J Ind Microbiol Biotechnol (2003) 30: 22–27
DOI 10.1007/s10295-002-0004-2

ORIGINAL PAPER

Christopher D. Skory

# Lactic acid production by *Saccharomyces cerevisiae* expressing a *Rhizopus oryzae* lactate dehydrogenase gene

Received: 11 March 2002 / Accepted: 28 August 2002


**Abstract** This work demonstrates the first example of a fungal lactate dehydrogenase (LDH) expressed in yeast. A L(+)-LDH gene, *ldhA*, from the filamentous fungus *Rhizopus oryzae* was modified to be expressed under control of the *Saccharomyces cerevisiae adh1* promoter and terminator and then placed in a 2μ-containing yeast-replicating plasmid. The resulting construct, pLdhA68X, was transformed and tested by fermentation analyses in haploid and diploid yeast containing similar genetic backgrounds. Both recombinant strains utilized 92 g glucose/l in approximately 30 h. The diploid isolate accumulated approximately 40% more lactic acid with a final concentration of 38 g lactic acid/l and a yield of 0.44 g lactic acid/g glucose. The optimal pH for lactic acid production by the diploid strain was pH 5. LDH activity in this strain remained relatively constant at 1.5 units/mg protein throughout the fermentation. The majority of carbon was still diverted to the ethanol fermentation pathway, as indicated by ethanol yields between 0.25–0.33 g/g glucose. *S. cerevisiae* mutants impaired in ethanol production were transformed with pLdhA68X in an attempt to increase the lactic acid yield by minimizing the conversion of pyruvate to ethanol. Mutants with diminished pyruvate decarboxylase activity and mutants with disrupted alcohol dehydrogenase activity did result in transformants with diminished ethanol production. However, the efficiency of lactic acid production also decreased.

**Keywords** Lactic acid · *Saccharomyces cerevisiae* · *Rhizopus oryzae* · Lactate dehydrogenase

C.D. Skory
Fermentation Biotechnology Research Unit,
National Center for Agricultural Utilization Research,
USDA Agricultural Research Service,
1815 N. University Street, Peoria IL 61604, USA
E-mail: skorycd@ncaur.usda.gov
Tel.: +1-309-6816275
Fax: +1-309-6816427

## Introduction

Lactic acid production has a global market in excess of 100,000 t/year [15] and predictions of increasing demand are quickly becoming a reality. Much of this growth is attributable to two emerging products, poly-lactic acid for biodegradable plastics [14] and the environmentally safe solvent ethyl lactate [23]. Both of these rely on lactic acid as a renewable raw material. With this increased demand and potential growth comes a greater interest in finding more efficient methods to produce lactic acid.

Lactic acid is often manufactured using *Lactobacillus* spp, which typically are fastidious in their growth requirements and unable to produce significant lactic acid below pH 4. The use of base or carbonates to maintain a more neutral pH can lead to decreased product solubility and requires further processing to regenerate the acid from the lactate salt [16, 17]. Several laboratories have attempted to develop genetically modified yeast capable of producing lactic acid at lower pH. *Saccharomyces cerevisiae* is generally considered a robust, acid-tolerant microorganism that is well accepted in an industrial process. In *S. cerevisiae*, the major flux of pyruvate metabolism is to ethanol, by way of pyruvate decarboxylase (PDC; EC 4.1.1.1) and alcohol dehydrogenase (ADH; EC 1.1.1.1). Providing an alternative route for regenerating $NAD^+$ through lactate dehydrogenase (LDH; E.C. 1.1.1.27), which catalyzes the reduction of pyruvate to lactate, can theoretically replace ethanolic fermentation. Dequin and Barre [6] were the first to demonstrate that *S. cerevisiae* could produce lactic acid with heterologous production of the *L. casei* LDH. While 20% of the utilized glucose was fermented to lactic acid, the majority of fermentation product was still ethanol. Minimizing PDC activity using *pdc* mutants was somewhat successful in reducing ethanol and increasing lactate, although the yields and productivities were still poor [1, 4, 18].

The goal of this research was to determine whether expression of a fungal LDH, as opposed to a bacterial

LDH, might be a more effective strategy in a yeast system. The cytoplasmic pH of *S. cerevisiae* is maintained around neutrality during growth on glucose, even with a low external pH [5]. This is in contrast to lactic acid bacteria that often respond to low external pH by allowing intracellular pH to decrease as low as pH 5 [20]. As such, bacterial LDH enzymes often have a lower pH optimum than eukaryotic LDH and, therefore, may not function as effectively in a yeast system. Furthermore, bacterial LDH frequently require fructose 1,6-diphosphate for allosteric activation [11]. Expression of bovine LDH has been evaluated in a yeast system with limited success [1, 4, 18, 19]. Currently, the only confirmed *ldh* gene isolated from a fungus is that from the L(+) lactic acid-producing *Rhizopus oryzae* [21]. In this study, results are presented to show the effectiveness of expressing this gene under control of the *S. cerevisiae adh1* promoter and terminator. Several different *S. cerevisiae* strains and fermentation conditions were tested with this modified plasmid.

## Materials and methods

### Strains and culture conditions

The yeast strains used in this study are listed in Table 1. Growth in defined medium was with 6.7 g yeast nitrogen base (YNB)/l without amino acids (Difco/Becton Dickinson, Sparks, Md.). All minimal media contained the complete amino acid supplemental mixture, CSM-minus uracil (Bio101, Carlsbad, Calif.) at the recommended concentration of 0.78 g/l. Carbon sources for defined medium were 5 g glucose/l or 30 g ethanol plus 30 g glycerol/l, respectively designated YNB-D and YNB-EG. Yeast cultures transformed according to the protocol described by Gietz and Woods [12] were selected and maintained on YNB-D. All growth was at 30 °C.

### Construction of LdhA expression vector

A 962-bp *ldhA* gene fragment [21] was altered by PCR amplification to introduce a *Bam*HI site adjacent to the ATG start codon. The modified sequence was 5′-*ggatcc*ATGGTA-3′ (the *Bam*HI site is in italics). An *Acc*I site, 39 bp downstream of the TGA stop codon, was chosen as the terminal 3′ end of the gene. The *Acc*I site was blunted by 5′→3′ T4 polymerase prior to ligation. Through subsequent subcloning, polylinker sequence was added to the 3′ end, such that the downstream sequence was 5′-*gtat*GATCCGCATGCGAGCTCGGTACCCCGGGTCGAC*ctgcag*-3′ with the *gtat* nucleotides representing the filled-in *Acc*I overhang and the *ctgcag* nucleotides being a *Pst*I site used for excising the *ldhA* fragment.

The *Bam*H1/*Pst*I *ldhA* fragment was cloned into the yeast expression plasmid pVT102-U [22], using the corresponding restriction sites found in the vector polylinker region. This vector contains the *S. cerevisiae adh1* promoter and transcription termination sequence flanking the coding region, but does not possess an ATG start codon. Therefore, translation should initiate at the ATG from the *R. oryzae ldhA*, resulting in no change from the native amino acid sequence. The pVT102-U vector also contains the yeast 2μ origin of replication and *ura3* for selection on uracil-deficient medium. All constructs were sequenced to confirm that no mutations had occurred during amplification.

### Fermentation studies

Fermentations were performed in 500-ml bioreactors constructed similarly to those described by Beall et al. [3]. Seed cultures were prepared in 250-ml baffled flasks containing 50 ml YNB-D or YNB-EG and were shaken for 18 h. The fermentation vessels, having a working volume of 300 ml, were then inoculated with 5% v/v of this seed. The fermentation media used in this study were YNB-D, adjusted to approximately 90 g glucose/l, or YPD (10 g yeast extract/l, 20 g peptone/l, 90 g glucose/l). Each medium was supplemented with 0.1 ml Antifoam-289/l (Sigma, St. Louis, Mo.) at the time of inoculation. Cultures were mixed with 25 mm magnetic stirrers at 300 rpm and the pH was maintained at 5.5, unless specified otherwise, by automatic addition of 5 M NaOH. Cultures typically were not aerated unless specifically described.

Fermentations in a Biostat B (B. Braun Biotech, Allentown, Pa.) were performed with a working volume of 1.2 l. Seed cultures were prepared and used for inoculation as before, except with YPD medium. After addition of antifoam, cultures were agitated at 630 rpm and aerated with sterile compressed air at 8 l/min. A mixture of equal volumes of 2.5 M NaOH and 2.5 M KOH was used to maintain pH 5.5. Dissolved oxygen was measured as percent saturation using a $pO_2$ electrode (model 341003057; Ingold, Columbus, Ohio). Probes were calibrated with uninoculated medium sparged with air for the 100% saturation and sparged with nitrogen (purity >99.99%) for the 0% saturation.

### Analytical procedures

Lactic acid, glycerol and glucose concentrations in the medium were analyzed by HPLC, using an HPX-87H column (Bio-Rad Laboratories) and a differential refractometer (model 410; Waters, Milford, Mass.). Concentrations of ethanol were determined by gas chromatography, using a flame ionization detector and a Porapak Q column (Supelco, Bellefonte, Pa.). At the end of the fermentation phase when glucose was completely consumed, yields were calculated by determining the total amount of accumulated product per amount of starting sugar. The product yield incorporated changes in volume due to the addition of base for pH control. The total product yield represented a carbon balance that incorporated all of the detected products and biomass production. Cellular dry weight estimates were obtained from optical densities at 600 nm (data not shown). Additionally, carbon dioxide production from ethanol fermentation was assumed to be 0.49 g carbon dioxide for each 0.51 g ethanol formed.

For the measurement of LDH activity, protein extracts were prepared in LDH extraction buffer (0.1 M Mops, pH 7.2, 10% glycerol, 1 mM dithiothreitol), to maintain protein stability. Yeasts were homogenized using a FastPrep System (Bio101, Vista, Calif.) with 0.5-mm zirconia–silica beads and then centrifuged at 15,800 *g* for 15 min at 4 °C. LDH activities were assayed at 32 °C by measuring the first-order change in absorbance at 340 nm resulting

**Table 1** Strains of *Saccharomyces cerevisiae* used in this study

| Strain | Genotype | Source | Reference |
|---|---|---|---|
| Day4 | Haploid: *ura3-52, trp1, leu2, his4, ser1* | D.R. Appling (University of Texas, Austin, Tex.) | [2] |
| InvSc1 | Diploid: *Matα, his3Δ1, leu2, trp1-289, ura3-52* | Invitrogen Corp. (San Diego, Calif.) | – |
| YSH 4.123.–1C | Haploid: *Matα, leu2-3/112, trp1-92, ura3-52, pdc1-14* | S. Hohman (Göteborg University, Göteborg, Sweden) | [8] |
| Adh1 | Haploid: *Matα, can1-100, ade2-1, lys2-1, ura3-52, leu2-3/112, trp1-*Δ901*, adh1-0* | M. Grey (Institut fur Mikrobiologie, Frankfurt am Main, Germany) | [13] |

from the oxidation of NADH after initiating the reaction by the addition of sodium pyruvate to a final concentration of 4 mM. All protein concentrations were adjusted to ensure that the change in absorbance followed first-order kinetics for a minimum of 3–5 min. Assays were performed in triplicate and 1 unit of activity was defined as the amount of enzyme necessary to convert 1 μmol NADH to $NAD^+$ in 1 min. Protein concentrations were determined using a protein assay kit (BioRad, Hercules, Calif.).

## Results and discussion

### Lactic acid production in haploid and diploid strains

*S. cerevisiae* strains Day4 and InvSc1 were each transformed with plasmids pVT102 and pLdhA68X. The genotypes of these strains were very similar, except Day4 was a haploid isolate and contained a *ser1* mutation. *S. cerevisiae* InvSc1 grew more rapidly and was expected to be better suited for fermentation applications. Inocula were grown in YNB supplemented with ethanol and glycerol, to minimize expression of the *ldhA* gene prior to initiating the fermentation.

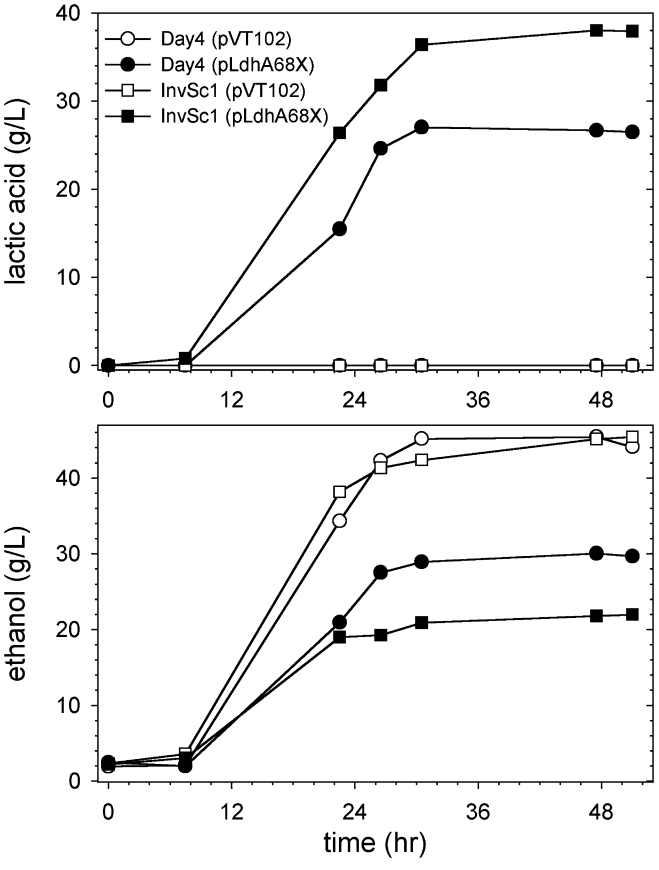


**Fig. 1** Accumulation of lactic acid and ethanol by modified strains of *Saccharomyces cerevisiae* in YPD (yeast extract/peptone/glucose) medium containing 92 g glucose/l. *hr* Hours, *L* liter

Strains transformed with pLdhA68X produced lactic acid, while none was detected by control strains that were transformed with the vector pVT102-U (Fig. 1). The accumulation of ethanol paralleled lactic acid production. This was expected, since expression of the *ldhA* is controlled by the *S. cerevisiae adh1* promoter. All of the strains utilized the initial 92 g glucose/l within 30.5 h of transfer to YPD medium. The diploid InvSc1 cultures accumulated approximately 40% more lactic acid and 27% less ethanol than the haploid Day4 strain. Cultures containing plasmid pLdhA68X appeared to produce lactic acid at the expense of ethanol, as demonstrated by the decrease in ethanol yield and increase in lactic acid production (Table 2). Ethanol yields for the Day4 and InvSc1 strains transformed with vector pVT102 were near-theoretical, which is 0.51 g ethanol/g glucose. The shift from ethanol to lactic acid production demonstrates that *R. oryzae* LDH is effective in competing with PDC for the available pyruvate pool.

### Effects of aeration, pH and medium composition on lactic acid production

Aerating cultures decreased lactic acid and ethanol accumulation by approximately 10–20% (data not shown). Therefore, fermentations in this study were performed without aeration. It was presumed that cultures were primarily anaerobic during the production phase, since the ethanol fermentation resulted in significant formation of carbon dioxide.

*S. cerevisiae* InvSc1 (pLdhA68X) was chosen for further optimization, since it performed better than the Day4 strain in preliminary studies. Fermentations to determine optimal pH were performed as before, except seed cultures were grown in YNB-D. The pH set-points for the YPD fermentation medium were maintained at several different values, from pH 3.5 to pH 6.0 (Fig. 2). Glucose was completely utilized in all of the fermentors by 32 h. The rate of lactic acid production for each of the tested pH values was similar, except for pH 3.5 and

**Table 2** Accumulation of fermentation products by strains of *S. cerevisiae*. Measurements were obtained 30.5 h after starting fermentation. The lactic acid yield is for medium supplemented with 92 g glucose/l. Total product yield includes estimated $CO_2$ (see Materials and methods)

| Strain | Maximum lactic acid (g/l) | Yield lactic acid (g/g) | Yield ethanol (g/g) | Yield glycerol (g/g) | Total product yield (g/g) |
|---|---|---|---|---|---|
| Day4 (pVT102) | 0.0 | 0.0 | 0.501 | 0.069 | 1.09 |
| Day4 (pLdhA68X) | 27.0 | 0.308 | 0.330 | 0.062 | 1.04 |
| InvSc1 (pVT102) | 0.0 | 0.0 | 0.474 | 0.075 | 1.04 |
| InvSc1 (pLdhA68X) | 38.0 | 0.445 | 0.255 | 0.058 | 1.02 |

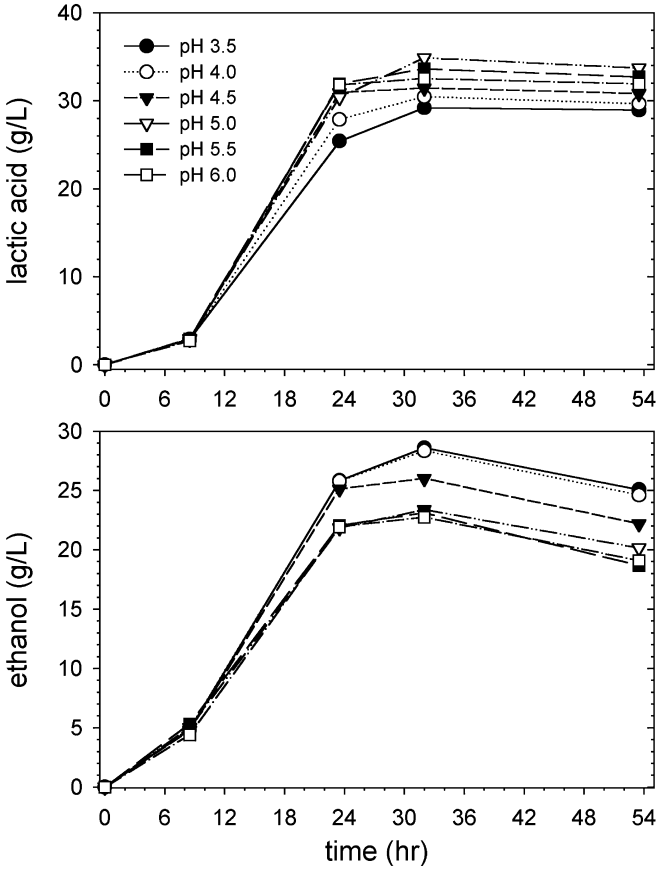


**Fig. 2** Accumulation of lactic acid and ethanol by *S. cerevisiae* InvSc1 (pLdhA68X) in YPD medium containing 92 g glucose/l maintained at varying pHs

pH 4.0. The pK of lactic acid is 3.8, so it is not surprising that some inhibition occurred at the lower pH values. The maximum accumulation of lactic acid occurred in fermentations performed at pH 5.0, with yields greater than 0.4 g lactic acid/g glucose (Table 3). Decreasing pH also resulted in increased ethanol yield and decreased glycerol yield.

The strain was next tested in a minimal medium, which would ensure that the auxotrophic selection for plasmid pLdhA68X was continuous throughout the fermentation. Inocula were grown in YNB-D medium and transferred to either YNB-D or YPD. Rates of glucose utilization and lactic acid productivity were greater in YPD medium (Fig. 3). Fermentations were completed in 31 h, compared to approximately 50 h for the minimal medium. Lactic acid yields were approximately 50% higher with YPD than with the minimal medium, while there was not much difference in ethanol production. The apparent carbon loss for strains grown in minimal medium was primarily due to increased glycerol production, which was approximately 27% higher in YNB-D (data not shown).

LDH activity was tested throughout the experiment to determine whether the difference in lactic acid production was associated with varying levels of LdhA expression. Activity was approximately 1.5 units/mg protein from cells harvested from either YNB-D or YPD. Furthermore, levels of LDH activity remained relatively constant throughout the experiment. These data suggest that plasmid loss during fermentation in YPD is not a significant factor.

## Lactic acid production by *S. cerevisiae* impaired in ethanol production

Plasmid pLdhA68X was transformed into *pdc* mutant *S. cerevisiae* YSH 4.123.–1C, in an attempt to divert more carbon to lactic acid. This strain contains a *pdc1-14* mutation that results in production of approximately 2% of wild-type PDC activity [8]. Some PDC activity is necessary, because the acetaldehyde produced from the PDC conversion is required for acetyl-CoA production [9, 10]. Seed cultures for transfer to YPD fermentation medium were grown in YNB-EG because there were uncertainties regarding the stability of the transformed strain in glucose. The rate of glucose utilization was markedly slower and fermentations resulted in slightly less lactic and ethanol production than that obtained with previous strains (Fig. 4). The reduced efficiency of fermentation may be a result of insufficient acetyl-CoA or possibly an inability of the *R. oryzae* LdhA to regenerate cytoplasmic $NAD^+$ at a sufficient rate.

A *S. cerevisiae* ADH mutant was also tested in hopes that it would minimize ethanol production, while still allowing acetyl-CoA to be synthesized from acetaldehyde. Plasmid pLdhA68X was transformed into *S. cerevisiae* Adh1, a null mutant with a *his3* replace-

**Table 3** Accumulation of fermentation products by *S. cerevisiae* InvSc1 (pLdhA68X) at different pHs. Measurements were obtained 30 h after starting fermentation with 92 g glucose/l. The lactic acid yield is for medium supplemented with 92 g glucose/l. Total product yield includes estimated $CO_2$ (see Materials and methods)

| pH | Maximum lactic acid (g/l) | Yield lactic acid (g/g) | Yield ethanol (g/g) | Yield glycerol (g/g) | Total product yield (g/g) |
|---|---|---|---|---|---|
| 3.5 | 29.2 | 0.319 | 0.327 | 0.027 | 1.01 |
| 4.0 | 30.4 | 0.338 | 0.315 | 0.028 | 1.01 |
| 4.5 | 31.4 | 0.360 | 0.298 | 0.031 | 1.00 |
| 5.0 | 34.9 | 0.432 | 0.290 | 0.035 | 1.06 |
| 5.5 | 33.6 | 0.404 | 0.278 | 0.037 | 1.01 |
| 6.0 | 32.5 | 0.398 | 0.279 | 0.042 | 1.01 |

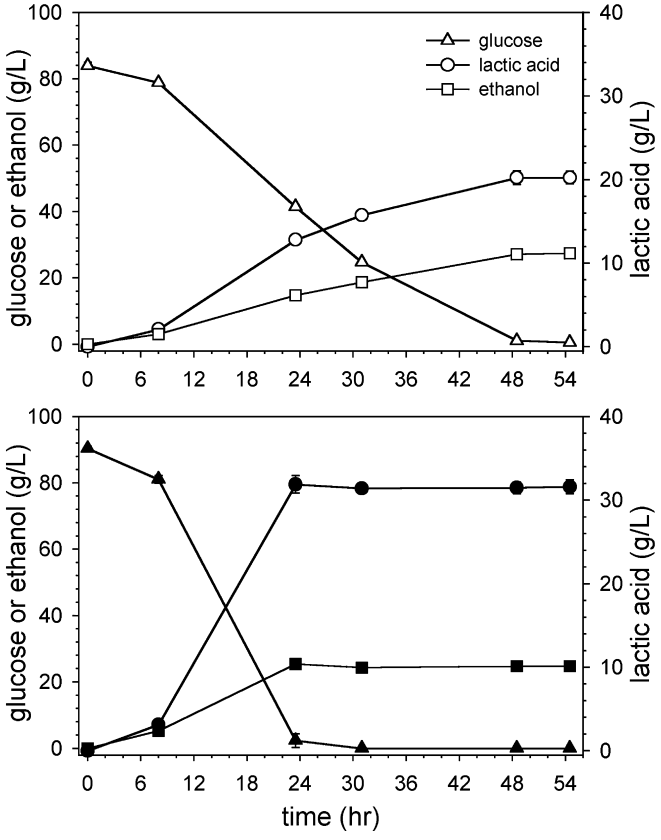


**Fig. 3** Comparing fermentations by *S. cerevisiae* InvSc1 (pLdh A68X) in minimal YNB-D (yeast nitrogen base with glucose) and complex YPD media. *Top panel* YNB-D. *Bottom panel* YPD

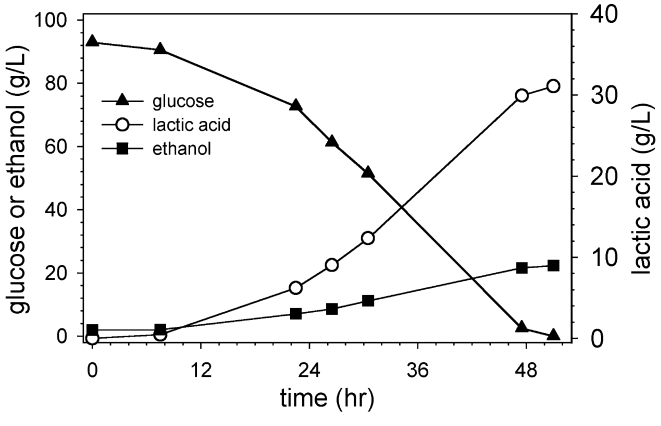


**Fig. 4** Fermentation by the *S. cerevisiae pdc* mutant (pLdhA68X) in YPD medium

ment of *adh1* [13]. The seed culture for fermentation was YPD, since growth in YNB was very poor, regardless of the carbon source. Initial fermentations failed when the inoculum was unable to grow in the fermentors. It was thought that perhaps acetaldehyde was accumulating to toxic levels, as a result of the *adh1* mutation, thus preventing further growth. Aeration of the fermentors allowed some additional growth

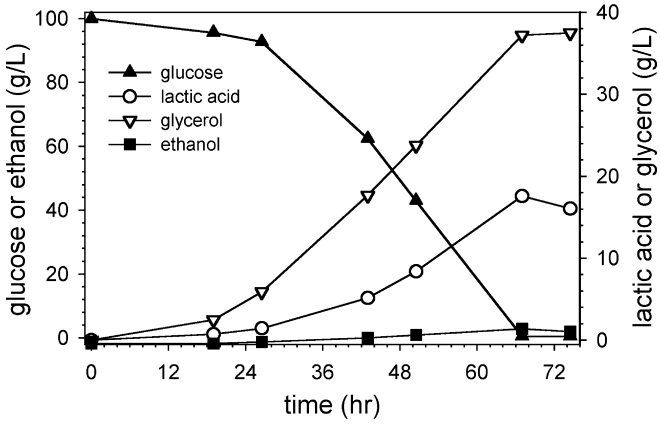


**Fig. 5** Fermentation by the *S. cerevisiae adh-0* mutant (pLdhA68X) in YPD medium

and lactic acid production, but fermentations were still unable to utilize all of the available glucose. Fermentations were finally repeated using a Braun bioreactor, which allowed for better aeration. We did not expect to suppress the alcoholic fermentation pathway, but hoped that increased oxygen availability would reduce the accumulation of acetaldehyde by accelerating the conversion to acetyl-CoA.

Fermentations with the increased aeration and stirring were such that dissolved oxygen was maintained at approximately 85% saturation. Under these conditions, strains were able to utilize all of the available glucose within 67 h (Fig. 5) and accumulated lactic acid to levels approximately 50% of that seen in *S. cerevisiae* InvSc1 (pLdhA68X) fermentations. Some ethanol was still produced during the fermentation, even though the *adh1* was disrupted. This was expected, since strains of *S. cerevisiae* deficient in functional Adh1, Adh2, Adh3 and Adh4 are still able to form ethanol, as a result of mitochondrial-pathway enzymes [7]. Interestingly, the majority of the carbon flux resulted in glycerol production with a yield of approximately 0.39 g glycerol/g glucose. Production of excess glycerol by ADH mutants was also observed by Wills and Phelps [24], who proposed that the glycerol 3-phosphate shuttle allowed regeneration of cytoplasmic $NAD^+$. The excess glycerol production seems to suggest that the LDH activity is not sufficiently meeting the cellular demand for $NAD^+$ in this strain.

This is the first demonstration of a fungal LDH expressed in yeast. Expression of the *R. oryzae* LdhA protein resulted in production of lactic acid by recombinant *S. cerevisiae* strains, although ethanol still continued to be the predominant fermentation product. The best strain was able to accumulate up to 38 g lactic acid/l with a yield of 0.44 g lactic acid/g glucose. Fermentations could be performed at pH values down to pH 3.5, but lactic acid productivity was highest around pH 5.0. Ethanol formation was minimized with the use of *pdc* or *adh* mutants. However, the efficiency of lactic acid production was also reduced in these strains. The rate and

yield of lactic acid produced by our strains was comparable with that obtained in other laboratories. Thus, it appears that there is no significant advantage of using a fungal LDH, compared with genes derived from bacterial or bovine sources.

**Acknowledgements** I thank Kristina Glenzinski for her invaluable technical contribution to this project. I also acknowledge Dr. Dean Appling, Dr. Stefan Hohmann and Dr. Martin Grey for graciously providing me with yeast strains crucial for this study. Lastly, I am grateful for Dr. Bruce Dien's assistance with the assembly of the fermentors used in this study and for his valuable review of the manuscript. Names are necessary to report factually on available data, but the USDA neither guarantees nor warrants the standard of named products; and the use of names by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

## References

1. Adachi E, Torigoe M, Sugiyama M, Nikawa JI, Shimizu K (1988) Modification of metabolic pathways of *Saccharomyces cerevisiae* by expression of lactate dehydrogenase and deletion of pyruvate decarboxylase genes for the lactic acid fermentation at low pH. J Ferment Bioeng 86:284–289
2. Barlowe CK, Williams ME, Rabinowitz JC, Appling DR (1989) Site-directed mutagenesis of yeast C1-tetrahydrofolate synthase: analysis of an overlapping active site in a multifunctional enzyme. Biochemistry 28:2099–2106
3. Beall DS, Ohta K, Ingram LO (1991) Parametric studies of ethanol production from xylose and other sugars by recombinant *Eschericia coli*. Biotechnol Bioeng 38:296–303
4. Bianchi MM, Brambilla L, Protani F, Liu CL, Lievense J Porro D (2001) Efficient homolactic fermentation by *Kluyveromyces lactis* strains defective in pyruvate utilization and transformed with the heterologous LDH gene. Appl Environ Microbiol 67:5621–5625
5. Breeuwer P, Abee T (2000) Assessment of the intracellular pH of immobilized and continuously perfused yeast cells employing fluorescence ratio imaging analysis. J Microbiol Methods 39:253–264
6. Dequin S, Barre P (1994) Mixed lactic acid–alcoholic fermentation by *Saccharomyces cerevisiae* expressing the *Lactobacillus casei* L(+)-LDH. Biotechnology 12:173–177
7. Drewke C, Thielen J, Ciriacy M (1990) Ethanol formation in *adh0* mutants reveals the existence of a novel acetaldehyde-reducing activity in *Saccharomyces cerevisiae*. J Bacteriol 172:3909–3917
8. Eberhardt I, Cederberg H, Li H, Konig S, Jordan F, Hohmann S (1999) Autoregulation of yeast pyruvate decarboxylase gene expression requires the enzyme but not its catalytic activity. Eur J Biochem 262:191–201
9. Flikweert MT, Van Der ZL, Janssen WM, Steensma HY, Dijken JP van, Pronk JT (1996) Pyruvate decarboxylase: an indispensable enzyme for growth of *Saccharomyces cerevisiae* on glucose. Yeast 12:247–257
10. Flikweert MT, Swaaf M de, Dijken JP van, Pronk JT (1999) Growth requirements of pyruvate-decarboxylase-negative *Saccharomyces cerevisiae*. FEMS Microbiol Lett 174:73–79
11. Garvie EI (1980) Bacterial lactate dehydrogenases. Microbiol Rev 44:106–139
12. Gietz RD, Woods RA (2001) Genetic transformation of yeast. Biotechniques 30: 816–826, 828
13. Grey M, Schmidt M, Brendel M (1996) Overexpression of ADH1 confers hyper-resistance to formaldehyde in *Saccharomyces cerevisiae*. Curr Genet 29:437–440
14. Hester A (2000a) IB market forecast. Ind Bioprocess 22 (5):3–5
15. Hester A (2000b) IB market forecast. Ind Bioprocess 22 (7):4–5
16. Kascak JS, Kominek J, Roehr M (1996) Lactic acid. In: Rehm HJ, Reed G, Puhler A, Stadler P (eds) Biotechnology. VCH, Weinheim, pp293–306
17. Litchfield JH (1996) Microbiological production of lactic acid. Adv Appl Microbiol 42:45–95
18. Porro D, Bianchi MM, Brambilla L, Menghini R, Bolzani D, Carrera V, Lievense J, Liu CL, Ranzi BM, Frontali L, Alberghina L (1999) Replacement of a metabolic pathway for large-scale production of lactic acid from engineered yeasts. Appl Environ Microbiol 65:4211–4215
19. Porro D, Brambilla L, Ranzi BM, Martegani E, Alberghina L (1995) Development of metabolically engineered *Saccharomyces cerevisiae* cells for the production of lactic acid. Biotechnol Prog 11:294–298
20. Siegumfeldt H, Bjorn RK, Jakobsen M (2000) Dynamic changes of intracellular pH in individual lactic acid bacterium cells in response to a rapid drop in extracellular pH. Appl Environ Microbiol 66:2330–2335
21. Skory CD (2000) Isolation and expression of lactate dehydrogenase genes from *Rhizopus oryzae*. Appl Environ Microbiol 66:2343–2348
22. Vernet T, Dignard D, Thomas DY (1987) A family of yeast expression vectors containing the phage f1 intergenic region. Gene 52:225–233
23. Watkins K (2002) A solvent business. Chem Eng News 80:15–16
24. Wills C, Phelps J (1975) A technique for the isolation of yeast alcohol dehydrogenase mutants with altered substrate specificity. Arch Biochem Biophys 167:627–637