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Induction of Xylose Reductase and Xylitol Dehydrogenase Activities in *Pachysolen tannophilus* and *Pichia stipitis* on Mixed Sugars

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The induction of xylose reductase and xylitol dehydrogenase activities on mixed sugars was investigated in the yeasts *Pachysolen tannophilus* and *Pichia stipitis*. Enzyme activities induced on D-xylose served as the controls. In both yeasts, D-glucose, D-mannose, and 2-deoxyglucose inhibited enzyme induction by D-xylose to various degrees. Cellobiose, L-arabinose, and D-galactose were not inhibitory. In liquid batch culture, *P. tannophilus* utilized D-glucose and D-mannose rapidly and preferentially over D-xylose, while D-galactose consumption was poor and lagged behind that of the pentose sugar. In *P. stipitis*, all three hexoses were used preferentially over D-xylose. The results showed that the repressibility of xylose reductase and xylitol dehydrogenase may limit the potential of yeast fermentation of pentose sugars in hydrolysates of lignocellulosic substrates.

D-Xylose is an aldopentose which occurs primarily in polymer form as xylan in the hemicellulose portion of plant cell walls (25). Next to D-glucose, D-xylose is the second most abundant renewable sugar in nature, forming up to 25% of the total dry weight of some forestry and agricultural residues (8). The efficient utilization of pentose sugars is, therefore, important in the overall bioconversion of plant biomass for the production of chemicals and liquid fuels.

Current interest in D-xylose bioconversion stems largely from the discovery of pentose-fermenting yeasts in the early 1980s (7, 11, 19, 22, 24). It is worth noting, however, that fermenting D-xylose is practicable only when it is performed in conjunction with the overall utilization of lignocellulose (12). Potential substrates envisioned for the application of pentose-fermenting technologies include hydrolysates of plant biomass or waste liquors of pulp- and paper-processing industries. Such materials often contain a variety of sugars such as D-glucose, D-mannose, D-galactose, D-xylose, L-arabinose, L-rhamnose, and cellobiose (5, 20).

It is, therefore, desirable that microorganisms used for the fermentation of biomass-derived carbohydrates should be able to convert a mixture of hexose and pentose sugars to ethanol. However, yeasts that ferment D-xylose efficiently in batch culture may ferment the pentose poorly when other, more readily metabolized hexoses are present. The preferential utilization of D-glucose over D-xylose has been noted in cultures of *Candida utilis* (10), *Pachysolen tannophilus* (3, 23), and *Candida shehatae* and *Pichia stipitis* (5). This phenomenon has been postulated to arise from glucose repression of enzymes involved in xylose metabolism. It is known that xylose reductase (XR) and xylitol dehydrogenase (XDH) activities in *P. tannophilus* are induced in xylose-grown but not glucose-grown cells (1, 4). However, since these studies were done on single sugars, it was not certain whether low enzyme activities found in glucose-grown cells resulted from catabolite repression or simply an inability of glucose to induce such activities.

The present study was undertaken to investigate whether D-glucose represses the induction of XR and XDH in the presence of D-xylose. Also tested for repressive activity were several sugars that can occur in lignocellulosic hydrolysates and 2-deoxyglucose, which is a nonmetabolizable glucose analog often used to select for nonrepressible mutants (6). The results indicate that the glucose effect is indeed operative in *P. tannophilus* and *P. stipitis*. The repression of XR and XDH activities by D-glucose and D-mannose was correlated with the ability of these hexoses to inhibit D-xylose utilization by the two yeasts.

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MATERIALS AND METHODS

Microorganisms. *P. tannophilus* NRRL Y2460 and *P. stipitis* NRRL Y7124 were maintained at 4°C on slants of potato glucose agar (Difco Laboratories, Detroit, Mich.) in 20-ml screw-cap bottles.

Preparation of inocula. A loopful of cells from a potato glucose agar slant was transferred to 20 ml of medium containing 0.67% (wt/vol) yeast nitrogen base (YNB; Difco) without amino acids and 2% (wt/vol) glycerol. The culture was kept at 30°C in a loosely capped 125-ml Erlenmeyer flask which was agitated at 200 rpm in a New Brunswick model G24 bench-top Gyrotory shaker for 48 h (14).

Induction of XR and XDH activities. A 2-ml sample of inoculum culture was transferred to 100 ml of medium containing 0.67% YNB without amino acids and 2% glycerol in a 250-ml loosely capped flask which was incubated as above. After 16 h, a concentrated solution of D-xylose was added to a final concentration of 4% (wt/vol). Incubation was continued for 8 h before being terminated. The 8-h induction period was chosen on the basis of preliminary experiments which indicated that maximum induction of XR and XDH activities in both yeasts was achieved within 6 h and maintained for an additional 2 h (data not shown). Cells grown for 16 h on glycerol alone served as the control for the xylose induction experiments.

To study the effect of another sugar on enzyme induction by D-xylose, we added that select sugar at the same time as D-xylose. The sugars tested included D-glucose, D-mannose, D-galactose, L-arabinose, cellobiose, and 2-deoxyglucose. Each test sugar was added to a final concentration of 4%

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TABLE. 1. Induction of NADPH-dependent XR and NAD-dependent XDH activities in *P. stipitis* NRRL Y7124 cultures by different carbohydrates

Carbohydrate(s)	No. of trials	Relative activity (%)[a]	
		XR	XDH
D-Xylose	4	100	100
Glycerol	1	7	0
D-Glucose	4	5 ± 3	13 ± 4
D-Xylose + D-glucose	4	19 ± 1	17 ± 7
D-Xylose + D-mannose	3	54 ± 8	38 ± 7
D-Xylose + D-galactose	3	160 ± 40	90 ± 40
D-Xylose + L-arabinose	3	120 ± 20	110 ± 10
D-Xylose + cellobiose	3	112 ± 8	81 ± 8
D-Xylose + 2-deoxyglucose	2	50 ± 20	50 ± 20

[a] Values shown are average percentages ± standard error. Average specific activities for XR and XDH induced by D-xylose were 0.5 ± 0.1 and 0.7 ± 0.1 nmol of cofactor converted per min per μg of protein, respectively.

(wt/vol) except for 2-deoxyglucose, which was added to 0.5% (wt/vol).

Preparation of cell extracts. Upon termination of incubation, cells were harvested by centrifugation at 8,000 × *g* with a Sorvall refrigerated centrifuge (GSA rotor). The cell pellet was suspended in 2 to 4 ml of ice-cold 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA. Cells were broken either by sonication (16) or by mechanical disruption (21). Both methods provided adequate cell breakage, as ascertained by microscopic examination. Cell extracts were then obtained by centrifugation (14).

Enzyme assays. NADPH-dependent XR and NAD-dependent XDH activities were determined as described previously (14) with D-xylose and xylitol as the respective substrates. Specific activities were based on protein determined by the method of Lowry et al. (15) with bovine serum albumin as the standard.

Characterization of sugar utilization in liquid cultures. A 2-ml sample of inoculum culture was transferred to 100 ml of medium containing 0.67% YNB without amino acids and approximately 2% of one of the following sugars: D-xylose, D-mannose, D-glucose, and D-galactose. To study the effect of a hexose on D-xylose utilization, we used a medium containing both the select hexose and D-xylose. The cultures were kept in 250-ml Erlenmeyer flasks and incubated as above. At various times, 1-ml samples were removed for metabolite analyses. The samples were clarified by centrifugation at 8,000 × *g* for 2 min. The supernatants were stored frozen until analyses. All experiments were repeated two to four times.

Sterilization. All solid media were sterilized by autoclaving. For preparation of liquid media, concentrated solutions of YNB without amino acids and sugars were filter sterilized separately and then added to the autoclaved water to desired concentrations.

Analytical methods. Ethanol concentrations were measured by gas chromatography with isopropanol as the standard (17). Sugars and xylitol were determined by high-pressure liquid chromatography with either a Bio-Rad HPX-87P column (13) or an amino-silica column (Jones Chromatography, Inc., Mississauga, Ontario, Canada). With the latter, the mobile phase was 75% (vol/vol) acetonitrile which was operated at a flow rate of 0.8 ml/min at 30°C. With both columns, base-line resolution between D-xylose and the hexoses was not achieved. Quantitation was, therefore, based on the ratio between the peak height of the sugar and that of 1% glycerol added as a standard.

RESULTS

Enzyme induction. In both *P. tannophilus* and *P. stipitis*, XR and XDH activities were induced by D-xylose. Cells grown on glycerol alone for 16 h exhibited low activities of these enzymes (Tables 1 and 2). A number of sugars, including some that are commonly found in lignocellulosic hydrolysates, were tested for their ability to repress the induction of XR and XDH activities by D-xylose. Specific enzyme activities induced by D-xylose alone for 8 h were used as the control values. They were arbitrarily expressed as 100%, with which specific activities induced by other carbohydrates were compared. Induction by D-glucose alone yielded very low activities compared with those induced by D-xylose (Tables 1 and 2).

The glucose effect was clearly evident in both *P. stipitis* and *P. tannophilus*. D-Glucose, D-mannose, and 2-deoxyglucose were capable of repressing the induction of XR and XDH activities in both yeasts (Tables 1 and 2). However, a closer examination revealed that the patterns of repression differ between the two yeasts.

In *P. stipitis*, D-glucose was the most effective repressor. XR and XDH activities were induced to 19 and 17% of the control values, respectively (Table 1). D-Mannose and 2-deoxyglucose were about equally effective in repressive activity. The amount of enzyme activities induced ranged from 38 to 54% of the controls in the presence of these sugars.

In *P. tannophilus*, induction of XDH was repressed to a greater extent than that of XR (Table 2). D-Mannose was as effective as D-glucose in repressing enzyme induction. In the presence of one of these sugars, XR activity was induced to about 30% of the control values, while XDH activity was found in trace amounts only. With 2-deoxyglucose, the induction of XDH activity but not that of XR was repressed.

None of the other sugars tested, L-arabinose, D-galactose, and cellobiose, repressed enzyme induction. With D-galactose, a stimulatory effect was observed with the induction of XR in *P. stipitis* and XDH in *P. tannophilus*. The reason for this effect was not investigated.

Single sugar utilization. The performance of *P. stipitis* and *P. tannophilus* on four different sugars was studied. The fermentation patterns found conformed generally to those described earlier (14, 18) except *P. stipitis* showed longer fermentation times compared with published values (5). Briefly, D-glucose and D-mannose were the best substrates for growth and fermentation in both yeasts. These hexoses were consumed at faster rates than D-xylose. D-Galactose

TABLE 2. Induction of NADPH-dependent XR and NAD-dependent XDH activities in *P. tannophilus* NRRL Y2460 cultures by different carbohydrates

Carbohydrate(s)	No. of trials	Relative activity (%)[a]	
		XR	XDH
D-Xylose	4	100	100
Glycerol	2	32 ± 6	20 ± 10
D-Glucose	3	15 ± 8	0 ± 0
D-Xylose + D-glucose	4	34 ± 9	7 ± 5
D-Xylose + D-mannose	3	30 ± 20	4 ± 4
D-Xylose + D-galactose	3	107 ± 5	180 ± 80
D-Xylose + L-arabinose	3	110 ± 10	80 ± 10
D-Xylose + cellobiose	3	70 ± 20	80 ± 10
D-Xylose + 2-deoxyglucose	3	94 ± 8	11 ± 7

[a] Values shown are average percentages ± standard error. Average specific activities for XR and XDH induced by D-xylose were 0.079 ± 0.006 and 0.38 ± 0.08 nmol of cofactor converted per min per μg of protein, respectively.

was poorly utilized by *P. tannophilus*, while *P. stipitis* consumed D-galactose readily. Ethanol was the major fermentation product in both yeasts. The yields and final concentrations of ethanol were higher on readily utilized hexoses than those on D-xylose. Xylitol was also produced on D-xylose by *P. tannophilus* cultures.

Mixed sugar utilization. The ability of *P. stipitis* and *P. tannophilus* to utilize and ferment D-xylose in the presence of a hexose was examined. The hexoses tested included D-glucose and D-mannose, both of which could repress the induction of XR and XDH, and D-galactose, which could not (Tables 1 and 2). When both D-xylose and a hexose were present in the medium, a sequential pattern of sugar consumption was generally observed. In cultures of both yeasts, the presence of D-xylose did not affect hexose utilization. Hexoses were consumed at rates similar to those found when they were the sole carbon sources. However, the utilization of D-xylose was clearly affected by the hexoses.

In *P. stipitis* NRRL Y7124, D-xylose consumption was slow and incomplete in the presence of a hexose. This is illustrated in Fig. 1 and 2 for glucose-xylose and galactose-xylose mixtures, respectively. The utilization of a mannose-xylose mixture followed the pattern of a glucose-xylose mixture closely except that a short lag period for D-xylose was found in the latter but not in the former culture (data not shown). In all instances, D-xylose consumption commenced before all the hexoses were consumed. This perhaps reflected the finding that XR and XDH activities were not completely repressed by the hexose sugars in *P. stipitis* (Table 1). Ethanol was produced rapidly, and peak concentrations were observed at the times when the hexoses were depleted, suggesting that most of the ethanol was derived from the hexoses.

In *P. tannophilus* NRRL Y2460, D-xylose consumption was affected by D-mannose and D-glucose only. An example for a mannose-xylose mixture is shown in Fig. 3. D-Mannose was utilized rapidly, and D-xylose consumption began when most of the hexose had disappeared from the medium. A lag period of about 16 h for D-xylose was apparent. The pentose sugar was completely consumed, although at a rate slightly slower than that when it was the sole carbon source. Ethanol and xylitol were the major fermentation end products. Ethanol values continued to rise after depletion of the hexose. Peak concentrations were obtained after most of the D-xylose was consumed, suggesting that the pentose sugar contributed to ethanol production. Xylitol accumulation occurred shortly after D-xylose consumption began. The amount accumulated remained fairly constant throughout the course of the culture (Fig. 3). Essentially the same utilization pattern was observed with a glucose-xylose mixture (data not shown).

With a galactose-xylose mixture, consumption of D-xylose preceded that of D-galactose in *P. tannophilus* (Fig. 4). The pattern and rate of D-xylose utilization in such a mixture were similar to those found when the pentose was the sole carbon source. The amounts of ethanol and xylitol formed were also similar to values obtained on D-xylose alone.

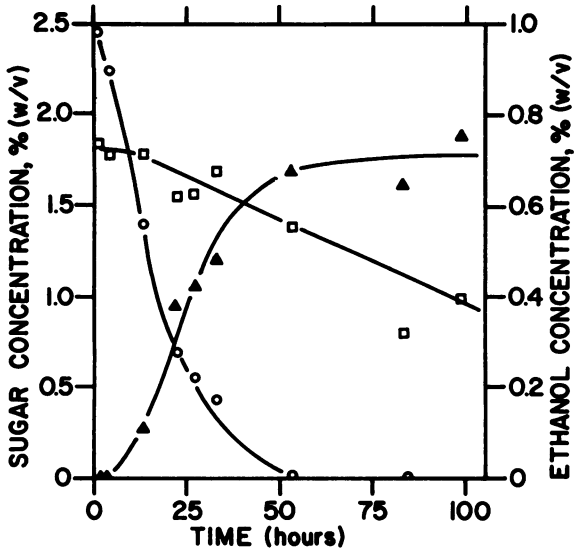

FIG. 1. Utilization and fermentation of a glucose-xylose mixture by *P. stipitis* NRRL Y7124. Symbols: ○, D-glucose; □, D-xylose; ▲, ethanol.

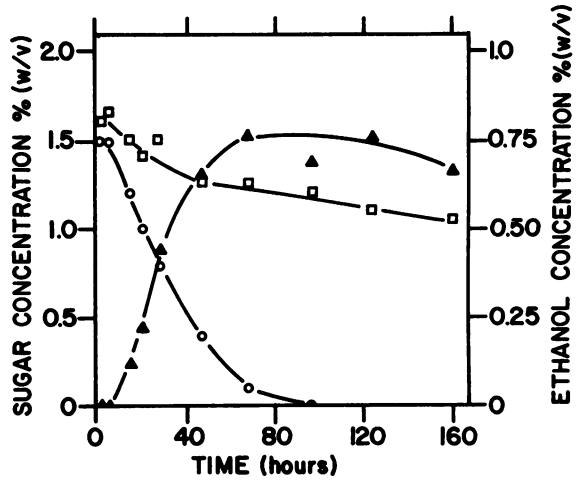

FIG. 2. Utilization and fermentation of a galactose-xylose mixture by *P. stipitis* NRRL Y7124. Symbols: ○, D-galactose; □, D-xylose; ▲, ethanol.

DISCUSSION

Previous work with *P. tannophilus* has shown that XR and XDH activities are induced in xylose-grown but not glucose-

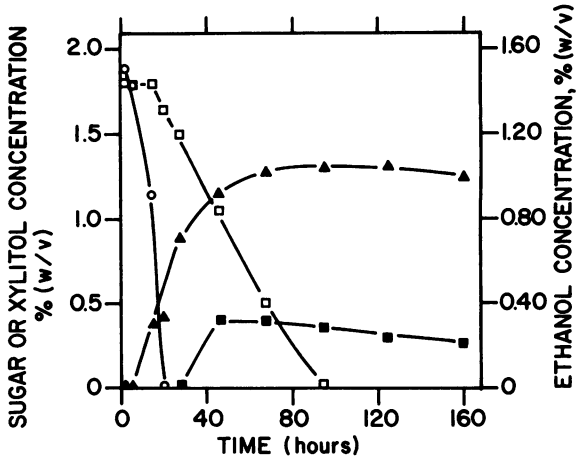

FIG. 3. Utilization and fermentation of a mannose-xylose mixture by *P. tannophilus* NRRL Y2460. Symbols: ○, D-mannose; □, D-xylose; ▲, ethanol; ■, xylitol.

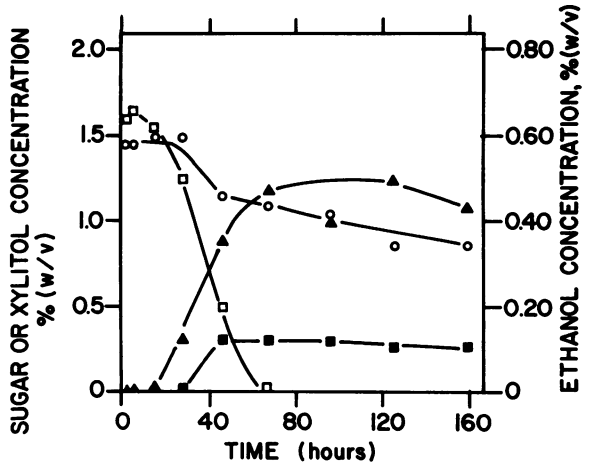

FIG. 4. Utilization and fermentation of a galactose-xylose mixture by *P. tannophilus* NRRL Y2460. Symbols: ○, D-galactose; □, D-xylose; ▲, ethanol; ■, xylitol.

grown cells (1, 4). The results presented here confirm these observations and extend them to *P. stipitis* as well (Table 1). In addition, our results with mixed substrates provided clear and direct experimental evidence that these enzymes are repressible in both yeasts. This repression of synthesis of inducible enzymes in the presence of D-glucose or closely related carbon sources is characteristic of the glucose effect or catabolite repression (6, 9). The present study focused on XR and XDH because of their regulatory role in channelling D-xylose into intermediary metabolism. It is possible that other xylose-metabolizing enzymes can be regulated by glucose repression as well.

The patterns of glucose repression of xylose-metabolizing enzymes differ between the two yeasts. In *P. stipitis*, D-glucose was a more effective repressor than either D-mannose or 2-deoxyglucose. However, XR and XDH activities were repressed to a similar extent in the presence of a particular repressor. In *P. tannophilus*, D-glucose and D-mannose were equally effective. XDH activity was repressed to a much greater extent than XR activity in *P. tannophilus*. This suggests that the two enzymes are not under coordinate control. This view is also supported by the finding that 2-deoxyglucose could repress XDH but not XR activity in *P. tannophilus*. Likely, uptake of 2-deoxyglucose or its phosphorylation or both triggered a mechanism which inhibited the synthesis of XDH in *P. tannophilus*. However, the repression of XR requires further catabolism of the hexose sugar.

The performance of *P. stipitis* on various sugars was less efficient than that of *P. tannophilus* in this study. Moreover, D-xylose was poorly and incompletely consumed when present in sugar mixtures. These observations contrasted with other studies which showed better fermentation performance of *P. stipitis* on D-xylose (2, 5). The poor fermentation characteristics may be attributed to the use of a defined YNB medium without amino acids in the study reported here. Yeast extract can stimulate the growth of *P. stipitis* on D-xylose (2). Furthermore, fermentation rates on the pentose were enhanced by supplementation of the medium with biotin and thiamine (5).

Despite this, patterns of mixed sugar utilization generally correlated well with studies on enzyme induction. In *P. tannophilus*, the repressive sugars, D-glucose and D-mannose, caused a noticeable lag in D-xylose utilization, while the nonrepressive sugar, D-galactose, did not. In *P. stipitis*, the three hexoses were utilized preferentially over D-xylose. A short lag period for pentose consumption was apparent only with a glucose-xylose mixture. D-Xylose utilization commenced while considerable hexoses were still present in the medium. This confirmed an earlier study that *P. stipitis* could utilize D-xylose, D-mannose, and D-galactose concurrently (5). The finding is also consistent with the observation that none of the hexoses caused complete inhibition of XR and XDH activities in *P. stipitis* (Table 1).

Enzyme repression in the presence of D-glucose is a major regulatory system in yeasts. From a practical point of view, this type of regulation is important in some industrial fermentations involving substrates such as starch, sucrose, and lactose in which D-glucose released can repress the process. The present study shows that this phenomenon can also limit the yeast fermentation of pentose sugars in lignocellulosic substrates. As with other glucose-repressible systems, inducibility and repression of XR and XDH are likely controlled by different mechanisms.

A general approach to obtaining mutants with nonrepressible enzymes has been the use of gratuitous repressors such as 2-deoxyglucose (6). However, the incomplete repression of XR and XDH activities by this compound suggests that it is only partially effective in selecting for nonrepressible mutants in *P. tannophilus* and *P. stipitis*.

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