Efficient Production of L-Lactic Acid by Metabolically Engineered *Saccharomyces cerevisiae* with a Genome-Integrated L-Lactate Dehydrogenase Gene

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We developed a metabolically engineered yeast which produces lactic acid efficiently. In this recombinant strain, the coding region for pyruvate decarboxylase 1 (*PDC1*) on chromosome XII is substituted for that of the L-lactate dehydrogenase gene (*LDH*) through homologous recombination. The expression of mRNA for the genome-integrated *LDH* is regulated under the control of the native *PDC1* promoter, while *PDC1* is completely disrupted. Using this method, we constructed a diploid yeast transformant, with each haploid genome having a single insertion of bovine *LDH*. Yeast cells expressing *LDH* were observed to convert glucose to both lactate (55.6 g/liter) and ethanol (16.9 g/liter), with up to 62.2% of the glucose being transformed into lactic acid under neutralizing conditions. This transgenic strain, which expresses bovine *LDH* under the control of the *PDC1* promoter, also showed high lactic acid production (50.2 g/liter) under nonneutralizing conditions. The differences in lactic acid production were compared among four different recombinants expressing a heterologous *LDH* gene (i.e., either the bovine *LDH* gene or the *Bifidobacterium longum LDH* gene): two transgenic strains with 2μm plasmid-based vectors and two genome-integrated strains.

Plant- and crop-based renewable plastics (23), including polylactic acid, are polymeric materials that could be produced with a fermentation process and would be biodegraded to H_2O and CO_2 finally (25). In the sustainable society to come, renewable plastics should be available at a lower price than petroleum-based plastics currently in use. Lactic acid, which is used as a monomer for polymerization into polylactic acid, has a global market in excess of 100,000 tons per year (11), and further increased demand is predicted. Lactic acid is generally produced with lactic acid bacteria (22), such as *Lactobacillus* species, which are hard to cultivate at high density and show high auxotrophy regarding growth (13). During lactic acid fermentation, a low pH has inhibitory effects on cell growth and lactic acid production. While chemicals ($CaCO_3$, NaOH, or NH_4OH) are added to neutralize lactic acid, the processes are limited by the difficulty in the regeneration of precipitated lactates. With the potential demand for an increase in lactic acid production comes a greater interest in finding another approach to producing lactic acid.

Yeasts, such as *Saccharomyces cerevisiae*, are more tolerant to low pHs than lactic acid bacteria. Recently, new methods for producing lactic acid with genetically engineered yeast have been developed and applied for large-scale production on a trial basis. In ethanol fermentation, pyruvic acid is converted into acetaldehyde by pyruvate decarboxylase (PDC; EC 4.1.1.1), and then the acetaldehyde is converted into ethanol by alcohol dehydrogenase (EC 1.1.1.1) (29). A transgenic yeast expressing exogenous L-lactate dehydrogenase (L-LDH; EC 1.1.1.27) could produce lactic acid from pyruvic acid. Such metabolically engineered yeasts were first reported by Dequin and Barre (7) and Porro et al. (4, 27), who showed that the recombinants yielded about 10 to 20 g of lactate/liter. In both cases, a considerable amount of ethanol was produced concurrently because *S. cerevisiae* predominantly produces ethanol under anaerobic conditions. The by-product ethanol has become a problem in lactic acid fermentation with a transgenic yeast.

There are three structural *PDC* genes, *PDC1*, *PDC5* (33, 34), and *PDC6* (15), in the *S. cerevisiae* genome. To increase the metabolic flow from pyruvic acid to lactic acid, a mutant strain, such as the *pdc1*, *pdc5* (1), or *adh1* (35) mutant strain, was utilized as the genetic background for obtaining an L-LDH gene-expressing yeast. However, a remarkable improvement in L-lactic acid production has not been observed. In addition to the fact that the PDC activity in yeast is due mainly to *PDC1*, *PDC5* was observed to compensate for a PDC1 deficit, because *PDC1* deletion led to a great increase in *PDC5* promoter-driven mRNA expression (14). A double mutant strain with *pdc1* and *pdc5* exhibited significantly impaired growth on glucose medium (9, 10). The other approach for lactic acid production involved the use of a Crabtree-negative yeast, such as *Kluyveromyces lactis* (28). *K. lactis* has a single *PDC1* gene, *KlPDC1*, expressing PDC activity (2). Bianchi et al. used *K. lactis* strains lacking either PDC activity or PDC and PDH activities, transformed with the *LDH* gene placed under the control of the promoter of *KlPDC1* gene and cloned into stable multicopy vector. Transgenic *K. lactis* strains showed remarkable improvement under the fed-batch condition (3).

To achieve mass production of lactic acid by using *S. cerevi-*

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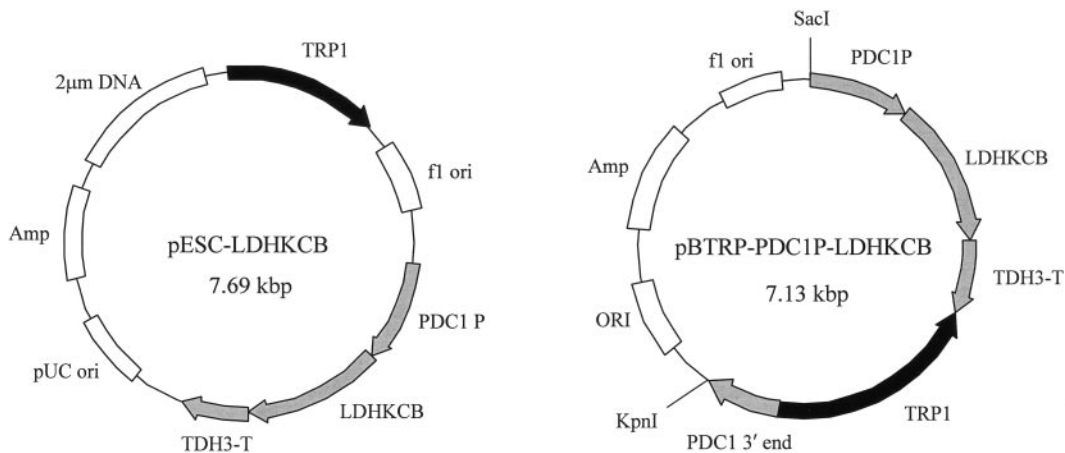† N.I. and S.S. contributed equally to this work.

FIG. 1. Maps of the plasmid vectors used for LDH expression. PDC1 P, *PDC1* promoter from *S. cerevisiae* OC-2T. This fragment was −705 bp upstream from the *PDC1* ORF start codon. TDH3-T, *TDH3* terminator fragment. LDHKCB, bovine *LDH*. PDC1 3′ end, *PDC1* downstream fragment of 518 bp in length was between +501 and +1,018 from the *PDC1* ORF start codon. Two other vectors (pESC-LDHKCY and pBTRP-PDC1P-LDHKCY) were obtained by substituting the LDHKCB in these vectors for LDHKCL (*B. longum LDH*).

siae, we have developed a recombinant strain that produces lactic acid efficiently. In this study, we found, firstly, genome-integrated *LDH* was regulated under the control of the *PDC1* promoter. Secondly, PDC1 was completely inactive. Also, two LDHs, i.e., bovine and *Bifidobacterium longum* LDHs, were compared, and they were compared with lactic acid-producing yeasts reported so far.

MATERIALS AND METHODS

Strains and media. The *Escherichia coli* strain used for molecular cloning was JM109 (TOYOBO, Osaka, Japan). *E. coli* cultivation and medium were as described previously (32). The *S. cerevisiae* OC-2T (**a**/α *trp1/trp1*) was derived from the wine yeast strain IFO2260 (30). The culture medium used for *S.*

cerevisiae was YPD medium (1% Bacto yeast extract, 2% Bacto peptone, 2% glucose, wt/vol).

Synthesis of an L-LDH gene. Bovine (GenBank accession number D90141) (17) and *B. longum* (GenBank accession number M33585) (24) *LDH* sequences were designed based on major codon usage according to the database on *S. cerevisiae* (Codon Usage Database [http://www.kazusa.or.jp/codon/]). The amino acids sequences encoded by these sequences were the same as those in the database. The experimental method was that of Horton et al. (16). Each oligonucleotide primer was synthesized at 100-mer intervals (QIAGEN GmbH, Hilden, Germany) and then fused with the overlapping region by PCR. In the PCR, KOD DNA polymerase (TOYOBO) was used for amplification. Each fragment was subcloned into the pBluescript II SK(+) vector (Stratagene, La Jolla, Calif.) according to a previous report (32). The ligase reaction was performed with a Lig Fast rapid DNA ligation system (Promega, Madison, Wis.), and the competent cells used for transformation were of the *E. coli* JM109 strain.
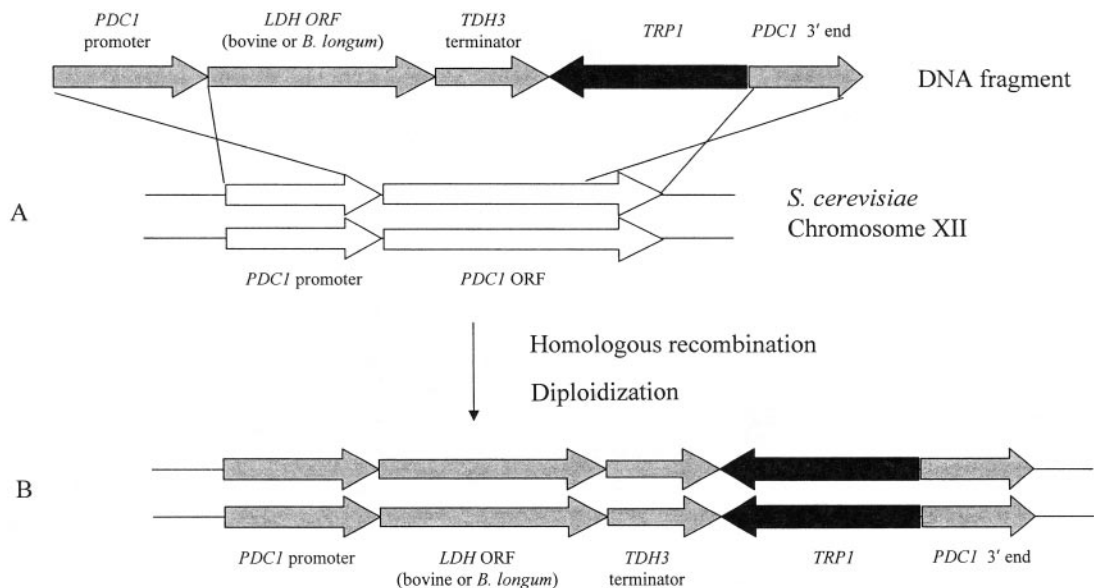

FIG. 2. The improved method for *LDH* gene expression. (A) The constructed vector was integrated into the *PDC1* locus of *S. cerevisiae* chromosome XII through homologous recombination. (B) Firstly, one copy of the target *LDH* was introduced into the host, the OC-2T strain. Secondly, diploidization occurred through spore formation. The introduced *LDH* was amplified into two copies, and the *PDC1* coding region was completely disrupted.

TABLE 1. Yeast strains used in this study

Strain	Relevant genotype	*LDH* expression cassette	Description or characteristic	Reference or source
OC-2T	**a**/α *trp1/trp1*		Host strain	31
YEBL-8A	**a**/α *pdc1/pdc1* Phl[r]	P_{PDC1}− *B. longum LDH*	YEp (2μm DNA)	This study
YEBO-1B	**a**/α *pdc1/pdc1* Phl[r]	P_{PDC1}− bovine *LDH*	YEp (2μm DNA)	This study
YIBL-2D	**a**/α *pdc1/pdc1*	P_{PDC1}− *B. longum LDH*	YIp (*PDC1* locus)	This study
YIBO-7A	**a**/α *pdc1/pdc1*	P_{PDC1}− bovine *LDH*	YIp (*PDC1* locus)	This study

To confirm the subcloning of the vector, the nucleotide sequence was determined with an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, Calif.). The resulting fragments were named LDHKCB (bovine *LDH*) and LDH-KCL (*B. longum LDH*).

Construction of plasmid vectors. Maps of the plasmids used in this study are shown in Fig. 1, and the vectors in which the L-LDH gene was replaced were pBTRP-PDC1P-LDHKCB and pBTRP-PDC1P-LDHKCL. 2μm-based multicopy plasmid vectors, including pESC-LDHKCB (Fig. 1A) and pESC-LDH-KCY, consisted of the *PDC1* promoter and *LDH* within the pESC-TRP vector (Stratagene). Also, the pBBLE-Δpdc1 vector consisted of the *PDC1* promoter, the Tn*5 BLE* gene cassette, and a *PDC1* downstream fragment. The phleomycin resistance gene was the bleomycin gene (Tn*5 BLE*) of bacterial transposon Tn*5* (11), which was fused downstream from the *S. cerevisiae TDH3* (glyceraldehyde-3-phosphate dehydrogenase 3) promoter. Integration vectors, including pBTRP-PDC1-LDHKCB (Fig. 1B) and pBTRP-PDC1-LDHKCY, consisted of the *PDC1* promoter, *LDH*, *TRP1* (tryptophan requiring 1), and the *PDC1* downstream fragment. *TRP1* was obtained by treating pRS404 (Stratagene) with AatII and SspI and ligating it to the pBluescript II SK(+) vector treatment with T4 DNA polymerase. Each fragment was isolated by PCR using the genomic DNA of the *S. cerevisiae* OC-2T strain as a template. Genomic DNA was prepared using a Fast DNA kit (Qbiogene, Carlsbad, Calif.), and the concentration was determined with an Ultraspec 300 spectral photometer (Pharmacia Biotech, Uppsala, Sweden). KOD DNA polymerase was used for PCR amplification, and the primers and their oligonucleotide sequences were as follows. For the *PDC1* promoter fragment, PDC1P-U had the sequence 5′-ATATAT<u>GGATCC</u>GCGT TTATTTACCTATCTC-3′, containing a BamHI restriction site (underlined), and PDC1P-D had the sequence 5′-ATATAT<u>GAATTC</u>TTTGATTGATTTGA CTGTG-3′, containing an EcoRI restriction site (underlined). For the *PDC1* downstream fragment, PDC1D-U had the sequence 5′-ATATAT<u>CTCGAG</u>G CAGCTAACTTCTTGGTCGAC-3′, containing a XhoI restriction site (underlined). This fragment was upstream −705 bp from the *PDC1* open reading frame (ORF) start codon. PDC1D-D had the sequence 5′-ATATAT<u>GGGCCC</u>CTCG TCAGCAATAGTGGTCAAC-3′, containing an ApaI restriction site (underlined). For the *PDC1* 3′ end, the *PDC1* downstream fragment of 518 bp in length was between +501 and +1,018 from the *PDC1* ORF start codon. The amplification fragments were treated with each restriction enzyme (TaKaRa BIO, Otsu, Japan) and were ligated to a vector, and all plasmids constructed in this work were obtained by using standard techniques (32).

Yeast transformation. *S. cerevisiae* transformation was performed by the lithium acetate procedure of Ito et al. (18). Host strain OC-2T is a diploid and homothallic strain (30). Spore formation was performed on sporulation plates (1% acetate, 0.1% D-glucose, 0.1% yeast extract, and 2% agar, wt/vol). Diploid formation was performed using the homothallic property, and tetrads were dissected under an optical microscope (Olympus, Tokyo, Japan) and a micro-

manipulator (Narishige Science, Tokyo, Japan). After the colonies had been isolated, the target gene introduction was confirmed by PCR.

Construction of YEp multicopy control strains. Recombinant strains, such as YEBL-8A and YEBO-1B, were constructed in the following way. By use of the pBBLE-Δpdc1 vector, a *PDC1* deletion strain was constructed. After the colonies had been isolated, the *PDC1* deletion was confirmed by PCR. Next, the pESC-LDHKCY and pESC-LDHKCB vectors were introduced into these strains, and the recombinants were named YEBL-8A and YEBO-1B, respectively.

Construction of PDC1 integration strains. Recombinant strains, such as YIBL-2D and YIBO-7A, were constructed in the following way (Fig. 2A). By use of the pBTRP-PDC1-LDHKCB vector, the heterozygous integrated strain was constructed. But the *PDC1* gene in this transformant was not completely deleted, because the OC-2T strain is a homothallic yeast. The *LDH* gene on one side of the chromosome could be amplified, and the *PDC1* gene was completely deleted upon spore formation (Fig. 2B).

Enzyme-specific activity in cell extracts. Cell extracts were prepared with a SONIFIER 250 (Branson) as described previously (29). PDC-specific activity in freshly prepared extracts, as described by Pronk et al. (29), was determined by using 6a Ubest-55 spectrophotometer at 340 nm (Japan Spectroscopic, Tokyo, Japan). LDH-specific activity was determined in freshly prepared extracts as described by Minowa et al. (24). Protein concentrations in cell extracts were determined with a DC protein assay kit (Bio-Rad, Richmond, Calif.) by using bovine serum albumin (Sigma, St. Louis, Mo.) as a standard.

Fermentation. Fermentation experiments were performed at 30°C in 100-ml flasks with working volumes of 40 ml in YPD10 medium (1% Bacto yeast extract, 2% Bacto peptone, 10% D-glucose) containing 3% sterilized calcium carbonate. The inoculum was prepared by transferring strains from stock cultures to flasks containing 5 ml of YPD medium. Each culture was incubated for 72 h at 30°C on a shaker and then transferred to the fermentation medium of 0.1% packed cell volume at the inoculum size. The glucose, lactic acid, and ethanol concentrations were measured with a biosensor BF-4 S instrument (Oji Keisoku Kiki, Hyogo, Japan).

RESULTS

Lactate production with multicopy plasmids. We constructed two kinds of recombinant yeasts expressing a heterologous *LDH* gene, bovine *LDH* or *B. longum LDH* (Table 1), under the control of the *PDC1* promoter using a 2μm plasmid-based vector (Fig. 1A). The recombinants with the *PDC1* deletion showed no significant differences in either PDC or LDH activities, and the level of PDC activity was one-third of that in the case in which OC-2T was used as the host strain (Table 2). The YEBO-1B strain, which expresses bovine *LDH*, was cultivated for 72 h, and the yields of lactate and ethanol were 16.3 g/liter and 34.3 g/liter, respectively (Fig. 3A). The yield of lactate for YEBL-2D, which expresses *B. longum LDH*, was almost the same as that for YEBO-1B (Fig. 3B), with no difference in yield being seen between the two kinds of LDH.

Lactate production with *PDC1* integration. We constructed two other lactate-producing yeast recombinants, YIBL-2D (*B. longum LDH*) and YIBO-7A (bovine *LDH*), with integration in the *PDC1* locus (Table 1). For the initial physiological characterization, the growth rates of these *PDC1*-disrupted recombinants were determined in shake flask cultures on YPD medium. On analysis of the absorbency at an optical density at

TABLE 2. Specific activities of PDC and LDH

Strain	Mean specific activity ± SD (mU/mg of protein)[a]	
	PDC	LDH
OC-2T	225.0 ± 2.5	ND
YEBL-8A	87.0 ± 1.2	4.6 ± 3.3
YEBO-1B	88.2 ± 3.9	5.1 ± 4.8
YIBL-2D	84.0 ± 3.1	12.7 ± 2.2
YIBO-7A	89.7 ± 2.5	26.7 ± 3.1

[a] These strains were cultivated for 24 h. Enzyme-specific activity in freshly prepared cell extracts was determined. The average and standard deviation were determined for three independent experiments. ND, not determined.

TABLE 3. L-Lactic acid and ethanol productions from wild-type and transgenic strains during batch growth on 10% (wt/vol) glucose-based YPD medium

Strain	Vector	*LDH* gene type	Neutralization		Yield of lactic acid[b] (%)	Nonneutralization		Yield of lactic acid (%)
			Lactate (g/liter)	EtOH (g/liter)[a]		Lactic acid (g/liter)	EtOH (g/liter)	
OC-2T				40.8			40.5	
YEBL-8A	YEp	*B. longum*	14.1	33.9	17.2	14.7	34.8	17.6
YEO-1B	YEp	Bovine	16.3	34.3	19.2	16.1	33.1	19.6
YIBL-2D	YIp	*B. longum*	25.7	31.1	29.5	24.0	27.6	30.3
YIBO-7A	YIp	Bovine	55.6	16.9	62.2	50.6	13.8	64.7

[a] EtOH, ethanol.
[b] Yields are expressed as grams of lactate produced per liter divided by grams of glucose consumed per liter.

600 nm, both transgenic strains were found to grow exponentially, with a growth rate of 1.73/h, while that of OC-2T was 1.85/h. Compared to the host strain, suppression of the growth rate was not observed, although the *PDC1* gene was disrupted. The LDH-specific activities of both YIBL-2D and YIBO-7A were clearly higher than those of the transgenic strains including a multicopy plasmid, although these recombinants have only two copies of the *LDH* genes (Table 2). The PDC-specific activities of the four recombinant strains were one-third of that of the OC-2T host strain and seemed to be the same. The YIBO-7A strain expressing bovine *LDH* under the control of the *PDC1* promoter was observed to produce both lactate (55.6 g/liter) and ethanol (16.9 g/liter), with up to 62.2% of the glucose being transformed into lactic acid (Fig. 4B). YIBL-2D, the other strain which expresses the *B. longum* L-LDH gene, produced 25.7 g of L-lactate/liter and 31.1 g of ethanol/liter (Fig. 4A). Two genome integration recombinants, YIBL-2D and YIBO-7A, exhibited high levels of production of lactate compared with those of the YEBO-1B and YEBL-8A strains including a multicopy plasmid, although the genome-integrated strains maintained only two *LDH* genes. However, the yield of lactate with YEBO-7A expressing bovine *LDH* was almost twice that with YEBO-1B, and an effect of bovine *LDH* and *B. longum LDH* was observed clearly for the two genome-integrated strains. This difference in production on account of the different *LDH* genes was not observed for the multicopy strain.

Lactic acid production under nonneutralizing conditions. The production of lactic acid was examined by cultivation under nonneutralizing conditions without CaCO₃. The accumulations of lactic acid and ethanol are shown in Fig. 5. The YIBO-7A strain, which had integrated the bovine *LDH* gene, gave yields of 50.2 g of lactic acid/liter and 16.7 g of ethanol/liter. The glucose was completely consumed in 72 h in the case of neutralizing conditions. The final pH was 2.8. The effect of changing the initial glucose concentration from 100 to 150 g/liter on the yield of the lactic acid production was examined. However, as judged by fermentation analysis, under the nonneutralizing conditions, an improvement of the lactic acid production was not observed; it did not reach 50 g/liter or more (data not shown).

DISCUSSION

In this study, we developed a recombinant yeast exhibiting efficient lactate production by substituting the coding region of *PDC1* on chromosome XII for that of *LDH* through homologous recombination (Fig. 2). This method allowed robust gene expression under the control of the native *PDC1* promoter. Steady lactate production was also maintained with the recom-
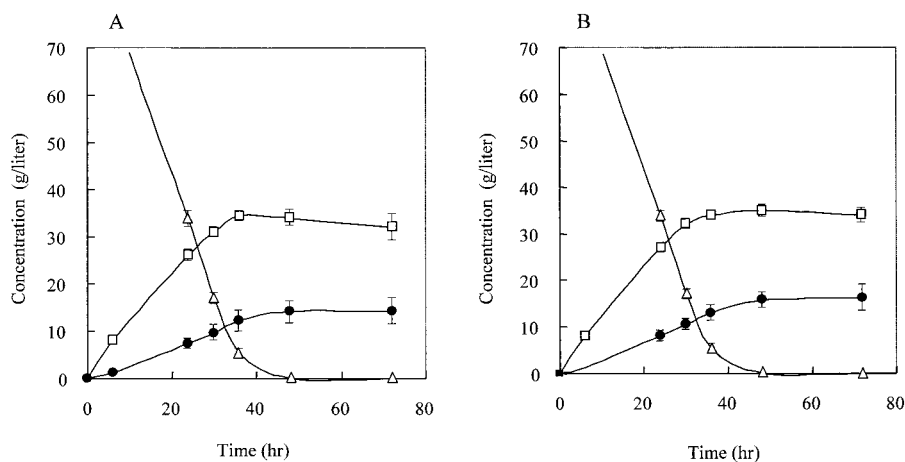

FIG. 3. Comparison of fermentation with the *S. cerevisiae* YEBL-8A (A) and YEBO-1B (B) strains in YPD medium containing 100 g of glucose/liter and 50 g of CaCO₃/liter. ●, lactate; □, ethanol; △, glucose. Each strain was cultivated for 72 h under microaerobic conditions at 30°C. The averages and standard deviations (error bars) for three independent experiments are presented.
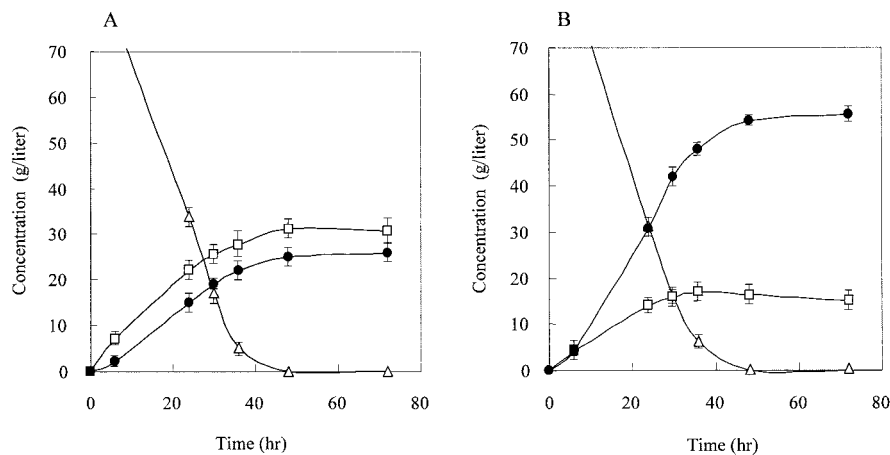
FIG. 4. Comparison of fermentation with the *S. cerevisiae* YIBL-2D (A) and YIBO-7A (B) strains in YPD medium containing 100 g of glucose/liter and 50 g of CaCO$_3$/liter. ●, lactate; □, ethanol; △, glucose. Each strain was cultivated for 72 h under microaerobic conditions at 30°C. The averages and standard deviations (error bars) for three independent experiments are presented.

binant strain that was subcultured two times in nonselective medium (data not shown). For *LDH* expression in yeast, a YEp multicopy plasmid is generally used, but remarkable improvement of lactate production was not observed in this study (Table 3). In the case of multicopy strains, it was expected that unstable *LDH* expression would decrease the number of intracellular plasmids in nonselective medium. However, genome integration does not affect the number of *LDH* genes during fermentation; therefore, steady expression according to the promoter occurs. The comparison with transgenic yeast is shown in Table 4. In the batch culture condition, it can be confirmed that the strain of this report shows a high yield of lactic acid. A transgenic strain in which the *Lactobacillus plantarum LDH* gene was integrated into the genome was developed recently by Colombié et al.; this recombinant yields about 58 g of lactate/liter (6). The *ADH1* promoter was used for the expression of the heterogeneous gene. In this study, we used the native *PDC1* promoter on chromosome XII. *PDC1* encoded pyruvate decarboxylase, which converts pyruvic acid to

acetaldehyde and plays an important role in the production of ethanol. In general, host strain OC-2T was able to produce a high level of ethanol, even with a high glucose concentration; besides, only *PDC1* usually worked for the production of ethanol. The choice to use this promoter was based on the fact that the expression of *PDC1* is strongly induced by glucose (20), and glucose-responding elements in *S. cerevisiae* have already been reported (5, 21). It is advantageous to use the *PDC1* promoter for fermentation with a high initial glucose level. Similarly, Bianchi et al. used *KlPDC1* promoter for bovine *LDH* expression. Transgenic *K. lactis* strains showed 60 g of lactic acid/liter under the fed-batch condition, but it expended 500 h (28). Our *S. cerevisiae* strain disrupted only *PDC1* and not *PDC5*, and genome-integrated *LDH* was regulated under the control of the native *PDC1* promoter.

In the experiment using *B. longum* and bovine LDH, an improvement in lactate production was observed in the case of the integrated bovine LDH recombinant; however, a remarkable change was not observed in the case of a YEp-based multicopy plasmid (Table 4). The two LDHs were found to differ in amino acid level (40.3% identity; the calculation was performed with genetic analysis software GENETYX version 7.0.2). In order to determine the substrate affinity for pyruvic acid, bovine LDH was examined (K_m, 0.13 mM; V_{max}, 3,333 U/mg of protein) (data not shown). Also, the K_m value for pyruvate of *B. longum* LDH has already been reported (K_m, 1.05 mM; V_{max}, 1,670 U/mg of protein) (19). The two LDHs are different in substrate affinities and enzyme activity levels. Because steady gene expression can be expected in the genome-integrated strain compared with a recombinant constructed with the YEp vector, it is considered that the differences in enzyme characteristics between the two LDHs were reflected by the lactic acid production. The combination of bovine *LDH* and the native *PDC1* promoter on the genome is important for the efficient production of lactic acid.

The maximal concentration of lactate with YIBO-7A, which expresses bovine *LDH* under the control of the *PDC1* promoter, was 58.8 g/liter under neutralizing conditions (Table 4). This recombinant, including two copies of the *LDH* gene on
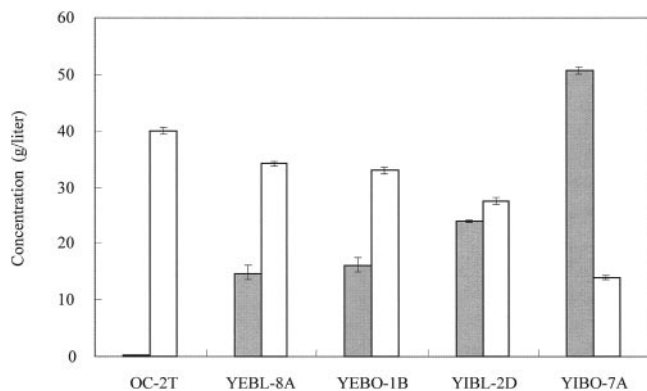

FIG. 5. Accumulations of lactic acid (gray bars) and ethanol (white bars) by *S. cerevisiae* recombinants in YPD medium containing 100 g of glucose/liter but without CaCO$_3$. Each strain was cultivated for 72 h under microaerobic conditions at 30°C. The glucose had been completely consumed after 72 h. The averages and standard deviations (error bars) for three independent experiments are presented.

TABLE 4. Study of L-lactic acid production by transgenic yeast

Host	Vector	Promoter	*LDH* gene type	Maximum production (g/liter)	Yield of lactate (%)	Reference or source
Batch culture						
S. cerevisiae	YEp	*ADH1*	*Lactobacillus casei*	12.0	24.0	7
S. cerevisiae (pdc1, pdc5, pdc6)	YEp	*ADH1*	Bovine	7.9	26.3	1
S. cerevisiae (wine yeast)	YEp	*ADH1*	*Lactobacillus casei*	8.6	4.3	8
S. cerevisiae (pdc1 and adh1)	YEp	*ADH1*	*Rhizopus oryza*	38.0	41.3	35
S. cerevisiae	YIp	*ADH1*	*L. plantarum*	58.0	29.0	6
S. cerevisiae (wine yeast)	YIp	*PDC1*	Bovine	55.6	62.2	This study
Fed-batch culture						
S. cerevisiae	YEp	*ADH1*	Bovine	20.0		27
K. lactis (klpdc1)	YRp	*KLPDC1*	Bovine	26.7		3
K. lactis (klpdc1 and klpda1)	YRp	*KLPDC1*	Bovine	60.0		28

a For batch culture, the yields are expressed as grams of lactate produced per liter divided by grams of glucose consumed per liter.

the genome, showed higher production than the transgenic strain, which had integrated one copy of the *LDH* gene on the genome (data not shown). It will be possible to improve the lactate production by increasing the number of *LDH* genes through genome integration. An analysis of a recombinant with an increased copy number of *LDH* is described elsewhere (31). On the other hand, if lactic acid is obtained directly under nonneutralizing conditions without a desalination process, lowering the cost on a manufacturing scale is possible. It has been pointed out that for a conventional method involving lactic acid bacteria, the process of desalination of lactate is a factor that increases the cost. In the case of lactic acid bacteria, it has been reported that the use of the genome-shuffling method improves the low pH tolerance (26). *S. cerevisiae* is well known to grow and survive at low pHs compared with lactic acid bacteria. Lactic acid production under nonneutralizing conditions was attempted by using a transgenic yeast (1, 7, 28, 35), but this production has not been reported so far. The present strain, which expresses bovine *LDH* under the control of the *PDC1* promoter, exhibited high lactic acid production (50.2 g/liter) without pH control (Table 4). This result might lead to lactic acid fermentation that does not need the desalination process. But the level of lactic acid production did not become 50 g/liter or more. The final pH was 2.8, and it was expected that the low pH suppressed the lactic acid production. To improve the production under nonneutralizing conditions, it is important to make more progress regarding the low pH tolerance of intracellular LDH or host cells. *S. cerevisiae* is a suitable microorganism for an industrial scale. This strategy could be useful for applications for lactic acid production other than the use of lactic acid bacteria.

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