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L-Arabinose transport and catabolism in yeast

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Two yeasts, *Candida arabinofermentans* PYCC 5603^T and *Pichia guilliermondii* PYCC 3012, which show rapid growth on L-arabinose and very high rates of L-arabinose uptake on screening, were selected for characterization of L-arabinose transport and the first steps of intracellular L-arabinose metabolism. The kinetics of L-arabinose uptake revealed at least two transport systems with distinct substrate affinities, specificities, functional mechanisms and regulatory properties. The L-arabinose catabolic pathway proposed for filamentous fungi also seems to operate in the yeasts studied. The kinetic parameters of the initial L-arabinose-metabolizing enzymes were determined. Reductases were found to be mostly NADPH-dependent, whereas NAD was the preferred cofactor of dehydrogenases. The differences found between the two yeasts agree with the higher efficiency of L-arabinose metabolism in *C. arabinofermentans*. This is the first full account of the initial steps of L-arabinose catabolism in yeast including the biochemical characterization of a specific L-arabinose transporter.

Lignocellulose biomass is regarded as a highly promising feedstock for a rapidly expanding alcohol fuel industry in response to a pressing energy problem ([1] and references therein). The industrial fermentative yeast *Saccharomyces cerevisiae* lacks the ability to metabolize five-carbon sugars such as D-xylose and L-arabinose, which are the most abundant hemicellulose-derived pentoses. For lignocellulose ethanol to become an economically competitive feedstock, all sugars in the raw material must be fermented [2], which has caused a surge of interest in microbial pentose metabolism.

Sugar transport across the plasma membrane is the first reaction in pentose metabolism. Very little information exists about L-arabinose transport in natural arabinose-utilizing yeasts. To the best of our knowledge, the only reference to the presence of an L-arabinose/proton symporter is in work on the xylose-fermenting yeast *Candida shehatae* [3]. D-Xylose transport, in contrast, has been characterized in various yeast species, including the nonmetabolizing *S. cerevisiae* [4–6]. In this yeast, L-arabinose is known to be a very poor substrate of the D-galactose transporter Gal2p [7–9]. With respect to the transport of sugar monomers, many yeasts display, in addition to the facilitated diffusion transport system, an active sugar/proton symport which allows sugar accumulation in the cell and is tightly regulated by the sugar concentration in the environment [3,10–13]. In general, compared with the facilitated diffusion mechanism, active transport systems show one to two orders of magnitude higher affinities and 80–90% lower capacities. It is noteworthy that, in xylose-metabolizing yeasts, D-xylose uptake by either system appears mostly associated with D-glucose transport.

The initial L-arabinose metabolism in bacteria is distinct from the pathway usually proposed for filamentous

Abbreviations

AR, L-arabinose reductase; LAD, L-arabitol-4-dehydrogenase; LXR, L-xylulose reductase; PYCC, Portuguese Yeast Culture Collection; XDH, xylitol dehydrogenase; XK, D-xylulose kinase; XR, D-xylose reductase.

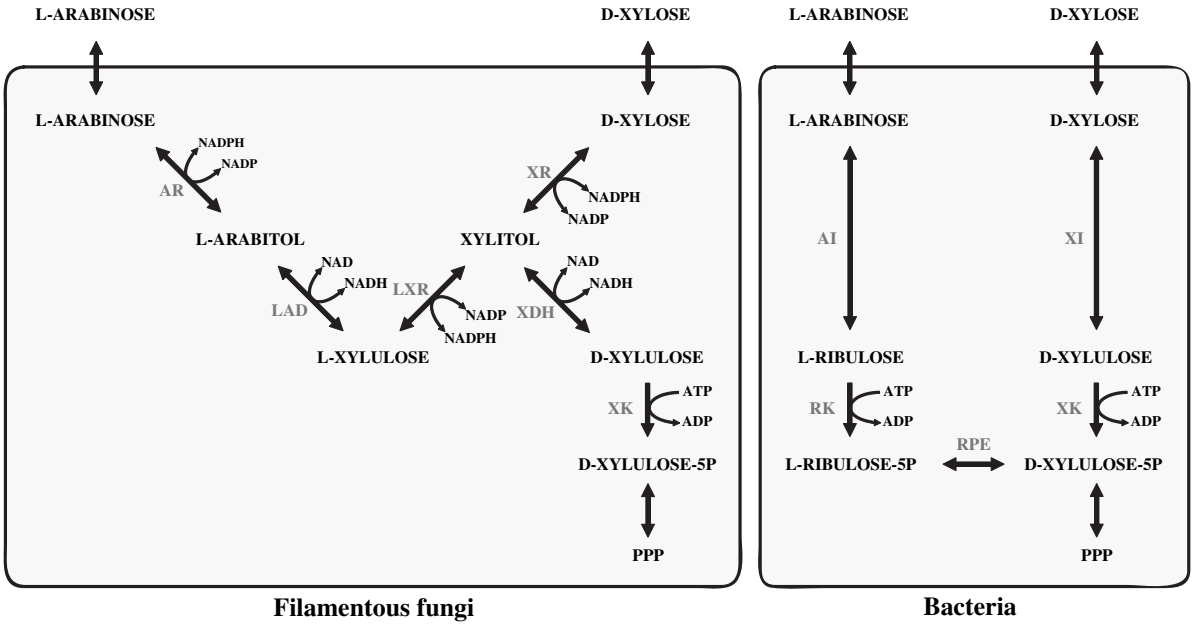

Fig. 1. Initial steps of pentose metabolism in filamentous fungi and bacteria. XI, D-xylose isomerase; XK, D-xylulose kinase; AI, L-arabinose isomerase; RK, L-ribulokinase; RPE, L-ribulose-5-phosphate 4-epimerase; XR, D-xylose reductase; XDH, xylitol dehydrogenase; AR, L-arabinose reductase; LAD, L-arabitol 4-dehydrogenase; LXR, L-xylulose reductase.

fungi (Fig. 1). Bacteria can convert L-arabinose directly into L-ribulose using a specific isomerase [14,15]. In the fungal pathway, L-arabinose has to be first converted into the corresponding polyol, and L-arabitol is subsequently oxidized to L-xylulose. L-arabinose utilization requires additional reduction by an L-xylulose reductase which converts L-xylulose into xylitol, the intermediate compound common to the catabolic pathways of both pentoses. In filamentous fungi, the L-arabinose and D-xylose reductases prefer NADPH as cofactor, whereas the sugar alcohol dehydrogenases are strictly dependent on NAD (Fig. 1). Cellular capacity to regenerate NAD under low oxygen conditions is limited and this may result in the accumulation of arabitol [16]. Alternative pathways for bacterial L-arabinose metabolism, involving an L-arabinose 1-dehydrogenase, are known but appear to be less common [17].

Whereas D-xylose metabolism has been intensively investigated in yeasts (reviewed, e.g. in [18,19]), the utilization of L-arabinose has received far less attention [20,21]. Despite the scarce biochemical data, a strong correlation between L-arabinose and D-xylose utilization in yeasts was observed a long time ago [22], already pointing to a partial overlap between the catabolic pathways of the two pentoses. In the first step of the D-xylose-metabolizing pathway, conducted by a broad-spectrum aldose reductase, a few yeasts (e.g. the xylose-fermenter *Pichia stipitis*) can use both NADPH and NADH as cofactors, although showing a preference for the former. As the second reaction is catalysed by a strictly NAD-dependent xylitol dehydrogenase, the dual cofactor specificity of the aldose reductase might be important to avoid excessive xylitol formation due to the alleviation of cofactor imbalance under oxygen-limited conditions. Recently, and in contrast with what has been described for *Penicillium chrysogenum* and Aspergilli, the yeast *Ambrosiozyma monospora* was found to produce an L-xylulose reductase (Alx1p) that uses NADH as cofactor [21]. The *ALX1*-encoded protein has a striking high similarity to D-arabitol dehydrogenases reported for *P. stipitis*, *Candida albicans* and *Candida tropicalis* but a low similarity to the L-xylulose reductase previously identified in *Hypocrea jecorina* (*Trichoderma reesei*). Although particular enzymatic reactions involved in the fungal L-arabinose pathway (Fig. 1) have been shown to occur in various yeast species, the initial steps of the catabolic sequence have not been systematically investigated. Many yeast species are able to utilize L-arabinose as sole carbon and energy source [1,23,24], mainly aerobically to produce biomass. Under conditions of reduced aeration, several of these yeasts convert L-arabinose into arabitol with high yields and traces of xylitol [25,26], suggesting that the fungal pathway also functions in yeasts, as these polyols are not intermediates of the bacterial pathway (Fig. 1). However, only

four species, including *Candida arabinofermentans* and *A. monospora*, have been reported to produce trace amounts of ethanol in yeast extract medium with a high L-arabinose content [24,27].

Our interest in L-arabinose fermentation in yeast led us to screen a collection of strains and recent isolates from wood-rich environments. Two yeast strains, *C. arabinofermentans* PYCC 5603^T and *P. guilliermondii* PYCC 3012, stood out, as they combined the capacity to ferment glucose with relatively high growth rates in L-arabinose medium and superior rates of L-arabinose uptake. They were both selected for the first comprehensive study on the characteristics of L-arabinose transport and the initial steps of L-arabinose catabolism.

Results

Yeast screening and selection

With a view to elucidating L-arabinose metabolism in yeast, we screened (not shown) strains from the Portuguese Yeast Culture Collection (PYCC) for which the recorded phenotypic data indicated a good ability to grow in mineral medium with L-arabinose as the sole carbon source, as well as recent isolates obtained from enrichment cultures in L-arabinose medium, under low oxygen conditions, of tree exudates and other material collected in a wood-rich environment. In the first round of experiments, semiquantitative results from standard liquid assimilation tests used in yeast identification [23] were obtained. A subset of strains combining rapid growth in mineral medium with L-arabinose and the capacity to ferment D-glucose were tested further. To assess the relative capacity of L-arabinose utilization, the specific growth rate in L-arabinose medium and the initial rate of uptake of 20 mM L-[1-^{14}C]arabinose were estimated. In addition, L-arabinose/proton symport activity was evaluated by determining the rate of proton influx associated with the transport of 5 mM L-arabinose. The strains *C. arabinofermentans* PYCC 5603^T and *P. guilliermondii* PYCC 3012 presented a unique combination of features: the highest growth rates (0.23 h^{-1} and 0.19 h^{-1}, respectively, at 25 °C), the highest rates of uptake of labelled L-arabinose [8 and 40 mmol·h^{-1}·(g dry mass)$^{-1}$, respectively], and an apparent active transport system for L-arabinose. As *C. arabinofermentans* had previously been reported to be one of the very few yeasts with a weak capacity to ferment L-arabinose [24,27] and *P. guilliermondii* is often referenced as a particularly good L-arabitol producer [25,28], we considered it appropriate to investigate both yeasts.

Characterization of L-arabinose transport

Patterns of L-arabinose transport using ^{14}C-labelled L-arabinose were similar in *C. arabinofermentans* PYCC 5603^T and *P. guilliermondii* PYCC 3012 cells grown in 0.5% L-arabinose medium and harvested from mid-exponential cultures. The kinetics of arabinose uptake were of the Michaelis–Menten type but not linear, suggesting the presence of at least two transport components with clearly distinct substrate affinities (Fig. 2). The experimental data were analysed by a nonlinear regression method and the estimated kinetic constants are presented in Table 1. For both yeasts, the K_m of the low-affinity components was about 125 mM, three orders of magnitude higher than the value found for the high-affinity systems (0.12–0.14 mM). A similar, but inverse, ratio was obtained for the estimated V_{max} values, although the apparent capacity of the low-affinity transport system in *P. guilliermondii* is about threefold higher than the capacity of the corresponding transport system in *C. arabinofermentans* (Table 1).

Proton influx signals elicited by the addition of L-arabinose to aqueous cell suspensions of *C. arabinofermentans* PYCC 5603^T and *P. guilliermondii* PYCC 3012 grown in 0.5% (33.3 mM) L-arabinose were observed (Fig. 3). The transient extracellular alkalification indicates that a sugar/proton symport activity is present. The proton influx concomitant with arabinose uptake was abolished in the presence of a 0.1 mM concentration of the protonophore carbonyl cyanide *m*-chlorophenylhydrazone, as would

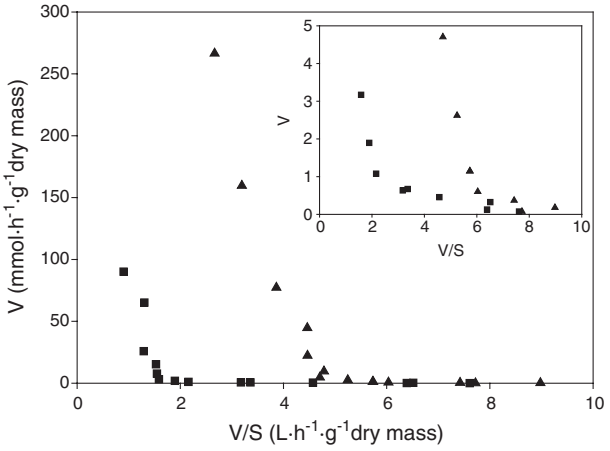

Fig. 2. Kinetics of L-arabinose uptake. Eadie–Hofstee plots of initial rates of uptake of L-[1-^{14}C]arabinose in L-arabinose-grown cells of (■) *C. arabinofermentans* PYCC 5603^T and (▲) *P. guilliermondii* PYCC 3012. Inset: magnification (*y*-axis) of the area corresponding to high-affinity transport.

Table 1. Kinetic parameters of L-arabinose uptake in L-arabinose-grown cells. Cells were grown in 0.5% (w/v) L-arabinose medium, and cell suspensions were assayed at 25 °C, pH 5. Data are mean ± SEM for duplicates from at least two independent experiments.

	L-[1-^{14}C]arabinose uptake				L-arabinose/proton symport activity	
Yeast	K_{m1} (mM)	V_{max1} [mmol·h^{-1}·(g dry mass)$^{-1}$]	K_{m2} (mM)	V_{max2} [mmol·h^{-1}·(g dry mass)$^{-1}$]	K_{m2}' (mM)	V_{max2}' [mmol·h^{-1}·(g dry mass)$^{-1}$]
C. arabinofermentans PYCC 5603^T	125 ± 25	205 ± 35	0.14 ± 0.03	1.1 ± 0.2	0.09 ± 0.02	1.39 ± 0.08
P. guilliermondii PYCC 3012	123 ± 15	574 ± 58	0.12 ± 0.06	1.2 ± 0.5	0.08 ± 0.01	1.17 ± 0.03

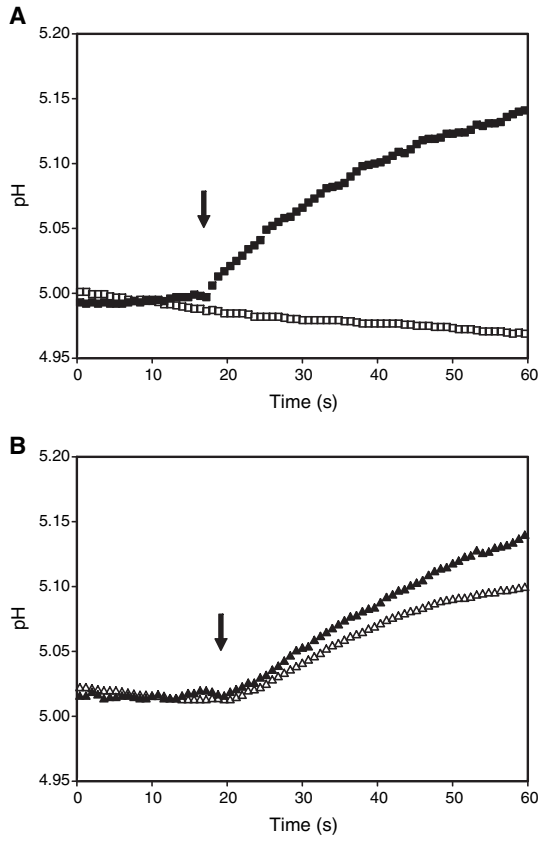

Fig. 3. L-Arabinose/proton symport activity. Proton influx elicited by the addition of L-arabinose to aqueous cell suspensions of *C. arabinofermentans* PYCC 5603^T (A) and *P. guilliermondii* PYCC 3012 (B) grown in 0.5% (■, ▲) and 4% (□, △) L-arabinose medium. The arrow indicates time of sugar addition (5 mM, final concentration).

be expected for the presumed transport mechanism. Eadie–Hofstee plots (not shown) of the initial rates of proton uptake as a function of the L-arabinose concentration in the assay yielded K_m and V_{max} values similar to those estimated for the high-affinity components when using radiolabelled sugar (Table 1). These results suggest that the high-affinity transport components represent L-arabinose/proton symporters and that one proton is cotransported with each L-arabinose molecule. On the other hand, the low-affinity/high-capacity component observed for both yeasts (Fig. 2) seems to represent a facilitated diffusion transport system for L-arabinose.

Changing the L-arabinose concentration in the culture medium to 4% (267 mM) resulted in different behaviours of the strains under study. Whereas the symporter activity was roughly maintained in *P. guilliermondii*, the proton influx associated with L-arabinose uptake could no longer be observed in *C. arabinofermentans* (Fig. 3), suggesting negative regulation of the active transport system by the substrate. In contrast, the low-affinity components were not affected by the increase in L-arabinose concentration in the growth medium (not shown).

Inhibition studies were conducted, as a first approach, taking into consideration the relative affinities of known transporters for pentoses and D-glucose. Neither 50 mM D-xylose nor 20 mM D-glucose inhibited labelled L-arabinose transport via the facilitated diffusion system in *C. arabinofermentans* (Fig. 4). Similar results were obtained with *P. guilliermondii* cells. The range of sugars tested as inhibitors was extended and their concentration in the transport assay increased to 200 mM. Under these conditions, the uptake of 5 mM labelled L-arabinose was determined for both yeasts in the absence and presence of each sugar compound tested (Fig. 5). In *C. arabinofermentans* PYCC 5603^T the control rate, obtained in the absence of inhibitor, was reduced to ≈50% by D-ribose and to 65% by D-fucose. No significant changes were observed with D-xylose, D-glucose, D-galactose, D-arabinose or L-fucose. The pattern for *P. guilliermondii* PYCC 3012 was slightly different as D-galactose clearly inhibited L-arabinose uptake by 50%, an effect similar to that observed with D-ribose, and D-fucose reduced transport of the substrate to 65% of the reference value. None of the other sugars caused significant inhibition. These results reveal facilitated diffusion transporters with a high specificity towards L-arabinose.

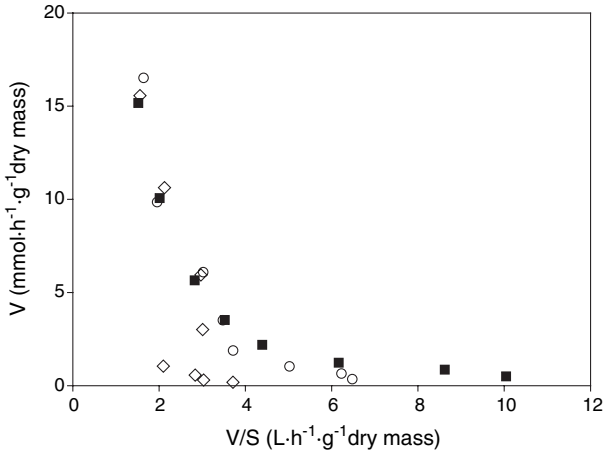

Fig. 4. Effect of D-xylose and glucose on L-arabinose uptake. Eadie–Hofstee plots of initial rates of uptake of L-[1-^{14}C]arabinose in 0.5% L-arabinose-grown cells of *C. arabinofermentans* PYCC 5603^{T}, in the absence (■) and in the presence of 50 mM D-xylose (◇) or 20 mM D-glucose (○).

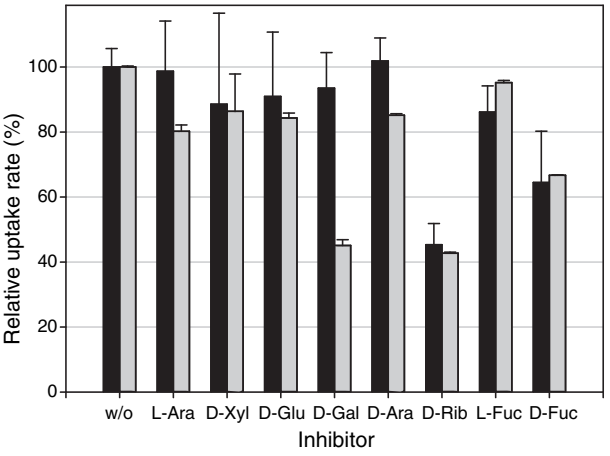

Fig. 5. Inhibiton of L-arabinose uptake. Effect of different sugars (200 mM) on the initial rate of uptake of 5 mM L-[1-^{14}C]arabinose in 0.5% L-arabinose-grown cells of *C. arabinofermentans* PYCC 5603^{T} (black) and *P. guilliermondii* PYCC 3012 (grey). L-Ara, L-arabinose; D-Xyl, D-xylose; D-Glu, D-glucose; D-Gal, D-galactose; D-Ara, D-arabinose; D-Rib, D-ribose; L-Fuc, L-fucose; D-Fuc, D-fucose.

Inhibition kinetics of the L-arabinose/proton symporter did not provide clear results because of the very low initial uptake rates displayed by this transport system. However, it could be observed that both D-xylose (50 mM) and D-glucose (20 mM) reduced the transport rates corresponding to the high-affinity component in 0.5% L-arabinose-grown cells (Fig. 4), suggesting that the hexose and the two pentoses might share a common transport system. It was not possible though to infer anything about the type of inhibition. It can be seen in Fig. 4 that, in *C. arabinofermentans* PYCC 5603^{T}, D-xylose has a stronger inhibitory effect in the putative L-arabinose/proton symporter than D-glucose. In fact, radioactive D-glucose was hardly taken up at all by *C. arabinofermentans* L-arabinose-grown cells. On the basis of the initial rates of proton uptake, a K_m of 0.6 mM for D-xylose was obtained, a value $\approx$ 10 times higher than for L-arabinose (Table 1). In contrast, the L-arabinose symporter in *P. guilliermondii* PYCC 3012, estimated from initial proton influx rates, seems to have similar affinities for the same three sugars (K_m values between 0.05 and 0.1 mM). The L-arabinose active transport system thus seems to be less specific than its passive counterpart.

Trehalose-grown cells of *C. arabinofermentans* PYCC 5603^{T} were also tested to better evaluate regulatory mechanisms governing the expression of both transport systems. In these cells, only the facilitated diffusion component appears to be operating, but its extrapolated maximum velocity was reduced to one half of the values obtained in L-arabinose-grown cells: 91 ± 13 and 205 ± 80 mmol·h^{-1}·(g dry mass)$^{-1}$, respectively. Results in D-xylose-grown cells were similar. The available evidence for this yeast indicates that the L-arabinose facilitator is constitutive, although its activity may vary with the growth substrate, and the L-arabinose symporter is inducible and subject to repression by high L-arabinose concentrations. The data obtained with trehalose-grown cells of *P. guilliermondii* PYCC 3012 were different, as no uptake of labelled L-arabinose was detected. In this yeast, both the facilitator and the symporter appear to be inducible, the latter not being regulated by the L-arabinose concentration in the medium.

When grown in 2% D-glucose, equivalent to carbon catabolite repressed conditions, neither yeast transported L-arabinose, pointing to glucose repression as a regulatory mechanism for all transport systems observed.

L-Arabinose catabolic pathway

The presence in yeast of a functional L-arabinose metabolic pathway analogous to that described for filamentous fungi [29,30] was evaluated. The pathway for converting L-arabinose into D-xylulose 5-phosphate was investigated in *C. arabinofermentans* PYCC 5603^{T} and *P. guilliermondii* PYCC 3012. Extracts of L-arabinose-grown cells were assayed for reductase and dehydrogenase activities, using both NAD(H) and NADP(H) in the assay reaction with each substrate: D-xylose reductase (XR), L-arabinose reductase (AR) and L-xylulose reductase (LXR),

Table 2. Kinetic parameters for enzymes in the initial L-arabinose catabolic pathway. Cells were grown in 2% (w/v) L-arabinose medium, and cell-free extracts assayed at 25 °C. Data are mean ± SEM for duplicates from at least two independent experiments. ND, not determined.

		C. arabinofermentans PYCC 5603^T			*P. guilliermondii* PYCC 3012		
Enzyme	Cofactor	K_m (mM)	V_{max} [U·(mg protein)$^{-1}$]	Catalytic efficiency (V_{max}/K_m)	K_m (mM)	V_{max} [U·(mg protein)$^{-1}$]	Catalytic efficiency (V_{max}/K_m)
AR	NADPH	33 ± 5	2.1 ± 0.1	0.063	164 ± 31	0.71 ± 0.06	0.004
XR	NADPH	68 ± 11	1.9 ± 0.1	0.028	126 ± 20	0.45 ± 0.03	0.004
LAD	NAD^+	57 ± 13	0.93 ± 0.07	0.016	43 ± 20	0.15 ± 0.02	0.003
LXR	NADPH	19 ± 2	5.8 ± 0.3	0.310	89 ± 34	0.6 ± 0.2	0.007
	NADH	115 ± 26	2.9 ± 0.4	0.025	ND	ND	ND
XDH	NAD^+	9 ± 2	1.9 ± 0.1	0.198	23 ± 2	0.49 ± 0.01	0.021
XK	ATP	0.40 ± 0.06	1.1 ± 0.1	2.849	0.13 ± 0.13[a]	0.08 ± 0.02	0.658

[a] Enzyme activities too low to determine with greater accuracy.

L-arabitol 4-dehydrogenase (LAD) and xylitol dehydrogenase (XDH). As a key enzyme in the pentose-metabolizing pathway, D-xylulose kinase (XK) activity was also analysed. The kinetic parameters were determined for all enzymes and the results are presented in Table 2. For both yeasts, reductases showed a higher or even absolute preference for NADPH, whereas dehydrogenases used NAD rather than NADP.

Cofactor specificity was further investigated by comparing the two yeasts used in the present study with *A. monospora*, one of the best L-arabinose-utilizing yeasts [24] and reported to have an L-xylulose reductase with a striking, and uncommon, preference for NADH as cofactor [21]. The data obtained for enzyme activities at specific substrate concentrations are shown in Fig. 6. LXR activity was estimated using a much lower substrate concentration because of the high cost of L-xylulose.

Growth of *C. arabinofermentans* PYCC 5603^T on L-arabinose induced aldopentose reductases (AR and XR) with higher affinity for the substrates and with higher V_{max} values than those of *P. guilliermondii* PYCC 3012. To clarify whether a single broad-substrate aldose reductase could be involved in both reduction reactions, cells of *C. arabinofermentans* PYCC 5603^T were grown on different carbon sources, and the activities determined using 266 mM L-arabinose or D-xylose. AR and XR activities were twofold to threefold higher in L-arabinose-grown cells [1.7 and 1.3 U·(mg protein)$^{-1}$, respectively] compared with D-xylose-grown cells [0.7 and 0.5 U·(mg protein)$^{-1}$, respectively]. Residual activities of ≈0.07 U·(mg protein)$^{-1}$ were found in D-glucose-grown cells, and the titres increased only to values around 0.12 U·(mg protein)$^{-1}$ when the cells were derepressed for 3 h in the same medium without glucose. The same very low activities were found in trehalose-grown cells. These results suggest the existence of a low-specificity aldose reductase, which is more effectively induced by L-arabinose than by D-xylose and showing a relative AR activity 35–50% higher than the corresponding XR activity. Similar observations were made in *P. guilliermondii*, except that, in this yeast, the reductase prefers D-xylose to L-arabinose in contrast with what was

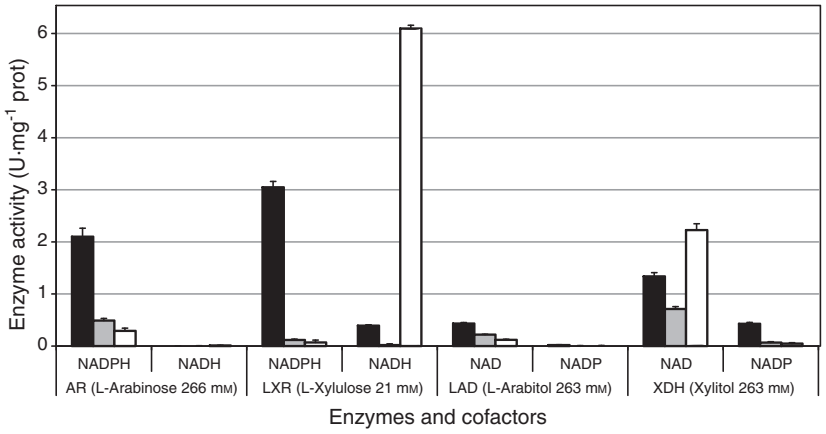

Fig. 6. Cofactor specificity of enzymes in L-arabinose catabolism of *C. arabinofermentans* PYCC 5603^T (black), *P. guilliermondii* PYCC 3012 (grey) and *A. monospora* PYCC 4390^T (light). Enzymes were assayed in cell-free extracts, at 25 °C, using the indicated substrate at the specified concentration. Abbreviations of enzymes as in Fig. 1.

observed in *C. arabinofermentans*. The ketopentose reductase, LXR, showed higher activities than AR and XR in both yeasts (Table 2), possibly resulting from a higher affinity for the substrate (observed in both *C. arabinofermentans* and *P. guilliermondii*) and an increased enzyme capacity (only in the case of *C. arabinofermentans*).

No apparent relationship exists between enzymes responsible for the oxidation of L-arabitol (LAD) or xylitol (XDH). When the yeasts were grown in L-arabinose, both LAD and XDH activities were detected (Table 1), the former displaying similar K_m values in the two yeasts, and XDH showing higher affinity for xylitol in *C. arabinofermentans*. However, the maximum activity of both enzymes was significantly higher in *C. arabinofermentans* PYCC 5603^T (sixfold for LAD and fourfold for XDH).

XK activity was $\approx$14 times higher in *C. arabinofermentans* than in *P. guilliermondii*. The very low activity found in cell extracts of the latter yeast prevented accurate determination of the respective K_m value.

AR and LAD activities were determined during growth of both yeasts in L-arabinose medium. Whereas AR activities were fairly constant throughout the exponential and early-stationary phases, LAD increased with decreasing aeration and concomitant excretion of arabitol into the medium (data not shown), suggesting induction by the enzyme's substrate.

Discussion

The comparison of different steps of L-arabinose catabolism in two yeast strains belonging to distinct species allowed us to gain a more general insight into pentose metabolism in this group of eukaryotic microorganisms and to identify potential constraints along the pathway followed by this sugar. Both *C. arabinofermentans* PYCC 5603^T and *P. guilliermondii* PYCC 3012 have the ability to ferment D-glucose and grow efficiently on L-arabinose under aerobic conditions, showing comparable specific growth rates and biomass yields, but *P. guilliermondii* accumulates substantially more arabitol at low oxygen than *C. arabinofermentans*. Circumstantially, traces of ethanol are produced from D-xylose by both yeasts and from L-arabinose in the case of *C. arabinofermentans* [25].

The co-utilization of D-xylose and L-arabinose in mixtures by *Candida entomaea* and *P. guilliermondii* had already provided good indications of separate transport systems for the two pentoses [28], but no further studies had been undertaken. Our investigations of L-arabinose uptake revealed two mechanistically distinct transport systems operating simultaneously and differing in substrate affinity (half-saturation constants of $\approx$125 mM and 0.1 mM for low-affinity and high-affinity uptake, respectively). Low-affinity L-arabinose transport was clearly induced by L-arabinose in *P. guilliermondii* but only partially in *C. arabinofermentans*, repressed by D-glucose, not significantly inhibited by either D-glucose or D-xylose, the predominant sugars in hemicellulose hydrolysates, and it apparently corresponds to facilitated diffusion. D-ribose, and to a lesser extent D-fucose (6-deoxy-D-galactose), was a weak inhibitor of this uptake system in both yeasts, suggesting that the C2–C4 configuration is important for transport activity. However, D-galactose, which has the same stereoconfiguration, only affected low-affinity transport in *P. guilliermondii* (see Fig. 5). A concurrent influx of L-arabinose and protons indicated that the high-affinity system corresponds to an L-arabinose/proton symporter, which is repressible by D-glucose and negatively regulated (in *C. arabinofermentans* but not in *P. guilliermondii*) by an increased substrate concentration in the growth medium. The symporter showed weak activity and was significantly inhibited by both D-xylose and D-glucose in *P. guilliermondii* and particularly by D-xylose in *C. arabinofermentans*, demonstrating its lower specificity in comparison with the L-arabinose facilitator. In *C. arabinofermentans*, the symport system had an apparently higher affinity for L-arabinose followed by D-xylose, whereas in *P. guilliermondii* the affinities were very similar for L-arabinose, D-xylose and D-glucose. Yeast D-xylose/proton symporters described so far usually show a higher affinity for D-glucose than for D-xylose [10,12]. The *C. arabinofermentans* L-arabinose/proton symporter displays a preference for pentoses rather than D-glucose. The K_m values and velocities of L-arabinose uptake by the low-affinity transport system were exceptionally high compared with similar transport systems described in yeast, namely the Hxt transporters in *S. cerevisiae* for which the best reported V_{max} values for D-glucose transport are less than 10% those obtained for L-arabinose in *C. arabinofermentans* and *P. guilliermondii*. This characteristic associated with the absence of the transport system observed in D-glucose-grown cells and its relatively high specificity may become very useful for isolating the encoding gene(s) and improving fermentation of xylose/arabinose mixtures in recombinant *S. cerevisiae*. It is noteworthy that *P. guilliermondii* displays a three times higher L-arabinose transport activity than *C. arabinofermentans* [574 $\pm$ 58 mmol·h^{-1}·(g dry mass)$^{-1}$ versus 205 $\pm$ 35 mmol·h^{-1}·(g dry mass)$^{-1}$], and the two yeasts grow at comparable rates in

L-arabinose medium, which suggests that L-arabinose uptake does not limit the metabolic flux. Overall, L-arabinose transport seems to be more strictly regulated in *C. arabinofermentans* than in *P. guilliermondii*.

The reported data on accumulation of arabitol and traces of xylitol [25] and the results presented here on key enzymes involved in L-arabinose metabolism are consistent with the presence in the yeasts examined of the predominant catabolic pathway described for filamentous fungi [30,31]. This means that D-xylose and L-arabinose metabolism are intrinsically related. All enzymes required to convert D-xylose into D-xylulose, which is then phosphorylated to D-xylulose 5-phosphate which enters the phosphate pentose pathway, are present in the L-arabinose-metabolizing strains. A supposedly single unspecific aldose reductase, as proposed for *C. albicans* [32], *Candida (Pichia) guilliermondii* [33], and *P. stipitis* [34], can either convert L-arabinose into L-arabitol or D-xylose into xylitol. The catabolic sequence for L-arabinose degradation involves two additional redox reactions as compared with D-xylose metabolism (Fig. 1). L-arabitol is oxidized to L-xylulose by an L-arabitol 4-dehydrogenase, and L-xylulose is converted into xylitol, the first metabolite common to the catabolic pathway of both pentoses, by a L-xylulose reductase. The cofactor dependence varied with the enzyme and with the yeast. The ARs from both yeasts and LXR from *P. guilliermondii* exclusively used NADPH, whereas the LXR produced by *C. arabinofermentans* used both NADPH and NADH, although preferring the former. In contrast, dehydrogenases were almost strictly NAD-dependent. The exception was again *C. arabinofermentans* which apparently showed dual cofactor specificity for XDH, with a preference for NAD. However, it could not be excluded that the activity obtained was the result of a retroconversion by LXR of xylitol into L-xylulose. We therefore determined D-xylulose reductase activity using NADPH as cofactor. The value obtained was relatively low, although it clearly indicates that NADP can also be used as a cofactor in the XDH reaction.

A. monospora followed the pattern observed for *P. guilliermondii*, except for LXR cofactor dependence. Our results confirm that this enzyme is NADH-dependent (Fig. 6). As to the higher activity of LXR in *A. monospora*, it is possible that it contributed to an apparently high XDH activity as xylitol can also be oxidized to L-xylulose using NAD^+ as cosubstrate. It is noteworthy that the relative LAD activities in the three yeasts (Fig. 6) correlate with their maximum specific growth rates under aerobic conditions at 25 °C (0.23 h^{-1}, 0.19 h^{-1} and 0.16 h^{-1} for *C. arabinofermentans*, *P. guilliermondii* and *A. monospora*, respectively). The cofactor imbalance resulting from AR/XR/LXR and LAD/XDH requirements leads to arabitol and xylitol secretion under oxygen limitation. It is likely that the extent to which both metabolites accumulate depends on the specific enzyme activities in the pathway.

The relative enzyme activities and kinetic parameters determined in cell extracts of *C. arabinofermentans* PYCC 5603^T and *P. guilliermondii* PYCC 3012 fall within the range of values found for other yeasts [33,35–41] and provide support for the behaviour of the yeast strains studied in mineral medium with D-xylose or L-arabinose as carbon source [25]. The first intracellular enzyme (AR) showed higher activities in *C. arabinofermentans*, where it prefers L-arabinose to D-xylose ($K_m = 33 \pm 5$ and 68 ± 11 mM, respectively), than in *P. guilliermondii,* where the pentoses are equivalent substrates. Moreover, the conversion of L-arabitol into L-xylulose by LAD proceeds at approximately six times higher rates in *C. arabinofermentans* than in *P. guilliermondii*. These are key steps for more effectively regulated L-arabinose utilization by *C. arabinofermentans* and may account for the higher accumulation of arabitol observed in *P. guilliermondii*. The same explanation holds for xylitol accumulation, although to a different degree, in L-arabinose medium and low oxygen [25]. Only traces of xylitol were detected in *P. guilliermondii* PYCC 3012 but not in *C. arabinofermentans* PYCC 5603^T. The catalytic efficiency (V_{max}/K_m) of all enzymes tested in the catabolic sequence was significantly higher in *C. arabinofermentans*, in agreement with its more effective L-arabinose pathway. The 10-fold higher activity of XK in *C. arabinofermentans* may be particularly relevant. This yeast seems to represent a natural case of a combined 'pushing/pulling' strategy to increase the metabolic flux of the pentose, potentially leading to ethanol formation [42]. The analogies with D-xylose fermentation by *P. stipitis* are striking, although the specificity of cofactors is even more problematic in L-arabinose catabolism as the number of redox reactions linked to distinct cofactors in the initial steps of the pathway doubles (Fig. 1) and restrains clearly visible ethanol production.

The fermentation of D-xylose provides a good illustration of what can be achieved in terms of ethanol production in fungi unable to produce a xylose isomerase that converts D-xylose directly into D-xylulose. The introduction of the bacterial xylose isomerase pathway reduced xylitol formation in recombinant yeast [43,44], and, more recently, the successful expression of a fungal xylose isomerase XylA in *S. cerevisiae* circumvented the cofactor imbalance derived from

usage of different cofactors by the reductase and the dehydrogenase and led to improvement in the ethanol yield [45,46]. Accordingly, it seems that the best strategy for efficient L-arabinose conversion into ethanol is to engineer *S. cerevisiae* using the bacterial L-arabinose pathway. This strategy has already been tested with promising results by expressing in *S. cerevisiae* the enzymes AraA (L-arabinose isomerase) from *Bacillus subtilis*, AraB (L-ribulokinase) and AraD (L-ribulose-5P 4-epimerase) from *Escherichia coli*, and simultaneously overexpressing the homologous galactose permease encoded by *GAL2* [47]. The increased expression of Gal2p, which also accepts L-arabinose as a (weak) substrate, improved L-arabinose metabolism in the newly engineered *S. cerevisiae*, highlighting the crucial role of transport in the recombinant strain. Recent metabolic control analysis conducted in *Aspergillus niger* suggests that the flux control is strongly dependent on the intracellular L-arabinose concentration [48]. Our finding of a highly active and specific L-arabinose transporter is of interest for improving L-arabinose fermentation in yeast.

Experimental procedures

Strains and maintenance

C. arabinofermentans PYCC 5603^T (NRRL YB-2248; originally provided by the ARS Culture Collection, Peoria, IL, USA), *P. guilliermondii* PYCC 3012 and *A. monospora* PYCC 4390^T were obtained from PYCC, Faculty of Sciences and Technology, New University of Lisbon, Caparica, Portugal.

Both strains were maintained on YP medium (yeast extract 1%, peptone 2% and agar 2%) supplemented with either D-glucose or L-arabinose 2%, at 4 °C.

Assays of sugar transport

Strains were grown aerobically in shaking flasks with medium containing 0.67% yeast nitrogen base (Difco, Detroit, MI, USA) and 0.5% sugar (L-arabinose, D-xylose, D-glucose, D-galactose or α,α-trehalose), except when a different concentration is indicated, at 25 °C and 150 r.p.m. on a rotary shaker (Gallenkamp, Leicester, UK).

Cells from exponentially growing cultures (D_{640} 0.6–1.0) were harvested by centrifugation at 8000 ***g*** for 5 min at 4 °C and washed twice with ice-cold demineralized water. Cells were resuspended in water to a final concentration of about 25 mg dry mass·mL^{-1}. The cell dry mass was determined at least in duplicate for each sample by placing 100 μL cell suspension in preweighed aluminium foil and dried for 24 h at 80 °C.

Initial uptake rates were determined using a previously described method [49] and ^{14}C-labelled sugars: L-[1-^{14}C]arabinose (American Radiolabeled Chemicals Inc., St Louis, MO, USA), D-[U-^{14}C]xylose and D-[U-^{14}C]glucose (Amersham, Little Chalfont, UK). In 5-mL Röhren tubes, 20 μL 100 mM Tris/citrate buffer (pH 5.0) and 20 μL cell suspension were mixed and incubated for ≈5 min at 25 °C. The reaction was started with the addition of 10 μL radiolabelled sugar (specific radioactivity 10^2–10^4 cpm/nmol), at various concentrations, and stopped after 5 s by vigorous dilution with 3.5 mL ice-cold demineralized water. The resulting suspension was immediately filtered through Whatman GF/C membranes (2.5 cm diameter) and the filter washed twice with 10 mL ice-cold demineralized water. The filter was then transferred to a scintillation vial containing 6 mL liquid-scintillation cocktail Wallac OptiPhase 'HiSafe' 2 (Walla, Turku, Finland). Radioactivity was measured in a Tri-Carb^{TM} liquid-scintillation analyzer 1600 CA (Packard, Downers Grove, IL, USA). The control time point (0 s) was performed in a similar manner but the cell suspension was diluted with ice-cold water before addition of the labelled sugar. Kinetic parameters were estimated from Eadie–Hofstee plots or by nonlinear Michaelis–Menten regression analysis using the GRAPHPAD PRISM 3.0 software.

For inhibition studies, a solution of the sugar to be tested as inhibitor was prepared in 100 mM Tris/citrate buffer (pH 5.0). Then 20 μL was mixed in the assay tube with 10 μL labelled sugar, and the reaction was initiated by adding 20 μL cell suspension.

Proton symport activity was estimated by determining initial rates of proton uptake elicited by the addition of sugar to the yeast cell suspension [49] using a combined Crison pH electrode (Crison, Alella, Spain) and a pHM240 pH/Ion meter (Radiometer-Copenhagen, Lyon, France). A connection to a computer allowed pH measurements to be registered every 0.4 s. The pH electrode was immersed in a water-jacketed chamber of 2 mL capacity kept at 25 °C and provided with magnetic stirring. To the chamber were added 1.32 mL demineralized water and 150 μL cell suspension. The pH was adjusted to 5.0, using 1 M HCl or NaOH, until a suitable baseline was obtained. The sugar solution (30 μL) in various concentrations was added to the aqueous suspension, and the subsequent alkalification followed. The slope from the initial part of the pH trace, which lasted ≈5–10 s, was used to calculate the initial rate of proton uptake for each sugar concentration tested. Calibration was performed with HCl. Assays were run at least in duplicate for two independent cultures of the same yeast.

Enzymatic assays

Strains were grown in 500-mL shaking flasks containing 100 mL mineral medium [50] supplemented with 0.1% yeast

extract and 2% sugar (D-glucose, D-xylose or L-arabinose) at 25 °C and 180 r.p.m. on an orbital shaker. Cells from a late-exponential phase culture (D_{640} 5–7) were harvested by centrifugation at 8000 ***g*** for 5 min at 4 °C in a Sorvall SLA-1500 rotor and washed twice with ice-cold demineralized water.

Pelleted cells, ≈0.6 g wet mass, were collected in a 2-mL Eppendorf tube, and 1.5 mL Y-PER® Yeast Protein Extraction Reagent (Pierce, Rockford, IL, USA) was added [51]. The mixture was incubated for 1 h at room temperature on a Rocking Platform (Biometra, Göttingen, Germany). Crude cell-free extract was obtained by recovering the supernatant after spinning down cell debris. This preparation was used to estimate enzyme activities.

Enzyme activities were determined at 25 °C using an Ultrospec 3100 pro UV/visible spectrophometer (Biochrom Ltd., Cambridge, UK). Enzymatic assays were performed as previously described [52] with the following reaction mixtures (the indicated concentrations represent final concentrations): reductase activities, AR (EC 1.1.1.21) and LXR (EC 1.1.1.10), were determined with triethanolamine buffer (100 mM, pH 7.0), NADPH or NADH (0.2 mM) and L-arabinose, D-xylose or L-xylulose solution to the desired concentration as starting reagent; dehydrogenase activities, LAD (EC 1.1.1.12) and XDH (EC 1.1.1.9), were determined with glycine buffer (100 mM, pH 9.0), $MgCl_2$ (50 mM), NAD or NADP (3.0 mM) and L-arabitol or xylitol solution to the desired concentration as starting reagent; XK (EC 2.7.1.17) was determined with Tris/HCl (50 mM, pH 7.5), $MgCl_2$ (2.0 mM), NADH (0.2 mM), phosphoenolpyruvate (0.2 mM), pyruvate kinase (10 U), lactate dehydrogenase (10 U) and D-xylulose to the desired concentration. ATP (2.0 mM) was added as starting reagent. The reaction occurring before the addition of ATP (D-xylulose reductase activity) was subtracted from the conversion observed in the presence of ATP.

The production or consumption of NAD(P)H was followed at 340 nm. A value of 5.33 $mM^{-1} \cdot cm^{-1}$ was used for the absorption coefficient of NAD(P)H. One unit produced 1 μmol NAD(P)H per min.

The protein concentration was determined using the Bicinchoninic Acid (BCA) Protein Quantitation Assay (Pierce) with BSA as standard.

The specific enzyme activities are given in units (U) per mg protein.

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References

1 Hahn-Hägerdal B, Karhumaa K, Fonseca C, Spencer-Martins I & Gorwa-Grauslund MF (2007) Towards industrial pentose-fermenting yeast strains. *Appl Microbiol Biotechnol* **74**, 937–953.
2 Galbe M & Zacchi G (2002) A review of the production of ethanol from softwood. *Appl Microbiol Biotechnol* **59**, 618–628.
3 Lucas C & van Uden N (1986) Transport of hemicellulose monomers in the xylose-fermenting yeast *Candida shehatae*. *Appl Microbiol Biotechnol* **23**, 491–495.
4 Hamacher T, Becker J, Gárdonyi M, Hahn-Hägerdal B & Boles E (2002) Characterization of the xylose-transporting properties of yeast hexose transporters and their influence on xylose utilization. *Microbiology* **148**, 2783–2788.
5 Lee WJ, Kim MD, Ryu YW, Bisson LF & Seo JH (2002) Kinetic studies on glucose and xylose transport in *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* **60**, 186–191.
6 Sedlak M & Ho NW (2004) Characterization of the effectiveness of hexose transporters for transporting xylose during glucose and xylose co-fermentation by a recombinant *Saccharomyces* yeast. *Yeast* **21**, 671–684.
7 Kou SC, Christensen MS & Cirillo VP (1970) Galactose transport in *Saccharomyces cerevisiae*. II. Characteristics of galactose uptake and exchange in galactokinaseless cells. *J Bacteriol* **103**, 671–678.
8 Ramos J, Szkutnicka K & Cirillo VP (1989) Characteristics of galactose transport in *Saccharomyces cerevisiae* cells and reconstituted lipid vesicles. *J Bacteriol* **171**, 3539–3544.
9 Tschopp JF, Emr SD, Field C & Schekman R (1986) *GAL2* codes for a membrane-bound subunit of the galactose permease in *Saccharomyces cerevisiae*. *J Bacteriol* **166**, 313–318.
10 Gárdonyi M, Österberg M, Rodrigues C, Spencer-Martins I & Hahn-Hägerdal B (2003) High capacity xylose transport in *Candida intermedia* PYCC 4715. *FEMS Yeast Res* **3**, 45–52.
11 Nobre A, Lucas C & Leão C (1999) Transport and utilization of hexoses and pentoses in the halotolerant yeast *Debaryomyces hansenii*. *Appl Environ Microbiol* **65**, 3594–3598.
12 Spencer-Martins I (1994) Transport of sugars in yeasts: implications in the fermentation of lignocellulosic materials. *Bioresource Technol* **50**, 51–57.
13 Stambuk BU, Franden MA, Singh A & Zhang M (2003) D-xylose transport by *Candida succiphila* and *Kluyveromyces marxianus*. *Appl Biochem Biotechnol* **105**, 255–263.
14 Englesberg E (1961) Enzymatic characterization of 17, L-arabinose negative mutants of *Escherichia coli*. *J Bacteriol* **81**, 996–1006.

15 Englesberg E, Anderson RL, Weinberg R, Lee N, Hoffee P, Huttenhauer G & Boyer H (1962) L-arabinose-sensitive, L-ribulose 5-phosphate 4-epimerase-deficient mutants of *Escherichia coli*. *J Bacteriol* **84**, 137–146.
16 Bruinenberg PM, Debot PHM, van Dijken JP & Scheffers WA (1983) The role of redox balances in the anaerobic fermentation of xylose by yeasts. *Eur J Appl Microbiol Biotechnol* **18**, 287–292.
17 Watanabe S, Kodaki T & Makino K (2006) Cloning, expression, and characterization of bacterial L-arabinose 1-dehydrogenase involved in an alternative pathway of L-arabinose metabolism. *J Biol Chem* **281**, 2612–2623.
18 Hahn-Hägerdal B, Wahlbom CF, Gárdonyi M, van Zyl WH, Cordero Otero RR & Jonsson LJ (2001) Metabolic engineering of *Saccharomyces cerevisiae* for xylose utilization. *Adv Biochem Eng Biotechnol* **73**, 53–84.
19 Jeffries TW (2006) Engineering yeasts for xylose metabolism. *Curr Opin Biotechnol* **17**, 320–326.
20 Richard P, Verho R, Putkonen M, Londesborough J & Penttilä M (2003) Production of ethanol from L-arabinose by *Saccharomyces cerevisiae* containing a fungal L-arabinose pathway. *FEMS Yeast Res* **3**, 185–189.
21 Verho R, Putkonen M, Londesborough J, Penttilä M & Richard P (2004) A novel NADH-linked L-xylulose reductase in the L-arabinose catabolic pathway of yeast. *J Biol Chem* **279**, 14746–14751.
22 Barnett JA (1976) The utilization of sugars by yeasts. *Adv Carbohyd Chem Biochem* **32**, 125–234.
23 Barnett JA, Payne RW & Yarrow D (2000) *Yeasts: Characteristics and Identification*, 3rd edn. Cambridge University Press, Cambridge.
24 Dien BS, Kurtzman CP, Saha BC & Bothast RJ (1996) Screening for L-arabinose fermenting yeasts. *Appl Biochem Biotechnol* **57–58**, 233–242.
25 Fonseca C, Spencer-Martins I & Hahn-Hägerdal B (2007) L-arabinose metabolism in *Candida arabinofermentans* PYCC 5603^T and *Pichia guilliermondii* PYCC 3012: influence of sugar and oxygen on product formation. *Appl Microbiol Biotechnol* (2007) Epub ahead of print.
26 McMillan JD & Boynton BL (1994) Arabinose utilization by xylose-fermenting yeasts and fungi. *Appl Biochem Biotechnol* **45–46**, 569–584.
27 Kurtzman CP & Dien BS (1998) *Candida arabinofermentans*, a new L-arabinose fermenting yeast. *Antonie Van Leeuwenhoek* **74**, 237–243.
28 Saha BC & Bothast RJ (1996) Production of L-arabitol from L-arabinose by *Candida entomaea* and *Pichia guilliermondii*. *Appl Microbiol Biotechnol* **45**, 299–306.
29 Chiang C & Knight SG (1961) L-arabinose metabolism by cell-free extracts of *Penicillium chrysogenum*. *Biochim Biophys Acta* **46**, 271–278.
30 Witteveen CFB, Busink R, van de Vondervoort P, Dijkema C, Swart K & Visser J (1989) L-arabinose and D-xylose catabolism in *Aspergillus niger*. *J Gen Microbiol* **135**, 2163–2171.
31 Chiang C & Knight SG (1960) A new pathway of pentose metabolism. *Biochim Biophys Res Commun* **3**, 554–559.
32 Veiga LA, Bacila M & Horecker BL (1960) Pentose metabolism in *Candida albicans*. I. The reduction of D-xylose and L-arabinose. *Biochem Biophys Res Commun* **2**, 440–444.
33 Sugai JK & Delgenes JP (1995) Induction of aldose reductase activity in *Candida guilliermondii* by pentose sugars. *J Ind Microbiol* **14**, 46–51.
34 Verduyn C, van Kleef R, Frank J, Schreuder H, van Dijken JP & Scheffers WA (1985) Properties of the NAD(P)H-dependent xylose reductase from the xylose-fermenting yeast *Pichia stipitis*. *Biochem J* **226**, 669–677.
35 Bruinenberg PM, Debot PHM, van Dijken JP & Scheffers WA (1984) NADH-linked aldose reductase: the key to anaerobic alcoholic fermentation of xylose by yeasts. *Appl Microbiol Biotechnol* **19**, 256–260.
36 du Preez JC, van Driessel B & Prior BA (1989) Effect of aerobiosis on fermentation and key enzyme levels during growth of *Pichia stipitis*, *Candida shehatae* and *Candida tenuis* on D-xylose. *Arch Microbiol* **152**, 143–147.
37 Hahn-Hägerdal B, Jeppsson H, Skoog K & Prior BA (1994) Biochemistry and physiology of xylose fermentation by yeasts. *Enzyme Microb Technol* **16**, 933–943.
38 Machová E (1992) Induction of aldose reductase and polyol dehydrogenase activities in *Aureobasidium pullulans* by D-xylose, L-arabinose and D-galactose. *Appl Microbiol Biotechnol* **37**, 374–377.
39 Neuhauser W, Haltrich D, Kulbe KD & Nidetzky B (1997) NAD(P)H-dependent aldose reductase from the xylose-assimilating yeast *Candida tenuis*. Isolation, characterization and biochemical properties of the enzyme. *Biochem J* **326**, 683–692.
40 Winkelhausen E & Kuzmanova S (1998) Microbial conversion of D-xylose to xylitol. *J Ferment Bioeng* **86**, 1–14.
41 Yablochkova EN, Bolotnikova OI, Mikhailova NP, Nemova NN & Ginak AI (2003) The activity of xylose reductase and xylitol dehydrogenase in yeasts. *Microbiology* **72**, 414–417.
42 Cornish-Bowden A, Hofmeyr JHS & Cardenas ML (1995) Strategies for manipulating metabolic fluxes in biotechnology. *Bioorg Chem* **23**, 439–449.
43 Träff KL, Otero Cordero RR, van Zyl WH & Hahn-Hägerdal B (2001) Deletion of the *GRE3* aldose reductase gene and its influence on xylose metabolism in recombinant strains of *Saccharomyces cerevisiae* expressing the *xylA* and *XKS1* genes. *Appl Environ Microbiol* **67**, 5668–5674.

44 Walfridsson M, Bao X, Anderlund M, Lilius G, Bulow L & Hahn-Hägerdal B (1996) Ethanolic fermentation of xylose with *Saccharomyces cerevisiae* harboring the *Thermus thermophilus xylA* gene, which expresses an active xylose (glucose) isomerase. *Appl Environ Microbiol* **62**, 4648–4651.

45 Kuyper M, Harhangi HR, Stave AK, Winkler AA, Jetten MS, de Laat WT, den Ridder JJ, Op den Camp HJ, van Dijken JP & Pronk JT (2003) High-level functional expression of a fungal xylose isomerase: the key to efficient ethanolic fermentation of xylose by *Saccharomyces cerevisiae*? *FEMS Yeast Res* **4**, 69–78.

46 Kuyper M, Toirkens MJ, Diderich JA, Winkler AA, van Dijken JP & Pronk JT (2005) Evolutionary engineering of mixed-sugar utilization by a xylose-fermenting *Saccharomyces cerevisiae* strain. *FEMS Yeast Res* **5**, 925–934.

47 Becker J & Boles E (2003) A modified *Saccharomyces cerevisiae* strain that consumes L-arabinose and produces ethanol. *Appl Environ Microbiol* **69**, 4144–4150.

48 de Groot MJ, Prathumpai W, Visser J & Ruijter GJ (2005) Metabolic control analysis of *Aspergillus niger* L-arabinose catabolism. *Biotechnol Prog* **21**, 1610–1616.

49 Spencer-Martins I & van Uden N (1985) Catabolite interconversion of glucose transport systems in the yeast *Candida wickerhamii*. *Biochim Biophys Acta* **812**, 168–172.

50 Verduyn C, Postma E, Scheffers WA & van Dijken JP (1992) Effect of benzoic acid on metabolic fluxes in yeasts: a continuous-culture study on the regulation of respiration and alcoholic fermentation. *Yeast* **8**, 501–517.

51 Katz M, Hahn-Hägerdal B & Gorwa-Grauslund MF (2003) Screening of two complementary collections of *Saccharomyces cerevisiae* to identify enzymes involved in stereo-selective reductions of specific carbonyl compounds: an alternative to protein purification. *Enzyme Microb Technol* **33**, 163–172.

52 Eliasson A, Christensson C, Wahlbom CF & Hahn-Hägerdal B (2000) Anaerobic xylose fermentation by recombinant *Saccharomyces cerevisiae* carrying *XYL1*, *XYL2*, and *XKS1* in mineral medium chemostat cultures. *Appl Environ Microbiol* **66**, 3381–3386.