

Engineering of *Saccharomyces cerevisiae* for Efficient Anaerobic Alcoholic Fermentation of L-Arabinose[∇]

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For cost-effective and efficient ethanol production from lignocellulosic fractions of plant biomass, the conversion of not only major constituents, such as glucose and xylose, but also less predominant sugars, such as L-arabinose, is required. Wild-type strains of *Saccharomyces cerevisiae*, the organism used in industrial ethanol production, cannot ferment xylose and arabinose. Although metabolic and evolutionary engineering has enabled the efficient alcoholic fermentation of xylose under anaerobic conditions, the conversion of L-arabinose into ethanol by engineered *S. cerevisiae* strains has previously been demonstrated only under oxygen-limited conditions. This study reports the first case of fast and efficient anaerobic alcoholic fermentation of L-arabinose by an engineered *S. cerevisiae* strain. This fermentation was achieved by combining the expression of the structural genes for the L-arabinose utilization pathway of *Lactobacillus plantarum*, the overexpression of the *S. cerevisiae* genes encoding the enzymes of the nonoxidative pentose phosphate pathway, and extensive evolutionary engineering. The resulting *S. cerevisiae* strain exhibited high rates of arabinose consumption ($0.70 \text{ g h}^{-1} \text{ g [dry weight]}^{-1}$) and ethanol production ($0.29 \text{ g h}^{-1} \text{ g [dry weight]}^{-1}$) and a high ethanol yield (0.43 g g^{-1}) during anaerobic growth on L-arabinose as the sole carbon source. In addition, efficient ethanol production from sugar mixtures containing glucose and arabinose, which is crucial for application in industrial ethanol production, was achieved.

In the past decades, it has become clear that for future sustainable and cost-effective production of fuel ethanol from plant biomass, not only the readily degradable starch and sucrose fractions but also the much more resistant lignocellulosic fractions of plant biomass should be used. Although glucose and xylose are often the predominant sugars in these feedstocks, the economically efficient production of ethanol also requires the conversion of smaller carbohydrate fractions, such as L-arabinose, at high rates and yields (9, 23).

Saccharomyces cerevisiae is presently the organism of choice for industrial ethanol production. Although wild-type *S. cerevisiae* strains rapidly ferment hexoses with high efficiency, they cannot grow on or use pentoses, such as D-xylose and L-arabinose (3). In addition to the development of pentose-consuming bacteria such as *Zymomonas mobilis*, *Escherichia coli*, and *Klebsiella oxytoca* as alternative biocatalysts for ethanol production (5), this situation has inspired various studies to expand the substrate range of *S. cerevisiae*. The combination of metabolic and evolutionary engineering with the heterologous expression of either yeast xylose reductase and xylitol dehydrogenase (14, 32, 34, 35, 41) or a fungal xylose isomerase (19–22) has already enabled the anaerobic fermentation of D-xylose by *S. cerevisiae*. The next challenge is the fermentation of other pentoses, such as L-arabinose. Although several yeasts and fungi can utilize L-arabinose as a carbon and energy source, most of them are unable to ferment it into ethanol. At best,

very low ethanol yields and production rates are accompanied by the formation of L-arabinitol under microaerophilic or oxygen-limited conditions (6, 18, 26). The rarity of ethanolic arabinose fermentation may be due to a redox imbalance in the fungal arabinose pathway as a result of the use of NADPH for the reductive reactions and the production of NADH in the oxidation reactions (6, 36) (Fig. 1). Consistent with this, the overexpression of all the structural genes of the fungal L-arabinose pathway (*XYL1*, *lad1*, *lxr1*, *XYL2*, and *XKS1*) in *S. cerevisiae* does not result in fast and efficient fermentation of L-arabinose into ethanol (29). Although the engineered strain produced only $0.35 \text{ mg of ethanol g}^{-1} \text{ h}^{-1}$ under anaerobic conditions, it provided the first example of ethanolic arabinose fermentation by *S. cerevisiae*.

An alternative approach to constructing an L-arabinose-fermenting *S. cerevisiae* strain is the overexpression of the bacterial L-arabinose pathway. In the bacterial pathway, the enzymes L-arabinose isomerase (AraA), L-ribulokinase (AraB), and L-ribulose-5-phosphate 4-epimerase (AraD) are involved in converting L-arabinose into L-ribulose, L-ribulose-5-P, and D-xylulose-5-P, respectively (Fig. 1). This approach circumvents the intrinsic redox imbalances associated with the expression of the fungal pathway. A first attempt to introduce this pathway into *S. cerevisiae* by expressing the *E. coli* *araA*, *araB*, and *araD* genes was only partly successful, with L-arabinose consumption resulting in the accumulation of arabinitol instead of the desired production of ethanol (33). Becker and Boles (4), who followed essentially the same strategy but used the *Bacillus subtilis* *araA* gene, were more successful. Combined with evolutionary engineering (31), the expression of this pathway eventually resulted in a strain capable of aerobic

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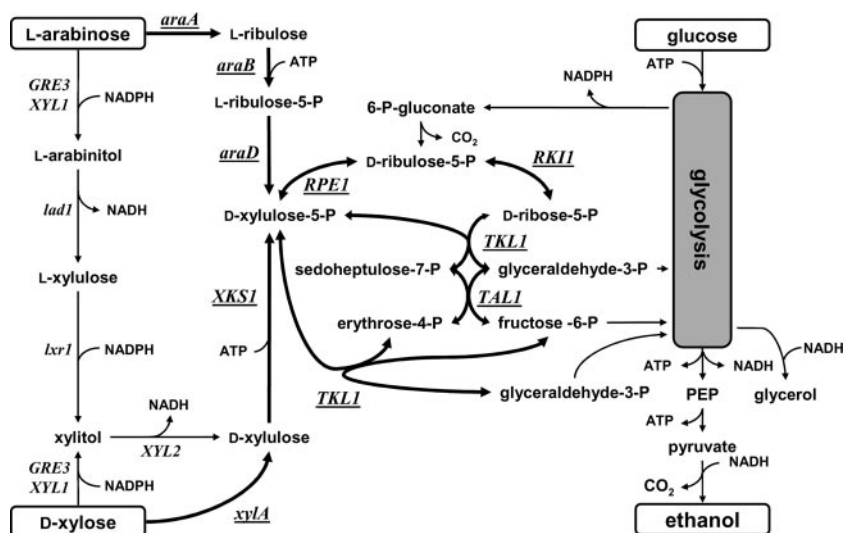


FIG. 1. Schematic representation of D-xylose and L-arabinose catabolism in metabolically engineered *S. cerevisiae* strains described in the literature. The engineering steps involved in this work are indicated by the underlined gene names. Components of the catabolism are as follows: aldose/xylose reductase (*GRE3/XYL1*), xylitol dehydrogenase (*XYL2*), xylulokinase (*XKS1*), D-xylose isomerase (*xyt1*), arabinitol 4-dehydrogenase (*lad1*), L-xylulose reductase (*lxr1*), L-arabinose isomerase (*araA*), L-ribulokinase (*araB*), L-ribulose-5-phosphate 4-epimerase (*araD*), transaldolase (*TAL1*), transketolase (*TKL1*), D-ribulose-5-phosphate 3-epimerase (*RPE1*), and ribose-5-phosphate ketol-isomerase (*RK11*). PEP, phosphoenolpyruvate.

growth on L-arabinose (4). Although the anaerobic fermentation of arabinose was not observed, the evolved *S. cerevisiae* strain produced ethanol from arabinose at a specific rate of 60 to 80 mg h⁻¹ g (dry weight)⁻¹ under oxygen-limited conditions. The evolved strain was reported to have acquired a mutation in the L-ribulokinase gene (*araB*) that resulted in reduced activity of the L-ribulokinase enzyme. Enhanced transaldolase (*TAL1*) activity was also reported to be required for L-arabinose fermentation. Moreover, the authors found that, although it is not essential for growth on arabinose, the overexpression of the gene encoding the *S. cerevisiae* galactose permease (*GAL2*)—also known to transport arabinose (17)—improved growth on arabinose (4). Thus, Becker and Boles have convincingly demonstrated that the overexpression of the bacterial L-arabinose pathway is a promising basis for achieving anaerobic L-arabinose fermentation by *S. cerevisiae*.

The present study aimed for fast and efficient fermentation of arabinose by *S. cerevisiae* under anaerobic conditions. To this end, a genetically engineered *S. cerevisiae* strain was constructed and subjected to extensive evolutionary engineering for improved anaerobic arabinose utilization.

MATERIALS AND METHODS

Strains and maintenance. The *S. cerevisiae* strains used in this work were derived from the xylose-fermenting strain RWB217 (20) and are listed in Table 1. During construction, *S. cerevisiae* strains were maintained on complex medium, consisting of 10 g liter⁻¹ yeast extract (BD Difco) and 20 g liter⁻¹ peptone (BD Difco), or synthetic medium for yeast (MY) (39) supplemented with D-glucose (2%) as a carbon source (yeast extract-peptone-D-glucose [YPD] or MYD, respectively). Agar (1.5%) was added to the plates. *S. cerevisiae* cells were plated onto MYD after transformation with plasmids. *S. cerevisiae* stock cultures were prepared after growth in shake flasks at 30°C in MYD or in MY supplemented with 2% (wt/vol) L-arabinose (MYA) by the addition of sterile glycerol to 30% (vol/vol) in the stationary-growth phase and were stored as 2-ml aliquots at -80°C. Plasmids were amplified in *E. coli* strain XL1-Blue (Stratagene, La Jolla,

CA). *E. coli* was grown on Luria-Bertani plates or in liquid Terrific broth medium for the isolation of plasmids (30).

In order to get a high level of expression, the *L. plantarum araA* and *araD* genes were ligated into the *xylA*-bearing plasmid pAKX002. The *araA* expression cassette was constructed by amplifying the *S. cerevisiae TDH3* promoter (P_{TDH3}) with oligonucleotides SpeI5'Ptdh3 and 5'AraAPtdh3, the *araA* gene with oligonucleotides Ptdh5'AraA and Tadh3'AraA, and the *ADH1* terminator (T_{ADH1}) with oligonucleotides 3'AraATadh1 and 3'Tadh1SpeI. The three fragments were extracted from gel and mixed in roughly equimolar amounts. With this mixture, a PCR using the SpeI5'Ptdh3 and 3'Tadh1SpeI oligonucleotides was performed. The resulting P_{TDH3} -*araA*- T_{ADH1} cassette was gel purified, cut at the 5' and 3' SpeI sites, and then ligated into *NheI*-digested pAKX002, resulting in plasmid pRW230.

The *araD* construct was made by first amplifying a truncated version of the *HXT7* promoter (P_{HXT7}) with oligonucleotides SalI5'Phtx7 and 5'AraDPhtx, the *araD* gene with Phtx5'AraD and Tpgi3'AraD, and the *PGII* terminator region (T_{PGI}) with the 3'AraDTpgi and 3'TpgiSal oligonucleotides. The resulting fragments were extracted from gel and mixed in roughly equimolar amounts, after which a PCR using oligonucleotides SalI5'Phtx7 and 3'TpgiSal was performed. The resulting P_{HXT7} -*araD*- T_{PGII} cassette was gel purified, cut at the 5' and 3' Sal sites, and then ligated into XhoI-digested pRW230, resulting in plasmid pRW231 (Fig. 2).

TABLE 1. *S. cerevisiae* strains and plasmids used in this study

Strain or plasmid	Characteristics ^a	Reference
Strains		
RWB217	<i>MATa ura3-52 leu2-112 loxP-P_{TP1}::(-266, -1)TAL1 gre3::hphMX pUGP_{TP1}-TKL1 pUGP_{TP1}-RPE1 kan-loxP-P_{TP1}::(-40, -1)RKII</i> (p415ADHXXKS, pAKX002)	Kuyper et al. (20)
RWB219	<i>MATa ura3-52 leu2-112 loxP-P_{TP1}::(-266, -1)TAL1 gre3::hphMX pUGP_{TP1}-TKL1 pUGP_{TP1}-RPE1 kan-loxP-P_{TP1}::(-40, -1)RKII</i> (p415ADHXXKS1, pAKX002)	This work
RWB220	<i>MATa ura3-52 leu2-112 loxP-P_{TP1}::(-266, -1)TAL1 gre3::hphMX pUGP_{TP1}-TKL1 pUGP_{TP1}-RPE1 loxP-P_{TP1}::(-40, -1)RKII</i>	This work
IMS0001	<i>MATa ura3-52 leu2-112 loxP-P_{TP1}::(-266, -1)TAL1 gre3::hphMX pUGP_{TP1}-TKL1 pUGP_{TP1}-RPE1 loxP-P_{TP1}::(-40, -1)RKII</i> (pRW231, pRW243)	This work
IMS0002	<i>MATa ura3-52 leu2-112 loxP-P_{TP1}::(-266, -1)TAL1 gre3::hphMX pUGP_{TP1}-TKL1 pUGP_{TP1}-RPE1 loxP-P_{TP1}::(-40, -1)RKII</i> (pRW231, pRW243); selected for anaerobic growth on L-arabinose	This work
Plasmids		
pSH47	CEN <i>URA3</i> ; Cre recombinase gene behind P _{GAL1}	Güldener et al. (10)
pRS305	Integration plasmid; <i>LEU2</i>	Gietz and Sugino (7)
pAKX002	2μm <i>ori URA3</i> P _{TP1} - <i>xylA</i> _{Piromyces} -T _{CYC1}	Kuyper et al. (19)
p415ADHXXKS1	CEN <i>LEU2</i> P _{ADH1} - <i>XKS1</i> -T _{CYC1}	Kuyper et al. (20)
pRW229	Integration plasmid; <i>LEU2</i> P _{ADH1} - <i>XKS1</i> -T _{CYC1}	This work
pRW230	2μm <i>ori URA3</i> P _{TP1} - <i>xylA</i> _{Piromyces} -T _{CYC1} P _{TDH3} - <i>araA</i> _{L. plantarum} -T _{ADH1}	This work
pRW231	2μm <i>ori URA3</i> P _{TP1} - <i>xylA</i> _{Piromyces} -T _{CYC1} P _{TDH3} - <i>araA</i> _{L. plantarum} -T _{ADH1} P _{HXT7} - <i>araD</i> _{L. plantarum} -T _{PGI1}	This work
pRW243	Integration plasmid; <i>LEU2</i> P _{ADH1} - <i>XKS1</i> -T _{CYC1} P _{PGI1} - <i>araB</i> _{L. plantarum} -T _{ADH1}	This work

^a Numbers in parentheses refer to areas described in reference 20.

For the expression of *araB*, the integration plasmid pRS305 was used. Aside from *araB*, the *S. cerevisiae* *XKS1* gene was also included on this vector. For this construction, the P_{ADH1}-*XKS1*-T_{CYC1}-containing PvuI fragment from p415ADHXXKS was ligated into the PvuI-digested vector backbone from the integration plasmid pRS305, resulting in pRW229. For the expression of *araB*, a cassette containing the *L. plantarum* *araB* gene between the *PGI1* promoter (P_{PGI1}) and the *ADH1* terminator (T_{ADH1}) was constructed by PCR amplification. The *L. plantarum* *araB* expression cassette was made by amplifying the *PGI1* promoter with the oligonucleotides SacI5'Ppgi1 and 5'AraBPpgi1, the *araB* gene with Ppgi5'AraB and Tadh3'AraB, and the *ADH1* terminator with 3'AraBTadh1 and 3'Tadh1SacI. A PCR with an equimolar mixture of the three gel-purified PCR fragments was performed using primers SacI5'Ppgi1 and 3'Tadh1SacI. The resulting P_{PGI1}-*araB*-T_{ADH1} cassette was gel purified, digested at the 5' and 3' SacI sites, and then ligated into SacI-digested pRW229, resulting in plasmid pRW243 (Fig. 2).

Plasmids pRW243 and pRW231 were used to transform *S. cerevisiae* strain

RWB220, a strain that is derived from strain RWB219. Strain RWB219 was obtained by the selection of RWB217 colonies for improved growth on D-xylose, similar to the way in which strain RWB218 was developed (21), with the difference that RWB218 was obtained by plating and restreaking onto MYD plates while RWB219 was selected from solid synthetic medium supplemented with D-xylose. Strain RWB219 was grown nonselectively on YPD in order to facilitate the loss of plasmids pAKX002 and p415ADHXXKS1 (20), harboring the *URA3* and *LEU2* selective markers, respectively. After plating onto YPD, single colonies were screened for plasmid loss by testing for uracil and leucine auxotrophy. In order to remove a KANMX cassette, still present after the integration of the *RKII* overexpression construct (20), a strain that had lost both plasmids was transformed with pSH47, containing the Cre recombinase gene (10). Transformants containing pSH47 were resuspended in complex medium with 1% D-galactose and incubated for 1 h at 30°C. Cells were plated onto YPD, and colonies were screened for the loss of the KANMX marker (G418 resistance) and pSH47 (*URA3*). A strain that had lost both the KANMX marker and the

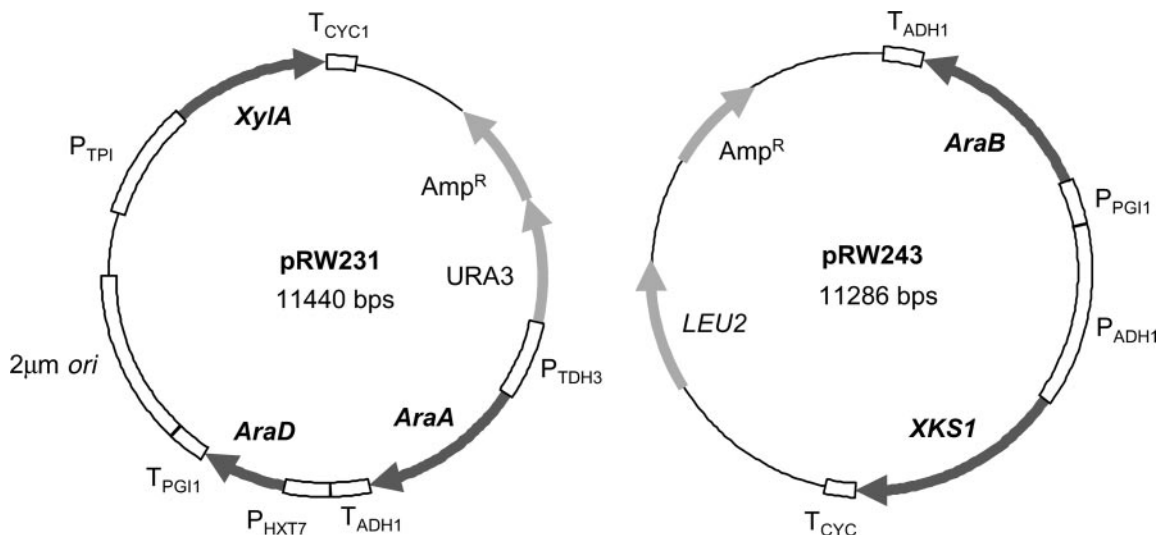


FIG. 2. Maps of plasmids pRW231 and pRW243 containing the genes encoding L-arabinose isomerase (*araA*), L-ribulokinase (*araB*), and L-ribulose-5-phosphate epimerase (*araD*) from *L. plantarum*, the *Piromyces* sp. strain E2 xylose isomerase gene (*xylA*), and the yeast xylulokinase gene (*XKS1*).

pSH47 plasmid was designated RWB220. Strain RWB220 was transformed with pRW231 and pRW243 (Table 1; Fig. 2), resulting in strain IMS0001. The selection of strain IMS0001 for (anaerobic) growth on L-arabinose, as described in Results, yielded strain IMS0002.

Cultivation. Shake flask and fermenter cultivations were performed at 30°C in MY containing 5 g liter⁻¹ (NH₄)₂SO₄, 3 g liter⁻¹ KH₂PO₄, 0.5 g liter⁻¹ MgSO₄ · 7H₂O, 0.05 ml liter⁻¹ silicon antifoam, and trace elements (39). For the cultivation in shake flasks, the pH of the medium was adjusted to 6.0 with 2 M KOH prior to sterilization. After heat sterilization (121°C for 20 min), a filter-sterilized vitamin solution (39) and an appropriate carbon and energy source were added. Shake flask cultures and precultures for anaerobic batch cultivations were prepared by inoculating 100 ml of medium containing the appropriate sugar into a 500-ml shake flask with a frozen stock culture. After incubation at 30°C in an orbital shaker (200 rpm), precultures were used to inoculate either another 500-ml shake flask containing MY with an appropriate carbon source or an anaerobic fermenter. For anaerobic cultivation in fermenters, the synthetic medium was supplemented with 0.01 g liter⁻¹ ergosterol and 0.42 g liter⁻¹ Tween 80 dissolved in ethanol (1, 2). Anaerobic (sequencing) batch cultivation was carried out at 30°C in 2-liter laboratory fermenters (Applikon, Schiedam, The Netherlands) with a working volume of 1 liter. The culture pH was maintained at 5.0 by the automatic addition of 2 M KOH. Cultures were stirred at 800 rpm and sparged with 0.5 liter min⁻¹ nitrogen gas (<10 ppm oxygen). To minimize the diffusion of oxygen, fermenters were equipped with Norprene tubing (Cole-Palmer Instrument Company, Vernon Hills, IL). Dissolved oxygen was monitored with an oxygen electrode (Applisens, Schiedam, The Netherlands). Oxygen-limited conditions in the same experimental setup were achieved by headspace aeration at approximately 0.05 liter min⁻¹.

Determination of biomass dry weight. Culture samples (10.0 ml) were filtered over preweighed nitrocellulose filters (pore size, 0.45 µm; Gelman Laboratory, Ann Arbor, MI). After filtration of the broth, the biomass was washed with demineralized water, dried in a microwave oven for 20 min at 360 W, and weighed. Duplicate determinations varied by less than 1%.

Gas analysis. Exhaust gas was cooled in a condenser (2°C) and dried with a type MD-110-48P-4 dryer (Perma Pure, Toms River, NJ). Oxygen and carbon dioxide concentrations were determined with an NGA 2000 analyzer (Rosemount Analytical, Orrville, OH). The exhaust gas flow rate and specific carbon dioxide production rates were determined as described previously (37, 42). In calculating the cumulative carbon dioxide production, volume changes caused by withdrawing culture samples were taken into account.

Metabolite analysis. Glucose, xylose, arabinose, xylitol, organic acids, glycerol, and ethanol were analyzed by high-performance liquid chromatography (HPLC) using an Alliance 2690 HPLC system (Waters, Milford, MA) supplied with an HPX 87H column (Bio-Rad, Hercules, CA), a Waters 2410 refractive-index detector, and a Waters 2487 UV detector. The column was eluted at 60°C with 0.5 g liter⁻¹ sulfuric acid at a flow rate of 0.6 ml min⁻¹.

Rate calculations. For calculation of the specific rates of arabinose consumption and ethanol production, the time-dependent arabinose and ethanol data were fitted with Boltzmann sigmoidal equations. For each time point, the specific arabinose consumption rate and the ethanol production rate were calculated by dividing the derivative of the fitted curve by the dry weight.

Carbon recovery. Carbon recoveries were calculated as the amount of carbon in the products formed divided by the total amount of sugar carbon consumed and were based on a carbon content of the biomass of 48% (38). To correct for ethanol evaporation during the fermentations, the amount of ethanol produced was assumed to be equal to the measured cumulative amount of CO₂ produced minus the amounts of the CO₂ produced due to biomass synthesis (5.85 mmol of CO₂ per g of biomass) (38) and the CO₂ associated with acetate formation.

Total RNA extraction and cDNA synthesis. Strains RWB219, IMS0001, and IMS0002 were cultivated in shake flasks containing MYD. At an optical density at 660 nm (OD₆₆₀) of approximately 2, when glucose was not yet depleted, cells were harvested by centrifuging 40 ml of the culture at 0°C. Total RNA extraction was performed as described previously (28). Isolated RNA was DNase I treated according to the RNeasy mini kit (QIAGEN) protocol for RNA cleanup. First-strand cDNA synthesis was carried out using 2 µg of DNase-treated RNA, random hexamer primers, and Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA).

Expression profiling using real-time quantitative PCR. Quantitative real-time PCR was run on a DNA engine Opticon I system (Bio-Rad, Hercules, CA) with the following settings: 94°C for 2 min, 94°C for 10 s, 66°C for 10 s, 72°C for 10 s, and plate reading. The denaturation, annealing, elongation, and reading steps were repeated for 39 cycles. A melting curve from 50 to 94°C was generated at the end of the reaction. The 20-µl reaction mixture consisted of 10 µl of SYBR green Taq ready mix (catalog no. S1816; Sigma), 0.2 µM forward primer, 0.2 µM

TABLE 2. Oligonucleotides used in this work

Function and oligonucleotide designation	5' → 3' DNA sequence
Amplification of <i>araA</i> expression cassette	
SpeI5'Ptdh3	GACTAGTCGAGTTTATCATTTCAATACTGC
5'AraAPtdh	CTCATAATCAGGTACTGATAACATTTGTTTGTTTA TGTGTGTTTATTC
Ptdh5'AraA	GAATAAACACACATAAACAAACAAATGTTATCAGT ACCTGATTATGAG
Tadh3'AraA	AATCATAAATCATAAGAAATTCGCTTACTTTAAGAA TGCTTAGTCAT
3'AraATadh1	ATGACTAAGGCATTCCTAAAGTAAGCGAATTTCTTA TGATTTATGATT
3'Tadh1SpeI	CACTAGTCTCGAGTGTGGAAGAAGCATTACAACAGG
Amplification of <i>araB</i> expression cassette	
SacI5'Ppgi1	CGAGCTCGTGGGTGTTTGGATTATAGGAAG
5'AraBPpgi1	TTGGGCTGTTTCACTAAATTCATTTTAGGCTGGT ATCTTGATTCTA
Ppgi5'AraB	TAGAAATCAAGATACCGCCTAAATTAAGTTAGTT GAACAGCCCAA
Tadh3'AraB	AATCATAAATCATAAGAAATTCGCTCTAATATTGTA TTGCTTGCCGAG
3'AraBTadh1	CTGGGCAAGCAATCAATATTAGAGCGAATTTCTTA TGATTTATGATT
3'Tadh1SacI	TGAGCTCGTGTGGAAGAAGCATTACAACAGG
Amplification of <i>araD</i> expression cassette	
SalI5'Phxt7	ACGCGTCGACTCGTAGGAACAATTTTCGG
5'AraDPhxt	CTTCTTGTGTTTAAATGCTTCTAGCATTTTGTGATTA AATTAACAACTT
Phxt5'AraD	AAGTTTTTTTAAATTTAATCAAAAAATGCTAGAAGC ATTAAACAAGAAG
Tpgi3'AraD	GGTATATATTTAAGAGCGATTGTTTACTTGCGAAC TGCATGATCC
3'AraDTpgi	GGATCATGCGATTTCGCAAGTAAACAAATCGCTCTTA AATATATACC
3'TpgiSalI	CGCAGTCGACCTTTTAAACAGTTGATGAGAACC
Quantitative real-time PCR	
QAraA-FW	GCCAGAAGCCGAACACCTA
QAraA-RV	ACCACCAGCTTGCATCCATT
QAraB-FW	TGTCGGCAAACTGCAATGA
QAraB-RV	TCCGGTCTTGGACTCAACG
QAraD-FW	GCAATTGACCCGTGCAAGTA
QAraD-RV	AGGCATTCGACCATGCTT
QXylA-FW	GAACGGTGAACCAAGCAAA
QXylA-RV	TGCGGCCCTCTAGTTATTGG
QXKS1-FW	CATGGCATGTAATGGAAGCA
QXKS1-RV	GCTTAAGGGGACAATCTTGA
3' FW ACT1	GGCTTCTTTGACTACCTTCCA
3' RV ACT1	AGAAACACTTGTGGTGAACGA

reverse primer, and cDNA (20, 2, 0.2, or 0.02 ng per PCR). The primers used are listed in Table 2. The cycle threshold value was calculated with the Opticon Monitor software version 1.08 (Bio-Rad, Hercules, CA) by setting the threshold for significant detection levels to 10 times the standard deviation over the cycle range from 3 to 7. Each PCR was carried out in triplicate. The relative expression of *xylA*, *XKS1*, *araA*, *araB*, and *araD* was quantified by using the comparative 2^{-ΔΔC_T} method (24) with *ACT1* as the reference gene.

RESULTS

Expression of the *L. plantarum* arabinose pathway in *S. cerevisiae*. In this work, *araA* (L-arabinose isomerase), *araB* (L-ribulokinase), and *araD* (L-ribulose-5-P 4-epimerase) from the lactic acid bacterium *L. plantarum* were expressed in *S. cerevisiae*. An analysis of the codon adaptation indices (CAI)

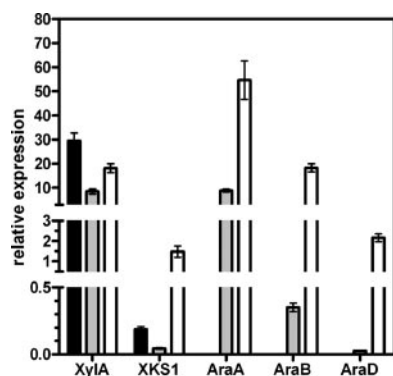


FIG. 3. Expression of *xylA*, *XKS1*, *araA*, *araB*, and *araD* in strains RWB219 (black bars), IMS0001 (gray bars), and IMS0002 (white bars). The levels of expression displayed are relative to the level of expression of the reference gene *ACT1* and are the averages calculated from cycle threshold values measured in triplicate reactions with mixtures containing 0.02, 0.2, 2, and 20 ng of cDNA. The error bars indicate the standard deviations calculated by using standard propagation of error methods.

using codonw (<http://bioweb.pasteur.fr/seqanal/interfaces/codonw.html>) indicated that the *L. plantarum* *araA* (CAI, 0.213), *araB* (CAI, 0.112), and *araD* (CAI, 0.159) genes better matched the codon usage in *S. cerevisiae* than the previously used *B. subtilis* *araA* (CAI, 0.057) and *E. coli* *araB* (CAI, 0.075) and *araD* (CAI, 0.098) genes (4). To achieve fermentation of L-arabinose in combination with other sugars, such as D-xylose, two plasmids that together harbored the genes encoding both the L-arabinose and D-xylose pathways were constructed according to the procedure described above (Fig. 2). Strain RWB220 (Table 1), a strain overexpressing the *TAL1*, *TKL1*, *RPE1*, and *RKI1* genes (20, 21), was transformed with both plasmids, resulting in strain IMS0001.

Since several relevant substrates for enzymes encoded by *araA*, *araB*, and *araD* are not commercially available (16), thus precluding enzyme activity determination, the expression of these genes in strain IMS0001 at the mRNA level was analyzed by real-time quantitative PCR. Figure 3 displays the levels of expression of the genes relative to the expression of *ACT1*. This highly expressed housekeeping gene encoding actin is commonly used as an internal standard for quantitative mRNA

analysis (24). The highest levels of expression were found to be those of *xylA* and *araA*, encoding the D-xylose isomerase and L-arabinose isomerase, respectively. Both genes were transcribed at levels approximately 8.5-fold higher than the reference gene *ACT1*. However, the levels of expression of *araB*, *araD*, and *XKS1* were lower than that of *ACT1*, at 0.35-, 0.02-, and 0.04-fold the level of *ACT1* expression, respectively. Although these quantitative PCR results confirm the transcription of *araA*, *araB*, and *araD*, strain IMS0001 was not capable of growing on solid MYA.

Selection for growth on L-arabinose. As described above, functional expression of the *L. plantarum* *araA*, *araB*, and *araD* genes did not result in the immediate growth of strain IMS0001 on arabinose. Evolutionary engineering (31) has been shown to be a powerful tool in gain-of-function situations, as has been shown previously, for instance, for the serial transfer of *S. cerevisiae* RWB202 cultures in shake flasks, resulting in improved growth on xylose (22). Therefore, a similar approach was applied for the selection of cells of strain IMS0001 with an improved specific rate of growth on arabinose. Prior to the selection in synthetic medium supplemented with 2% arabinose, cells were grown in synthetic medium with galactose, as it has been shown previously that galactose-induced *S. cerevisiae* cells can transport L-arabinose via the galactose permease GAL2p (17). Galactose-grown cells of both strains RWB219 and IMS0001 were transferred into shake flasks containing MY supplemented with 0.1% D-galactose and 2% L-arabinose. After approximately 800 h of cultivation in a single initial shake flask, the culture of strain IMS0001 showed growth after the depletion of the galactose, albeit very slow (data not shown), in contrast to the reference strain RWB219, which did not grow after the depletion of galactose. Cells of both cultures were transferred into fresh MYA. After approximately 400 h of cultivation in MYA, cells in the culture inoculated with strain IMS0001 grew with an estimated doubling time of 90 h, while strain RWB219 did not grow. At an OD_{660} of 2 to 3, cells were sequentially transferred into fresh MYA with a starting OD_{660} of approximately 0.05. Figure 4A shows that, indeed, the specific growth rate of the sequentially transferred cultures increased from approximately 0.01 to 0.15 h^{-1} in 17 transfers. The utilization of arabinose was confirmed by occasionally

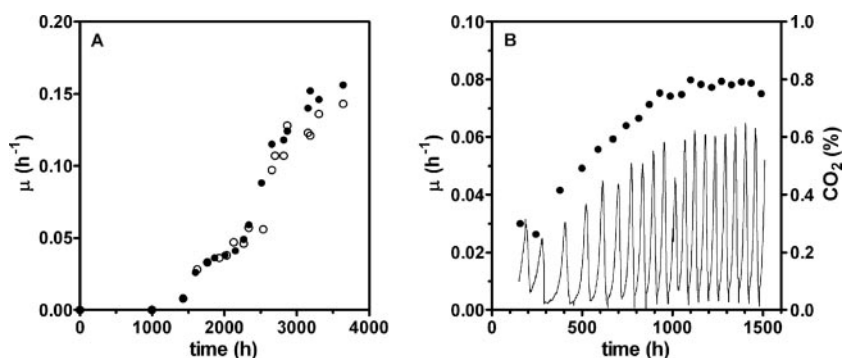


FIG. 4. (A) Specific growth rate (μ) of *S. cerevisiae* IMS0001 in shake flask cultures containing synthetic medium with 2% (wt/vol) L-arabinose during serial transfers. Each point represents the growth rate estimated from the OD_{660} measured during (exponential) growth. The closed and open circles represent results from duplicate serial transfer experiments. (B) Growth rate and CO_2 profile during an anaerobic SBR fermentation of *S. cerevisiae* IMS0001 in synthetic medium with 2% (wt/vol) L-arabinose. Each point (closed circle) represents the growth rate estimated from the CO_2 profile (lines) during exponential growth.

TABLE 3. Growth parameters, final substrate and product concentrations, and product yields determined for anaerobic batch cultivations of strain IMS0002^a

Parameter	Value(s) for strain cultivated on:	
	L-Arabinose (20 g liter ⁻¹)	D-Glucose (20 g liter ⁻¹) and L-arabinose (20 g liter ⁻¹)
Specific growth rate(s) (h ⁻¹)	0.051 ± 0.001	0.14 ± 0.01, ^b 0.025 ± 0.001 ^c
Biomass yield ^d (g g ⁻¹ of sugar)	0.087 ± 0.004	0.075 ± 0.001
Ethanol yield (g g ⁻¹ of sugar)	0.43 ± 0.00	0.42 ± 0.00 (0.39 ± 0.00, ^b 0.45 ± 0.00 ^c)
Carbon recovery (%)	105.1 ± 0.1	101.9 ± 0.3
Amt (mmol liter ⁻¹) of indicated sugar consumed		
Glucose	NA	112 ± 1
Arabinose	138 ± 1	137 ± 0
Amt of indicated product formed		
Biomass ^d (g liter ⁻¹)	1.81 ± 0.04	3.06 ± 0.05
CO ₂ (mmol liter ⁻¹)	205 ± 2	392 ± 3
Ethanol ^e (mmol liter ⁻¹)	194 ± 2	370 ± 3
Glycerol (mmol liter ⁻¹)	15.3 ± 0.0	30.5 ± 0.02
Acetate (mmol liter ⁻¹)	1.86 ± 0.00	5.5 ± 0.02
Lactate (mmol liter ⁻¹)	1.46 ± 0.00	0.96 ± 0.01
Pyruvate (mmol liter ⁻¹)	0.23 ± 0.02	0.27 ± 0.04
Succinate (mmol liter ⁻¹)	0.76 ± 0.02	1.42 ± 0.00
Xylitol (mmol liter ⁻¹)	<0.1	<0.1
Arabinitol (mmol liter ⁻¹)	<0.1	<0.1

^a Values presented are the averages ± the standard deviations of results from two independent anaerobic batch fermentations.

^b Value determined for the glucose growth phase.

^c Value determined for the arabinose growth phase.

^d As growth ceased after approximately 48 to 52 h, the maximum observed dry weight was used for calculating biomass yields.

^e Calculated from the cumulative CO₂ production.

measuring arabinose concentrations by HPLC (data not shown). In these aerobic shake flask cultures, no ethanol production was observed.

To further improve the rate of arabinose consumption by the aerobically evolved arabinose-grown *S. cerevisiae* cells, these cells were cultivated in an anaerobic batch fermenter with synthetic medium supplemented with 2% L-arabinose. Under anaerobic conditions, which are required for efficient ethanol production from L-arabinose, the ATP yield is 1½ mol of ATP per mol of arabinose that is fermented into ethanol, which is much lower than the 13½ mol of ATP per mol of arabinose produced under respiratory conditions, assuming a ratio of ATP produced per oxygen atom of 1.0 for all redox equivalents in *S. cerevisiae* (40). Therefore, under anaerobic (fermentative) conditions, an 8-fold-higher arabinose influx is required to achieve a specific ATP production rate equal to that achieved under aerobic (respiratory) conditions. In batch cultures, cells are growing at the maximum growth rate, and the sequential transfer of these batch cultures should select for cells with an increasingly higher specific growth rate and thus a higher arabinose consumption rate. In contrast, chemostat cultures usually select for cells with a lower saturation constant (K_s) for the specific substrate (27). To automate the sequential transfers of the culture, a sequencing batch reactor (SBR) setup was used (21, 22).

The first cycle of anaerobic batch fermentation was initiated by inoculating synthetic medium supplemented with 2% L-arabinose with aerobically evolved L-arabinose-grown *S. cerevisiae* cells. During the aerobic serial transfer protocol, the max-

imum aerobic growth rate of these cells on arabinose had increased to approximately 0.12 h⁻¹. Initially, growth under anaerobic conditions was not observed. To allow for a more gradual transfer to anaerobic conditions, the first cycle of SBR fermentation was performed under oxygen-limited conditions. When growth was observed, the culture was switched to anaerobic conditions in the next batch cycle. Upon arabinose depletion, indicated by the decrease of the CO₂ percentage to below 0.05% after the CO₂ production peak (Fig. 4B), a new cycle was initiated by either manual or automated replacement of approximately 90% of the culture with fresh synthetic medium containing 20 g liter⁻¹ L-arabinose. For each cycle, the maximum specific growth rate was estimated from the CO₂ profile (Fig. 4B). In 13 cycles, the anaerobic specific growth rate increased from 0.025 to 0.08 h⁻¹. In the next seven cycles, the growth rate did not increase noticeably. After a total of 20 cycles, single colonies were isolated on solid MYA. A culture originating from a single colony grown in a shake flask containing MYA was designated strain IMS0002 and was used for further characterization.

The analysis of the expression of *araA*, *araB*, *araD*, *xylA*, and *XKS1* in strain IMS0002 by real-time quantitative PCR revealed that all five genes were expressed at higher levels than they were in the parental strain IMS0001 (Fig. 3). In particular, *araB* and *araD* were expressed at much higher levels: 52- and 90-fold higher than in strain IMS0001. The levels of expression of the genes displaying the highest relative expression levels, *araA* and *xylA*, increased only six- and twofold, respectively.

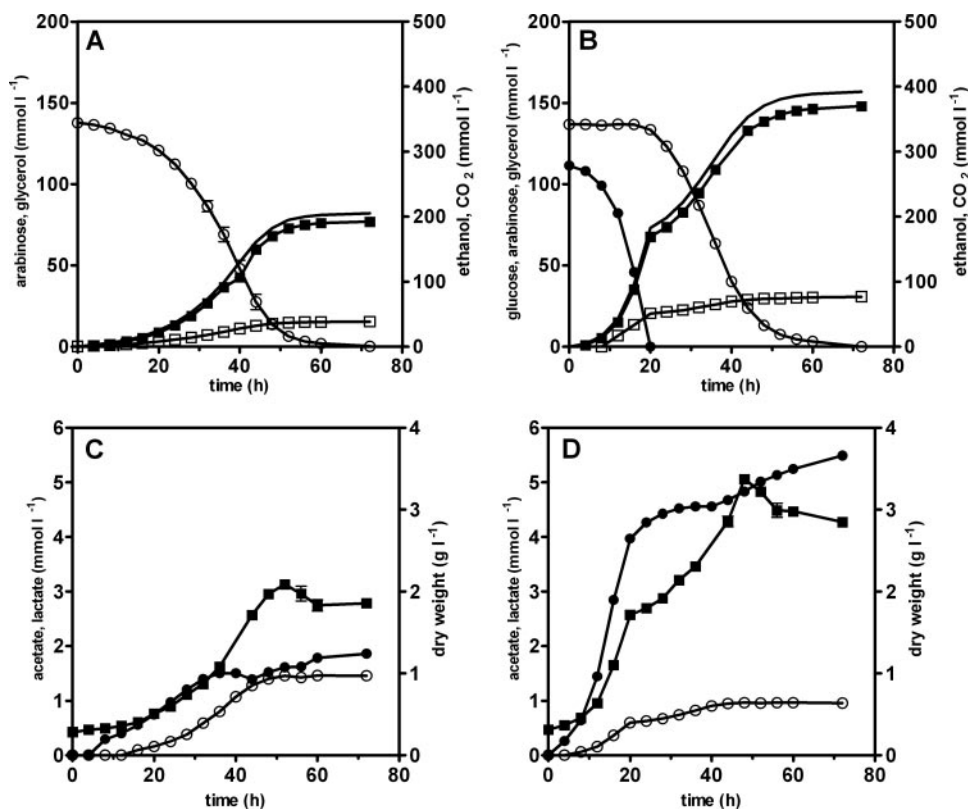


FIG. 5. Sugar consumption and product formation by strain IMS0002 during anaerobic batch fermentations in synthetic medium supplemented with 20 g liter⁻¹ L-arabinose (A and C) and 20 g liter⁻¹ glucose and 20 g liter⁻¹ L-arabinose (B and D). Data points represent the averages of results from two independent anaerobic batch fermentations. (A and B) Levels of glucose (●), L-arabinose (○), ethanol (calculated from cumulative CO₂ production) (■), glycerol (□), and cumulative CO₂ (—) produced. l, liter. (C and D) Levels of acetate (●) and lactate (○) produced and biomass dry weight (■).

Surprisingly, the expression of *XKS1*, encoding xylulokinase, also increased by 34-fold in strain IMS0002.

Characterization of an *S. cerevisiae* strain engineered and selected for anaerobic growth on L-arabinose. To characterize the growth of and product formation by the evolved strain IMS0002, anaerobic batch cultivations of strain IMS0002 in synthetic medium with 20 g liter⁻¹ of L-arabinose as the sole carbon source were performed. Precultures for these anaerobic batch fermentations were prepared in aerobic shake flasks containing 100 ml of MYA. With L-arabinose as the sole carbon source, strain IMS0002 was capable of growing anaerobically at a specific growth rate of 0.051 ± 0.001 h⁻¹ (Table 3). After 52 h, no further increase of the biomass was observed (Fig. 5C). With an initial biomass of 0.28 g (dry weight) liter⁻¹, strain IMS0002 completely consumed 20 g liter⁻¹ L-arabinose within 70 h (Fig. 5A) with a maximum specific arabinose consumption rate of 0.70 g h⁻¹ g (dry weight)⁻¹ (Table 4). The ethanol production was deduced from the CO₂ production to correct for ethanol evaporation during the batch fermentations (22). From 138 ± 1 mmol liter⁻¹ L-arabinose, 194 ± 2 mmol liter⁻¹ ethanol was produced, corresponding to a high ethanol yield of 0.43 g g⁻¹ of arabinose. The maximum specific ethanol production rate was determined to be 0.29 g h⁻¹ g (dry weight)⁻¹ (Table 4). By-products such as glycerol, lactate, and succinate were formed only in small amounts, similar to quantities observed previously in the fermentation of xylose by

engineered *S. cerevisiae* strains (20–22). The formation of arabinitol, which in other studies was found to be produced in substantial amounts by (engineered) yeasts fermenting arabinose (6, 16, 33), was not observed during the anaerobic growth of strain IMS0002 on L-arabinose.

Mixed-sugar fermentation. To investigate the capability of strain IMS0002 to ferment mixtures of sugars, anaerobic batch cultivations in synthetic medium with 20 g liter⁻¹ of glucose and 20 g liter⁻¹ of L-arabinose were performed. In these batch fermentations, glucose was consumed first, within 20 h. Upon glucose depletion, arabinose was consumed at a maximum observed rate of 0.42 g h⁻¹ g (dry weight)⁻¹ (Table 4), which is lower than the consumption rate during the batch fermentation with arabinose as the sole carbon source. The maximum growth rate observed during the glucose consumption phase was 0.14 h⁻¹, whereas the growth rate during the arabinose growth phase did not exceed 0.025 h⁻¹ (Table 3). Also, the biomass did not increase further after approximately 48 h (Fig. 5B). Within approximately 70 h, both glucose and arabinose were completely consumed and 370 ± 3 mmol liter⁻¹ ethanol was produced, corresponding to an overall ethanol yield of 0.42 g g⁻¹ (Table 3). The maximum observed ethanol production rate during growth on arabinose was 0.18 g h⁻¹ g (dry weight)⁻¹ (Table 4), which is consistent with the reduced arabinose consumption rate compared to that in the single-sugar fermentation.

TABLE 4. Arabinose consumption rates, ethanol production rates, and ethanol and arabinitol production yields for engineered arabinose-utilizing *S. cerevisiae* strains^a

Strain	Description	Cultivation	Sugar(s)	Rate (g h ⁻¹ g [dry wt] ⁻¹) of:		Yield (g g ⁻¹ of L-arabinose) of:		Reference
				Arabinose consumption	Ethanol production ^b	Arabinitol	Ethanol	
H2561	Carries <i>Pichia stipitis</i> <i>XYL1</i> and <i>XYL2</i> , <i>Trichoderma reesei</i> <i>lad1</i> and <i>bxl</i> , and <i>XKS1</i>	Anaerobic	L-Arabinose (50 g liter ⁻¹)	–	0.35 × 10 ⁻³	–	–	Richard et al. (29)
JBY25-4M	Carries <i>B. subtilis</i> <i>araA</i> and <i>E. coli</i> <i>araB</i> and <i>araD</i>	Oxygen limited	L-Arabinose (20 g liter ⁻¹)	–	0.06–0.08	–	0.3	Becker and Boles (4)
BWY02.XA	Carries <i>P. stipitis</i> <i>XYL1</i> and <i>XYL2</i> , <i>XKS1</i> , <i>B. subtilis</i> <i>araA</i> , and <i>E. coli</i> <i>araB</i> and <i>araD</i>	Anaerobic	Glucose (20 g liter ⁻¹) and L-arabinose (20 g liter ⁻¹)	0.03 ± 0.00	–	0.77 ± 0.22	ND	Karhumaa et al. (16)
		Anaerobic	Glucose (20 g liter ⁻¹), D-xylose (20 g liter ⁻¹), and L-arabinose (20 g liter ⁻¹)	0.035 ± 0.014	–	1.14 ± 0.18	–	Karhumaa et al. (16)
TM3063	Carries <i>P. stipitis</i> <i>XYL1</i> and <i>XYL2</i> , <i>XKS1</i> , <i>B. subtilis</i> <i>araA</i> , and <i>E. coli</i> <i>araB</i> and <i>araD</i>	Anaerobic	Glucose (20 g liter ⁻¹), D-xylose (20 g liter ⁻¹), and L-arabinose (20 g liter ⁻¹)	0.029 ± 0.002	–	0.68 ± 0.17	–	Karhumaa et al. (16)
IMS0002	Carries <i>Pirromyces</i> <i>xylA</i> , <i>XKS1</i> , and <i>L. plantarum</i> <i>araA</i> , <i>araB</i> , and <i>araD</i>	Anaerobic	L-Arabinose (20 g liter ⁻¹)	0.70 ± 0.02	0.29 ± 0.01	<0.001	0.43 ± 0.00	This work
		Anaerobic	Glucose (20 g liter ⁻¹) and L-arabinose (20 g liter ⁻¹)	0.42 ± 0.01 ^c	0.18 ± 0.00 ^d	<0.001	0.45 ± 0.00	This work

^a Except for rates of ethanol production by strains H2561 and JBY25-4M, yields of arabinitol production by strain IMS0002, and the yield of ethanol production by strain JBY25-4M, values presented are the averages ± the standard deviations as presented within the cited references. ND, not detected; –, not determined.

^b Ethanol production rate during arabinose consumption.

^c Maximum arabinose consumption rate during the arabinose consumption phase.

^d Maximum ethanol production rate during the arabinose consumption phase.

To test the capability for cofermentation of D-glucose, D-xylose, and L-arabinose, strain IMS0002 was grown anaerobically in a mixture of 30 g liter⁻¹ D-glucose, 15 g liter⁻¹ D-xylose, and 15 g liter⁻¹ L-arabinose. Despite high levels of expression of both the xylose isomerase and xylulokinase genes (Fig. 3), strain IMS0002 was not able to ferment xylose into ethanol during an anaerobic batch fermentation with mixed substrates. Instead, a considerable amount of xylitol was produced (data not shown).

DISCUSSION

The functional expression of the bacterial genes encoding the L-arabinose pathway has proven to be the most successful approach to obtain an L-arabinose-fermenting *S. cerevisiae* strain. Becker and Boles (4) have shown previously that the combination of the expression of the *B. subtilis* L-arabinose isomerase gene (*araA*) and the *E. coli* ribulokinase (*araB*) and ribulose-5-phosphate epimerase (*araD*) genes and sequential selection experiments results in an *S. cerevisiae* strain capable of growing on L-arabinose and fermenting it into ethanol under oxygen-limited conditions. In this work, strain RWB220, a strain with increased levels of expression of genes encoding enzymes of the pentose phosphate pathway (*TAL1*, *TKL1*, *RPE1*, and *RKII*), was transformed with plasmids pRW231 and pRW243, containing the *L. plantarum* *araA*, *araB*, and *araD* genes under the control of yeast promoters expected to be constitutively strong. Kuyper and coworkers (20) have demonstrated that the increased expression of *TAL1*, *TKL1*, *RPE1*, and *RKII* in a D-xylose isomerase-expressing *S. cerevisiae* strain dramatically improves growth on D-xylose and its fermentation into ethanol. Since the bacterial pathways for D-xylose and L-arabinose metabolism both enter the pentose pathway via the intermediate xylulose-5-phosphate, one would expect that the increased expression of the genes encoding the enzymes of the pentose phosphate pathway would also benefit growth on L-arabinose. This idea is supported by the work of Becker and Boles (4), who found that the expression of *TAL1* (transaldolase) in particular is increased in an *S. cerevisiae* strain selected for growth on L-arabinose. Despite the increased expression levels of the pentose phosphate pathway genes and the functionally expressed *araA*, *araB*, and *araD* genes (Fig. 3), no immediate growth on L-arabinose was observed. This may imply that the level of expression of one or more of these genes, and thus the activity of the corresponding enzyme(s), was not sufficient for growth on L-arabinose. In particular, the level of expression of *araD* in strain IMS0001 was very low (Fig. 3). Since enzyme activities could not be determined due to the unavailability of the required biochemicals, no clear conclusion can be drawn here.

Long-term selection in synthetic medium with L-arabinose as the sole carbon source resulted in an efficiently L-arabinose fermenting strain (IMS0002) that was able to grow on L-arabinose as the sole carbon source under anaerobic conditions with a specific growth rate of 0.05 h⁻¹ (Table 3), deduced from measurements of dry weight. The increase of 52- and 90-fold in (relative) levels of *araB* and *araD* transcripts, respectively, after the extensive selection on arabinose strongly supports the idea that an increase in the activity of L-ribulokinase and L-ribulose-5-phosphate 4-epimerase contributed to the improved (anaerobic)

growth on arabinose and that these enzymes may have been limiting the arabinose utilization in the parental strain, IMS0001. In addition, the expression of *araA* increased, indicating that increased isomerase activity also contributed to the improved utilization of arabinose. The increased level of *araB* expression, however, is not consistent with the data in an earlier report of Becker and Boles, who described an engineered *S. cerevisiae* strain selected for growth on L-arabinose (4). Their evolved strain had acquired a mutation in the *araB* gene, resulting in decreased L-ribulokinase activity compared to that in the original (unevolved) strain. This finding was interpreted as an effect of a high (uncontrolled) level of expression of sugar kinases such as L-ribulokinase and D-xylulokinase on yeast physiology (e.g., rapid ATP depletion and/or toxic levels of phosphorylated sugars) (15). It seems plausible that the levels of activity of the enzymes of the pentose phosphate pathway in strain IMS0002, which were much higher than those in the strain constructed by Becker and Boles, may have prevented the accumulation of the phosphorylated sugars L-ribulose-5-phosphate and/or D-xylulose-5-phosphate. In addition, a higher level of flux through the nonoxidative pentose phosphate pathway, resulting in an increased flux through glycolysis and thereby increased ATP generation, may allow for higher in vivo activity of the initial phosphorylating enzyme L-ribulokinase.

The evolved strain IMS0002 displayed promising kinetics with respect to arabinose consumption and ethanol production during anaerobic cultivation on arabinose or mixed sugars. To our knowledge, the maximum specific arabinose consumption rate of 0.70 g h⁻¹ g (dry weight)⁻¹, the ethanol production rate of 0.29 g h⁻¹ g (dry weight)⁻¹, and the yield of 0.43 g g⁻¹ are the highest rates and yields reported for engineered *S. cerevisiae* strains (Table 4). In contrast to previously described yeast strains or *S. cerevisiae* strains engineered for growth on arabinose, strain IMS0002 did not form any detectable arabinitol (Table 3), and this lack of arabinitol formation contributed to high ethanol yields.

Although the arabinose in the anaerobic cultures was completely consumed, a decreasing arabinose consumption rate over time at arabinose concentrations below approximately 50 mmol liter⁻¹ was observed (Fig. 4 and 5). This decrease may indicate a low affinity for arabinose of the arabinose transporter. Alternatively, the relatively high *K_m* of the *L. plantarum* arabinose isomerase for arabinose (28 mM) (11) may have contributed to the decreasing arabinose consumption rates. In addition, strain IMS0002 exhibited a reduced arabinose consumption rate when grown in mixtures of arabinose and glucose compared to that in the single-sugar fermentation with arabinose as the sole carbon source. This observation is similar to that for xylose consumption by the engineered *S. cerevisiae* strain RWB217 in mixtures of glucose and xylose (20) and may indicate that the expression of the transporter(s) responsible for arabinose transport (possibly Gal2p [4, 17]) is hampered by the preceding growth on glucose.

The xylose-fermenting *S. cerevisiae* strain RWB218, which was used as a platform for the present study on L-arabinose fermentation, was shown to retain its xylose fermentation characteristics during several cycles of nonselective batch cultivation on glucose (M. Kuyper et al., unpublished results). Nevertheless, the ability to ferment xylose in anaerobic cultures

was lost during the long-term selection for improved L-arabinose fermentation. This finding should not be interpreted to indicate a specific, inherent instability of this xylose-fermenting strain background. During long-term (200-generation) glucose-limited, aerobic chemostat cultivation of wild-type *S. cerevisiae*, even the high glycolytic capacity of wild-type *S. cerevisiae* is strongly reduced (13, 25). The latter observation has been interpreted as the result of selection for spontaneous mutants that synthesize fewer "excess," energetically expensive glycolytic proteins. Prolonged cultivation (>1,000 generations) on a carbon source other than xylose may well exert a similar selective pressure on the expression of genes involved in xylose metabolism. Future work will focus on functionally expressing the two pentose pathways in a single strain and on further improvement of the kinetics of mixed-sugar consumption and ethanol production.

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