

Lactic acid production in *Saccharomyces cerevisiae* is modulated by expression of the monocarboxylate transporters Jen1 and Ady2

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Introduction

Lactic acid is commonly used in food, cosmetic and pharmaceutical industries, and new uses for this compound are emerging. That is true for production of polylactic acid, a biodegradable and biocompatible polymer, with biomedical and environmental applications that represent an annual industrial investment of several million dollars (van Maris *et al.*, 2004; Sauer *et al.*, 2010). Lactic acid can be produced by sugar fermentation, namely by lactic acid bacteria. However, these microorganisms are sensitive to low pH, and lactic acid production at industrial level requires the addition of large amounts of neutralizing agents, such as CaCO₃, NaOH and NH₄OH (Benninga, 1990). This limits lactic acid bioproduction, because it compromises the regeneration of the precipitated lactate salts, and higher costs are involved. Yeast cells are promising organisms for the production of carboxylic acids as they have simple nutritional requirements

Abstract

We aimed to manipulate the metabolism of *Saccharomyces cerevisiae* to produce lactic acid and search for the potential influence of acid transport across the plasma membrane in this process. *Saccharomyces cerevisiae* W303-1A is able to use L-lactic acid but its production in our laboratory has not previously been detected. When the L-LDH gene from *Lactobacillus casei* was expressed in *S. cerevisiae* W303-1A and in the isogenic mutants *jen1Δ*, *ady2Δ* and *jen1Δ ady2Δ*, all strains were able to produce lactic acid, but higher titres were achieved in the mutant strains. In strains constitutively expressing both LDH and *JEN1* or *ADY2*, a higher external lactic acid concentration was found when glucose was present in the medium, but when glucose was exhausted, its consumption was more pronounced. These results demonstrate that expression of monocarboxylate permeases influences lactic acid production. *Ady2* has been previously characterized as an acetate permease but our results demonstrated its additional role in lactate uptake. Overall, we demonstrate that monocarboxylate transporters *Jen1* and *Ady2* are modulators of lactic acid production and may well be used to manipulate lactic acid export in yeast cells.

and are easily manipulated, being widely used as cell factories. Additionally, they are able to grow at lower pH than bacteria, therefore tolerating the production of acids to higher levels. In attempts to produce lactic acid, several yeast species have already been metabolically manipulated through the heterologous expression of lactate dehydrogenases (Ldh; EC 1.1.1.27), namely *Saccharomyces cerevisiae* (Dequin & Barre, 1994; Porro *et al.*, 1995), *Kluyveromyces lactis* (Porro *et al.*, 1999a; Bianchi *et al.*, 2001), *Pichia spitiipis* (Ilmen *et al.*, 2007), *Zygosaccharomyces bailii* (Branduardi *et al.*, 2004), *Torulaspora delbruekii* (Porro *et al.*, 1999b), *Candida utilis* (Ikushima *et al.*, 2009) and *Candida boidinii* (Osawa *et al.*, 2009).

Saccharomyces cerevisiae was the first and most widely engineered yeast species used to produce lactic acid, by the heterologous expression of LDH genes, creating novel metabolic pathways for lactic acid production (Dequin & Barre, 1994; Porro *et al.*, 1995). Several approaches arose to improve lactic acid yield by suppressing the activity of

enzymes involved in ethanol formation, for example pyruvate decarboxylase (Pdc) and alcohol dehydrogenase (Adh), channelling the pyruvate produced in glycolysis to the synthesis of lactate (van Maris *et al.*, 2004; Ishida *et al.*, 2006; Tokuhiko *et al.*, 2009). Although inhibition of alcoholic fermentation has been achieved, accumulation of the lactic acid produced inside the cell promotes intracellular acidification and Ldh inhibition, leading to a decrease in lactic acid yield (Branduardi *et al.*, 2006; Sauer *et al.*, 2010). Efflux of the acid is, in this way, one possible target to improve lactic acid titres. Understanding in detail the metabolism and transport of short-chain monocarboxylic acids across the plasma membrane can be important in this regard. In *S. cerevisiae*, two monocarboxylate permeases were identified, Jen1 (Casal *et al.*, 1999) and Ady2 (Paiva *et al.*, 2004): Jen1 mediates the transport of lactate, pyruvate, acetate and propionate whereas Ady2 mediates the transport of acetate, propionate, formate and, according to our results, lactate, being both induced by non-fermentable carbon sources, and repressed in the presence of glucose. An additional role of Jen1 in the export of lactic acid has been demonstrated when it accumulates inside the cell (Porro *et al.*, 1999b; Branduardi *et al.*, 2006). In fact, it has been reported that overexpression of *JEN1* improved lactic acid yield (Porro *et al.*, 1999b; Branduardi *et al.*, 2006). In this work we engineered yeast strains and demonstrated the role of the monocarboxylate transporters Jen1 and Ady2 as modulators for lactic acid export by the cell.

Material and methods

Yeast strains, plasmids and media

All the yeast strains used here were derived from W303-1A and are listed in Table 1. Growth of the strains was performed at 30 °C in synthetic minimal media with 0.67%

(w/v) yeast nitrogen base, 2% (w/v) glucose and supplemented with amino acids to meet auxotrophic requirements. Yeast strains were maintained in yeast-potato-dextrose media and bacteria strains in solid Luria–Bertani medium supplemented with ampicillin (100 mg L⁻¹).

Expression of LDH, *JEN1* and *ADY2* in *S. cerevisiae*

Lactobacillus casei LDH, amplified from the vector pVT100-LDH harbouring the gene (Dequin & Barre, 1994), was cloned into the plasmid p426-GPD (Mumberg *et al.*, 1995) by the gap-repair method (Orr-Weaver *et al.*, 1983). The primers used to amplify LDH are listed in Table 2. The plasmid p426-GPD was digested with EcoRI, dephosphorylated with alkaline phosphatase and co-transformed with the PCR product. Correct clones were verified by colony PCR and sequencing.

Saccharomyces cerevisiae *JEN1* and *ADY2* were transferred from the plasmid p416-GPD, where they were formerly cloned, to p415-GPD, in order to co-express LDH and *JEN1* or *ADY2* in *S. cerevisiae*. The two genes were isolated from the plasmids where they were inserted by digestion with BamHI and XhoI, and ligated to p415-GPD digested with the same enzymes. The ligation product was used directly to transform the appropriate yeast strains. The LiAc/PEG/ss-DNA protocol was used in all assays of yeast transformations (Gietz & Woods, 2002). The vectors were rescued from the transformed strains using glass beads and commercial minipreps and transformed to *Escherichia coli*, and correct integration of the genes was verified.

Transport assays

Saccharomyces cerevisiae *jen1Δ ady2Δ*-ADY2 glucose-grown cells were harvested at mid-exponential phase, washed

Table 1. *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype	Reference
W303-1A	MATa <i>ade2 can1 ura3 leu2 his3 trp1</i>	Thomas and Rothstein (1989)
<i>jen1Δ</i>	W303-1A <i>jen1::KanMX4</i>	M. Casal collection
<i>ady2Δ</i>	W303-1A <i>ady2::KanMX4</i>	Paiva <i>et al.</i> (2004)
<i>jen1Δ ady2Δ</i>	W303-1A <i>jen1::HphMx4 ady2::KanMX4</i>	Soares-Silva <i>et al.</i> (2007)
W303-1A-LDH	W303-1A transformed with p426-LDH	This work
<i>jen1Δ</i> -LDH	<i>jen1Δ</i> transformed with p426-LDH	This work
<i>Ady2Δ</i> -LDH	<i>ady2Δ</i> transformed with p426-LDH	This work
<i>jen1Δ ady2Δ</i> -LDH	<i>jen1Δ ady2Δ</i> transformed with p426-LDH	This work
<i>jen1Δ</i> -JEN1-LDH	<i>jen1Δ</i> transformed with p426-LDH and with p415-JEN1	This work
<i>ady2Δ</i> -ADY2-LDH	<i>jen1Δ</i> transformed with p426-LDH and with p415-ADY2	This work
<i>jen1Δ ady2Δ</i> -p416-GPD	<i>jen1Δ</i> transformed with p416-GPD	This work
<i>jen1Δ ady2Δ</i> -ADY2	<i>jen1Δ</i> transformed with p416-ADY2	This work
W303-1A-LDH-p415	W303.1A transformed with p426-LDH and p415-GPD	This work
<i>jen1Δ ady2Δ</i> -LDH-p415	<i>jen1Δ ady2Δ</i> transformed with p426-LDH and p415-GPD	This work

Table 2. Primers used in this study; the sequence homologous to the vector p426-GPD is underlined

Primer	Sequence (5'–3')
LDH-p426fwd	TCTAGA <u>ACTAGTGGATCCCCGGGCTGCAGAA</u> TGGCAAGTATTACGGATAAGG
LDH-p426rev	CGACGGTATCGATAAGCTTGATATCGAATTCGACGGGTTTCGATG

twice with ice-cold water and transferred to YNB acetic acid medium (0.5% acetic acid, pH 6.0). After 6 h incubation, the derepressed cells were harvested by centrifugation, washed twice with ice-cold water and resuspended in ice-cold water to a final biomass concentration of 30–40 mg dry wt. mL⁻¹. Uptake rates of labelled lactic acid were determined as described by Casal & Leão (1995). Briefly, 10 µL of this cell suspension was mixed with 30 µL of 0.1 M KH₂PO₄ (pH 5.0) in a 10-mL conical tube. The reaction mixture was incubated for 2 min in a bath at 26 °C. The reaction was started by adding 10 µL of the radiolabelled substrate (D,L-[U-¹⁴C] lactic acid, sodium salt (CFB97) or [1-¹⁴C] acetic acid, sodium salt (CFA13), both purchased from Amersham Biosciences) at the desired concentration and pH, and stopped after 5 s by dilution, adding 5 mL of ice-cold water. The sample was immediately filtered under vacuum through a Whatman GF/C membrane and washed on the filter with 10 mL of ice-cold water. The membrane filter was transferred to a vial containing 5 mL of scintillation liquid (Opti-phase HiSafe II, LKB FSA Laboratory Supplies, Loughborough, UK) and radioactivity incorporated by the cells was measured in a Packard Tri-Carb 2200 CA liquid scintillation counter. The nonspecific adsorption of the acid to the cells or to the filters was measured by adding 5 mL of ice-cold water to the reaction mixture, before the addition of the radioactive acid. The transport kinetics best fitting the experimental values of initial uptake rates, as well as the kinetic parameters, were determined using a computer-assisted nonlinear regression analysis (GraphPad Software, San Diego, CA). Inhibition studies were assayed adding simultaneously nonlabelled lactic acid at the desired concentration and the labelled acetic acid. The pHs used for the uptake assays were chosen according to the different pKas for lactic acid (pK₁ = 3.85) and acetic acid (pK_a = 4.75), ensuring that more than 90% of the acid is in the anionic form.

Measurement of lactate, glucose and ethanol

During growth of the cultures of the strains listed in Table 1, samples of the media were collected over time and analysed for different metabolites. Lactate, glucose and ethanol were measured using diagnostic enzymatic kits (lactate assay kit, Spinreact; glucose GOD-POD assay kit, Spinreact; ethanol assay kit, Megazyme K-EtOH),

according to the manufacturers' instructions and using the appropriate dilutions. Cell growth (OD₆₀₀) was also evaluated with time.

Statistical analysis

All the experiments were repeated at least three times and the data presented are averages.

Results and discussion

Heterologous expression of *L. casei* LDH in the isogenic yeast mutants *jen1Δ*, *ady2Δ* and *jen1Δ ady2Δ*

Saccharomyces cerevisiae W303-1A is able to consume lactic acid, but its production was below the detection level under our conditions tested (data not shown). Through heterologous expression of the *LDH* gene from the lactic acid bacterium *L. casei* we have created this metabolic pathway in yeast cells. As expected, the *LDH* transformant strain was able to produce lactic acid, but after exhaustion of glucose the titres of lactic acid decreased significantly, probably due to its metabolism (Fig. 1). We therefore investigated whether the *JEN1* and *ADY2* genes, coding for monocarboxylate permeases, would influence lactic acid production and consumption profiles. To check this, the *LDH* gene from *L. casei* was also expressed in the isogenic *jen1Δ*, *ady2Δ* and *jen1Δ ady2Δ* mutants. These strains were grown on glucose and samples were collected over time to evaluate cell density, and lactic acid, glucose and ethanol concentration (Fig. 1). The growth profile and glucose consumption were very similar in the four strains (Fig. 1a and b), as well as the ability to convert glucose both to lactic acid (Fig. 1c) and ethanol (Fig. 1d). *JEN1* and *ADY2* disruption did not lead to significant differences in lactic acid production in the first few hours of growth, when glucose was still present in the medium. However, after glucose is exhausted, lactic acid concentration in the media was considerably higher in *jen1Δ* and *ady2Δ* mutants, leading to a reduction in acid consumption (Fig. 1c), an observation that was even more pronounced in the double mutant. The first step of acid metabolism is its entrance into the cell and these results highlight the important role of Jen1 and Ady2 transporters for efficient use of lactic acid.

Characterization of *Ady2* as a lactate transporter

Ady2 has previously been described as an acetate, propionate and formate transporter (Casal *et al.*, 1996; Paiva *et al.*, 2004), although its ability to transport lactate has not previ-

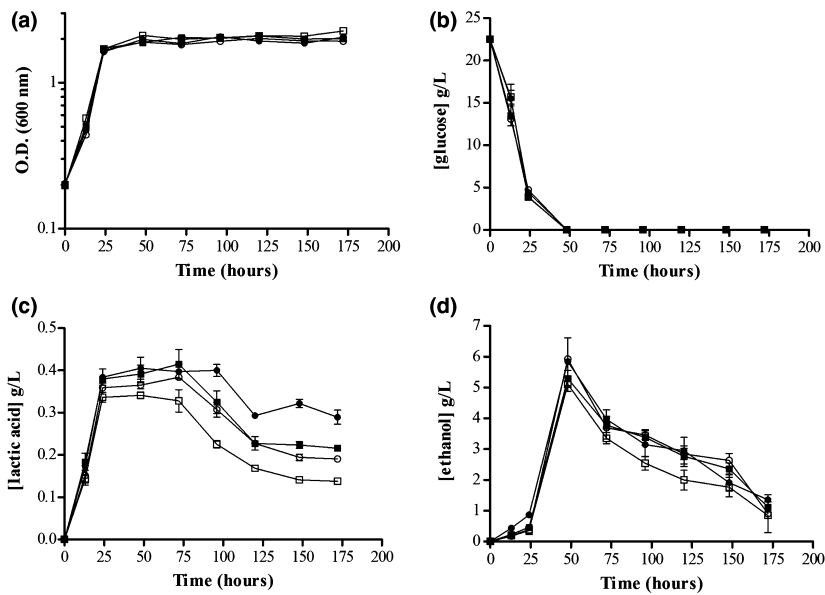


Fig. 1. Growth profile (a), and concentration of glucose (b), lactic acid (c) and ethanol (d) in the medium, for *Saccharomyces cerevisiae* W303-1A-LDH (□), *jen1*Δ-LDH (■), *ady2*Δ-LDH (○) and *jen1*Δ *ady2*Δ-LDH (●).

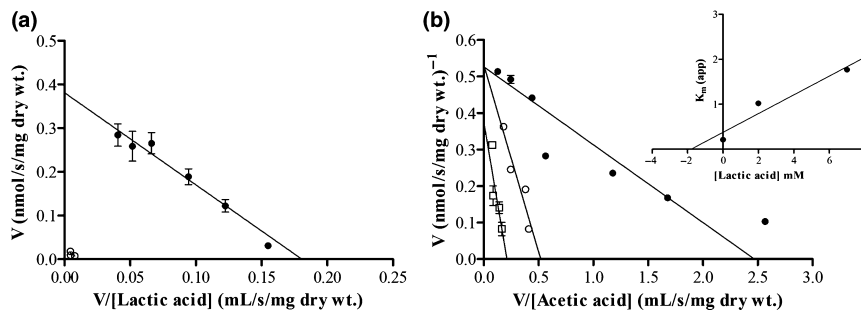


Fig. 2. Eadie–Hofstee plots of the initial uptake rates of labelled monocarboxylic acids as a function of the acid concentration in *Saccharomyces cerevisiae* strains. (a) Labelled lactic acid uptake, at pH 5.0, in *S. cerevisiae* *jen1*Δ *ady2*Δ-p416-GPD (○) and *jen1*Δ *ady2*Δ-ADY2 (●). (b) Labelled acetic acid uptake, pH 6.0, in *S. cerevisiae* *jen1*Δ *ady2*Δ-ADY2 cells. (●) Absence of other non-labelled substrate; presence of 2 (○) and 7 mM (□) cold lactic acid. Inset: competitive inhibition constant (K_i) of 1.8 mM for total lactic acid was determined, from the intercepts with the x-axis, plotting the apparent K_m of labelled acetic acid versus lactic acid concentration.

ously been reported. To clarify this, we constructed a strain lacking activity for Jen1 and constitutively expressing Ady2. The cells were grown on glucose, harvested during mid-exponential phase of growth and transferred to fresh media supplemented with acetic acid for 6 h. Michaelis–Menten kinetics were found for the initial uptake rates of labelled lactic acid, at pH 5.0, with a K_m of 2.11 ± 0.27 mM total lactic acid and a V_{max} of 0.38 ± 0.02 nmol total lactic acid s^{-1} per mg dry weight of cells (Fig. 2a). Under the same culture conditions, cells of the double mutant transformed with the empty vector p416-GPD exhibited no saturable kinetics and uptake values were negligible. Additionally, initial uptake rates of labelled acetic acid were competitively inhibited by lactic acid, demonstrating that both acids share the same permease (Fig. 2b). The kinetic parameters for acetic acid at

pH 6.0 were: K_m of 0.21 ± 0.03 mM total acetic acid and a V_{max} of 0.53 ± 0.02 nmol total acetic acid s^{-1} per mg dry weight of cells. The inhibition constant (K_i) for lactic acid was 1.8 mM, ranging from 1.4 to 2.5 mM (inset of Fig. 2b), as expected of the same order of magnitude as K_m . Together, these results indicate that Ady2 is a lactate transporter, but with a seven-fold lower affinity for lactate, when compared with Jen1 with a K_m of 0.3 mM (Casal *et al.*, 1999).

Constitutive expression of *L. casei* LDH and *JEN1* or *ADY2* in *S. cerevisiae*

The *JEN1* and *ADY2* genes were also constitutively expressed in *S. cerevisiae* *jen1*Δ-LDH and *ady2*Δ-LDH, respectively using the p415-GPD vector. Lactic acid and

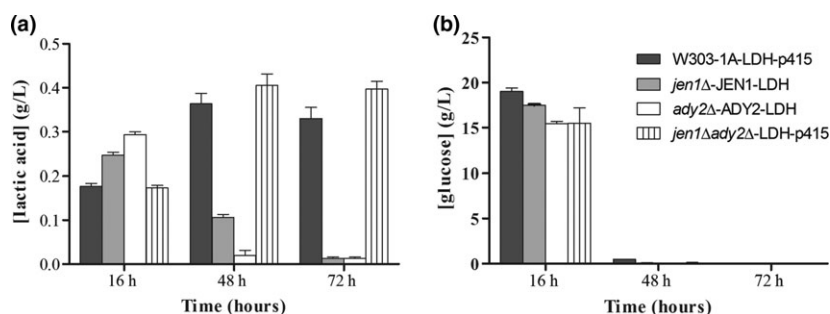


Fig. 3. Effect of *Jen1* and *Ady2* constitutive expression on lactic acid production (a) and on glucose consumption (b) over time in *Saccharomyces cerevisiae* strains constitutively expressing LDH: W303-1A-LDH-p415, W303-1A *jen1*Δ-JEN1-LDH, W303-1A *ady2*Δ-ADY2-LDH and W303-1A1A *jen1*Δ *ady2*Δ-LDH-p415.

glucose were measured over time in glucose-grown cells (Fig. 3). Constitutive expression of either *JEN1* or *ADY2* leads to higher external lactic acid concentrations when glucose is present. With regard to *JEN1* expression, these results are in agreement with those previously published (Porro *et al.*, 1999b; Branduardi *et al.*, 2006). *Jen1* is involved in lactic acid uptake, but when the acid accumulates within the cell, it can also mediate its efflux. Expression of *ADY2* gave similar results to those obtained with the constitutive expression of *JEN1*. When glucose is absent, lactic acid is totally consumed in both strains expressing *JEN1* or *ADY2*, probably due to the activity of the two transporters. Although other transporters could

be involved, here we show that the constitutive expression of *JEN1* and *ADY2* leads to higher external lactic acid concentration when glucose is present due to the role of these genes in the efflux of the acid, but when glucose is exhausted they are also involved in lactic acid uptake and consumption. As expected, and according to the data from Fig. 3, the strain *jen1*Δ*ady2*Δ-LDH-p415 did not consume lactic acid after glucose was exhausted.

Glucose is a preferential substrate for yeast cells and its presence represses metabolism of non-fermentable carbon sources, as is the case of lactic acid. In LDH-transformed cells, the fermented glucose is converted either to ethanol or to lactic acid, and the acid produced (most probably

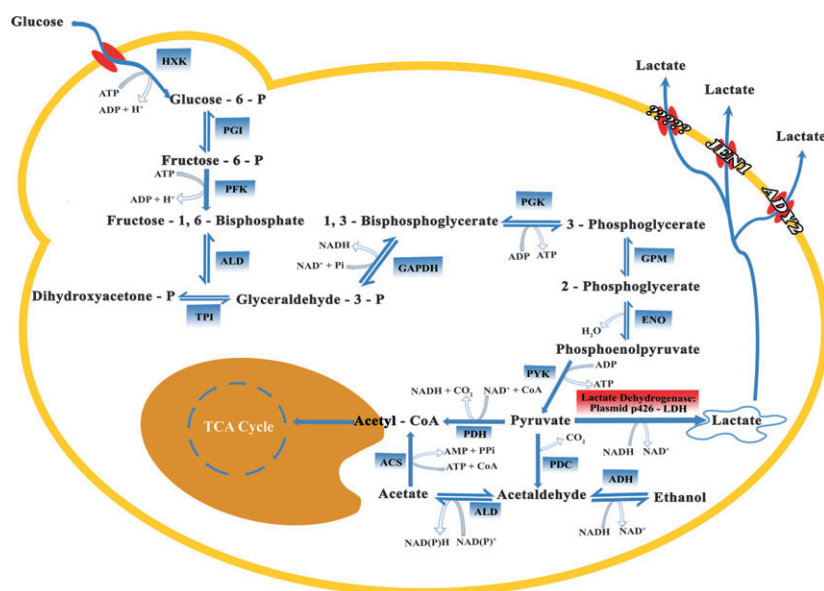


Fig. 4. Metabolic pathways involved in lactic acid production from glucose, in engineered *Saccharomyces cerevisiae* strains heterologously expressing *Lactobacillus casei* LDH. Abbreviations of enzymes: HXK, hexokinase; PGI, phosphoglucose isomerase; PFK, phosphofruktokinase; ALD, aldolase; TPI, triose phosphate isomerase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; GPM, phosphoglycerate mutase; ENO, enolase; PYK, pyruvate kinase; PDH, pyruvate dehydrogenase; PDC, pyruvate decarboxylase; ADH, alcohol dehydrogenase; ACS, acetyl-coA synthetase; LDH, lactate dehydrogenase.

the anionic form given the physiological pH of the cytoplasm) is exported via Jen1 and Ady2. However, after glucose utilization, lactic acid metabolism becomes operational and the expression of *JEN1/ADY2* also leads to a higher consumption of the acid. Figure 4 provides an overview of the main metabolic pathways involved in lactate production in the engineered yeast.

It is not yet clear whether Jen1 and Ady2 are the sole transporters carrying out efflux of the acid or if another lactic acid exporter (not yet identified) is also involved. The existence of other lactic acid exporters besides Jen1 has been previously proposed (Branduardi et al., 2006). Our results support the hypothesis that Jen1 acts as a lactic acid exporter and suggest a similar role for Ady2. However, the existence of other exporters cannot be ruled out as in the strain *jen1Δ ady2Δ*-LDH lactic acid efflux is still operational. A transcriptome analysis of *S. cerevisiae* cells constitutively expressing *JEN1* and *ADY2* could give valuable clues regarding this question.

Branduardi et al. (2006) demonstrated that the yield of lactic acid changed significantly depending on the strain and *LDH* gene used. The lactic acid titres achieved in the present study were much lower than those reported in other modified yeast strains (reviewed in Sauer et al., 2010), but higher than that for *S. cerevisiae* W303-1A transformed with a heterologous *LDH* (Branduardi et al., 2006). It is reported that *S. cerevisiae* W303-1A expressing a *Bacillus taurus* *LDH* has a lactic acid titre of 20 mg L⁻¹ after 30 h of growth in YNB glucose 2% medium (Branduardi et al., 2006). In our work we have used the *LDH* gene of *L. casei* and obtained a 15-fold higher level of lactic acid, in the same time frame. This clearly shows the influence of expression of monocarboxylate permeases in lactic acid titres. Additional metabolic manipulations, such as inactivation of *Pdc* and *Adh*, should be done in other *S. cerevisiae* strains to improve the yields of lactic acid produced by engineered strains.

As final remark, it is worth mentioning the work of Kok et al. (2012) that by using a laboratory evolution strategy of an *S. cerevisiae* *jen1Δ* strain have found two mutants with enhanced ability to grow on lactate, both located in Ady2 permease (C755G/Leu219Val and C655G/Ala252Gly), an achievement that further reinforces our observations on the role of Ady2 as a lactate transporter.

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