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Regulation of Phosphotransferases in Glucose- and Xylose-Fermenting Yeasts

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ABSTRACT

This research examined the titers of hexokinase (HK), phosphofructokinase (PFK), and xylulokinase (XUK) in *Saccharomyces cerevisiae* and two xylose fermenting yeasts, *Pachysolen tannophilus* and *Candida shehatae*, following shifts in carbon source and aeration. Xylose-grown *C. shehatae*, glucose-grown *P. tannophilus*, and glucose-grown *S. cerevisiae*, had the highest specific activities of XUK, HK, and PFK, respectively. XUK was induced by xylose to moderate levels in both *P. tannophilus* and *C. shehatae*, but was present only in trace levels in *S. cerevisiae*. HK activities in *P. tannophilus* were two to three fold higher when cells were grown on glucose than when grown on xylose, but HK levels were less inducible in *C. shehatae*. The PFK activities in *S. cerevisiae* were 1.5 to 2 times higher than in the two xylose-fermenting yeasts. Transfer from glucose to xylose rapidly inactivated HK in *P. tannophilus*, and transfer from xylose to glucose inactivated XUK in *C. shehatae*. The patterns of induction and inactivation indicate that the basic regulatory mechanisms differ in the two xylose fermenting yeasts.

Index Entries: *Saccharomyces cerevisiae; Pachysolen tannophilus; Candida shehatae;* 6-phosphofructokinase; hexokinase; D-xylulokinase; regulation; phosphotransferase.

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INTRODUCTION

Aside from the documented induction of xylose reductase and xylitol dehydrogenase *(1)*, very little is known about regulation of metabolism in the fermentation of xylose or glucose by xylose-fermenting yeasts. In *Pichia stipitis, C. shehatae,* and most other yeasts, glucose represses the utilization of xylose and other sugars. This is a critical problem in the utilization of the mixture of glucose and other sugars found in hemicellulosic hydrolysates. The mechanism by which repression occurs in yeasts that rapidly metabolize xylose has not been elucidated. There is some indication that the basic regulatory mechanism in *P. tannophilus* might be similar to that of *S. cerevisiae (2).*

In *S. cerevisiae,* the *HXK2* gene product, hexokinase PII, EC 2.7.1.1, (HK PII) mediates uptake of hexoses and plays a role in carbon catabolite repression *(3,4)*. Hexokinase PII possesses both hexokinase and protein kinase activities, and the protein kinase activity of HK PII is also regulated by glucose *(5)*. In *S. cerevisiae,* mutants with reduced HK PII acvtivity have increased levels of glucose repressible enzymes such as invertase *(6)*. A regulatory role for HK is also seen in other yeasts. In *Kluyveromyces lactis,* the product of the HK gene, *RAG5,* controls the expression of *RAG1,* a low-affinity glucose-fructose transporter *(7)*. *P. tannophilus* mutants deficient in HK do not repress enzymes specific to xylose metabolism *(2)*, and they are able to ferment xylose in the presence of glucose (8). Pardo et al. *(9)* isolated *P. stipitis* mutants defective in carbon catabolite repression by selecting for resistance to 2-deoxy-D-glucose, an antimetabolite known to select for HK-deficient cells. Therefore, HK appears to be important in regulating sugar uptake in many systems.

D-Xylulokinase (XUK), EC 2.7.1.17 is believed to be critical for the assimilation of pentose sugars *(10)*, but its regulation in xylose fermenting yeasts has not been reported previously. Phosphofructokinase (PFK), EC 2.7.1.11 is instrumental in the glycolytic flux of both pentoses and hexoses. In *S. cerevisiae,* allosteric inhibition of 6-phosphofructokinase (PFK) EC 2.7.1.11 kinetically regulates the glycolytic pathway *(11,12)*.

In *S. cerevisiae,* loss of respirative activity and onset of fermentation results from high glycolytic flux regardless of the carbon source *(13)*. In *Hanseniospora uvarum,* intracellular acetate accumulation appears to impede electron transport, thereby promoting the shift from respirative to fermentative metabolism *(14)*. In xylose-fermenting yeasts glycolysis does not appear to repress respirative activities *(15)*. Rather, fermentation is induced by restricting aeration *(16–20)*. It is possible that the glycolytic flux never becomes high enough to repress respiration in xylose-fermenting yeasts. A high glycolytic flux requires high phosphotransferase activities, so it is of interest to determine the relative activities of HK, XUK, and PFK in respirative and fermentative cells.

Biochemical studies with mutants of *P. tannophilus* and *C. shehatae* have shown that the level of D-xylulokinase (XUK, EC 2.7.1.17) is impor-

tant in determining growth and xylose fermentation rates *(10,21)*. The possible roles of HK and PFK have not been previously studied in these organisms. The carbon source can be very important in determining the levels of glycolytic enzymes present in the cell *(22,23)*. Cells able to ferment both glucose and xylose should show adaptive responses when switched from one carbon source to the other, and cells grown on different carbon sources might demonstrate substantially different regulatory responses when metabolically perturbed.

P. tannophilus ferments glucose much faster than xylose *(24)*, whereas *C. shehatae* metabolizes glucose and xylose at similar rates. *C. shehatae* is a much better xylose fermenter than *P. tannophilus*. Such differences might be reflected in the relative activities of HK and XUK, so the phosphotransferase regulation in each of these species are of interest.

The objectives of this research were to compare the titers of HK, XUK, and PFK in yeasts capable of xylose and glucose fermentations, to determine whether these titers shifted significantly with growth, aeration, and anaerobiosis, and to see whether the carbon source employed for growth affected the regulatory patterns. A strain of *S. cerevisiae* was included in order to compare xylose-fermenting and non-xylose-fermenting yeasts.

MATERIALS AND METHODS

Micro-organisms

S. cerevisiae ATCC 26785 and *C. shehatae* ATCC 22984 were obtained from the American Type Culture Collection, Beltsville, MD. *P. tannophilus* NRRL Y-2460 was obtained from the Northern Regional Research Laboratory, Peoria, IL. All stock cultures were maintained on yeast malt agar (YMA, Difco).

Culture Methods

Cells were grown in 50 mL of 0.17% yeast nitrogen base without ammonium sulfate or amino acids (YB, Difco) using urea (2.27 g/L) as a nitrogen source and either glucose or xylose (90 g/L) as a carbon source. The pH was 4.6. Cells grown on fresh YPD medium plates (yeast extract, 6 g/L; peptone, 12 g/L; dextrose, 12 g/L), were washed and resuspended with sterile water and used as an inoculum. Initial cell densities were ≈ 2.0 OD at 525 nm. Cultures were incubated with shaking at 100 rpm, 30°C until midethanol production phase (midphase cells), which varied with the organism. For glucose-grown *S. cerevisiae*, *P. tannophilus*, or *C. shehatae* 12-, 18-, or 24-h-old cells were used, respectively. For xylose-grown *P. tannophilus*, or *C. shehatae*, 48- or 24-h-old cells were used, respectively. Continuous-culture studies were performed as previously described *(16)*.

Induction Studies

S. cerevisiae would not grow on xylose, so HK levels were assayed following incubation in the presence of xylose for 4.5 h. Cells from the three yeast species were cultivated on either glucose or xylose. Each batch of cells was then harvested, washed, and divided into 5-mL aliquots. One aliquot was immediately frozen (initial cells). Two aliquots were suspended in 50 mL YB with 5.0 g/L of $(NH_4)_2SO_4$ (YNB). These served as carbon starved controls. Two aliquots were suspended in 50 mL YNB medium along with 60 g/L glucose and two were suspended in 50 mL YNB plus 60 g/L xylose. Of each set of two aliquots, one was placed in a ventilated 125-mL Erlenmeyer flask (aerated), and the other was placed in a 100-mL serum vial, sealed with a rubber serum stopper and flushed with nitrogen (anaerobic). All flasks and serum vials were incubated with shaking at 200 rpm for 4.5 h at 30°C. Cells were then harvested, washed, and frozen in –80°C freezer until enzyme assays could be performed on the homogenates. Separate, longer-term induction studies were performed under fermentative conditions in a similar manner except that cycloheximide was added to determine how inhibition of protein synthesis would affect enzyme activity.

Preparation of Homogenates

Cells were thawed, washed, and suspended in a minimum volume of 0.1*M* 3-[*N*-morphilino]propanesulfonic acid (MOPS) buffer (pH 6.8). Cell slurries (≈ 1.0 mL) were kept on ice and placed in a 13 mm (id) glass tube containing 1.0 g of 0.5 mm acid-washed glass beads and blended in a high speed vortex mixer for two 1-min bursts. Microscopic examination showed that approx 60% cell disruption was attained *(25)*. Cell homogenates were centrifuged at 3000*g* for 15 min, and supernatants were collected for enzyme assays.

Enzyme Assays

All assays were performed within 4 h of cell breakage. PFK was assayed by the method of Bruinenberg et al. *(26)*, but modified by the addition of 10 m*M* NH_4Cl, 1 m*M* 5′-AMP, and 0.02 m*M* fructose 2,6-bisphosphate. HK was assayed by the method of Bergmeyer *(27)* except that final concentrations of 11 m*M* glucose and 0.55 m*M* ATP were used. Activity was determined by observing the reduction of NADP of NADPH by glucose-6-phosphate dehydrogenase. XUK was assayed by a modification of the method of Simpson *(28)*. The reaction mixture contained 0.5*M* Tris/HCl buffer (with 0.01*M* EDTA), pH 7.8, 0.1 mL; freshly prepared 0.01*M* phospho-*enol*-pyruvate, 0.1 mL; 0.05*M* $MgCl_2$, 0.1 mL; 0.01*M* ATP 0.05 mL; 0.03*M* NADH, 0.03 mL; 0.01*M* D-xylulose, 0.1 mL; L-lactic acid dehydrogenase (≈10 U), 0.03 mL; and diluted enzyme sample to a final volume of 1.0 mL. Addition of D-xylulose started the reaction. XUK activity

was determined from the rate of NADH disappearance. Control assays for xylitol dehydrogenase (reaction mixture minus ATP) and NADH oxidation (reaction mixture minus ATP and D-xylulose) were performed, and the rates of these reactions were subtracted to obtain XUK activity.

Analytical Methods

Protein concentrations were determined by the method of Bradford *(29)* using bovine serum albumin as a standard. Specific activities are expressed in international units (μmoles of substrate consumed min^{-1}) per mg protein.

In this study, we cultivated all three organisms on glucose or xylose, induced them under six different conditions, prepared 21 different cell homogenates (including controls), and performed two to four enzyme assays of each homogenate. The experiment was repeated one to three times. Results were reported as average of the data obtained in these experiments, and we inferred effects of the variables only when the differences between the averages exceeded the sum of the observed ranges.

RESULTS AND DISCUSSION

Phosphofructokinase Activities

PFK activities were higher in *S. cerevisiae* than in the two xylose-fermenting yeasts (Table 1). The specific activities of PFK were similar in *P. tannophilus* and *C. shehatae*, when cells were grown on glucose. PFK levels were slightly higher in xylose-grown *C. shehatae* than on xylose-grown *P. tannophilus*. PFK levels in glucose-grown *P. tannophilus* were only one-third as high following anaerobic incubation in the presence of xylose as compared to glucose. Overall, the levels of PFK did not shift as much with *C. shehatae* as they did with *P. tannophilus*. These results indicate that the Embden-Meyerhoff Parnas pathway is probably functional in both glucose and xylose grown *P. tannophilus* and *C. shehatae*. This is consistent with the assumption that PFK plays a role in the metabolism of phosphorylated intermediates that are rearranged by the non-oxidative pentose phosphate pathway.

Hexokinase Activities

There was no significant difference between HK levels of *S. cerevisiae* and the two xylose fermenting yeasts when each was grown on glucose (Table 2). HK levels were higher in glucose-grown than in xylose-grown cells of *P. tannophilus* and. Less significant differences were seen with *C. shehatae*. Aerated incubation with xylose-strongly reduced HK titers in glucose grown *S. cerevisiae*, *P. tannophilus*, and *C. shehatae*. HK activity of xylose-grown cells of *C. shehatae* did not seem to be affected by this short-term incubation.

Table 1
Specific Activity of Phosphofructokinase[a] in *S. cerevisiae*, *P. tannophilus*, and *C. shehatae*

	Saccharomyces cerevisiae	*Pachysolen tannophilus*		*Candida shehatae*	
Incubation condition[b]	Glucose grown	Glucose grown	Xylose grown	Glucose grown	Xylose grown
Aerated					
YB + $(NH_4)_2SO_4$ + glucose	1.93 ±0.22	1.10 ±0.51	0.51 ±0.01	1.37 ±0.59	0.90 ±0.16
YB + $(NH_4)_2SO_4$ + xylose	1.99 ±0.12	1.07 ±0.18	0.59 ±0.10	1.17 ±0.22	1.05 ±0.01
Anaerobic					
YB + $(NH_4)_2SO_4$ + glucose	2.29 ±0.38	1.26 ±0.23	0.86 ±0.04	1.12 ±0.10	1.02 ±0.11
YB + $(NH_4)_2SO_4$ + xylose	1.86 ±0.34	0.42 ±0.15	0.89 ±0.09	1.24 ±0.16	1.21 ±0.12

[a]Specific activity IU/mg protein. Average ± range.
[b]YB = Yeast base.

Table 2
Specific Activity of Hexokinase[a] in *S. cerevisiae*, *P. tannophilus*, and *C. shehatae*

	Saccharomyces cerevisiae	*Pachysolen tannophilus*		*Candida shehatae*	
Incubation condition[b]	Glucose grown[c]	Glucose grown	Xylose grown	Glucose grown	Xylose grown
Aerated					
YB + $(NH_4)_2SO_4$ + glucose	2.23	2.98 ±0.90	1.05 ±0.03	1.99 ±0.35	1.49 ±0 43
YB + $(NH_4)_2SO_4$ + xylose	1.30	1.67 ±0.67	0.78 ±0.38	1.33 ±0.19	1.50 ±0.32
Anaerobic					
YB + $(NH_4)_2SO_4$ + glucose	1.90	2.65 ±1.00	0 96 ±0.28	1 91 ±0.23	1.63 ±0.22
YB + $(NH_4)_2SO_4$ + xylose	1.78	2.79 ±1.14	0.95 ±0.03	1.71 ±0.20	1.66 ±0.46

[a]Specific activity IU/mg protein. Average ± range.
[b]YB = Yeast base.
[c]Single determination.

Xylulokinase Activities

Xylose-induced XUK in *P. tannophilus* and *C. shehatae*. Specific activity was two to four times higher in xylose-grown cells than in glucose-grown cells (Table 3). Specific activity of XUK was only about one-third of HK (cf Table 2). Since no growth was obtained with *S. cerevisiae* in xylose, all induction experiments with this organism were performed with glucose-grown cells. XUK levels were barely detectable (Table 3). Xylose induced XUK activity in glucose-grown *P. tannophilus* and *C. shehatae* more under aerated conditions than under anaerobic conditions.

Time Course Studies

To examine induction and repression of HK and XUK in more detail, we grew cells of *C. shehatae* and *P. tannophilus* on either glucose or xylose to midphase then transferred the cells to the other carbon source.

Table 3
Specific Activity of D-xylulokinase[a] in *S. cerevisiae*, *P. tannophilus*, and *C. shehatae*

	Saccharomyces cerevisiae	*Pachysolen tannophilus*		*Candida shehatae*	
Incubation condition[b]	Glucose grown[c]	Glucose grown	Xylose grown	Glucose grown	Xylose grown
Aerated					
YB + $(NH_4)_2SO_4$ + glucose	0.023	0.09[c]	0.48 ±0.07	0.03[c]	0.59 ±0.08
YB + $(NH_4)_2SO_4$ + xylose	0.070	0.53±0.04	0.89 ±0.09	0.55±0.04	1.13 ±0.01
Anaerobic					
YB + $(NH_4)_2SO_4$ + glucose	0.029	0.25[c]	0.77 ±0.08	0 (nd)[d]	0.88 ±0 05
YB + $(NH_4)_2SO_4$ + xylose	0.026	0.27[c]	0.97 ±0.16	0.22[c]	1.03 ±0.05

[a]Specific activity IU/mg protein. Average ± range.
[b]YB = Yeast base.
[c]Single determination.
[d]Not detectable.

The specific activities of HK and XUK were periodically measured for up to 9 h.

Following a transfer of *C. shehatae* from glucose to xylose, XUK levels increased eight fold and HK levels decreased by half within 3 h (Fig. 1A). Following transfer from xylose to glucose, XUK was inactivated, but HK activity was essentially unchanged (Fig. 1B). When cycloheximide was added during induction by xylose, XUK activity did not increase, and HK activity dropped slowly with time (Fig. 1C). This indicated that in *C. shehatae*, xylose does not inactivate HK. When *C. shehatae* was transferred from xylose to glucose in the presence of cycloheximide, both XUK and HK activities dropped rapidly (Fig. 1D). The apparent loss of HK activity in the presence of cycloheximide (Fig. 1D), but not in its absence (Fig. 1B) indicates that new protein synthesis is necessary to maintain hexokinase activity in *C. shehatae* following transfer to glucose.

Different patterns of induction were observed with the HK and XUK titers of *P. tannophilus*. Following transfer from glucose to xylose, HK was rapidly (but incompletely) inactivated, and XUK activity was induced but to a lesser extent than in *C. shehatae* (cf Fig. 1A and 2A). In a transfer from xylose to glucose, XUK activity was not rapidly inactivated as in *C. shehatae* (cf Fig 1B and 2B). It did, however decrease gradually after several hours. This difference was also apparent when cells were transferred from xylose to glucose in the presence of cycloheximide. In marked contrast to *C. shehatae*, no inactivation of either HK or XUK was apparent in *P. tannophilus* (Fig. 2D).

Overall, the titers observed in the time-course induction experiments were consistent with those observed in earlier experiments with multiple variables (Tables 2 and 3).

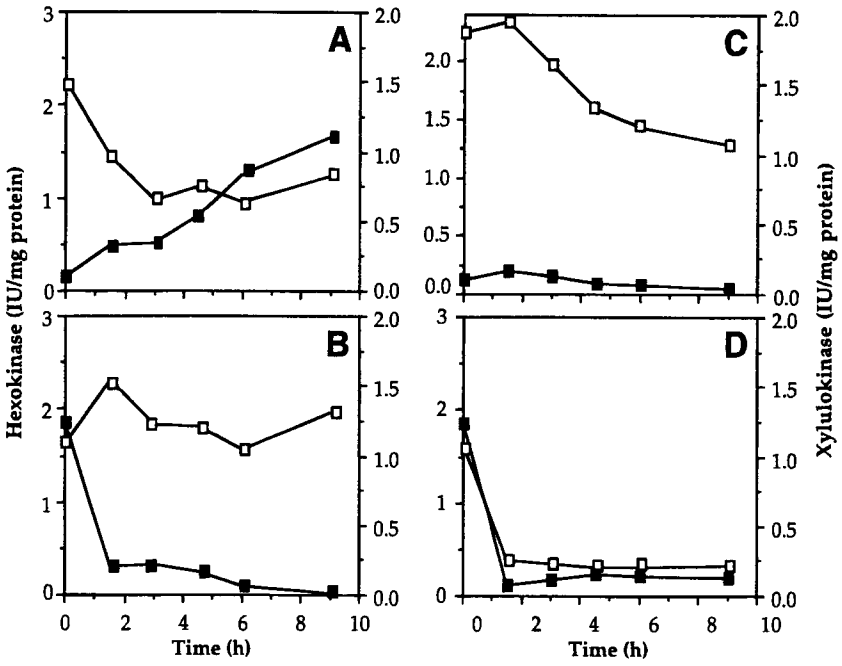

Fig. 1. Hexokinase (□) and D-xylulokinase (■) specific activities in *C. shehatae* following a shift from either **(A)** glucose to xylose or **(B)** xylose to glucose. Cells were grown to midethanol-production phase under aeration, then harvested, washed and transferred to fresh medium under similar conditions. HK and XUK specific activities in *C. shehatae* following a shift from either **(C)** glucose to xylose or **(D)** xylose to glucose with cycloheximide (final concentration, 6.8 μg/mL) added to the fresh medium. Time zero marks the transfer to fresh medium.

Continuous Culture Studies

To determine titers in steady-state cells, *C. shehatae* was cultivated under either fully aerobic or oxygen-limited (fermentative) conditions on xylose or glucose, and HK activities were measured. Four different dilution rates were examined, ranging between 0.05 and 0.22 h^{-1}, but no significant variation could be observed as a function of dilution rate, so the results are presented as averages. HK activity was slightly higher in glucose-grown than xylose-grown cells and somewhat higher in oxygen-limited than fully aerobic cells (Table 4). Studies with *S. cerevisiae* indicate that neither growth rate or cell cycle significantly affect HK or PFK specific activities *(30,31)*. Therefore, changes in enzyme titer likely represent regulatory adaptations to culture conditions.

Our data show that glucose induces HK activity and xylose induces XUK activity in both *P. tannophilus* and *C. shehatae* (Tables 2 and 3). There was no significant difference in PFK activity with these yeasts grown on glucose and incubated in glucose or xylose, and there was no difference when the yeasts were grown on xylose and incubated in glucose or xylose.

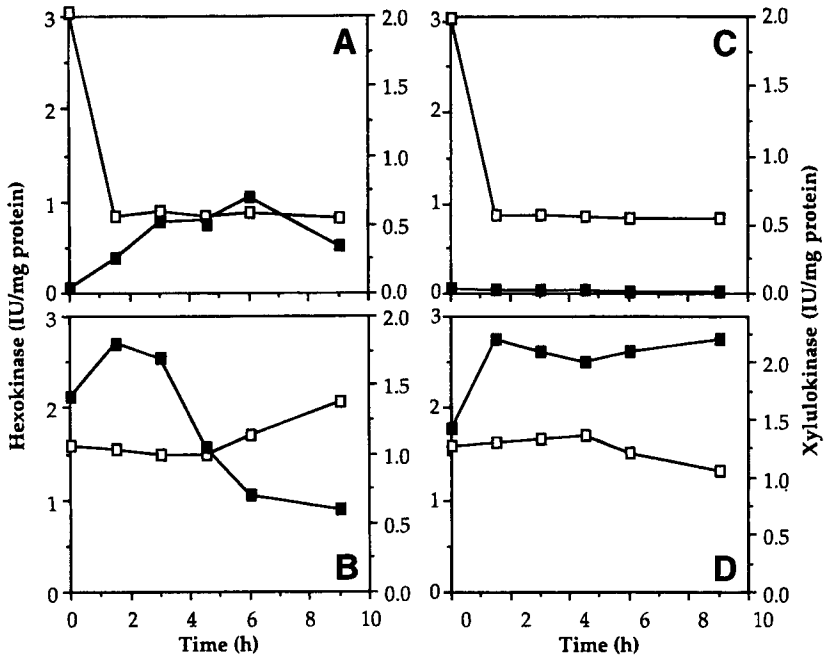

Fig. 2. Hexokinase (□) and D-xylulokinase (■) specific activities in *P. tannophilus* following a shift from either **(A)** glucose to xylose or **(B)** xylose to glucose. Cells were grown with aeration to midethanol production phase, then harvested, washed and transferred to fresh medium under similar conditions. HK and XUK specific activities in *P. tannophilus* following a shift from either **(C)** glucose to xylose or **(D)** xylose to glucose with cycloheximide added to the fresh medium. Time zero marks the transfer to fresh medium.

Table 4
Specific Activity of Hexokinase in *C. shehatae* Grown in Continuous Culture under Several Conditions

Growth condition	Carbon source	Hexokinase activity ave. ± SD (n)[a]	
Fully-aerobic	xylose	0.72 ± 0.16	(4)
Fully-aerobic	glucose	0.92[b]	
Oxygen-limited	xylose	1.07 ± 0.19	(11)
Oxygen-limited	glucose	1.84 ± 0.22	(4)

[a]Average ± standard deviation (number of replicates).
[b]Single determination.

C. shehatae HK activity observed in the presence and absence of cycloheximide, suggest the presence of two separate isozymes under different regulation in this organism. The total HK titer was maintained in growing cells by new protein synthesis, whereas in the presence of cycloheximide,

the HK present during growth on xylose was inactivated, and new protein synthesis or the second HK could not occur to replace it. These results suggest that HK was synthesized in response to glucose, even though no net increase in total activity was observed.

C. tropicalis (32) and *S. cerevisiae* contain three glucose-phosphorylating enzymes, HK PI, HK PII, and glucokinase. Hexokinase PI is constitutive, whereas, HK PII and glucokinase are regulated by the carbon source. The addition of D-xylose to homogenates of glucose-grown cells causes an irreversible inactivation of all three enzymes in *S. cerevisiae (33)*. In the study presented here, the HK of *P. tannophilus* was inactivated following transfer of glucose-grown cells to xylose, so these results are consistent with the previous findings with *S. cerevisiae*.

Hexokinase PII plays an important role in carbon catabolite repression in *S. cerevisiae (2,34–36)*; in *S. occidentalis*, an unusual HK with two differing catalytic sites appears to have a similar function *(37)*. Moreover, the xylose-induced decrease in HK PII activity confers resistance to carbon catabolite resistance in *Saccharomyces carlsbergensis (38)*. In our study, XUK activity developed shortly following inactivation of HK, so the observations with *P. tannophilus* are consistent with the carbon catabolite repression mechanism mediated by HK PII described for *S. cerevisiae (39)*. We do not know how important specific activities of these enzymes are to the overall metabolic activities of cells. The significance of PFK in determining the glycolytic flux has been demonstrated in erythrocytes *(40)* and more recently, in *S. cerevisiae (41)*. In the latter case, PFK did not limit the fermentation rate, but it was not present in great excess either. About 80% of the PFK present was utilized by the glycolytic pathway. In the present studies of xylose-fermenting yeasts, the levels of PFK observed ranged between about 50 and 75% of those present in *S. cerevisiae*, so it is possible that this enzyme could limit glycolysis in the xylose fermenting yeasts. HK levels in *P. tannophilus* and *C. shehatae* were comparable to those observed in *S. cerevisiae*. Even though *S. cerevisiae* possesses several metabolic activities important to xylose metabolism *(42)*, we suspect that the XUK levels present are insignificant, especially in light of the apparent importance of this enzyme in fermenting xylose *(20)*.

In conclusion, the elevated levels of PFK observed with *S. cerevisiae* are consistent with its good fermentative activity on glucose. However, in comparison to xylose-fermenting organisms, *S. cerevisiae* does not possess significant XUK activity. Both HK and XUK are inductively regulated by glucose and xylose in *P. tannophilus* and *C. shehatae*, but significant levels of each enzyme are also present under fermentative conditions, regardless of the carbon source. The slightly higher level of XUK in xylose-grown *C. shehatae* and the higher level of HK in glucose-grown *P. tannophilus* might help account for the different rates at which these two organisms metabolize these sugars.

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