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# Metabolic engineering for improved fermentation of L-arabinose

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Running title: L-arabinose fermentation

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**Abstract**

L-Arabinose, a five carbon sugar, has not been considered as important bioresource because most studies have focused on D-xylose; it is another type of a five carbon sugar that is prevalent as a monomeric structure of hemicellulose. In fact, L-arabinose is also an important monomer of hemicellulose, but its content is much more significant in pectin (3-22%, g/g pectin), which is considered as alternative biomass due to its low lignin content and mass production as juice-processing waste. This review presents native and engineered microorganisms that can ferment L-arabinose. *Saccharomyces cerevisiae* is highlighted as the most preferred engineering host for expressing a heterologous arabinose pathway for producing ethanol. Because metabolic engineering efforts has been limited so far, with this review as a momentum, more attention to research on the fermentation of L-arabinose as well as the utilization of pectin-rich biomass.

**Keywords**: L-arabinose, pectin, metabolic engineering, *Saccharomyces cerevisiae*, pentose

## Significance of L-arabinose as a bioresource

Bioconversion of lignocellulosic biomass such as agricultural residues and wood waste materials into fuels and value-added chemicals is technically challenging due to a variety of factors [1-4]. Lignocellulosic biomass is composed of 40-50 % cellulose, 25-30 % hemicellulose, and 15-20 % lignin, and the high lignin content requires strong physical and chemical pretreatment for its decomposition [5]. Also, hemicellulose is hydrolyzed into a mixture of glucose, xylose, and other minor sugars such as L-arabinose, and their fermentation is not efficiently done by any native industrial microorganisms [6]. For these reasons, industrial bioprocesses utilizing lignocellulosic biomass have not been realized so far, and the search for other alternative renewable biomass is continued [7-10].

Meanwhile, fruit processing wastes such as orange peels are becoming abundant with the growth of the fresh juice industry, but are not being efficiently utilized [11, 12]. Fruit processing wastes are high in pectin (12-35%, g/g dry weight) with much less lignin content (approximately 2%, g/g dry weight) than lignocellulosic biomass [13]. Therefore, this pectin-rich biomass can be easily broken down into monomers; however, pectin monomers, like hemicellulose monomers, are not easily metabolized by common industrial hosts [14].

The primary chemical structure of pectin is methylated polygalacturonic acid in an alpha-(1-4) chain with branched oligosaccharides consisting of arabinose, galactose, xylose, and some minor sugars [15]. Among them, L-arabinose is one of the most abundant pentose sugars in pectin [16]. Arabinose content in various fruits and vegetables ranges from 3.3 to 21. 6 g/L (summarized in Table 1) [14, 17, 18]. It is contradictory to lignocellulosic biomass which has the limited arabinose content (approximately 0.2%, g/g dry weight) [19].

L-Arabinose is a five-carbon sugar like xylose. Unlike other sugars that naturally occur in the D-form, such as D-xylose, L-arabinose is a component of pectin and hemicellulose, and it is

more common than D-arabinose in nature. Although studies have been conducted extensively for xylose metabolism to realize lignocellulosic bioprocesses [20], L-arabinose metabolism has not received much attention. In the present review, microbial strains that can natively metabolize L-arabinose are summarized. In some studies, the strains were engineered to produce useful products such as ethanol. Moreover, metabolic engineering efforts to develop efficient L-arabinose-fermenting strains using non-native but industrial hosts are discussed, focusing on *Saccharomyces cerevisiae*.

**Native L-arabinose-fermenting microorganisms**

Arabinose catabolic pathways of native strains can be divided into the oxidoreductase (fungal) and isomerase (bacterial) pathways (Fig. 1). In both pathways, L-arabinose is converted into D-xylulose-5-phosphate, which is then canonically metabolized by the non-oxidative pentose phosphate pathway [21], or alternatively by the phosphoketolase pathway such as that in *Clostridium acetobutylicum* [22].

In L-arabinose-fermenting native fungi, L-arabinose is converted into D-xylulose by two reduction and two oxidation reactions, which are composed of NAD(P)H-specific aldose reductase (AR or XR), $NAD^+$-specific L-arabitol-4-dehydrogenase (LAD), NAD(P)H-specific L-xylose reductase (LXR), and $NAD^+$-specific xylitol dehydrogenase (XDH) [21]. Then, D-xylulose is phosphorylated into D-xylulose-5-phosphate by D-xylulokinase (XK). As highlighted in Fig. 1, fungal pathways of L-arabinose and D-xylose share three enzymes: AR (XR), XDH and XK. Although the cofactor preferences of AR and LXR vary among fungal species, the first reductase enzyme usually prefers NADPH, while the two dehydrogenases strictly use $NAD^+$. Therefore, redox balance of the pathway leading to efficient cell growth is achieved under

aerobic conditions [23, 24]. Under oxygen-limited conditions, L-arabitol might be produced due to $NAD^+$ limitation, which is not found in the bacterial pathway of L-arabinose metabolism [25-27]. Because the oxygen-dependent nature of the fungal pathway, ethanol production by these native strains is marginal [28, 29].

As early as 1990, a few native arabinose-fermenting fungi strains were identified, but the strains yielded a trace level of ethanol or even no ethanol production [26, 28]. In detail, 116 different yeast strains were screened for the ability to catabolize arabinose or xylose aerobically. As a result, four yeast strains (*Ambroxiozyma monospora,* and three *Candida spp.*) were found to ferment L-arabinose as a sole carbon source. Additionally, the ethanol yield was at most 0.18 (g/g consumed arabinose) with *A. monospora* and *C. succiphila* (Table 2).

Meanwhile, 15 xylose-fermenting microorganisms were screened to evaluate the ability to ferment L-arabinose to ethanol [26]. As a result, one bacterium (*Erwinia chrysanthemi*), six yeast strains (*C. tropicalis, C. shehatae, Pachysolen tannophilusY-2460, P. tannophilus Y-12891, Scheffersomyces stipitis,* and *Torulopsis sonorensis*), and one mold strain (*Aspergillus oryzae*) were confirmed to assimilate arabinose with xylose and glucose as co-substrates. While *E. chrysanthemi* and *C. tropicalis* consumed xylose and arabinose simultaneously, all other fungal strains preferred xylose over arabinose. During arabinose metabolism, *S. stipitis* was the only fungal strain producing ethanol at a yield of 0.15 (g/g consumed sugar) and arabitol at a yield of 0.24 (g/g consumed sugar).

In comparison to the above-mentioned fungal pathways, bacterial pathways of L-arabinose metabolism are relatively simple; 1) only three enzymes are needed to convert to D-xylulose-5-phosphate, and 2) no cofactor is involved. The bacterial pathway consistof L-arabinose isomerase (AI), L-ribulose kinase (RK), and L-ribulose-5-phosphate-4-epimerase

98 (R5PE) encoded by the *araA*, *araB*, and *araD* genes, respectively (Fig 1b) [30]. In addition,
99 native bacterial strains such as *Sarcina ventriculi* can ferment L-arabinose anaerobically and
100 produce ethanol efficiently at a yield of 0.3 (g/g consumed arabinose) [31]. For some bacterial
101 strains lacking the *pdc* and *adh* genes (encoding pyruvate decarboxylase and alcohol
102 dehydrogenase, respectively) such as *Klebsiella oxytoca*, introducing the genes from *Zymomonas*
103 *mobilis* enabled ethanol fermentation from L-arabinose [32]. For native L-arabinose-
104 metabolizing *Escherichia coli*, in contrast, deletion of lactate dehydrogenase (*ldh*) and pyruvate
105 formate lyase (*pfl*) genes was required to enable ethanol production from L-arabinose [33]. As
106 summarized in Table 2, native bacterial strains assimilating L-arabinose can be promising hosts
107 for ethanol fermentation.
108
109 **Engineering *Saccharomyces cerevisiae* for L-arabinose fermentation**
110 *S. cerevisiae*, the industrial host for bioethanol production, cannot utilize L-arabinose as
111 efficiently as it can utilize xylose. For cellulosic ethanol production, a great amount of efforts has
112 been focused on the development of xylose-fermenting *S. cerevisiae* strains, while there have not
113 been many examples for L-arabinose.
114 There are a limited number of studies on the development of *S. cerevisiae* expressing a
115 heterologous fungal pathway of L-arabinose metabolism compared to that expressing a
116 heterologous bacterial pathway [30, 34-38]. Usually, xylose-fermenting *S. cerevisiae* expressing
117 heterologous AR, XDH, and XK is first engineered by expressing *Scheffersomyces stipitis XYL1*,
118 *XYL2*, and *XYL3* genes, respectively, in most cases [20]. It has to be noted that an AR is
119 NAD(P)H-specific aldose reductase with specificity for both xylose and L-arabinose [39] with a
120 50% higher rate for L-arabinose metabolism when using NADPH as a cofactor [40]. Next, the

121 resulting strain is further engineered to express LAD and LXR, which are *T. reesei LAD1* and *A.*
122 *monospora ALX1* genes, respectively [24, 34]. The *S. cerevisiae* 424A(LNH-
123 ST)/pLXR$_{NAD}$_LAD strain, which was developed as above, produced 10 g/L ethanol from 45 g/L
124 L-arabinose [35] as summarized in Table 3. In another study, *T. reesei LXR1* gene was
125 expressed instead of *A. monospora ALX1* gene, but the resulting strain only produced 0.1 g/L
126 ethanol from 50 g/L L-arabinose [35, 41]. The low ethanol production can be explained by the
127 fact that *T. reesei LXR1* gene is now functionally identified as mannitol dehydrogenase [42].
128 Another factor determining L-arabinose fermentation efficiency is aeration. The fungal L-
129 arabinose pathway is not redox-neutral because of the dual cofactor preference of AR and LXR
130 (NADPH and NADH) while XDH and LAD are $NAD^+$-specific. Therefore, cofactor imbalance
131 issue could be more severe than xylose fermentation requiring just AR and XDH.
132 Meanwhile, the bacterial L-arabinose pathway is redox-neutral; thus, more studies have
133 been performed for heterologous expression in *S. cerevisiae* from genes of *Bacillus subtilis*,
134 *Escherichia coli* and *Lactobacillus plantarum*. The bacterial L-arabinose pathway consisting of
135 *araA, araB* and *araD* genes were tested with various combinations from different origins (Table
136 3). The *araA* gene from *Bacillus subtilis* [36], *Bacillus licheniformis* [43, 44] and *Lactobacillus*
137 *plantarum* [30, 37] and the *araB* and *araD* genes from *Escherichia coli* [43, 44] or *L. plantarum*
138 [30, 37] were tested. Ethanol production from the engineered strains varied between 6-9 g/L
139 from 20 g/L L-arabinose. Regardless of the origin of the heterologous genes, various approaches
140 to improve L-arabinose fermentation have been performed. In general, the overexpression of the
141 non-oxidative pentose phosphate pathway genes (*TAL1, TKL1, RPE1* and *RKI1*) and adaptive
142 evolution were required [30, 36, 43]. However, despite all optimizations, the fermentation

productivity is limited by a bacterial pathway possibly because unfavorable thermodynamic properties of L-arabinose isomerase under ambient conditions [45].

It should be noted that adaptive evolution is proven to be an effective metabolic engineering strategy to improve xylose fermentation by engineered *S. cerevisiae* strains for both fungal and bacterial pathways [20, 46]. However, for L-arabinose fermentation, only the engineered strains with a bacterial pathway have been subjected to adaptive evolution (Table 2). It can be explained by the fact that L-arabinose fermentation has not been performed systematically and extensively compared to xylose fermentation. It is also possible that the heterologous expression of a fungal pathway in *S. cerevisiae* requires multiple strategies to be optimized to overcome the severe redox imbalance issue.

Arabinose fermentation can be improved by expressing L-arabinose-specific sugar transporters (Table 4). *S. cerevisiae* could uptake arabinose through some hexose transporters such as Hxt5 and Hxt7 with low affinity [47]. *S. cerevisiae* Gal2 had the highest affinity to L-arabinose (57 mM) among other native hexose transporter [48, 49]. It is also reported that *S. cerevisiae* Gal2 contributed to anaerobic arabinose fermentation when arabinose is the sole carbon source [37]. Several heterologous arabinose transporters have been identified from *Neurospora crassa* [50], *Myceliophthora thermophile* [50], *Penicillium chrysogenum* [51], *Arabidopsis thaliana* [48], and *Scheffersomyces stipitis* [48], and their ability to uptake L-arabinose varies significantly (0.02-116.7 mmol/h/g DCW). *N. crassa* LAT-1 was the most efficient L-arabinose transporter reported with a rate of 116.7 (mmol/h/g DCW), which is 2 orders of magnitude higher than that of *S. cerevisiae* GAL2 0.13 (mmol/h/g DCW) [50]. *P. chrysogenum* AraT was a high-affinity arabinose transporter with no activity with glucose and xylose, it was still inhibited by the presence of glucose and xylose [51, 52]. At the present stage,

no heterologous sugar transporter was reported to either improve L-arabinose fermentation or allow simultaneous uptake of arabinose and glucose. In arabinose metabolism, as in xylose's case [53, 54], it can be assumed that arabinose catabolism is currently more limiting than non-specific arabinose uptake in engineered *S. cerevisiae*.

**Engineering of other microorganisms for arabinose fermentation**

Some non-native arabinose fermenting microorganisms have also been engineered to assimilate L-arabinose and produce ethanol or other products. *Z. mobilis* (pZB206), which natively carries *pdc* and *adhB* but lacked arabinose-assimilating enzymes, was constructed by introducing *E. coli araABD*, *talB, tktA* to metabolize arabinose to ethanol [55]. The resulting strain showed an ethanol yield of 0.49 by consuming 25 g/L L-arabinose. Unlike *S. cerevisiae*, *Corynebacterium glutamicum* expressing *E. coli araABD* was easily engineered to produce amino acids such as L-glutamate, L-lysine, L-ornithine and L-arginine with arabinose as the sole carbon source [56]. For example, one of engineered *C. glutamicum* strains produced L-glutamate at a yield of 0.07 from 75 g/L L-arabinose. *Rhodococcus opacus* expressing *Streptomyces cattleya araABD* fermented 16 g/L L-arabinose as the sole carbon source and produced fatty acids at a yield of 0.13 (g/g consumed arabinose). The fatty acids were mostly palmitic acid with some cis-10-heptadecenoic acid, oleic acid, myristic acid, pentadecanoic acid, palmitoleic acid, heptadecanoic acid, and stearic acid. Although the examples are limited, non-*Saccharomyces cerevisiae* strains can be also engineered to ferment L-arabinose and produce various value-added products other than ethanol.

**Future outlook**

Both crop biomass and cellulosic biomass do not support sustainable bioprocesses due to their low contribution to greenhouse gas reduction [57] and limited technologies to overcome the recalcitrance [58], respectively. Alternatively, pectin-rich biomass such as fruit-processing wastes can be an attractive choice due to its low lignin content and the growing demands for fresh juice. L-Arabinose is a primary sugar of pectin structure and its content is minimal in other biomass. As discussed in this review, research for L-arabinose fermentation is in an early stage. Thus, all options are open to either optimizing native arabinose-assimilating strains or engineering non-native strains such as *S. cerevisiae*, *Z. mobilis*, *C. glutamicum*, and *R. opacus* depending on the desired products. Engineered *S. cerevisiae* strains fermenting L-arabinose are still limited to ethanol production, however, various chemicals and value-added products are expected to be studied as well. Additionally, the development of strains fermenting other pectin-derived monomers such as galacturonic acid and L-rhamnose needs to be considered.

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375 Figure legends

376

377 Fig. 1. Arabinose metabolic pathways in fungi (A) and bacteria (B). AR(XR); Aldose reductase,

378 LAD; L-arabitol-4-dehydrogenase, ALX; L-xylulose reductase, XDH; D-xylulose reductase, XK;

379 Xylulokinase, AI; L-arabinose isomerase, RK; L-Ribulokinase, R5PE; L-Ribulose-5-P-4-

380 epimerase.

381

382 Table 1. Representative pectin-rich biomass and their arabinose contents

| Source | Arabinose, %[1)] | Other major sugars[2)] | Refs |
|---|---|---|---|
| Sugar beet pulp | 21.6 (0.28) | Glu | [17, 18] |
| Lime peel | 8.5 | Glu | [14] |
| Pear peel | 6.0 | Glu, Xyl, Fru | [14] |
| Orange peel | 5.6 (0.20) | Glu, Fru | [14, 17] |
| Apple pomace | 5.5 | Glu, Fru, Suc | [14] |
| Mandarin peel | 3.3 | Glu, Fru, Suc | [14] |

383 **1)** % Dry matter (g/g pectin).

384 **2)** Higher contents than arabinose. Glu, Glucose; Xyl, xylose; Fuc, Fucose; Suc, Sucrose.

385 Table 2. Native arabinose-assimilating microorganisms and their engineered strains producing ethanol

| Strain | Genotype[1)] | Fermentation conditions[2)] | | Ethanol production | | Refs |
|---|---|---|---|---|---|---|
| | | Arabinose (g/L) | Aeration | Titer (g/L) | Yield (g/g) | |
| Yeast | | | | | | |
| *Ambrosiozyma monospora* Y-1484 | Wild type | 80 | OL | 4.1 | 0.18 | [28] |
| *Candida succiphila* Y-11998 | Wild type | 80 | OL | 3.9 | 0.05 | [28] |
| Bacteria | | | | | | |
| *Sarcina ventriculi* | Wild type | 19 | AN | 4.7 | 0.31 | [31] |
| *Klebsiella oxytoca* P2 | *Zm_pdc*, *Zm_adhB* | 80 | OL | 27.2 | 0.34 | [32] |
| *Escherichia coli* FBR3 | *Zm_pdc*, *Zm_adhB*, *ldh⊿*, *pfl⊿* | 100 | OL | 44.4 | 0.46 | [33] |

386 [1)] *Zm*, *Zymomonas mobilis*; *pdc*, pyruvate decarboxylase gene; *adhB*, alcohol dehydrogenase gene; *ldh*, lactate dehydrogenase gene; *pfl*,

387 pyruvate formate lyase gene.

388 [2)] All fermentations were performed with complex media with an initial arabinose concentration as shown above. Arabinose was the

389 only carbon source available. OL, oxygen-limited conditions; AN, anaerobic conditions.

390

Table 3. Ethanol fermentation by engineered *Saccharomyces cerevisiae* using arabinose as the sole carbon source

| Strain names | Strain backgrounds | Arabinose pathways | Optimization strategies | Media | Arabinose (g/L) | Aeration | Titer (g/L) | Yield (g/g consumed) | Refs |
|---|---|---|---|---|---|---|---|---|---|
| Heterologous fungal pathways | | | | | | | | | |
| H2561 | CEN.PK2 | Ss*XYL1*, Ss*XYL2*, Sc*XKS1*, Tr*LAD1*, Tr*LXR1* | - | Minimal | 50 | AN | 0.1 | - | [34] |
| 424A(LNH-ST)/pLXR$_{NAD}$-LAD | 424A | Ss*XYL1*, Ss*XYL2*, Ss*XYL3*, Tr*LAD1,* Am*ALX1* | - | Complex | 45 | OL | 9.4 | 0.22 | [35] |
| Heterologous bacterial pathways | | | | | | | | | |
| JBY25-4M | CEN.PK2-1C | Bs*araA*, Ec*araB*, Ec*araD*, Sc*GAL2* | - | Minimal | 20 | OL | 6 | 0.3 | [36] |
| BWY1-4S | CEN.PK2-1C | Bs*araA*, Ec*araB*, Ec*araD*, Sc*GAL2* | Codon optimization<br>Adaptive evolution | Minimal | 30 | AN | 9 | 0.39 | [43] |
| IMS0002 | CEN.PK2-1C | Lp*araA*, Lp*araB*, Lp*araD* | PPP overexpression<br>Adaptive evolution | Complex | 20 | AN | 8.92 | 0.45 | [30] |
| BSW3AP | CEN.PK102-3A | Lp*araA*, Lp*araB*, Lp*araD* | PPP overexpression<br>Adaptive evolution | Minimal | 20 | OL<br>AN | 6.9<br>- | 0.43<br>0.42 | [37] |
| BSW3AG | CEN.PK102-3A | Lp*araA*, Lp*araB*, Lp*araD*, *ScGAL2* | PPP overexpression<br>Adaptive evolution | Minimal | 20 | AN | - | 0.43 | [37] |

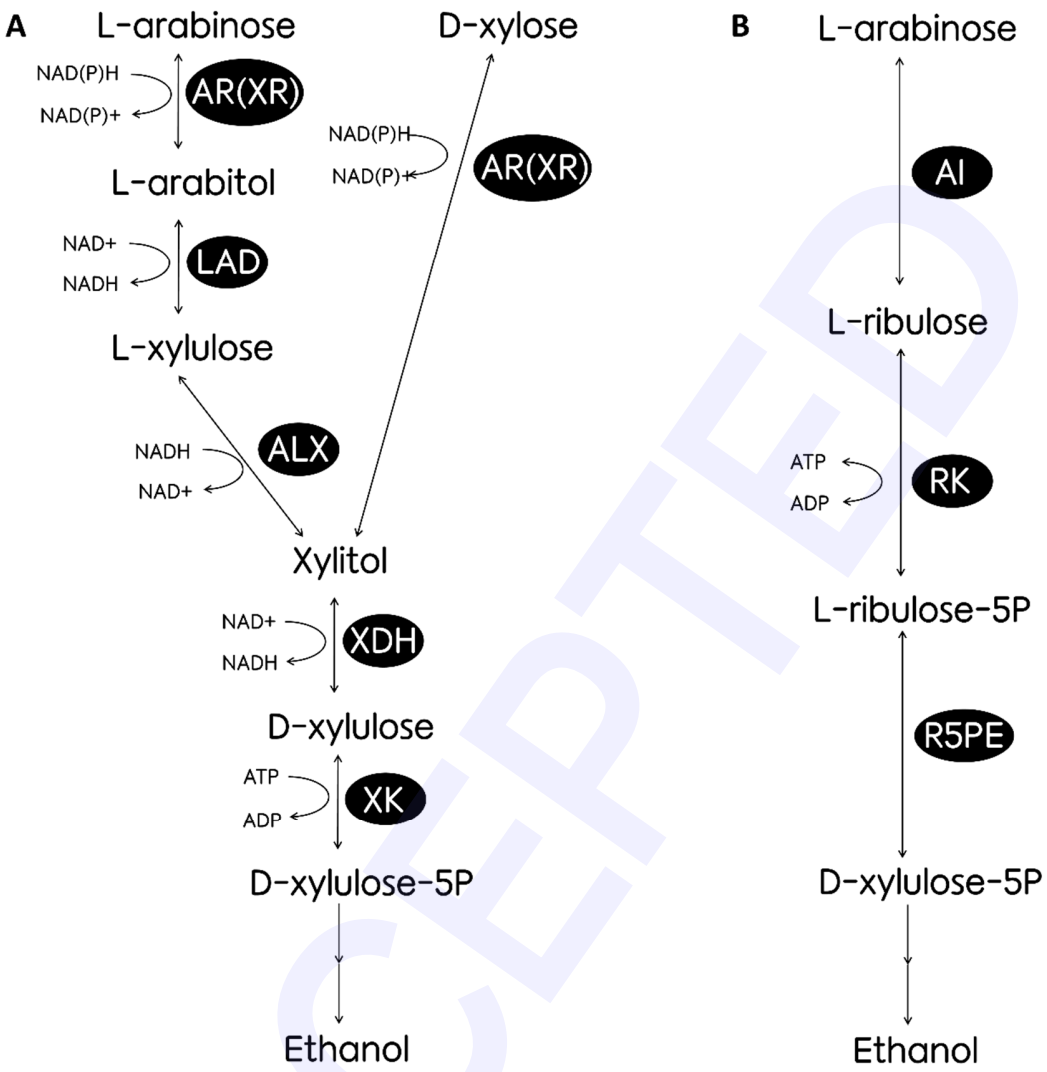
OL, oxygen-limited conditions; AN, anaerobic conditions; Bs, *Bacillus subtilis*; Ec, *Escherichia coli*; Sc, *Saccharomyces cerevisiae*; Tr, *Trichoderma reesei*; Am, *Ambrosiozyma monospora*; Pi, *Piromyces sp.*; Lp, *Lactobacillus plantarum*; Nc, *Neurospora crassa*; Mt, *Myceliophthora thermophile*; Ss*, Scheffersomyces stipitis*; PPP, pentose phosphate pathway.

395 Table 4. Characterization of putative arabinose transporters over-expressed in a hexose

396 transporter null mutant of *Saccharomyces cerevisiae*

| Transporter genes | Arabinose uptake rate (mmol/h/g DCW) | Arabinose affinity (mM) | References |
|---|---|---|---|
| *Neurospora crassa* LAT-1 | 116.7 | 58.12 | [50] |
| *Myceliophthora thermophile* LAT-1 | 10.29 | 29.39 | [50] |
| *Penicillium chrysogenum* AraT | 5.30 | 0.13 | [51] |
| *Saccharomyces cerevisiae* GAL2 | 0.13 | 57.00 | [48] |
| *Arabidopsis thaliana* Stp2 | 0.04 | 4.50 | [48] |
| *Scheffersomyces stipites* AraT | 0.02 | 3.80 | [48] |

397




1

2 Fig. 1.

3