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D-Xylose Transport by *Candida succiphila* and *Kluyveromyces marxianus*

Boris U. Stambuk,[*,1,2] Mary Ann Franden,[1] Arjun Singh,[1] and Min Zhang[1]

[1]National Bioenergy Center, National Renewable Energy Laboratory, 1617 Cole Boulevard, Golden, CO, 80401; and
[2]Departamento de Bioquímica, Universidade Federal de Santa Catarina, Florianópolis, SC 88040-900, Brazil, E-mail: bstambuk@mbox1.ufsc.br

Abstract

The kinetics and regulation of D-xylose uptake were investigated in the efficient pentose fermentor *Candida succiphila*, and in *Kluyveromyces marxianus*, which assimilate but do not ferment pentose sugars. Active high-affinity (K_m ~ 3.8 m*M*; V_{max} ~ 15 nmol/[mg·min]) and putative facilitated diffusion low-affinity (K_m ~ 140 m*M*; V_{max} ~ 130 nmol/[mg·min]) transport activities were found in *C. succiphila* grown, respectively, on xylose or glucose. *K. marxianus* showed facilitated diffusion low-affinity (K_m ~ 103 m*M*; V_{max} ~ 190 nmol/[mg·min]) transport activity when grown on xylose under microaerobic conditions, and both a low-affinity and an active high-affinity (K_m ~ 0.2 m*M*; V_{max} ~ 10 nmol/[mg·min]) transport activity when grown on xylose under fully aerobic conditions.

Index Entries: D-Xylose, transport kinetics, fermentation, *Candida succiphila*, *Kluyveromyces marxianus*.

Introduction

A substantial fraction (up to 25%) of the monosaccharides in lignocellulose hydrolysates consists of the pentose sugars D-xylose (5–20%) and L-arabinose (1–5%). Xylose is second only to glucose in natural abundance, and although this sugar can be fermented by some species of bacteria, yeast, and filamentous fungi, the ethanol yields are low. Thus, there has been a great emphasis in the last two decades on developing an efficient organism for xylose fermentation through metabolic engineering *(1–3)*. Although some bacteria (*Zymomonas mobilis* and *Escherichia coli*) seem to be the best-performing biocatalysts for xylose fermentation, the preferred organism for indus-

*Author to whom all correspondence and reprint requests should be addressed.

trial ethanol fermentation processes is the yeast *Saccharomyces cerevisiae*. Since wild-type strains of *S. cerevisiae* do not utilize D-xylose, several laboratories have attempted to engineer *S. cerevisiae* for xylose fermentation *(4,5)*. Results with such genetically engineered yeasts have been encouraging, although the xylose utilization rates and ethanol productivities are still low compared to glucose fermentation by this yeast *(5)*.

The first metabolic step in the fermentation of sugars by yeasts is the uptake through the plasma membrane, and several reports have shown that transport is the rate-limiting step for fermentation *(6–8)*. Recently, Eliasson et al. *(9)* have concluded from studies with chemostat cultures that xylose transport limits the xylose flux and metabolism by recombinant *S. cerevisiae* cells. Xylose is taken up in *S. cerevisiae* cells by the glucose transporters *(10–12)*, which mediate the uptake of xylose by facilitated diffusion with very low affinity (K_m > 100 m*M*). Thus, the transport step would pose a limitation on the flux, at least at low substrate concentrations. The specificity of the transporters is also of concern, since glucose inhibits xylose uptake when these two sugars are present in the fermentation medium *(13,14)*. Additionally, it is worth noting that xylose reductase (XR), the first enzyme in the xylose-utilizing pathway, has a low affinity toward xylose (K_m > 50–100 m*M*), which means that high intracellular concentrations of xylose are necessary for efficient utilization *(15,16)*. Thus, the properties of the *S. cerevisiae* transporter(s) suggest the need for the improvement of this metabolic step by genetic engineering *(5,17)*.

Although hexose transport by yeast has been extensively investigated, little attention has been given to pentose uptake, including the mechanisms and the regulation of the transport activity. Here we report studies on the kinetics and regulation of xylose transport activity in two species of yeast, *Candida succiphila* and *Kluyveromyces marxianus*. *C. succiphila* is one of the few yeasts capable of fermenting both D-xylose and L-arabinose *(18)*. Although it has been reported that some *K. marxianus* strains ferment xylose *(19)*, the *K. marxianus* (formerly *K. fragilis*) strain used in the present study assimilates but does not ferment this sugar.

Materials and Methods

Yeast Strains and Growth Conditions

C. succiphila (NRRL Y-11998) and *K. marxianus* (ATCC 52486) cells were grown at 30°C in YEP medium (1% Difco yeast extract, 2% Difco Bacto Peptone) to which the 2–5% carbon source was added and the pH adjusted to 5.0. Microaerobic conditions employed 50 mL of medium in a 125-mL unbaffled Erlenmeyer flask shaken at 100 rpm. Aerobic conditions employed 50 mL of broth in 250-mL baffled Erlenmeyer flasks shaken at 220 rpm.

Analytical Methods

Growth was followed by turbidity measurements at 600 nm. One absorbance unit corresponds to approx 0.25 mg (dry wt) of *C. succiphila*

cells/mL, or approx 0.35 mg (dry wt) of *K. marxianus* cells/mL. Substrate consumed and products formed were analyzed in the supernatants of samples of cultures removed periodically after cells were separated by centrifugation. Xylose, xylitol, ethanol, and acetate were determined by high-performance liquid chromatography using a Hewlett-Packard (HP) 1090L chromatograph equipped with an HP 1047A refractive index detector and a Bio-Rad HPX-87H organic acid column operating at 65°C with a 0.01 *N* sulfuric acid mobile phase flow rate of 0.6 mL/min *(20)*.

Transport Assays

Cells were harvested in mid–growth phase, centrifuged, washed twice with cold distilled water, and suspended in water to a cellular density of about 60 g (dry cell mass)/L. The uptake of D-(1-^{14}C)xylose (55 mCi/mmol; American Radiolabeled Chemicals) was measured as previously described *(10,21)*. As a modification, assays were performed with 50 m*M* succinate-Tris buffer, pH 5.0, and the uptake was measured during 30-s periods. Appropriate experiments had shown that uptake of labeled xylose was linear for at least 1 min. Transport activity is expressed as nanomoles of xylose transported per milligram (dry cell mass) per minute. Kinetic parameters were determined as described elsewhere *(22,23)* using 0.05–900 m*M* final substrate concentrations.

For assays in which the effect of inhibitors was evaluated, cell suspensions were incubated with the indicated concentration of the inhibitors for 15 min prior to the assay, and 10 m*M* labeled xylose was used as substrate, except for *K. marxianus* cells grown under aerobic conditions in which the substrate concentration was 1 m*M* (*see* Subheading "D-Xylose Transport by *K. marxianus*" and Table 2). The following compounds were dissolved in ethanol: diethylstilbestrol, 2,4-dinitrophenol (DNP), carbonyl-cyanide-*m*-chlorophenylhydrazine (CCCP), and dicyclohexyl-carbodiimide (DCCD). Ethanol did not inhibit the transport activity at the concentration used in the assays (<2% [v/v]). To determine the inhibitory effect of a sugar on the transport of xylose, an excess of the test sugar was added to the labeled xylose. All determinations were done at least in duplicate, which did not differ by more than 15%.

Results

Growth on D-Xylose

C. succiphila and *K. marxianus* differed in their mode of xylose utilization during growth on this carbon source under microaerobic conditions. *C. succiphila* showed low rates of growth and xylose consumption but fermented this sugar during growth producing significant amounts of ethanol and xylitol (Fig. 1). By comparison, *K. marxianus* grew and assimilated xylose faster, but almost no ethanol and only low quantities of xylitol were observed in the growth medium (Fig. 1), indicating that this yeast diverts almost all carbon and energy from xylose metabolism into cell growth. Both

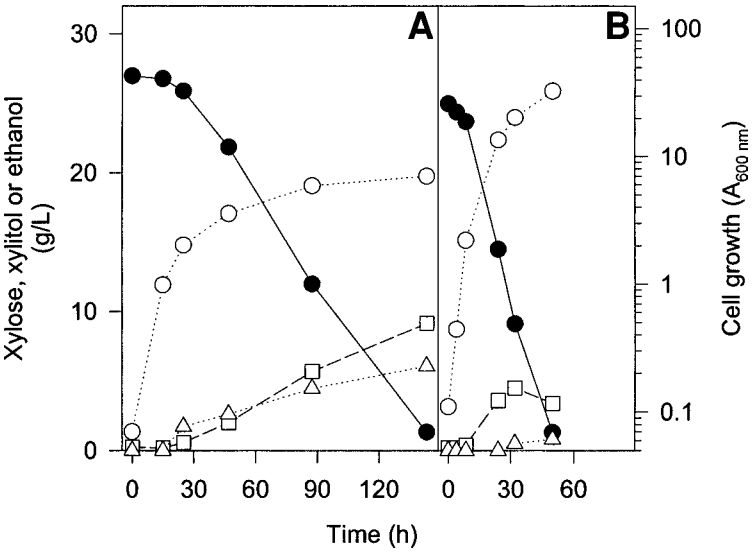

Fig. 1. Growth of *C. succiphila* **(A)** or *K. marxianus* **(B)** on 2.5% xylose under microaerobic conditions. At the indicated time points, (○)cell growth, (●)xylose, (□)xylitol, and (△)ethanol were determined as described in Materials and Methods.

strains produced low amounts of acetate (<0.8 g/L) at the end of the fermentations and consumed all these products from the medium after the sugar was exhausted. When grown under aerobic conditions, both yeasts grew faster than under microaerobic conditions, and higher cellular densities were obtained at the end of the incubations (data not shown). No ethanol was produced by *C. succiphila* during aerobic growth on xylose.

D-*Xylose Transport by* C. succiphila

Kinetic analysis showed that xylose-grown cells took up xylose by a single low-capacity (V_{max} = 15 nmol/[mg·min]) and high-affinity (K_m = 3.8 m*M*) transport system (Fig. 2). The low capacity of this transporter may explain the low sugar consumption rates observed when these cells are growing on xylose (Fig. 1). This transport system is an active transporter since the rate of xylose uptake in xylose-grown cells was significantly inhibited in the presence of protonophores (NaN_3, DNP, and CCCP) and the H^+–adenosine triphosphatase (ATPase) inhibitors diethylstilbestrol and DCCD (Table 1), indicating that the eletrochemical H^+ gradient across the plasma membrane is required for uptake of the sugar. Sugar competition studies indicated that a general monosaccharide transporter probably mediates this high-affinity system, since both an excess of either hexoses (glucose and galactose) or pentoses (L-arabinose) significantly inhibited the rate of xylose uptake (Table 1). Furthermore, control experiments using unlabeled xylose, under the same conditions of excess sugar as those used for glucose inhibition, showed that this sugar competed as well as glucose for the uptake of labeled xylose, indicating that this transport activity may have the same

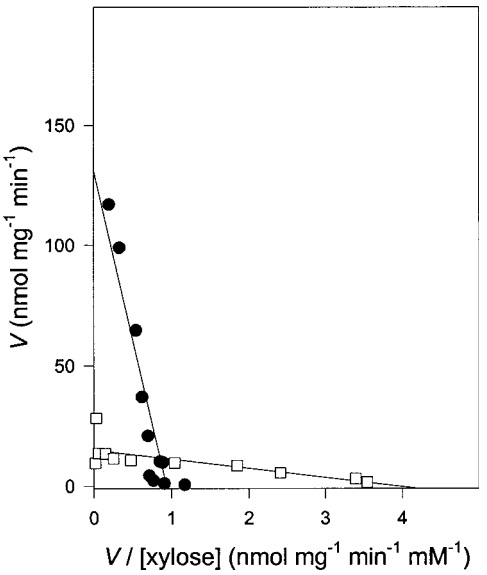

Fig. 2. Eadie-Hofstee plot of xylose transport by *C. succiphila*. The initial rates of labeled xylose uptake (0.4–900 m*M* final concentration) by (□) xylose-grown or (●) glucose-grown cells were determined as described in Materials and Methods.

Table 1
Effect of Inhibitors on Rate of Xylose Transport by *C. succiphila*

Inhibitor	Concentration (m*M*)	Relative xylose transport (%)[a]	
		Xylose-grown cells	Glucose-grown cells
None	—	100[b]	100[c]
NaN_3	10	5	107
DNP	2.5	2	86
CCCP	2.5	2	62
Diethylstilbestrol	5	31	68
DCCD	5	9	107
Glucose	250	2	2
Galactose	500	3	24
Arabinose	600	11	56

[a] Determined with 10 m*M* labeled xylose.
[b] Rate of xylose transport was 9.0 nmol/(mg·min).
[c] Rate of xylose transport was 8.5 nmol/(mg·min).

affinity for both sugars. However, further studies would be required to determine the affinity and/or K_i for each sugar.

Glucose-grown cells also transported xylose with a single and very low-affinity (K_m = 140 m*M*) uptake system, as indicated by the linear kinetics of xylose transport observed with these cells (Fig. 2). This transport activity had an approx 10-fold higher capacity (V_{max} = 130 nmol/[mg·min])

than that observed in xylose-grown cells and is probably mediated by a facilitated diffusion glucose transporter (Table 1). Unlabeled xylose did not affect the uptake of labeled xylose under the same conditions of excess sugar as those used for glucose inhibition, indicating that this transport activity probably has a higher affinity for glucose compared with that towards xylose.

D-*Xylose Transport by* K. marxianus

K. marxianus growing on xylose transported this sugar (Fig. 3) with a single transport system with low affinity (K_m = 103 m*M*) and high capacity (V_{max} = 190 nmol/[mg·min]). This transport activity was not significantly inhibited by protonophores and H^+-ATPase inhibitors (Table 2), indicating that, most likely, xylose transport is mediated by facilitated diffusion. The only sugar that strongly inhibited xylose transport was glucose (Table 2). Unlabeled xylose had almost no effect on the uptake of labeled xylose (<20% inhibition) under the same conditions of excess sugar as those used for glucose competition, indicating that this transport activity probably has a higher affinity for glucose.

The results just described were obtained when the cells were grown under microaerobic conditions. A very different pattern of xylose transport was obtained when the cells were grown under aerobic conditions. In this case, the Eadie-Hofstee plot was nonlinear (Fig. 3), indicating a multicomponent uptake mediated by at least two uptake systems: one transporting xylose with very high affinity (K_m = 0.2 m*M*) and low capacity (V_{max} = 10 nmol/[mg·min]), and another with kinetic properties similar to the transporter found in cells grown under microaerobic conditions (K_m = 110 m*M*; V_{max} = 190 nmol/[mg·min]). Both protonophores and H^+-ATPase inhibitors, as well as hexoses and pentoses, significantly reduced the rates of the high-affinity xylose uptake (measured with 1 m*M* labeled xylose) by aerobically grown cells (Table 2). Indeed, significant inhibition by all compounds tested was still observed even in the presence of a saturating concentration of the substrate (10 m*M*) for this high-affinity transport activity. Thus, our results indicate that growth on xylose under aerobic conditions induced production of a high-affinity active xylose transporter in *K. marxianus* cells.

Discussion

It is well known that efficient conversion of xylose to ethanol by most xylose-fermenting yeasts requires a limited amount of oxygen *(24–26)*. The explanation for this finding appears to lie in the specificity of the cofactor required for XR activity, with XR enzymes utilizing either NADPH or NADH as cofactor permitting more efficient fermentation of xylose under limited oxygen availability *(27)*. However, xylose transport into the cell may also limit fermentation of this sugar, as is the case with *Pichia stipitis* grown under different conditions *(28–30)*. Eadie-Hofstee plots of xylose uptake by several

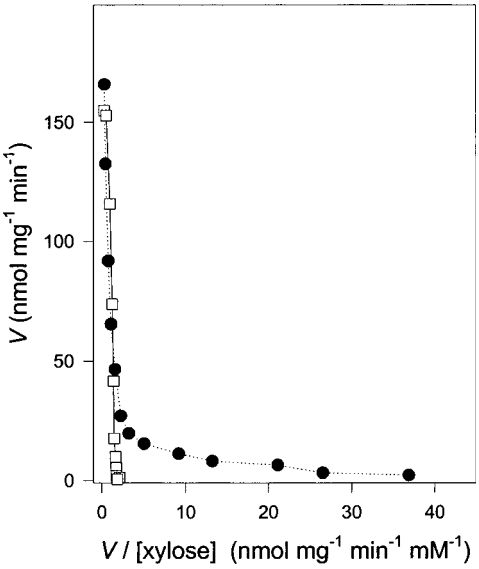

Fig. 3. Eadie-Hofstee plot of xylose transport by *K. marxianus*. The initial rates of labeled xylose uptake (0.05–600 m*M* final concentration) by xylose-grown cells under (□) microaerobic or (●) aerobic conditions were determined as described in Materials and Methods.

Table 2
Effect of Inhibitors on Rate of Xylose Transport
by Xylose-Grown *K. marxianus* Cells

Inhibitor	Concentration (m*M*)	Relative xylose transport (%)	
		Aerobic conditions	Microaerobic conditions
None	—	100[a]	100[b]
NaN_3	5	4	77
DNP	1.25	3	70
CCCP	1.25	2	68
Diethylstilbestrol	2.5	15	80
DCCD	2.5	18	ND[c]
Glucose	250	2	2
Galactose	500	9	53
Arabinose	600	6	89

[a] Rate of xylose transport was 9.9 nmol/(mg·min), determined with 1 m*M* labeled xylose.
[b] Rate of xylose transport was 21.2 nmol/(mg·min), determined with 10 m*M* substrate concentration.
[c] Not determined.

xylose-fermenting yeasts have revealed the presence of at least two kinetically distinct xylose transport systems *(17,21,30–33)*. The low-affinity transport component is usually a facilitated diffusion transport system, while the high-affinity components are proton symporters that use the proton motive

force to actively transport the sugar into the cells. While most of these xylose transport systems are shared with the structural sugar analog glucose, some transporters seem to be specific for xylose *(21,32)*.

Our data show that the rate of growth and xylose consumption by *C. succiphila* and *K. marxianus* under microaerobic conditions can be explained by the kinetics of xylose transport: the low capacity (V_{max}) of xylose uptake by *C. succiphila* determines slow growth and sugar consumption rates, while *K. marxianus* cells harbor a high-capacity transport system which allows rapid growth and sugar depletion from the medium. Neither the affinities (high vs low) nor the energetics of transport (active vs facilitated diffusion) could be correlated to the growth or fermentation performance of these yeasts.

Our results also indicate that *C. succiphila* shares a common characteristic with the other *Candida* species (*C. shehatae* and *C. utilis*) for which xylose transport data are available; that is, the kinetics of xylose transport by xylose-grown cells show the presence of a single high-affinity transporter, while after growth on glucose they exhibit a low-affinity transport activity, which is probably owing to the marginal xylose uptake by glucose permeases *(21,32)*. All other yeast species usually have complex nonlinear kinetics of transport, harboring both high- and low-affinity xylose permeases during growth on this sugar. Since none of the yeast high-affinity xylose transporters have been characterized at the molecular level thus far, these *Candida* species harboring single xylose transporters could be a good source for developing strategies for molecular cloning of suitable xylose transporters.

K. marxianus demonstrated facilitated diffusion transport activity with very low affinity for xylose. This activity is probably mediated by a previously characterized hexose transporter with very low affinity for xylose *(34)*. These results and the data recently published on xylose (and glucose) transport by *P. stipitis (35)*, indicate that oxygen availability plays a role in the regulation of the expression of high-affinity active sugar transporters. In addition, our results indicate that, in yeast, there are a variety of D-xylose transporters with different patterns of expression regulated by both the sugar substrates and growth conditions. Further investigations should unravel the molecular basis for the regulated expression of pentose transporter gene(s) in response to oxygen availability by yeasts.

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