Appl Microbiol Biotechnol (2004) 64: 237–242
DOI 10.1007/s00253-003-1480-7

ORIGINAL PAPER

C. D. Skory

Lactic acid production by *Rhizopus oryzae* transformants with modified lactate dehydrogenase activity

Received: 31 July 2003 / Revised: 25 September 2003 / Accepted: 10 October 2003 / Published online: 18 November 2003

Abstract *Rhizopus oryzae* is capable of producing high levels of lactic acid by the fermentation of glucose. Yields typically vary over 60–80%, with the remaining glucose diverted primarily into ethanol fermentation. The goal of this work was to increase lactate dehydrogenase (LDH) activity, so lactic acid fermentation could more effectively compete for available pyruvate. Three different constructs, pLdhA71X, pLdhA48XI, and pLdhA89VII, containing various lengths of the *ldhA* gene fragment, were transformed into *R. oryzae*. This fungus rarely integrates DNA used for transformation, but instead relies on extra-chromosomal replication in a high-copy number. Plasmid pLdhA48XI was linearized prior to transformation in order to facilitate integration into the *pyrG* gene used for selection. Isolates transformed with *ldhA* containing plasmid were compared with both the wild-type parent strain and the auxotrophic recipient strain containing vector only. All isolates transformed with pLdhA71X or pLdhA48XI had multiple copies of the *ldhA* gene that resulted in *ldhA* transcript accumulation, LDH specific activity, and lactic acid production higher than the controls. Integration of plasmid pLdhA48XI increased the stability of the strain, but did not seem to offer any benefit for increasing lactic acid production. Since lactic acid fermentation competes with ethanol and fumaric acid production, it was not unexpected that increased lactic acid production was always concomitant with decreased ethanol and fumaric acid. Plasmid pLdhA71X, containing a large *ldhA* fragment (6.1 kb), routinely yielded higher levels of lactic acid than the smaller region (3.3 kb) used to construct plasmid pLdhA48XI. The greatest levels of *ldhA* transcript and enzyme production occurred with isolates transformed with plasmid pLdhA89VII. However, these transformants always produced less lactic acid and higher amounts of ethanol, fumaric, and glycerol compared with the control.

Disclaimer: Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of a name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

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 (Hester 2000) 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 (Hester 2000) and the environmentally benign solvent ethyl lactate (Watkins 2002). With this increased demand and potential growth comes a greater interest in finding more efficient and less expensive methods to produce lactic acid.

Fermentations with the fungus *Rhizopus* are often preferred to bacterial systems, because the fastidious nature of *Lactobacillus* spp requires that considerable amounts of supplement (i.e. yeast extract) be added to the growth medium, adding additional cost and making product purification more difficult. Furthermore, *Rhizopus* only synthesizes L-(+)-lactic acid, which is desired for most polymer applications, while *Lactobacillus* spp typically produce mixed isomers. Recombinant yeast capable of producing stereoisometrically pure lactic acid have been developed, but poor yields make this approach only moderately successful (Adachi et al. 1988; Dequin et al. 1994; Porro et al. 1999; Skory 2003b).

One limitation with using *Rhizopus* for lactic acid synthesis is that productivity rates and yields are lower than those obtained for commercial *Lactobacillus* fermentations. Yields of lactic acid are compromised primarily because *Rhizopus* also produces ethanol with fermentative growth. In order to better understand the fermentative pathways of this fungus, the lactate dehydrogenase and

pyruvate decarboxylase genes were recently cloned (Skory 2000, 2003a). This work showed that one gene, *ldhA* (for lactate dehydrogenase; LDH), is primarily responsible for the conversion of pyruvate to lactic acid. In contrast, two different genes, *pdcA* and *pdcB* (for pyruvate decarboxylase; PDC), are required for converting pyruvate to acetaldehyde, which can then be converted to ethanol by way of alcohol dehydrogenase.

Competition and the regulation of pyruvate reduction ultimately control the fermentative flux of this organism and it was the goal of this work to direct more flow into lactic acid production. It was hypothesized that increasing LdhA activity would divert more pyruvate away from ethanol fermentation and increase the overall yield of lactic acid. Previous work confirmed that multi-copy replication of a *R. oryzae* genomic 6.1-kb fragment containing *ldhA* could increase the production of lactic acid in this fungus as a result of increased LdhA activity, compared with the parent strain (Skory 2001). However, it was unknown whether such a large fragment was necessary or even beneficial for increased lactic acid production. Therefore, this study tested several plasmid constructs containing various lengths of 5′ and 3′ untranslated region. Recognizing that plasmids typically replicate as multi-copy high-molecular-weight concatenated structures (Skory 2002), this work also included transformants having chromosomal integration of the plasmid. In most cases, increased LdhA activity achieved the goal of increased lactic acid production, while decreasing the byproducts, ethanol and fumaric acid.

Materials and methods

Construction of LdhA plasmids and strain

R. oryzae (syn. *R. arrhizus*) NRRL 395 was the source strain for all *Rhizopus* DNA used in this study. Several different *ldhA*-containing plasmids were constructed from a 6.1-kb *R. oryzae* genomic DNA fragment described by Skory (2000, 2001). This entire fragment was removed from the Lambda Zap Express vector (Stratagene, La Jolla, Calif.), utilizing the *Sal*I and *Cla*I restriction endonuclease sites present in the polylinker region. It was then ligated to plasmid pPyr225 (Skory 2002) linearized with the restriction enzymes *Xho*I and *Cla*I, to make plasmid pLdhA71X (Fig. 1). Plasmid pLdhA89VII was constructed by ligating the 4.4-kb *Hin*dIII fragment, partially filled-in with adenosine and guanine, into the *Xba*I site of pPyr225 filled-in using the same nucleotides. Finally, plasmid pLdhA48XI was made by ligating the 3.3-kb *Bsm*I fragment, blunted with T4 polymerase, into the *Sal*I and *Cla*I sites of pPyr225, treated in the same manner.

All plasmids were transformed into the *R. oryzae pyrG* mutant using microprojectile particle bombardment (BioRad, Hercules, Calif.), as described by Skory (2002). Plasmid pLdhA48XI was linearized with *Nde*I prior to transformation, to facilitate integration into the *pyrG* locus. Vector plasmid pPyr225 was also transformed and served as one of the controls, while the parent strain, *R. oryzae* NRRL 395 was used as the primary control throughout this study. Approximately 5–7 days following bombardment, spores were collected and diluted in sterile water to obtain single-spore isolates. Only one isolate per plate was used for further analysis to avoid multiple progeny originating from the same transformation event.

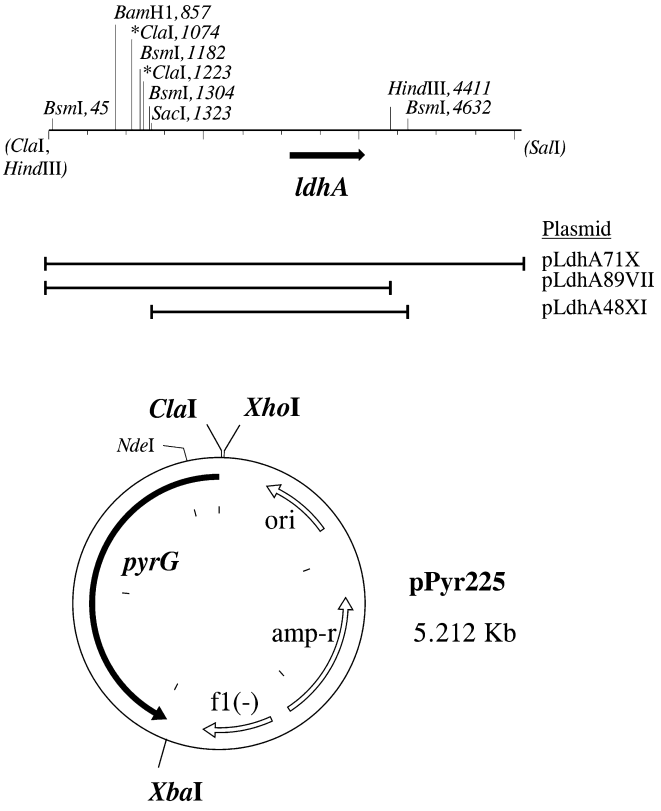

Fig. 1 Design of plasmids containing *Rhizopus oryzae ldhA*. A 6.1-kb genomic fragment was used for the construction of all plasmids in this study. Restriction enzyme sites shown in parentheses were located in the polylinker region of the Lambda zap express vector. *Asterisks* denote *Cla*I sites that are blocked from cleavage due to methylation. The *solid black arrow* for the *ldhA* gene shows the coding region. *Capped bars* represent the region of genomic DNA cloned into the selection plasmid, pPyr225, for each of the labeled constructs

Molecular analyses

Southern analyses were performed using the Genius (Boehringer Mannheim, Indianapolis, Ind.) system according to the manufacturer's recommendations. Genomic DNA was prepared as described by Skory (2002) and digested with *Bst*B1 prior to electrophoretic separation. Digoxigenin-labeled lambda DNA, cleaved with *Hin*dIII, was included in Southern hybridizations as a DNA molecular weight marker. Preparation of the *pyrG* probe for the analysis of transformants used primers PP1 (5′-ata gcg agc gtg cca aac aac-3′) and PP2 (5′-ttc aag ata tgc gtc cca acc a-3′) for PCR amplification and labeling of an internal 848-nt region of this gene. Northern analysis was performed similar to previous work (Skory 2000) with a 1,000-bp PCR-labeled *ldhA* probe, prepared using primers LP1 (5′-aag cgg tcc atg tct ctg tgt gt-3′) and LP2 (5′-ttc aac tga ttt caa tgc ctc tac a-3′). The copy number of the plasmids was estimated by comparing hybridization signal intensities for DNA isolated at the end of the bubble column fermentation. Total DNA was analyzed using a slot-blot apparatus and hybridized with the *pyrG* probe described above. Hybridization with the *pdcA* gene was used to normalize the amount of DNA bound to the membrane (Skory 2003a). All detections of hybridization blots were performed with an Image Station 1000 (Kodak, Rochester, N.Y.).

Fermentation studies

Fermentations were performed in quadruplicate with shake-flasks and in triplicate with bubble columns. Fermentation inocula were prepared in a RZ medium (Skory 2000), containing (per liter): 5 g glucose, 5 g Trypticase peptone (Becton Dickinson, Cockeysville, Md.), and 0.1 g Triton X100. *R. oryzae* spores at a final concentration of 10^5 spores/ml were grown for 24 h with shaking at 200 rpm and 30°C. It was previously shown that these conditions resulted in a dispersed reproducible inoculum that can be used for numerous applications. Shake-flask fermentations were initiated by transferring 2.5 ml inoculum to 125-ml flasks containing 50 ml RZ medium plus 100 g glucose/l and 30 g calcium carbonate/l for pH control. Incubation continued as before, except the speed of shaking was decreased to 150 rpm. The dry weight of the mycelium was calculated at the end of the fermentation by solubilizing excess calcium carbonate in 0.75 M HCl prior to harvesting the biomass by filtration.

Bubble column fermentations were similar to that previously described (Skory 2003a). Fermentors contained 760 ml RZ medium plus glucose, so that the addition of 40 ml inoculum adjusted the final concentration of glucose to 20 g/l. Mycelium was removed periodically and processed immediately to analyze the LDH specific activity and conduct a Northern analysis, as described by Skory (2000). Assays were performed in triplicate; and one unit of enzyme activity was defined as the activity necessary to convert 1 μmol NADH to NAD^+ in 1 min. Protein concentrations were determined using the BioRad Protein Assay kit. Additionally, DNA was isolated at the end of study (at 48 h) for the estimation of plasmid copy number. Lactic acid, glycerol, ethanol, and glucose concentrations in the medium were analyzed as described by Skory (2000).

Results

Isolation of *ldhA* transformants

Numerous single-spore isolates were obtained from biolistic transformation with each of the *ldhA*-containing constructs. Southern analyses confirmed that all but one of the transformants, isolate pLdhA48XI-B, contained plasmids replicating in a high-molecular-weight multi-copy concatenated structure, similar to that described by Skory (2002). None of the plasmids contained a recognition site for the *Bst*B1 used for Southern analysis and could be compared in size to the native *pyrG*, at 4.68 kb (Fig. 2). Isolates transformed with pLdhA89VII appeared to have a considerably higher copy number of plasmid than any of the other isolates.

Transformants obtained with *Nde*I-linearized pLdhA48XI were specifically screened for evidence of integration into the *pyrG* locus. Isolate pLdhA48XI-A contained both the replicating plasmid and a single-copy integration into the native *pyrG*. Isolate pLdhA48XI-B had three copies of the plasmid integrated in tandem into the *pyrG* locus, as determined by the molecular weight shift of the *pyrG* band from 4.68 kb to approx. 30 kb. Hybridization analyses with the *ldhA* and pBluescript probes did not reveal any indication of integration into the *ldh* genes or other non-specific loci for any of the transformants (data not shown).

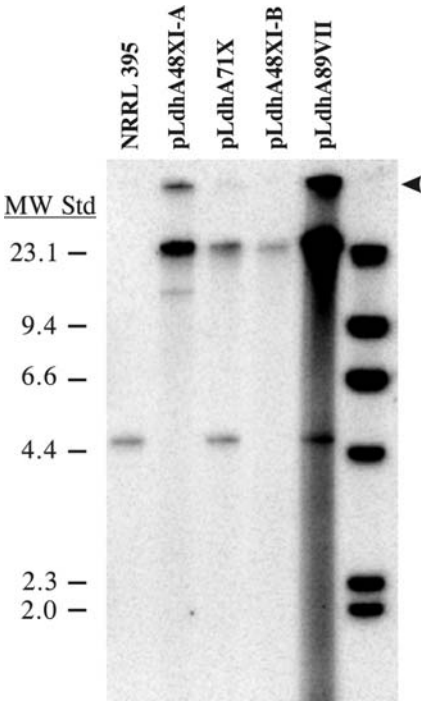

Fig. 2 Southern hybridization of *Bst*B1-digested DNA from recombinant *R. oryzae* and control strains. An internal region of the *pyrG* coding region was used as probe. Labeled *Hin*dIII fragments of mDNA were used as molecular weight standards (*MW Std*); and their sizes, in kilobases, are *shown on the left*. The *arrow on the right* marks the location of the wells. *NRRL 395* Control

Shake-flask fermentations

All but one of the transformants, *R. oryzae* pLdhA89VII, accumulated higher levels of lactic acid, compared with the wild-type *R. oryzae* NRRL 395 control (Fig. 3). Production rates for these transformants were only slightly different in the first 36 h of fermentation, while the most significant differences in the accumulation of product were after 48 h (Table 1). The biggest increase in lactic acid accumulation was from the *R. oryzae* pLdhA71X transformant, which contained the largest of the *ldhA* genomic fragments. There was approximately a 7% increase in lactic acid at both 47 h and 70.5 h, when compared with the control. Concomitant with increased lactic levels, there was a decrease in the by-products ethanol and fumaric acid in all of the transformants except *R. oryzae* pLdhA89VII. As before, the *R. oryzae* pLdhA71X transformant exhibited the greatest difference from the control, with almost a 20% decrease in ethanol and a 150% decrease in fumaric acid. *R. oryzae* transformant pLdhA89VII performed very poorly and produced approximately 42% of the lactic acid obtained with the control. The fermentations were considerably slower for this transformant and took approximately 24 h longer to completely utilize all of the glucose. Furthermore, the levels of the accumulated byproducts ethanol, fumaric acid, and glycerol increased by 130%, 900%, and 50%, respectively. All of the isolates produced 40–60 mg

Table 1 Accumulation of fermentation products by strains of *Rhizopus oryzae* at 47.0 h and 70.5 h after starting shake-flask fermentation. Data from four individual sets are shown (with SD in parentheses)

	NRRL 395	pLdh89VII	pLdhA71X	pLdhA48XI-A	pLdhA48XI-B
Product (g/l) at 47.0 h					
Lactic acid	70.9 (1.8)	27.2 (10.0)	76.0 (1.4)	74.9 (1.1)	72.8 (2.8)
Ethanol	11.2 (0.4)	18.4 (3.7)	9.0 (0.3)	9.8 (0.3)	10.5 (0.5)
Fumaric acid	0.199 (0.016)	1.652 (0.258)	0.059 (0.009)	0.099 (0.009)	0.094 (0.005)
Glycerol	2.21 (0.08)	2.69 (0.24)	1.96 (0.14)	2.16 (0.05)	2.16 (0.20)
Product (g/l) at 70.5 h					
Lactic acid	72.6 (0.7)	32.1 (8.0)	77.5 (0.7)	75.7 (0.7)	74.0 (1.7)
Ethanol	10.6 (0.7)	24.2 (1.6)	8.7 (0.6)	9.1 (0.3)	9.9 (1.0)
Fumaric acid	0.206 (0.008)	2.066 (0.152)	0.055 (0.009)	0.095 (0.010)	0.101 (0.013)
Glycerol	2.21 (0.08)	3.25 (0.17)	2.10 (0.14)	2.19 (0.05)	2.16 (0.05)

dry weight biomass/g glucose with no statistically significant differences.

Bubble column fermentations

Lactic acid accumulation for the bubble column fermentations followed the same general trends as the shake-flask fermentations (data not shown). All but one of the transformants, *R. oryzae* pLdhA89VII, accumulated higher levels of lactic acid, compared with the wild-type *R. oryzae* NRRL 395 control. Variability in lactic acid production was greater for cultures grown with bubble columns than for cultures grown with shake-flasks. However, bubble column fermentors were required so that molecular and enzymatic analyses could be performed on the culture without the interference of calcium carbonate. Only 20 g glucose/l was used, since higher amounts typically resulted in too much growth variability (e.g. clumping, pellet size) near the end of the fermentation (data not shown).

The LDH specific activity was clearly higher in all of the transformants, compared with the control (Table 2). Activity peaked early in the study and was highest at 7.5 h and 24 h. This was during the period of the highest lactic acid productivity. When lactic acid accumulation reached a plateau at 32 h, activity was decreasing for all isolates. Surprisingly, the highest LDH specific activity observed at each time-point was for isolate *R. oryzae* pLdhA89VII, which peaked at 5.7 units/mg protein. This is nearly six and three times higher, respectively, than any averaged values obtained for the control and transformants.

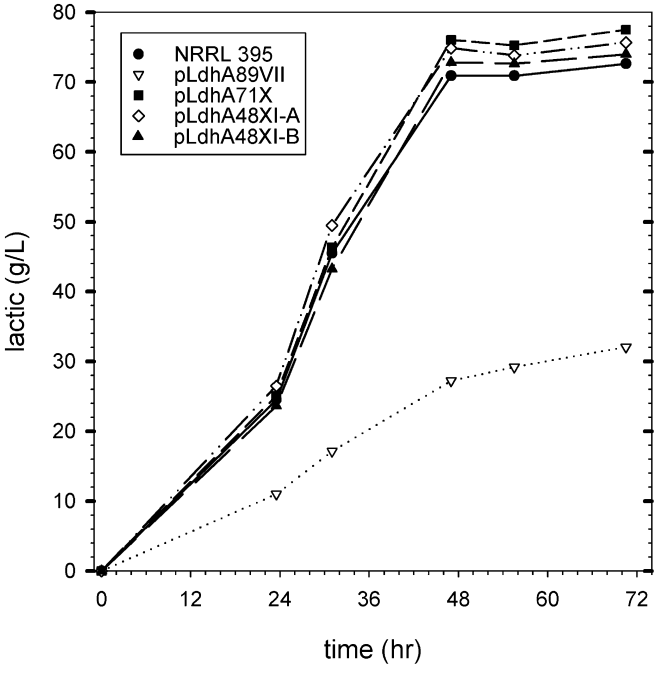

Fig. 3 Accumulation of lactic acid by modified and control strains during shake-flask growth. Each value is an average of four individual sets. *hr* Hours, *L* liter

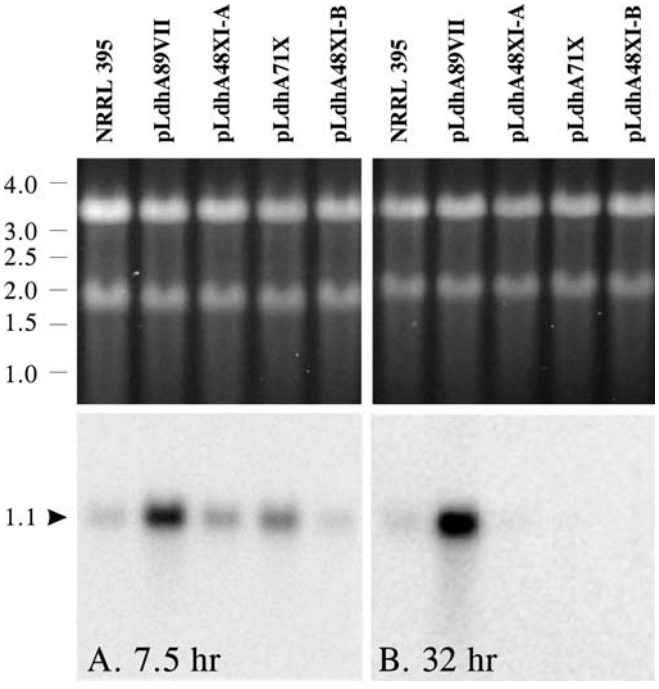

Fig. 4A, B Northern hybridization of *ldhA* transcript from recombinant *R. oryzae* and control strains during bubble column growth. RNA isolated from representative cultures at 7.5 h (**A**) and 32 h (**B**) after inoculation is *shown in the top panels*. The sizes of molecular weight standards, in kilobases, are *shown on the left*

Table 2 Lactate dehydrogenase (LDH) specific activity for strains of *R. oryzae* during bubble column fermentations. Data from three individual sets are shown (with SD in parentheses)

Time (h)	LDH specific activity (units activity/mg protein)				
	NRRL 395	pLdh89VII	pLdhA71X	pLdhA48XI-A	pLdhA48XI-B
0	0.32 (0.15)	0.83 (0.31)	0.60 (0.26)	0.70 (0.35)	0.40 (0.17)
7.5	0.88 (0.25)	3.57 (2.99)	1.70 (1.22)	2.10 (0.46)	1.45 (0.64)
24	1.07 (0.59)	5.67 (1.20)	1.13 (0.47)	1.77 (1.50)	1.27 (1.08)
32	0.30 (0.13)	3.90 (1.37)	0.80 (0.70)	0.83 (0.58)	0.73 (0.92)
48	0.17 (0.08)	1.87 (0.32)	0.27 (0.15)	0.23 (0.15)	0.17 (0.12)

Northern analysis at 7.5 h and 32 h showed that the *ldhA* transcript was considerably higher (more than ten times higher) for isolate *R. oryzae* pLdhA89VII when compared with the control (Fig. 4). Signal intensities for the remaining transformants paralleled that observed for the enzyme activities with approximately 1.5 to three times the amount of transcript, compared with the control. Transcript levels for *ldhA* were barely detectable at 32 h for all transformants, except *R. oryzae* pLdhA89VII. Analysis of the total DNA isolated at the end of the each bubble column replicate showed that the *pyrG* gene copy number was similar to that obtained in the previously described Southern analysis, with transformants having average additive copy numbers of 64, five, seven, and three for isolates pLdhA89VII, pLdhA71X, pLdhA48XI-A, and pLdhA48XI-B, respectively.

Discussion

Previous work demonstrated that *R. oryzae* could be transformed with plasmid pLdhA71X, which then resulted in multi-copy plasmid replication (Skory 2001). This increased copy number of the *ldhA* gene led to higher production of the *ldhA* transcript, higher LDH specific activity, and ultimately increased production of lactic acid. However, it was unknown whether such a large genomic *ldhA*-containing fragment was necessary to achieve this result. Therefore, one of the objectives of this study was to examine the influence of the length of the *ldhA*-flanking regions (i.e. 5′, 3′ untranslated regions), the copy number of the plasmids and the effects of integration with the recombinant construct.

The greatest challenge in this work was determining the effect of plasmid copy number, because of the lack of stability with extra-chromosomal replication (Skory 2002). It was previously determined that, while plasmids are maintained with selective conditions, their copy number can vary throughout growth and with subsequent generations (data not shown). For this reason, the plasmid copy number was determined at the end of the bubble column fermentation to ensure the continued presence of multiple copies of the *ldhA* gene in the isolates. This variability in plasmid copy number was also the rationale for using the parent strain, *R. oryzae* NRRL 395, as a control for most of these studies. Initial shake-flask fermentations were performed with isolates transformed with plasmid pPyr225. While growth and the production of lactic acid were typically near that observed for the parent strain, the variability resulted in average lactic acid levels approximately 10–20% less than *R. oryzae* NRRL 395 (data not shown). Since the goal of this study was to develop strains with lactic acid productivity higher than the parent strain, it was deemed realistic to use the wild-type isolate *R. oryzae* NRRL 395 as a control for comparison.

Isolates transformed with plasmid pLdhA71X, containing the larger *ldhA* fragment, always produced higher lactic acid levels than the control and other transformant strains. This greater efficiency could be due to regulatory factors encoded on the flanking regions of the *ldhA* gene, which may not be present in the other plasmids. However, neither *ldhA* transcription nor enzyme activity appeared to be significantly different between isolates transformed with pLdhA71X and the smaller pLdhA48XI. Increased stability of the plasmid, as a result of fragment length, is unlikely to be a sole explanation, since isolates with integrated plasmid pLdhA48XI, which are stable in *R. oryzae* without selection (Skory 2002), do not have production levels equal to that achieved with pLdhA71X.

It was anticipated that increasing LDH activity would result in decreased production of ethanol, as seen with isolates transformed with plasmids pLdhA71X and pLdhA48XI. Both LDH and PDC compete for available pyruvate and a higher metabolic flux toward lactic acid fermentation, which would understandably decrease ethanol formation. Since total mycelial biomass was similar among all of the isolates, it was concluded that these differences were due to changes in metabolic flux and not amount of growth. It was not anticipated that such a significant decrease in fumaric acid would also occur in these transformants. Fumaric acid is a product of the mitochondrial oxidation of pyruvate, but this type of high accumulation in *R. oryzae* is more likely a product of cytosolic enzymes (Friedberg et al. 1995; Kenealy et al. 1986; Osmani and Scrutton 1985; Peleg et al. 1989). This cytosolic pathway also competes for available pyruvate to form oxaloacetate with the enzyme pyruvate carboxylase. Malate dehydrogenase then catalyzes oxaloacetate to malic acid and eventually to fumaric acid by a cytosolic fumarase. It is interesting that increased LDH activity for isolates transformed with pLdhA71X and pLdhA48XI led to only a 20% decrease in ethanol, while fumaric acid was decreased by more than 75%.

It was most surprising to find that isolates transformed with pLdhA89VII produced significantly less lactic acid than any other isolates, in spite of having the highest LDH specific activity. While there was more than five times LDH specific activity compared with the control, this

certainly was not directly proportional to the high plasmid copy number and transcription. Further examination of additional isolates transformed with pLdh89VII confirmed that this plasmid always decreased lactic acid production by up to 80% (data not shown). Even more profound was that ethanol and fumaric acid increased by more than two and ten times, respectively, in this study. The increase in glycerol production is likely a result of glycerol shuttling to regenerate cellular NAD (Bakker et al. 2001; Taherzadeh et al. 2002) and may indicate a cofactor imbalance from insufficient fermentative conversion.

Because these pLdhA89VII isolates had such high levels of *ldhA* transcript and LDH specific activity associated with a poor production of lactic acid, it was speculated that either pyruvate was being depleted too rapidly (Bunch et al. 1997) or there was insufficient oxygen in the bubble columns to support the increased NADH/NAD turn-over. However, supplementation with 2.5 mM alanine, which can be directly converted to pyruvate through transamination, had a negligible effect on lactic acid production (data not shown). Additionally, bubble column fermentations with compressed oxygen did not significant improve lactic acid production. It is now thought that LDH activity may be increased to a level that compromises cofactor balancing and ultimately impairs the health of the transformant. Alternatively, LDH activity could be producing lactic acid at a rate in excess of the mechanisms to remove intracellular acid (i.e. monocarboxylic acid transporters). Such an imbalance would possibly be expected to result in cellular acidosis and general stress. Ethanol production would be more likely to continue, since ethanol fermentative enzymes have a more acidic optimal pH (Skory 2003a).

References

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

Bakker BM, Overkamp KM, Maris AJA van, Kotter P, Luttik MA H, Dijken JP van, Pronk JT (2001) Stoichiometry and compartmentation of NADH metabolism in *Saccharomyces cerevisiae*. FEMS Microbiol Rev 25:15–37

Bunch PK, Mat-Jan F, Lee N, Clark DP (1997) The *ldhA* gene encoding the fermentative lactate dehydrogenase of *Escherichia coli*. Microbiology 143:187–195

Dequin S, Barre P (1994) Mixed lactic acid-alcoholic fermentation by *Saccharomyces cerevisiae* expressing the *Lactobacillus casei*L(+)-LDH. Biotechnology 12:173–177

Friedberg D, Peleg Y, Monsonego A, Maissi S, Battat E, Rokem JS, Goldberg I (1995) The *fumR* gene encoding fumarase in the filamentous fungus *Rhizopus oryzae*: cloning, structure, and expression. Gene 163:139–144

Hester A (2000) IB Market Forecast. Ind Bioprocess 22:3–5

Kenealy W, Zaady E, DuPreez JC, Stieglitz B, Goldberg I (1986) Biochemical aspects of fumaric acid accumulation by *Rhizopus arrhizus*. Appl Environ Microbiol 52:128–133

Osmani SA, Scrutton MC (1985) The sub-cellular localisation and regulatory properties of pyruvate carboxylase from *Rhizopus arrhizus*. Eur J Biochem 147:119–128

Peleg Y, Battat E, Scrutton MC, Goldberg I (1989) Isozyme pattern and subcellular localisation of enzyme involved in fumaric acid accumulation by *Rhizopus oryzae*. Appl Microbiol Biotechnol 32:334–339

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

Skory CD (2000) Isolation and expression of lactate dehydrogenase genes from *Rhizopus oryzae*. Appl Environ Microbiol 66:2343–2348

Skory CD (2001) Fungal lactate dehydrogenase gene and constructs for the expression thereof. US Patent 6,268,189

Skory CD (2002) Homologous recombination and double-strand break repair in the transformation of *Rhizopus oryzae*. Mol Genet Genomics 268:397–406

Skory CD (2003a) Induction of *Rhizopus oryzae* pyruvate decarboxylase genes. Curr Microbiol 47:59–64

Skory CD (2003b) Lactic acid production by *Saccharomyces cerevisiae* expressing a *Rhizopus oryzae* lactate dehydrogenase gene. J Ind Microbiol Biotechnol 30:22–27

Taherzadeh MJ, Adler L, Liden G (2002) Strategies for enhancing fermentative production of glycerol—a review. Enzyme Microb Technol 31:53–66

Watkins K (2002) A solvent business. Chem Eng News 80:15–16