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The Effect of Pyruvate Decarboxylase Gene Knockout in *Saccharomyces cerevisiae* on L-Lactic Acid Production

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A plant- and crop-based renewable plastic, poly-lactic acid (PLA), is receiving attention as a new material for a sustainable society in place of petroleum-based plastics. We constructed a metabolically engineered *Saccharomyces cerevisiae* that has both pyruvate decarboxylase genes (*PDC1* and *PDC5*) disrupted in the genetic background to express two copies of the bovine L-lactate dehydrogenase (*LDH*) gene. With this recombinant, the yield of lactate was 82.3 g/liter, up to 81.5% of the glucose being transformed into lactic acid on neutralizing cultivation, although *pdc1 pdc5* double disruption led to ineffective decreases in cell growth and fermentation speed. This strain showed lactate productivity improvement as much as 1.5 times higher than the previous strain. This production yield is the highest value for a lactic acid-producing yeast yet reported.

Key words: lactic acid production; *Saccharomyces cerevisiae*; pyruvate decarboxylase; genome integration

Poly-lactic acid (PLA) has been receiving increasing attention due to its biodegradation into H_2O and CO_2, in comparison with petroleum-based plastics.[1)] Advancement toward a sustainable society has created an urgent need for large-scale production of L-lactic acid, which is used as a monomer for polymerization into PLA. The goal of this study is to establish efficient production of this monomer. L-Lactic acid is generally produced using lactic acid bacteria,[2)] whereas different approaches, such as genetically engineered yeasts, have been developed recently for large-scale production. Such metabolically engineered yeasts were first reported by Dequin and Barre[3)] and Porro *et al.*,[4)] who showed that the recombinants yielded approximately 10 to 20 g of lactate/liter in the end. In both cases, a considerable amount of ethanol was produced concurrently because *Saccharomyces cerevisiae* predominantly produces ethanol under anaerobic conditions. The by-product ethanol has become a problem in lactic acid fermentation with a transgenic yeast (Fig. 1).

In *S. cerevisiae*, there are three pyruvate decarboxylase (PDC) genes, *PDC1*, *PDC5*, and *PDC6*, that contribute directly to ethanol production.[5,6)] PDC activity in yeast is due mainly to the *PDC1* and *PDC5* genes,[7,8)] and the expression of these genes is controlled by an auto-regulation system, that is to say, *pdc1* deletion led to a great increase in *PDC5* promoter-driven mRNA expression.[9)] A PDC-negative mutant lacking all three genes exhibited a three-fold lower growth rate in complex medium containing glucose than the wild-type strain.[7)] Additionally, in the absence of PDC activity, it was proposed by Flikweert *et al.* that the reduction in the growth rate depended on regeneration of NADH.[10)] In lactic acid-producing yeast, Brambilla *et al.* tried to improve the regeneration of NADH in glycolysis using a recombinant strain expressing *Lactobacillus casei* L-lactate dehydrogenase (LDH). But it was found that NADH reoxidation does not control glycolytic flux during exposure of respiring *S. cerevisiae* cultures to glucose excess.[11)] On the other hand, to increase the metabolic flow from pyruvic acid to lactic acid, a mutant stain, such as the *pdc1 pdc5* double mutant[12)] or the *adh1* mutant strain,[13)] has been utilized as the genetic background to obtain an L-*LDH* gene-expressing yeast. No detailed analysis, however, has been reported, and no remarkable improvement in L-lactic acid production has been observed.

In a previous study, we attained efficient production of L-lactic acid with a recombinant wine yeast.[14)] Three key distinguishing features of our findings are as follows: Firstly, expression of mRNA for genome-integrated L-*LDH* is regulated by the native *PDC1* promoter. Also, the coding region for *PDC1* on chromosome XII was substituted for that of the L-*LDH* gene through homologous recombination. Secondly, the *PDC1* gene of this transformant was completely in-

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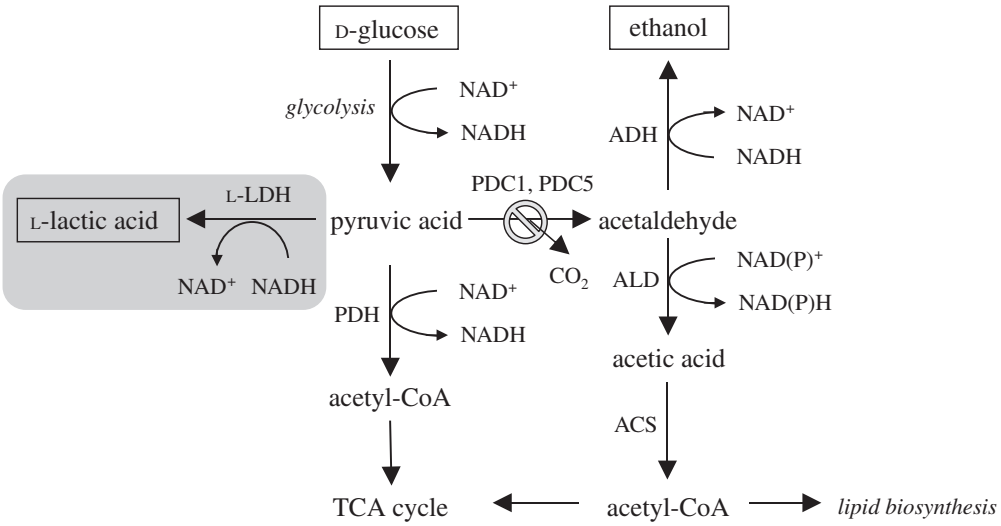


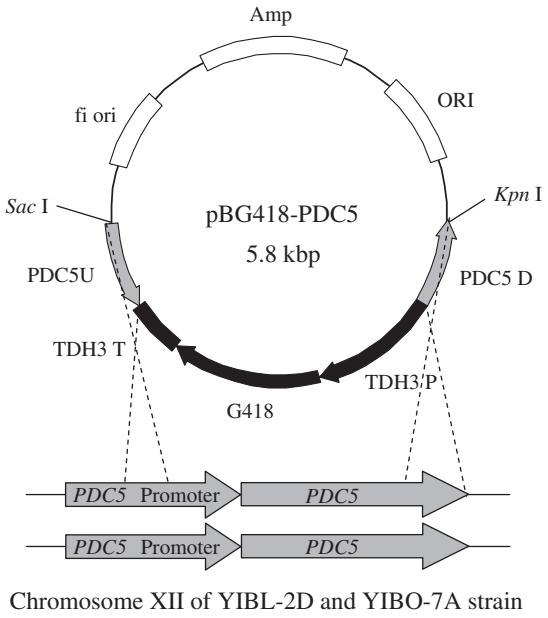
Fig. 1. Scheme of Pyruvate Metabolism in *S. cerevisiae* Expressing the L-*LDH* Gene.

Enzymes: ACS, acetyl-CoA synthetase; ADH, alcohol dehydrogenase; ALD, acetaldehyde dehydrogenase; L-LDH, L-lactate dehydrogenase; PDC, pyruvate decarboxylase; PDH, pyruvate dehydrogenase complex. The gray area indicates a new metabolite pathway in the transgenic strain.

active. Finally, the growth rate of this transgenic expressing genome-integrated L-*LDH* gene did not greatly decrease. *PDC1* deletion causes a decrease in PDC-specific activity, which leads to improvement in L-lactic acid production. But, the by-product ethanol is still produced, because the *PDC5* gene is overexpressed due to the auto-regulation system in *S. cerevisiae*. In this study, we explored a transgenic strain with *pdc1* and *pdc5* knockout in the genetic background to obtain L-*LDH* expression. *pdc1 pdc5* double disruption causes a profound decrease in ethanol production. It is expected that this effect leads to an improvement in L-lactic acid production by *S. cerevisiae*.

Materials and Methods

Strains and media. The *Escherichia coli* strain used for plasmid vector construction was JM109 (Toyobo, Osaka). *E. coli* cultivation and the medium were described previously.[15] The *S. cerevisiae* OC-2T strain (a/α, *trp1*/*trp1*) was derived from the wine yeast NBRC2260 strain,[16] and recombinant strains, YIBL-2D (*pdc1*::P_{pdc1}-*Bifidobacterium longum* L-*LDH*) and YIBO-7A (*pdc1*::P_{pdc1}-bovine L-*LDH*) producing L-lactic acid were described in detail in our previous report.[14] The culture medium used for *S. cerevisiae* was YPD medium (1% bacto yeast extract, 2% bacto peptone, and 2% D-glucose, wt/vol.).



Chromosome XII of YIBL-2D and YIBO-7A strain

Fig. 2. Map of the Plasmid Vector and Breeding of Transgenic *S. cerevisiae*.

The constructed DNA fragment, which was obtained by digesting the pBG418-PDC5 vector with *Sac* I and *Kpn* I, was integrated into the *PDC5* ORF region of the YIBL-2D strain (*pdc1*::P_{pdc1}-*B. longum* L-*LDH*) and the YIBO-7A strain (*pdc1*::P_{pdc1}-bovine L-*LDH*).[14]

Plasmid construction. Genome integration vectors, pBG418-PDC5 (Fig. 2), were constructed using the pBluescript II SK+ vector (Stratagene, La Jolla, CA, USA). This vector consisted of two *PDC5* gene fragments for homologous recombination and the *G418* gene cassette. The G418 (kanamycin) resistance gene is the aminoglycoside phosphotransferase (*APT*) gene,[17] which confers geneticin resistance on yeasts, fused downstream from the *S. cerevisiae* glyceraldehyde-3-phosphate dehydrogenase 3 (*TDH3*) promoter. Two *PDC5* gene fragments were 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 (Q-Biogene, Carlsbad, CA, USA), and the DNA concentration was determined with an Ultro Spec 300 spectral photometer (Pharmacia Biotech, Uppsala, Sweden). KOD DNA polymerase was used for PCR amplification (Toyobo), and the oligonucleotide sequences of the primers were as follows (Qiagen k.k., Tokyo). For the PDC5 upstream fragment, PDC5U, PDC5U-F: 5′-ATATATGAGCTCATGACGACGAGCCTGAAGCTGGCG-3′, containing a *Sac* I restriction site (underlined), and PDC5U-R: 5′-ATATATGGATCCTCTTGCAACACATTTTCTGATATA-3′, containing a *BamH* I site (underlined). And for the PDC5 downstream fragment, PDC5D, PDC5D-F: 5′-ATATATGTCGACCGTCCAAGTCTTGTGGGGTTC-3′, containing a *Sal* I restriction site (underlined), and PDC5D-R: 5′-ATATATGGGCCCGTTTAGCGTTAGTAGCGGCAGTC-3′, containing an *Apa* I site (underlined). Reactions were carried out using a Gene Amp PCR system 9600 (Applied Biosystems, Foster City, CA, USA) with preincubation at 96 °C for 1 min, and 25 cycles of 95 °C for 30 s, 53 °C for 30 s, and 72 °C for 30 s. These two amplification fragments were treated with each restriction enzyme (Takara Bio, Otsu), and then ligated to a vector. The ligase reaction was performed with a Lig-Fast Rapid DNA Ligation System (Promega, Madison, WI, USA), and the competent cells used for transformation were of the *E. coli* JM109 strain (Toyobo). To confirm subcloning of the vector, the nucleotide sequence was determined with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Breeding of yeasts. S. cerevisiae transformation was performed by the lithium acetate procedure,[18] and each transformant was selected on YPD medium containing 150 μg/ml G418 (Calbiochem, San Diego, CA, USA). The pBG418-PDC5 vector fragment (Fig. 2), which was digested with *Sac* I and *Apa* I, was transformed into each recombinant strain, YIBL-2D (*pdc1*::P_{pdc1}-*B. longum* L-*LDH*) and YIBO-7A (*pdc1*::P_{pdc1}-bovine L-*LDH*), which showed efficient production of lactic acid in the previous study.[14] The *PDC5* gene was completely disrupted through spore formation, because the host strain, OC-2T, is a diploid, homothallic strain.[16] Spore formation was performed on sporulation plates (1% potassium acetate, 0.05% 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) with a micromanipulator (Narishige Science, Tokyo). After colonies were isolated, target gene integration was confirmed by PCR. The primer sequences used were as follows: PDC5CH-F, 5′-ACTGCCATCACTAGAGAAG-3′; PDC5CH-R, 5′-CTAAAACTATATCTATGCC-3′. The two resulting recombinant strains in this study were named YIBL-*pdc1/5*Δ (*pdc1*::P_{pdc1}-*B. longum* L-*LDH*, *pdc5*::*G418*) and YIBO-*pdc1/5*Δ (*pdc1*::P_{pdc1}-bovine L-*LDH*, *pdc5*::*G418*) respectively.

Shake cultivation. Precultures were prepared by inoculating 4 ml of YPD medium with a few colonies from a plate. Cultures were incubated on an orbital shaker (130 rpm) at 30 °C for 1 d. For growth curves, 10 μl of preculture was inoculated into a 20 ml test tube containing 2 ml of YPD medium and then shaken (100 rpm) at 30 °C. Optical density measurements were performed with a Bio-photo recorder (Advantec, Tokyo).

Batch cultivation in fermenters. The fermentation experiment was performed at 30 °C in a 100 ml flask with a working volume of 40 ml in YPD10 medium (1% bacto yeast extract, 2% bacto peptone, and 10% D-glucose) containing 3% of sterilized calcium carbonate (wt/vol.). The inoculum was prepared by transferring a strain from a stock culture to a flask containing 5 ml of YPD medium. The culture was performed for 72 h at 30 °C on a shaker, followed by transfer to the fermentation medium at an inoculum size of 0.2% PCV (packed cell volume). D-glucose, L-lactic acid, and ethanol concentrations were measured with a Biosensor BF-4S (Oji Keisoku Kiki, Amagasaki).

Results and Discussion

Growth of the pdc1 pdc5 double knockout strain

According to the previous study,[14] two transgenic strains, YIBL-*pdc1/5*Δ and YIBO-*pdc1/5*Δ, that expressed L-*LDH* in the genetic background of *pdc1 pdc5* double mutant were constructed. Regarding the breeding of the YIBL-*pdc1/5*Δ strain, which expresses *B. longum* L-*LDH*, half of the 40 separated clones on spore formation hardly grew. Another 20 clones recovered cell growth and *PDC5* was not disrupted. Separation of the phenotype was confirmed as to cell growth. Most *pdc1 pdc5* double mutant strains could not be grown. Two clones did grow, but slowly. For the YIBO-*pdc1/5*Δ strain expressing bovine L-*LDH*, all 40 separated clones grew, and half of them were *pdc1 pdc5* double mutant strains. But the growth of the separated YIBO-*pdc1/5*Δ clones was also slow.

For initial physiological characterization, the growth rates of these recombinants were determined under aerobic conditions on YPD medium. As shown in Fig. 3, the growth rate of the parent strain, YIBO-7A, was $0.550\,h^{-1}$. The growth rate of another parent, YIBL-2D, was $0.544\,h^{-1}$ (data not shown). Compared to the host strain, OC-2T, no extreme suppression of the growth rate was observed, although the *PDC1* gene was disrupted (OC-2T = $0.640\,h^{-1}$). But the recombinant strain examined in this study, YIBO-*pdc1/5*Δ, which expresses bovine L-*LDH*, showed an up to five-fold reduction in growth rate as compared to the parent (YIBO-*pdc1/5*Δ = $0.138\,h^{-1}$). Although the growth rate of the YIBL-*pdc1/5*Δ strain, which expresses *B. longum* L-*LDH*, was $0.098\,h^{-1}$, it was still lower than that of the YIBO-*pdc1/5*Δ strain. From the

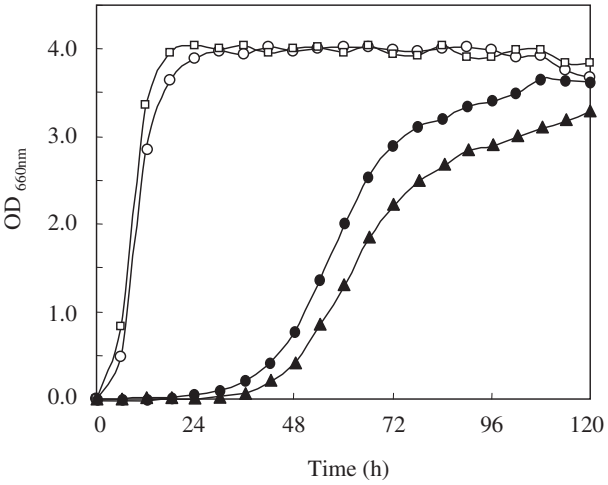


Fig. 3. Growth Curves for *pdc1* Mutant and *pdc1 pdc5* Double Mutant Strains with the Genetic Background to Obtaining an L-*LDH* Gene-Expressing Yeast.

One percent of precultured cells was inoculated into 2% of YPD medium. The strains were cultivated at 30 °C, and $OD_{660\,nm}$ was monitored. □, OC-2T (host strain); ○, YIBO-7A; ●, YIBO-*pdc1*/5Δ; ▲, YIBL-*pdc1*/5Δ. The averages and deviations for three independent experiments are presented.

viewpoint of enzyme activity in each parent strain, the *B. longum* LDH activity of YIBL-2D was approximately two times lower than that of YIBO-7A expressing bovine LDH.[14] The decrease in the growth rate of the YIBL-*pdc1*/5Δ strain expressing *B. longum* L-*LDH* appeared to be proportional to the difference in LDH activity.

Fermentation analysis

L-Lactate and ethanol production were examined under neutralizing micro-anaerobic conditions in a modified YPD medium containing 100 g/liter D-glucose as a carbon source. In our preliminary experiments, it was clarified that this micro-anaerobic condition is optimum for lactic acid-producing yeast (unpublished results). The YIBO-*pdc1*/5Δ strain expressing bovine L-*LDH* under the control of the *PDC1* promoter was observed to produce 82.3 g/liter of L-lactate, up to 81.5% of the glucose being converted into lactic acid in the end (Fig. 4A). In comparison with parent strain YIBO-7A, in which the *PDC5* gene was not deleted, the YIBO-*pdc1*/5Δ strain showed a final lactic acid production increase of 1.5 times. Additionally, this production yield was the highest value for a transgenic yeast yet reported. The YIBO-*pdc1*/5Δ strain produced 2.8 g/liter of ethanol (Fig. 4B). Ethanol production showed a decrease, but still occurred. It appeared that ethanol production by this strain was related to *PDC6* gene expression, but the effect on this ethanol production of *PDC6* was low. This indicates that PDC1 and PDC5 are key enzymes for ethanol production, as Hohmann previously stated,[9] in addition, that disruption of the two genes is strongly related to improvement in lactic acid production. But the production time for this recombinant was 192 h until the plateau phase was reached, although the target substance increased. This production speed was approximately 2.7 times slower than that of the parent strain, YIBO-7A, 72 h. High productivity for the YIBL-*pdc1*/5Δ strain expressing *B. longum* L-*LDH* was also observed, and the final concentration of lactic acid was 80.7 g/liter, but it took 216 h until the plateau phase was reached (Fig. 5). In comparison with the case of bovine L-*LDH*, a further decrease in the fermentation speed was observed. This finding indicates close relationship with the growth rate, which was lower than that of the YIBO-*pdc1*/5Δ strain that expresses bovine L-*LDH*. In addition, high LDH activity also has a profound effect on fermentation.

Adachi *et al.* also tried to develop a *pdc1 pdc5* recombinant expressing bovine L-*LDH* using 2 μm plasmid,[12] but the fermentation data for the *pdc1 pdc5* mutant strain was not presented in detail. In our research, we took a different approach to integrating a heterologous gene into the genome, not using 2 μm plasmid. Genome integration into the downstream of the *PDC1* promoter led to five times higher LDH activity than with the use of 2 μm plasmid.[14] The number of L-*LDH* genes did not change during fermentation under the nonselective medium as compared with multicopy plasmid. Therefore, steady expression according to the promoter occurs. The relation between LDH activity and cell growth is still unclear, hence further detailed analysis of the *pdc1 pdc5* double mutant introducing L-*LDH* genes with the use of 2 μm plasmid is necessary. Also, we constructed recombinant yeast exhibiting higher LDH activity by increasing the copy number of the *LDH* gene on the genome.[19] An interesting effect is expected on deletion of *PDC1* and *PDC5* genes in lactic acid-producing yeast exhibiting higher LDH activity.

The effect of the amount of initial inoculum at the fermentation stage of YIBO-*pdc1*/5Δ strain was examined in order to improve the decreased lactic acid production speed. The fermentation time can be shortened to approximately 20 h by increasing the amount of initial inoculum from 0.2 to 0.8 as PCV (data not shown), but it appears that such a large initial inoculum is impractical for industrial scale production, because such a large initial inoculum will lead to high costs for the production of lactic acid.

The aim of this research was to facilitate the advancement of L-lactic acid production using a metabolically engineered *S. cerevisiae*. With the *pdc1 pdc5* double knockout strain, the lactate production yield reached 81.5%, the highest value for a transgenic yeast yet reported. But disruption of these two genes led to ineffective decreases in cell growth and fermentation speed respectively. As shown in Fig. 1, suppression of the metabolic flow from pyruvate to acetaldehyde has a strong effect on the redox balance, because NADH derived through glycolysis is not consumed. The *pdc1 pdc5* double mutant strain expressing bovine L-*LDH*

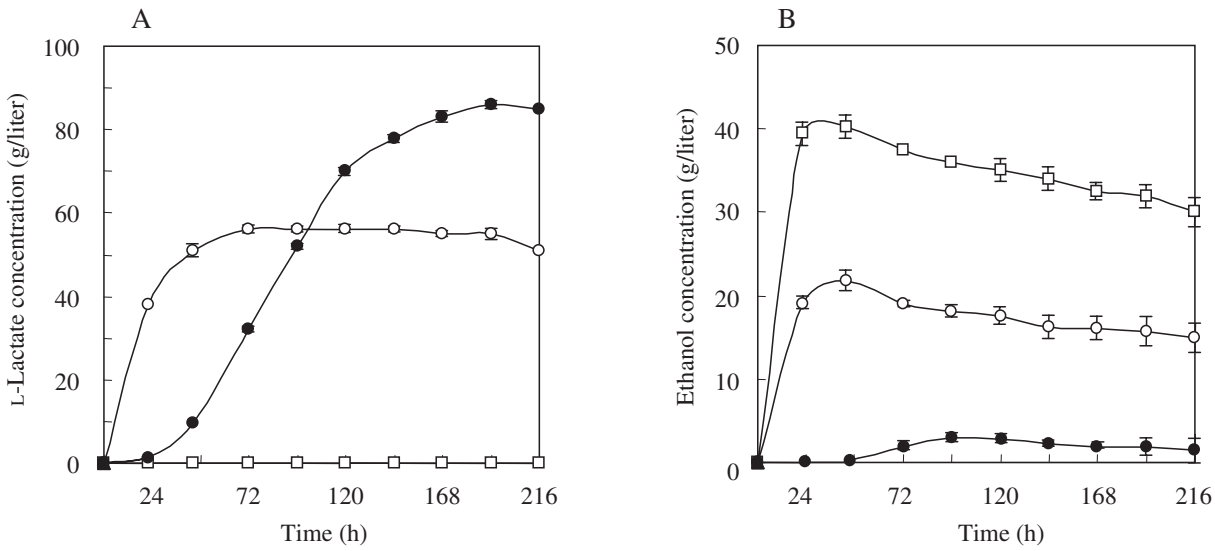


Fig. 4. Time Courses of Cultivation of YIBO-7A and YIBO-*pdc1/5*Δ Strains on a Flask Scale.
A, L-Lactate concentration; B, ethanol concentration; □, OC-2T (host strain); ○, YIBO-7A; ●, YIBO-*pdc1/5*Δ. The averages and deviations for three independent experiments are presented.

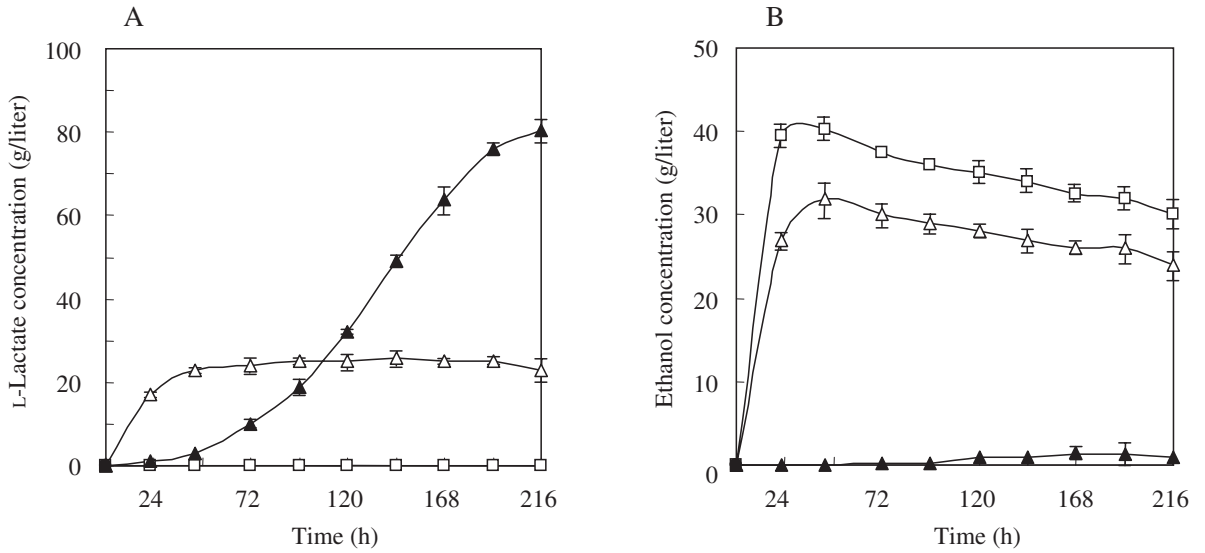


Fig. 5. Time Courses of Cultivation of YIBL-2D and YIBL-*pdc1/5*Δ Strains on a Flask Scale.
A, L-Lactate concentration; B, ethanol concentration. □, OC-2T (host strain); △, YIBL-2D; ▲, YIBL-*pdc1/5*Δ. The averages and deviations for three independent experiments are presented.

showed improvement in comparison with the case of the *B. longum* one, although growth and fermentation speed were still slow. It has also been pointed out by van Maris *et al.* that the acetyl-CoA supply to mitochondria and ATP production in the cytoplasm appear to be important factors.[20] To establish a high lactic acid production technology involving recombinant yeast, a basic examination that focuses on intracellular metabolic control will become more important in the future.

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