Enhancing ethanol yields through D-xylose and L-arabinose co-fermentation after construction of a novel high efficient L-arabinose-fermenting Saccharomyces cerevisiae strain

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Abstract

Lignocellulose contains two pentose sugars, L-arabinose and D-xylose, neither of which is naturally fermented by first generation (1G) ethanol-producing Saccharomyces cerevisiae yeast. Since these sugars are inaccessible to 1G yeast, a significant percentage of the total carbon in bioethanol production from plant residues, which are used in second generation (2G) ethanol production, remains unused. Recombinant Saccharomyces cerevisiae strains capable of fermenting D-xylose are available on the market; however, there are few examples of L-arabinose-fermenting yeasts, and commercially, there are no strains capable of fermenting both D-xylose and L-arabinose because of metabolic incompatibilities when both metabolic pathways are expressed in the same cell. To attempt to solve this problem we have tested D-xylose and L-arabinose co-fermentation. To find efficient alternative L-arabinose utilization pathways to the few existing ones, we have used stringent methodology to screen for new genes (metabolic and transporter functions) to facilitate L-arabinose fermentation in recombinant yeast. We demonstrate the feasibility of this approach in a successfully constructed yeast strain capable of using L-arabinose as the sole carbon source and capable of fully transforming it to ethanol, reaching the maximum theoretical fermentation yield (0.43 g g⁻¹). We demonstrate that efficient co-fermentation of D-xylose and L-arabinose is feasible using two different co-cultured strains, and observed no fermentation delays, yield drops or accumulation of undesired byproducts. In this study we have identified a technically efficient strategy to enhance ethanol yields by 10 % in 2G plants in a process based on C5 sugar co-fermentation.

INTRODUCTION

Second generation (2G) bioethanol is based on the use of lignocellulosic material from agricultural, forestry and urban residues; this material consists of lignocellulose and hemicellulose. While hydrolysis of cellulose yields C6 sugars, hydrolysis of hemicellulose yields glucose and several C5 sugars, such as D-xylose and L-arabinose, which first generation (1G) Saccharomyces cerevisiae (referred to from now on in this manuscript as yeast) is unable to ferment. The advantages of converting cellulosic waste residues into biofuel instead of using food-based material such as corn grains or sugarcane crops have been discussed before [1, 2]. Saccharification of cellulose and hemicellulose in lignocellulosic materials is rather recalcitrant, and requires relatively expensive complex enzymatic cocktails [3, 4]. In order for the 2G process to be economically feasible, high fermentation yields, including the complete fermentation of D-xylose and L-arabinose, are required [1, 2, 5, 6]. D-xylose and L-arabinose are metabolized by various organisms through the pentose phosphate pathway (PPP). For these sugars, there exist bacterial and fungal canonical pathways that converge at the chemical intermediate D-xyulose-5-P (Fig. 1) [7]. Fungal pathways include redox reactions, which come with a major challenge, namely co-factor imbalance. For example, the xylulose reductase favours NADPH consumption, while the xylitol dehydrogenase uses NAD⁺ as substrate which hampers fermentation efficiencies [8]. Thus, bacterial pathways seem more suitable for use in recombinant yeast, because they require fewer modifications. Regardless of the origin of the pathways, previous articles demonstrate that both C5 sugars can be efficiently fermented by genetically engineered yeast, and strains exist that are able to ferment them completely to ethanol [7, 9]. Yet, mainly due to metabolic incompatibilities between the two pathways (explained in detail in the Discussion), no strains currently exist that are...
able to simultaneously and fully transform both C5 sugars to ethanol [10–13].

Given the composition of lignocellulosic raw material, which comprises more D-xylose than L-arabinose, the number of characterized genes and strains constructed to ferment D-xylose outnumbers by far those designed to ferment L-arabinose [9, 14, 15]. In fact, there are few examples of gene pathways engineered in yeast to ferment L-arabinose, and the use of such pathways is patent-protected. This might turn out to be a limitation for the improvement of efficient 2G fermentation technologies. Successfully engineered L-arabinose-fermenting yeasts have been constructed through expression of the bacterial pathway comprising genes araA (L-arabinose isomerase), araB (ribulokinase) and araD (L-ribulose-5-phosphate 4-epimerase) [7, 9, 10, 12, 13, 16, 17]. In most instances, the ara genes used belonged to a chimeric pathway formed by araA from Bacillus subtilis and araBD from Escherichia coli [10, 16], or all three genes from Lactobacillus plantarum [17, 18]. The scenario is similar when it comes to specific L-arabinose transporters, for which there are only a few examples in the literature [19–21].

Our study aims to address this shortcoming by identifying novel L-arabinose pathways. To achieve this, we have designed a stringent protocol to determine functional L-arabinose genes leading us to identify the arabinose operon in Pedicoccus pentosaceus and use it to engineer a novel yeast strain capable of fermenting L-arabinose. Bearing this in mind we performed a similar approach to identify and incorporate an efficient L-arabinose yeast transporter from Spathaspora passalidarium. Furthermore, given that the major challenge of efficient D-xylose and L-arabinose co-fermentation is mainly due to substrate incompatibility, instead of using complex metabolic engineering, we hypothesized that metabolic compartmentalization may represent a simple solution to the problem. To test this hypothesis, we explored whether co-fermentation of two C5 sugars could be achieved using two different yeast strains at the same time. Indeed, using an existing D-xylose-fermenting strain together with our new L-arabinose-fermenting strain, we demonstrated that this alternative approach is effective at metabolizing L-arabinose and D-xylose without any metabolic hindrance or accumulation of undesired side-products. We propose the use of this strategy as a simple and cost-effective approach to improve fermentation yields in 2G ethanol production processes.

**METHODS**

**Strains and media**

All strains constructed in this study are derived from the Saccharomyces cerevisiae strain BY4741 [22]. The list of strains used can be found in Table S1 (available in the online Supplementary Material).

**Molecular biology**

The original sequences from Pedicoccus pentosaceus and Spathaspora passalidarium were codon optimized using IDT software technology. Genes were synthesized by GeneScript, and provided in a high-copy vector. All plasmids were constructed using Gibson assembly technology [23] provided by New England Biolabs. For the pACR3 plasmid, all fragments were added sequentially, beginning with the
pRS426 plasmid [24]. First, to ensure it would be an overexpressing vector, three promoters were introduced into the plasmid. The PKI promoter and terminator were amplified from the BY4741 yeast genome and assembled in the KpnI/Xhol-digested pRS426 plasmid, which introduced a KpnI site for gene cloning. Second, opposite to the PKI promoter we assembled the TDH3 promoter and terminator by linearizing the vector with Sall/HindIII. A new Sall site was then reintroduced between promoter and terminator for cloning. Third, the HKT7 promoter in combination with the PDC1 terminator was added using SmaI/NotI sites. Next, the NotI site was reconstructed between the promoter and the terminator and synthetic araABD genes were assembled sequentially at the KpnI, Sall and NotI sites, respectively, giving rise to the pACR3 plasmid (see Fig. 2). The araT gene was cloned into two different plasmids: pRS413GPD and pRS425GPD [25] to yield pACR4 and pACR5 plasmids, respectively. Once more, the synthetic araT gene was Gibson-assembled at BamHI sites.

The araABD integration construct was also derived from the plasmid pACR3 by adding the NTS1 sequence, amplified in two different parts and assembled in order to flank the genes, along with promoters and URA3 gene marker (see Fig. 7a). The 5’ fragment was inserted at BamHI and the 3’ fragment was inserted at SacI. The entire construct was linearized with XbaI/Sacl. The integration of araT required a new plasmid, pACR11, derived from pUC19. First, genes and promoters from pACR4 were amplified and assembled at the BamHI site of pUC19. Next, the marker gene HIS3 was amplified from pACR4 and inserted at the KpnI site. The δ sequences were amplified from the yeast genome using primers that incorporated a PacI site, then the amplified fragment was introduced at a reconstructed BamHI site and at an artificially added AscI site. The resulting construct was linearized with PacI. A list of primers used for the Gibson assembly cloning is provided in Table S2.

Strain evolutionary adaptations

To accomplish an arabinose-fermenting strain, after introducing the genetic pathway we performed an evolutionary strain selection forced through adaptation to the medium. Once the genetic construction was transferred to the strain’s background and confirmed by PCR, cells were exposed to arabinose as a major carbon source. The basal liquid culture used for the selection was Yeast Nitrogen Base (YNB Sigma #Y0626) minimal medium without uracil and histidine for plasmid stability, and either 10 or 20 g arabinose per litre. Volumes were set at 20 ml or 100 ml culture for aerobic and anaerobic selection, respectively, in a 100 ml flask, kept at 30 °C and agitated at 200 r.p.m. Cultures were monitored for one week, and samples were removed daily to check for colony formation on plates, OD measurement and HPLC analysis. After that, 5 ml of culture was removed, washed and used to inoculate fresh media. Plasmid stability was constantly surveyed by checking growth in selective medium with glucose.

Fermentation assays

Fermentation cultures were carried out at a final volume of 2 ml, in an Erlenmeyer flask sealed with a rubber septum to create hypoxia. The fermentation temperature was set at 30 °C and culture was agitated at 200 r.p.m. The fermentation medium was YNB minimal medium with ammonium sulfate (5 g l⁻¹), which was devoid of uracil and histidine for plasmid maintenance. L-Arabinose was added at either 10 or 20 g l⁻¹ according to the experiment. Cells for fermentation were propagated aerobically in rich medium YPD with glucose (40 g l⁻¹) for 48 h, after which cells were washed and inoculated in the fermentation medium through a syringe at a final OD₆₀₀ of 10. Samples were also extracted with a syringe and filtered for HPLC analysis.

Analytical methods

All the various substrates, glucose, L-arabinose, D-xylose, ethanol, glycerol and acetic acid, were analysed using an

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Fig. 2. Schematic of plasmid constructs used to overexpress L-arabinose genes. Four constitutive promoters (P) were used to express ara genes from *Pediococcus pentosaceous* and *Spathaspora passalidarum*. The gene sequences were optimized for translation in yeast. The backbone plasmids used were pRS426 for *araBD*, and pRS413GPD or pRS425GPD for *ARAT* (see Methods for more details). *Yeast codon optimized versions.*
Agilent 1260 Infinity high-performance liquid chromatograph (HPLC) with an RID detector and an anion exchange column (Aminex HPX-87H; BioRad). The chemical was eluted with 0.005 M H₂SO₄ at a constant flow rate of 0.6 ml min⁻¹ and glucose was detected at 8.9 min. All analyses were performed using culture supernatants.

RESULTS

Isolation of L-arabinose pathway genes

To be able to use an L-arabinose-fermenting strain with freedom to operate, we set out to identify new bacterial genes involved in L-arabinose metabolism and transport. The first step was to search for appropriate genes using a bioinformatics analysis. To ensure stringent gene selection, three requirements were established. First, we only selected genes that were between 60 and 80 % homologous to known genes. This range was selected to identify genes that were functional, but that had identities below the threshold that would otherwise impede their use due to existing patents. Secondly, only organisms that were known to metabolize L-arabinose were screened, and thirdly, we searched for araBDA operons that had intact genetic structure, because loss of the required genes might suggest that they exist as residual genes that possibly serve other functions.

As a gene template for the analysis, the araBDA operon of L. plantarum was used. Protein BLAST with the different Ara proteins (AraA, AraB and AraD) was performed. Using the screening rules described above, we identified several organisms with putative candidates that were sorted using an identity matrix generated with Clustal Omega multiple alignment (see Fig. S1). Using this approach, we selected the L-arabinose-fermenting pathway from Pediococcus pentosaceus, a Gram-positive lactic acid bacterium [26], which has the complete L-arabinose operon, including a putative proton-symporter and the three metabolic genes, araB (GenBank accession number WP_011672890), araD (WP_011672891) and araA (WP_002834340). As a gene template for the L-arabinose specific transporter, we used the AraT protein of Scheffersomyces stipitis [20, 27], which is a high-affinity, low capacity transporter. We identified a protein that was 79 % identical in the yeast Spathaspora pas-salidarum (Fig. S2). This yeast is a symbiont of wood-boring beetles, and is able to assimilate C5 sugars from hemicellulose, a property relevant to the 2G bioethanol production industry [28, 29].

The identified synthetic pathway genes were cloned into yeast plasmids, using modified versions of the genes that were codon optimized for Saccharomyces cerevisiae (for sequences see Fig. S3). To achieve this, first, we constructed a triple-promoter, high-copy vector, which we called pACR1, based on the high copy number pRS425 vector containing PGK1, TDH3 and HKT7 promoters (see Methods for details). We cloned the optimized versions of araA, araB and araD downstream from the promoters to generate the pACR3 plasmid (see Fig. 2). In parallel, we inserted the araT transporter, in low- and high-copy plasmids under the GPD1 promoter (see Fig. 2) to create pACR4 and pACR5 plasmids, respectively. Interestingly, several attempts to co-transform pACR3 and pACR5 in the BY4741 wild-type strain did not yield clones (Fig. S4), indicating that harbouring both of these high-copy plasmids was detrimental for the yeast. Therefore, we selected several transformants that harboured the pACR3 and pACR4 plasmids. Many of the selected clones displayed growth defects (Fig. S4); however, one clone, which we named ARACRN5, was selected due to its favourable growth behaviour in YNB-His-Ura minimal medium with glucose as a carbon source.

L-Arabinose-utilizing yeast

Yeast cannot use L-arabinose as a carbon source even when it bears heterologous L-arabinose catabolic and transporter genes, a characteristic that arises due to the metabolic limits of the pentose phosphate pathway (PPP) [30, 31]. The PPP cycle metabolizes C5 sugars via ribulose-5-phosphate to produce ATP and reducing agents. To overcome this, most of the existing C5 yeast strains developed for either D-xylose or L-arabinose were forced to pass through an adaptive phase to select for clones exhibiting PPP with higher activity [16, 32]. This was achieved either by forced evolution or by direct overexpression of PPP cycle genes such as TLK1 and TAL1 [33]. In our case, we relied on spontaneous adaptive evolution of ARACRN5 by selecting clones able to grow in L-arabinose after a process through which the ARACRN5 strain was grown aerobically in liquid cultures with minimal YNB-His-Ura medium (for plasmid selection) containing L-arabinose (20 g l⁻¹) as the only carbon source. To facilitate the first round of growth, these cultures were supplemented with 1 g l⁻¹ yeast extract (YE) and 2 g l⁻¹ peptone. Cultures were grown for one week, after which a small aliquot of cells were removed, washed and transferred to fresh media. Fig. 3 shows that ARACRN5 grew up to the fourth week in the presence of YE and peptone, but failed to use L-arabinose as a carbon source. In the fifth pass, we reduced the amount of YE and peptone by 10-fold (to 0.1 g l⁻¹ and 0.2 g l⁻¹, respectively). This caused a change in the growth trend, followed by the measurable consumption of L-arabinose. During the sixth to eighth passes, YE and peptone were completely removed, at which point growth depended on L-arabinose utilization. The final culture, which we labelled ARACRN52, was plated and single colonies were selected that were able to use L-arabinose aerobically as a carbon source. This result confirms that ara-ABD from Pediococcus pentosaceus and araT from Scheffer-sonymes stipitis were functional and can be used to construct a C5 yeast strain. Becker and Boles [16] showed that the galactose permease Gal2p is able to transport L-arabinose when overexpressed. We confirmed that our transporter is not a spontaneously up-regulated version of GAL2. The ARACRN52 strain was cured of the pACR4 plasmid through culture in non-selective medium (through the addition of histidine). After confirming loss of the plasmid (i.e. by showing that the strain was unable to grow in histidine-deficient medium), we verified that this strain lost its capacity to grow with L-arabinose (see Fig. S5), confirming that the araT gene product was required.
**L-Arabinose fermentation requires further adaptations**

After constructing the new strain, we checked L-arabinose fermentation under anoxic conditions and for ethanol accumulation. Cells grown with L-arabinose (20 g l\(^{-1}\)) under aerobic conditions were washed, concentrated and transferred to a rubber-septum-sealed flask. As shown in Fig. 4(a), we found that L-arabinose was not metabolized in the absence of oxygen. When oxygen access was provided under the same culture conditions, L-arabinose utilization commenced (Fig. 4b). Anaerobic conditions did not affect glucose fermentation, which was still efficiently transformed to ethanol (Fig. 4c), which suggests that failed L-arabinose catabolism under anaerobic conditions in ARACRN52 was not due to a generalized fermentation deficiency.

According to the literature, under aerobic conditions, one mole of L-arabinose produces 13\(\frac{1}{3}\) moles of ATP, whereas anaerobically, the same amount of L-arabinose yields only 1\(\frac{2}{3}\) moles of ATP. Thus, under anoxic conditions, an eight-fold-higher L-arabinose influx is required to achieve an ATP production rate equal to that achieved under aerobic conditions [17]. We hypothesized that in the ARACRN52 strain, a number of small improvements to the PPP cycle enabled growth in L-arabinose aerobically, but further PPP cycle enhancements were required to enable L-arabinose consumption and ATP production under anoxic conditions. Therefore, we performed a second round of evolutionary adaptation to L-arabinose catabolism under anoxic conditions.

The first round of anoxic cultivation did not show any strain improvement (Fig. 5a), and the initial growth was...
allowed until oxygen depletion. When oxygen was completely removed before inoculation (second pass), growth was completely abolished. Not until the ninth pass (~1000 h of culture) did we observe growth, oxygen consumption, and importantly, ethanol accumulation. Indeed, consecutive passes further improved growth rates, L-arabinose consumption and ethanol accumulation, leading to yields that reached the theoretical maximum of 0.43 grams of ethanol per gram of L-arabinose (Fig. 5d) as previously reported [18].

Using fermentation assays, we confirmed the efficiency of this new strain which we named 54.29A and found that 90% of the L-arabinose was fermented to ethanol within the first 30 h of the assay. In this assay, the yield also reached the theoretical maximum of 0.43 grams of ethanol per gram of L-arabinose (Fig. 6), further confirming the success of our engineered strain to efficiently ferment L-arabinose.

Integrated L-arabinose strain

Strains bearing plasmids are arguably unstable because these plasmids can be lost when selective conditions are not maintained. In fact, we confirmed that after 72 h in YPD rich medium without the selective pressure, 40% of ARACRN52 and 70% of 54.29A viable cells lost at least one of the two plasmids (Fig. S6).

Considering that industrial fermentation procedures do not typically provide the selective conditions required to maintain plasmid retention for such strains, we investigated whether the L-arabinose genes we identified would function when integrated in the genome. Our approach involved use of homologous recombination cassettes and the targeting of two highly repetitive sequences in the yeast genome. First, for the metabolic araABD construct, the NTS1 sequence region found within the rDNA loci was targeted, for which there exist around 200 copies in chromosome XII [34]. This strategy has been used before to clone L-arabinose metabolic genes [12]. In our case, we cloned fragments of the NTS1 region flanking the araABD genes and the URA3 marker into the pACR3 plasmid (see Fig. 7). Second, for araT, we targeted δ elements, which are long terminal repeats (LTRs) flanking Ty retrotransposons. The δ elements can also be found scattered around the genome as single fragments, which resulted from recombination events between the two LTRs of a full-length Ty element. Delta sequences are by far the most abundant repetitive DNA element in the yeast genome (~300 copies), and have therefore been used extensively to integrate sequences in high copy numbers [35, 36]. Both linear fragments were transformed into a strain named 54.29AΔ representing a derivative of the 54.29A strain that was cured of plasmids, but that retained the genomic evolutionary changes.

Fig. 7 shows that the new strain with the genome-integrated constructs, which we called NTACR303, is able to grow in L-arabinose with or without oxygen, and can ferment L-arabinose to ethanol with an efficiency of 0.43 grams of ethanol per gram of L-arabinose.

Substrate co-fermentation

Fermentation of C5 sugars derived from hemicellulose requires a yeast strain that can co-ferment D-xylose and L-arabinose [7]. No such strain is commercially available due to the compatibility caveats that exist between D-xylose and L-arabinose catabolic pathways, although there have been metabolic engineering efforts to build a complete C5 strain [13].

As an alternative solution, we explored whether the incompatibility described between the D-xylose and L-arabinose pathways occurs only when they are present in the same cell. Co-fermentation of L-arabinose and D-xylose involves use of two different strains during fermentation: one designed to ferment D-xylose and a second to ferment L-arabinose. We performed this fermentation using the NTACR303 strain combined together with the commercial yeast, CelluX from Leaf Technologies, which is able to ferment glucose and D-
xylose efficiently and tolerate high levels of inhibitors produced during the pre-treatment of the cellulolytic substrate (www.lesaffreadvancedfermentations.com). In order to test the performance of our co-fermentation approach under a scenario that is more representative of industrial production, we performed fermentation in the presence of the inhibitor acetic acid (1.5 g l\(^{-1}\)), under low pH (5.2), and in the presence of the three main sugars present in hydrolysed corn stover, namely glucose (20 g l\(^{-1}\)), D-xylose (30 g l\(^{-1}\)) and L-arabinose (10 g l\(^{-1}\)).

We also compared fermentation performance when the two strains were present in different proportions. Figs 8 and S7 show that, as expected, the strains that contain specific catabolic pathways only metabolize their corresponding substrates. When the two strains are combined, co-fermentation of both substrates occurs. At times, the D-xylose strain outperforms NTACR303; this is possibly due to its trained tolerance to fermentation inhibitors, a process that has not yet been carried out in NTACR303. Both strains can ferment glucose swiftly, and the mixed fermentation (in a ratio of 25:75 CelluX : NTACR303) enhanced ethanol productivity by 10% after 50 h, and 13.8% after 120 h compared to the commercial yeast strain which only metabolizes D-xylose. This productivity enhancement occurs even when NTACR303 only uses 60% of the available L-arabinose. The reduced efficiency of NTACR303 is not due to the

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**Fig. 5.** Anaerobic growth adaptation to L-arabinose. (a) Adaptive evolution of the L-arabinose-metabolizing strain. Cells were transferred for 13 different cycles prior to obtaining a strain that grew efficiently in L-arabinose as the sole carbon source anaerobically. L-Arabinose consumption (b) and ethanol production (c) in the 4th, 8th, 10th and 12th transfers are depicted, when a clear improvement trend was observed. (d) Evolution of the fermentation yields following the same trend.

**Fig. 6.** Time-course fermentation assay using cells pre-grown with L-arabinose. L-arabinose and ethanol concentrations were determined at the indicated times with close to 90% of the L-arabinose consumed in the first 30 h.
presence of CelluX because the same kinetics were observed in its absence, but is likely the result of the inhibitor acetic acid [37, 38]. Importantly, all the consumed sugars are efficiently transformed to ethanol, and we did not detect any of the dead-end products usually accumulated in strains designed to co-ferment D-xylose and L-arabinose. Moreover, the data shown in Figs 8 and S7 demonstrate that the presence of a second strain does not hinder in any way the fermentation capacity of the other strain; thus, there is no residual delay in either D-xylose or L-arabinose consumption due to co-fermentation. Taking these results into consideration and providing that strains capable of tolerating industrial environments are used, the use of co-fermentation to metabolize D-xylose and L-arabinose represents a promising alternative to accomplish the full fermentation of hemicellulose-derived sugars to bioethanol, and is one that may enhance the profitability of bioethanol energy plants.

**DISCUSSION**

Currently, bioethanol represents the most promising substitute to petroleum because it is relatively easy to produce and can be mixed with other liquid fuels for use in existing internal combustion engines. Having said this, it should be stressed that the consumption of large tracts of land crops to produce bioethanol can affect food prices, and calculations estimate that 1G biofuels do not significantly reduce the production of greenhouse gases. Therefore, a shift towards 2G from urban wastes or land crop residues that reduce up to 60% greenhouse gas emissions without consuming extra farmland [1] is a major goal for the biofuel sector. Economically speaking, co-fermentation of C5 sugars may represent a way to increase the profitability of 2G fermentation, which is currently hampered by hardly affordable costs because of lignocellulose material pretreatment and complex enzymatic cocktails required to extract sugar monomers. Estimates suggest that co-fermentation of C5 sugars from lignocellulose will reduce costs by 20% [39].

Since D-xylose is the main C5 constituent of hemicellulose, the development of an industrial D-xylose-fermenting yeast has been prioritized and achieved. But still the percentages of xylose and arabinose may vary depending on the substrate. For example, corn stover contains 19% xylans and just 3% arabinans, whereas wheat bran contains 19% xylans and 15% arabinans [9], which demonstrates the need to develop new strains capable of fermenting L-arabinose.

The few documented examples of characterized genes and recombinant L-arabinose-fermenting yeast [7] have limited access due to intellectual property protection. As shown in this paper, one of the problems associated with the discovery of new L-arabinose genes for the engineering of recombinant yeast is the difficulty in developing a strain that can efficiently metabolize this substrate. Principally, this is because once the genes are overexpressed, the background strain must be evolved to ferment C5 sugars, mainly...
through the accumulation of mutations in the PPP cycle and genes that encode sugar transporters. Thus, in order to maximize the chances of finding functional genes before carrying out the iterative adaptive changes in the yeast, one of the main contributions of this paper is the rational framework that we devised for screening new and alternative \textit{ara} genes. The stringent approach that we have developed considers gene homology, genetic architecture and the ecological niche. Given these requirements, we focused our efforts on \textit{L}-arabinose bacterial pathways, which have been shown to be highly resilient and do not require any specific modifications to compensate for co-factor imbalances (as is the case for fungal \textit{L}-arabinose metabolism). Our current findings successfully and positively support our approach to identify new genes, while demonstrating that functional \textit{ara} genes from the bacterial strain \textit{Pediococcus pentosaceous} can be engineered into yeast. The same strategy has also enabled us to identify new \textit{L}-arabinose specific transporters that had been previously reported in fungi [19, 21]. Using the engineered recombinant pathway, we have generated a strain capable of using \textit{L}-arabinose as the sole carbon source, and of fermenting it completely to ethanol under anoxic conditions.

We used the laboratory strain \textit{BY4741} as proof of concept to engineer an \textit{L}-arabinose-fermenting strain. This laboratory strain is not adapted to the complex and inhibitory conditions present in the fermentation medium; however, its high yields warrant that transfer of the pathway to one of the parental strains used in 2G ethanol production will result in a strain that will perform appropriately in co-fermentation (Fig. 8). An added advantage of using parental industrial strains for \textit{L}-arabinose fermentation is that these strains are polyploid and the number of copies of the \textit{ara}-metabolizing genes will be higher when compared to the haploid \textit{BY4741} background. Hence, we expect improved performance if we multiply the gene copy number in the industrial strain. Furthermore, we are convinced that projecting the performance of our approach to a fully optimized strain under industrial conditions will represent a promising method for improving fermentation yields.

When it comes to \textit{D}-xylose and \textit{L}-arabinose co-fermentation in the same strain, several reports in research articles have...
numbered the real complications behind the construction of a yeast strain capable of fermenting D-xylose and L-arabinose to ethanol. Accordingly, to our knowledge, there are no commercial strains developed with this capability. The different fungal/bacterial pathway combinations attempted so far have shown serious limitations. For example, the fungal D-xylose oxidoreductase pathway from *Pichia stipitis* has been combined with bacterial L-arabinose genes from *Escherichia coli* and *Bacillus subtilis* [12, 40]. In this case, during the first step of the D-xylose pathway, D-xylose reductase transforms L-arabinose into L-arabitol. This is a dead-end product, since it subsequently inhibits L-arabinose isomerase, the first enzyme of the L-arabinose catabolism pathway [10]. Therefore, as an alternative, L-arabinose and D-xylose fungal pathways have been overexpressed in yeast combining genes from *Pichia stipitis*, *Trichoderma reesei* and *Ambrosiozyma monospora* [11]. Co-factor balancing was identified as a key parameter to adjust when D-xylose and L-arabinose were co-fermented using fungal pathways. However, the resulting strain, which had improved versions of the pathways to balance co-factors, was still only able to consume 20% of the L-arabinose after 120 h. This underperformance is not surprising, as low rates of L-arabinose consumption are typically observed for the fungal L-arabinose pathway [41, 42]. To avoid these shortcomings, new efforts should explore the use of bacterial pathways for both D-xylose and L-arabinose. Early tests of these bacterial pathways showed that, while L-arabitol did not accumulate during fermentation, D-xylose metabolic rates were lower versus canonical fungal enzymes [10]. Wisselink and colleagues improved these results by developing a strain through intensive and carefully planned adaptive evolution [13, 17]. Interestingly, forcing strain adaptation towards metabolism of one of the sugars consistently results in loss of metabolic capacity for the second sugar. To address this, a careful and prolonged adaptation procedure was devised to obtain a strain capable of fermenting both C5 sugars efficiently [13], suggesting that extensive background changes are necessary to perform both fermentations. Given the extensive adaptation procedure required for the strain and that the genes are plasmid harboured, it would be interesting to learn how genetically stable the strain would be if used in longer term fermentations or under industrial conditions.

Due to the complexities explained above, we used an alternative and simpler approach, compartmentalizing the two pathways in two different strains. This approach overcame the problems associated with substrate co-fermentation, and the presence of one of the pathways in a given strain was shown not to affect the metabolism of the other. When combined in the same culture, both strains showed the same kinetics as if they were alone and without the accumulation of undesired side-products commonly described in previous co-fermentation attempts.

Regardless of the strain issues reported above, the proof of concept we have generated worked perfectly, suggesting that co-fermentation of xylose and arabinose can occur without any delay, incompatibility or product loss. It should be noted that a 10% increase in ethanol production in a 2G ethanol plant could result in enhancement of ethanol production from nominal values of 23 million gallons per year to 25.2 million gallons per year, and with a current price of 2.5 US$ per ethanol gallon, the results are highly beneficial for an industrial plant.

The use of multiple yeast strains or the use of yeast with other micro-organisms is a commonly accepted strategy in the fermentation sector. For example, it has been used to vary and improve wine flavours [43–45] and to improve other industrial biosynthesis processes [46]. In the case of the 2G bioethanol industry, given the lack of single co-fermenting yeast, we believe that our solution for combining D-xylose and L-arabinose fermentation is a promising option that should be further developed. In terms of costs, our strategy should carefully consider the expenses associated with the propagation of two yeast strains, rather than only one. Nevertheless, considering the difficulty in developing a single efficiently co-fermenting strain, there is great potential for the use of two separate strains in industrial settings.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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