0.18/**4.08–5.28**
III	*C. shehatae* Y-1632	5.02/**0.20**	2.31/**8.29**	3.40/**0.42**	4.93/**13.53**	4.81/**0.53**	3.10/**15.37**
	P. stipitis Y-2160	10.6/**0.56**	5.96/**17.50**	4.84/**0.27**	10.37/**8.37**	8.24/**0.42**	4.53/**9.92**

Note: The specific activities of xylitol dehydrogenase in the presence of $NADP^+$ and NAD^+ are shown in bold.

* One unit of enzymatic activity was defined as the amount of enzyme required for the reduction of 1 μmol of $NAD(P)^+$ or the oxidation of 1 μmol of NAD(P)H in 1 min.

** The oxygen transfer rates 0, 5, and 140 mmol O_2/(l h) correspond to anaerobic, microaerobic, and aerobic conditions, respectively. Experimental errors did not exceed 5%.

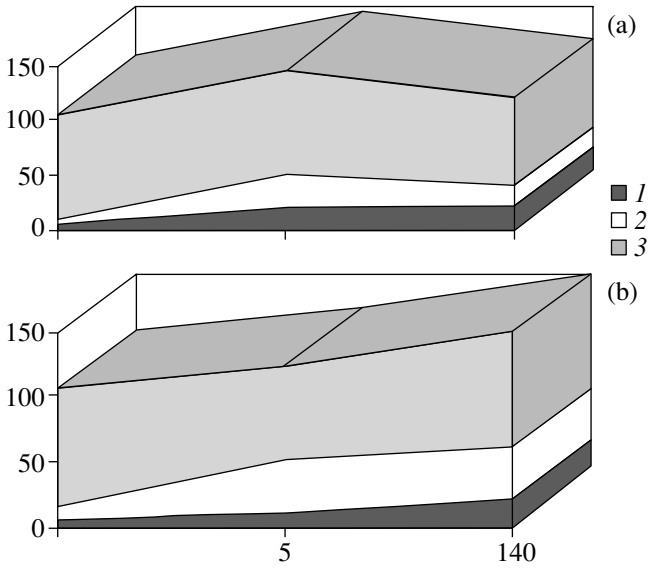

Fig. 1. The effect of aeration on the total activities of (a) xylose reductase and (b) xylitol dehydrogenase in the xylose-assimilating yeasts belonging to (*1*) group I, (*2*) group II, and (*3*) group III. The ordinates show enzymatic activities expressed as a percent of the maximum enzymatic activity observed in the particular experiment. The numbers 0, 5, and 140 on the abscissas show oxygen transfer rates to the fermentation medium expressed in mmol O_2/(l h).

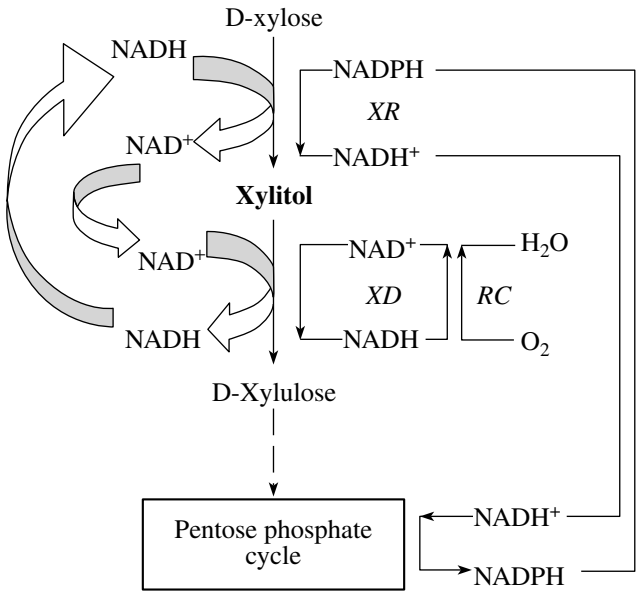

Fig. 2. Reaction scheme showing the regeneration of the pyridine nucleotide coenzymes involved in the transformation of D-xylose into D-xylulose. *XR*, xylose reductase; *XD*, xylitol dehydrogenase; *RC*, respiratory chain. The curved arrows show additional pathways of NAD^+ regeneration that probably exist in the ethanol-producing xylose-assimilating yeasts.

comprised 15.31 (*C. shehatae,* from group III), 6.25 (*C. tropicalis,* from group I), and 4.08–5.28 μmol/(mg min) (*Pa. tannophilus,* from group II). These results suggest that the yeasts contain only NAD^+-linked xylitol dehydrogenase, irrespective of aeration level.

The effect of OTR on the total activity of the key enzymes of D-xylose metabolism. The total activities of xylose reductase and xylitol dehydrogenase in the xylose-assimilating yeasts considerably varied, depending on aeration level (Fig. 1). In all the yeasts studied, the activity of xylose reductase was maximum under microaerobic conditions (Fig. 1a), whereas the activity of xylitol dehydrogenase was maximum under aerobic conditions (Fig. 1b). The activities of the key enzymes of D-xylose metabolism in the xylitol-producing yeasts (group I) were low, irrespective of aeration level. In *Pa. tannophilus* (group II), these activities were only two to three times higher than in the yeasts of group I. The activities of xylose reductase and xylitol dehydrogenase were relatively high and independent of OTR only in the ethanol-producing yeasts of group III, namely, *C. shehatae* and *P. stipitis* (Fig. 1).

DISCUSSION

The experimental data obtained in this work show that the tolerance of the initial stages of D-xylose metabolism to the intensity of aeration in *C. shehatae* and *P. stipitis* may be due to the high activity of NADH-linked xylose reductase in these yeasts. The effect of aeration level on the transformation efficiency of D-xylose into D-xylulose is likely to be related to induced changes in the activities of the NADPH- and NADH-linked forms of xylose reductase. The $NADP^+$ that is formed during xylitol formation can be reduced in the pentose phosphate cycle reactions [3, 8, 15]. The regeneration of NADH during the transformation of xylitol into D-xylulose requires an electron acceptor [16], whose role is probably played by oxygen (Fig. 2). According to this hypothesis, the regeneration of the pyridine nucleotide coenzymes that are involved in the initial stages of D-xylose metabolism disturbs a balance between NADPH and NAD^+ and leads to the formation of xylitol.

In the xylitol-producing yeasts, $NADP^+$ and NADH are regenerated independently. Under aerobic conditions, NADH is completely oxidized through the respiratory chain. Under microaerobic or anaerobic conditions, the intracellular pool of NADH increases, thereby retarding or completely blocking D-xylose catabolism at the stage of xylitol formation. In the ethanol-producing yeasts, however, the NADH that is produced during the transformation of xylitol into D-xylulose can be partially regenerated by NADH-linked xylose reductase. This additional regeneration cycle reduces the imbalance between NADH and NAD^+ and promotes the ethanol fermentation of D-xylose. There-

fore, the high activity of NADH-linked xylose reductase is favorable to the intracellular regeneration of NADH and NAD^+, which reduces the amount of xylitol produced from D-xylose (Fig. 2). This inference agrees well with the data of other researchers [1–8, 13–16].

It should be noted that the level of aeration that favors the production of xylitol (or ethanol) from D-xylose is very low, and its maintenance presents a difficult technical problem. For this reason, it will be more expedient to obtain promising yeast producers of xylitol and ethanol by the conventional methods of induced mutagenesis.

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