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Metabolic Engineering of *Saccharomyces cerevisiae* for Efficient Production of Pure L-(+)-Lactic Acid

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Abstract

We developed a metabolically engineered *Saccharomyces cerevisiae*, which produces optically pure L-lactic acid efficiently using cane juice-based medium. In this recombinant, the coding region of pyruvate decarboxylase *(PDC)1* was completely deleted, and six copies of the bovine L-lactate dehydrogenase *(L-LDH)* genes were introduced on the genome under the control of the *PDC1* promoter. To confirm optically pure lactate production in low-cost medium, cane juice-based medium was used in fermentation with neutralizing conditions. L-lactate production reached 122 g/L, with 61% of sugar being transformed into L-lactate finally. The optical purity of this L-lactate, that affects the physical characteristics of poly-L-lactic acid, was extremely high, 99.9% or over.

Index Entries: Cane juice-based medium; L-lactic acid production; optical purity; *Saccharomyces cerevisiae*.

Introduction

Poly lactic acid (PLA) is being developed as a renewable alternative for conventional petroleum-based plastics. The advancement of a sustainable society has created an urgent need for large-scale production of lactic acid, which is used as a monomer for polymerization into PLA. But it has been pointed out that this polymer was only thermostable up to approx 58°C *(1)*. The problem is that PLA is weak and therefore receives heat and has increased attention for the expanded use of this renewable plastic. Because it has been reported that optical purity affects the physical characteristics, such as crystallization, thermostability, the biodegradation rate,

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and performance (2), it is important to establish a processing technology for L-lactic acid with high optical purity.

The goal of this study is to establish efficient production of optically pure L-lactic acid. L-Lactic acid is generally produced using lactic acid bacteria such as *Lactobacillus* spp. The optical purity of this monomer is approx 95% (3), but this purity is not suitable for the high physical properties of PLA. To improve the purification of lactic acid, the separation of optical isomers through the crystallization has been reported (4,5). But it is important to obtain L-lactic acid of extremely high optical purity during the fermentation stage. *Lactobacillus* spp. are not able to produce lactic acid of extremely high optical purity, because some of them have both L- and D-lactate dehydrogenase (*L-, D-LDH*) genes. Even *Lactobacillus sakei*, which has only an *L-LDH*, also produces D-lactic acid owing to its lactate racemase activity (6). Recently, improvement of its optical purity with *Lactobacillus* spp. was reported (7–9), but it was not enough for industrial use.

On the other hand, yeasts, including *Saccharomyces cerevisiae*, hardly produce either L- or D-lactic acid, because they do not have lactate racemase or L- and D-LDH. Therefore, new methods for producing lactic acid with genetically engineered yeast have been developed, and applied for large-scale production on a trial basis. Such transgenic yeasts were first reported by Dequin and Barre (10) and Porro et al. (11), who showed that the recombinants yielded about 10–20 g of lactate/L. To increase the metabolic flow from pyruvic acid to lactic acid, a mutant strain, such as the *pdc1*, *pdc5* (12), or *adh1* (13) mutant, was utilized as the genetic background for obtaining an *L-LDH* expressing yeast. However, a remarkable improvement in L-lactic acid production has not been observed, and the analysis of the optical purity was not mentioned in those reports.

In a previous study, we attained efficient production of L-lactic acid with a recombinant wine yeast (14). To achieve mass production of L-lactic acid of extremely high optical purity by using *S. cerevisiae*, we have developed a more genetically modified yeast strain following the previous work. This new recombinant has six copies of the bovine *L-LDH* gene on the genome, and all the copies are expressed under the control of the *PDC1* promoter. In this study, we examined the optical purity of the lactic acid produced by this transgenic strain.

Additionally, fermentation analysis with an inexpensive medium, such as one including an unused resource, would also be significant for producing L-lactic acid of high purity on an industrial scale. Such examination with media containing cane juice (15,16), corn steep liquor (17), hydrolyzed sago starch (18), and biological pretreatment corncob (19) has been reported. However, the reported production involving the use of a transgenic yeast involved YPD medium rather than an inexpensive medium. In this study, we also examined the lactic acid productivity under the cane juice-based medium. It is expected that the achievement of the

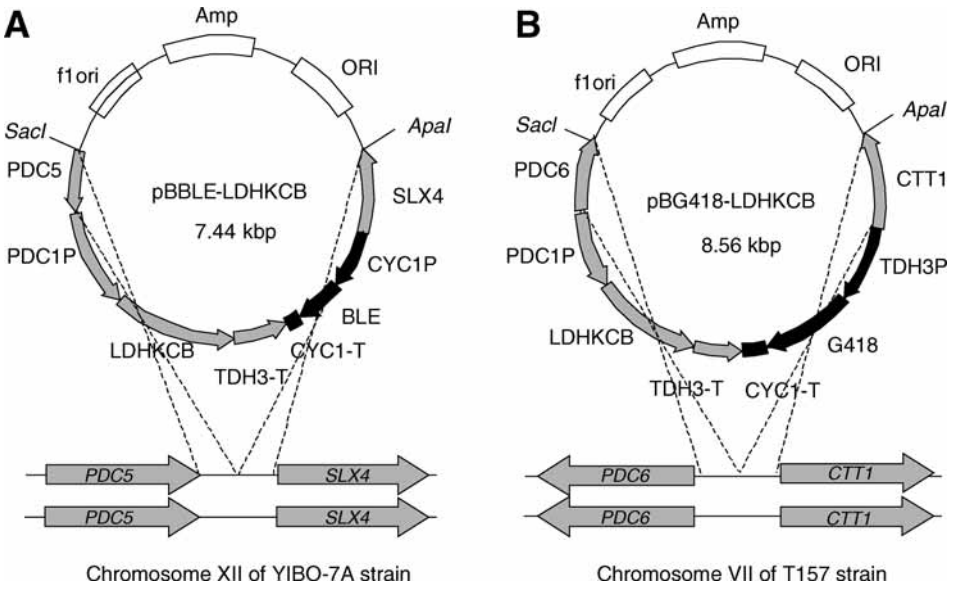

Fig. 1. Maps of the plasmid vectors and breeding of transgenic *S. cerevisiae*. **(A)** Construction of the T157 strain. The constructed DNA fragment, which was obtained by digesting the pBBLE-LDHKCB vector with *Sac*I and *Apa*I, was integrated into the *PDC5* downstream locus of chromosome XII in the YIBO-7A transgenic strain. **(B)** Construction of the T165 strain. The constructed DNA fragment, which was obtained by digesting the pBG418-LDHKCB vector with similar restriction enzymes, was integrated into the *PDC6* upstream locus of chromosome VII in the T157 transgenic strain.

efficient production of the lactic acid in an inexpensive medium contributes to low-cost production of PLA.

Materials and Methods

Strains and Media

The *Escherichia coli* strain used for molecular cloning was JM109 (Toyobo, Osaka, Japan). *E. coli* cultivation and the medium preparation were carried out by standard procedures *(20)*. The *S. cerevisiae* OC-2T strain (a/α *trp1*/*trp1*) was derived from the wine yeast IFO2260 strain *(21)*. The culture medium used for *S. cerevisiae* was YPD medium (1% bacto yeast extract, 2% bacto peptone, and 2% glucose, wt/vol).

Plasmid Construction

The plasmid vectors used in this study are shown in Fig. 1. The method used to modify the bovine *L-LDH* gene and the construction of the YIBO-7A recombinant strain were described in detail in the previous report *(14)*. Two genome integration vectors, pBBLE-LDHKCB (Fig. 1A) and pBG418-LDHKCB (Fig. 1B), were constructed using the pBluescript SKII$^+$ vector (Stratagene, La Jolla, CA).

Table 1
Primers Used for Constructing pBBLE-PDC1P-LDHKCB and pBG418G-PDC1P-LDHKCB

Primer	Sequence (5′–3′)[a]	Restiction site
PDC5-U	ATATATGAGCTCCATGATTAGATGGGGTTTGAAGCC	*Sac* I
PDC5-D	ATATATGCGGCCGCCTGGAAGACAGGACAGAAAAGT	*Not* I
SLX4-U	ATATATGTCGACGGTTAAAGATTAGCTTCTAATA	*Sal* I
SLX4-D	ATATATGGGCCCGGGCAACTGAACTACTGGTTATT	*Apa* I
PDC6-U	ATATATGAGCTCGTTGGCAATATGTTTTTGC	*Sac* I
PDC6-D	ATATATGCGGCCGCTTCCAAGCATCTTATAAACC	*Not* I
CTT1-U	ATATATGGGCCCGATGTACGATCGCCTGCACTAT	*Apa* I
CTT1-D	ATATATGGTACCGGGCAAGTAACGACAAGATTG	*Kpn* I

[a]Restrictions sites in the primer sequences are underlined.

In the pBBLE-LDHKCB vector (Fig. 1A), the phleomycin resistance gene casset was *Tn5 BLE* of bacterial transposon *Tn5 (22)*, which was fused downstream from the *S. cerevisiae* cytochrome-*c* (*CYC1*) promoter. The *PDC1* promoter, *PDC5* and *SLX4* 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), and the concentration was determined with an Ultro spec 300 spectral photometer (Pharmacia Biotech, Uppsala, Sweden). KOD DNA polymerase was used for PCR amplification, and the oligonucleotide sequences of the primers are shown in Table 1 (*see* the previous report for details of the primer sequence of the *PDC1* promoter fragment, *[14]*). The amplification fragments were treated with each restriction enzyme (Takara Bio, Otsu, Japan), and then ligated to a vector. The ligase reaction was performed with a Lig Fast Rapid DNA Ligation System (Promega, Madison, WI), 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, Foster City, CA).

In the pBG418-LDHKCB vector (Fig. 1B), the kanamycin (G418) resistance gene is the aminoglycoside phosphotransferase (*APT*) gene *(23)*, which confers geneticin resistance on yeasts, fused downstream from the *S. cerevisiae* glyceraldehyde-3-phosphate dehydrogenase 3 (*TDH3*) promoter. The *PDC6* and *CTT1* gene fragments were isolated by PCR using the genomic DNA of the *S. cerevisiae* OC-2T strain as a template, and the oligonucleotide sequences of the primers are shown in Table 1. The vector was constructed by a similar technique to that described earlier.

Breeding of Yeasts

S. cerevisiae transformation was performed by the lithium acetate procedure *(24)*, and each transformant was selected on YPD medium

Table 2
Transgenic Strains Used in this Study

Strain	Relevant genotype	Copy number of *LDH* gene	Reference
OC-2T (Host strain)	*trp1/trp1*	0 copies	*21*
YIBO-7A	*pdc1/pdc1*	2 copies	*14*
T157	*pdc1/pdc1, phlemycin^r*	4 copies	This study
T165	*pdc1/pdc1, phlemycin^r, kanamycin^r*	6 copies	This study
T165R	*pdc1/pdc1, phlemycin^r, kanamycin^r, EMS mutagenized*	6 copies	This study

containing 7.5 μg/mL phleomycin (Sigma, St. Louis, MO) or 150 μg/mL G418 (Calbiochem, San Diego, CA).

Table 2 shows the transgenic strains that were constructed in this study. First, the pBBLE-LDHKCB vector fragment, which had been digested with *Sac*I and *Apa*I, was transformed into the YIBO-7A recombinant strain (Fig. 1A), which showed high lactic acid production in the previous study *(12)*. Host strain OC-2T is a diploid and homotallic strain *(21)*. After transformation, the *L-LDH* caset was usually located on one side of a pair of chromosomes. The heterologous gene on one side of a chromosome could be duplicated through spore formation. 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) with a micro-manipulator (Narishige Science, Tokyo, Japan). After colonies had been isolated the target gene integration was confirmed by PCR. The primer sequences used were as follows; F2,5′-ACCAGCCCATCTCAATCCATCT-3′;R2,5′-ACACCCAATCTTTCACCCATCA-3′. The resulting recombinant yeast was named the T157 strain, which included four copies of the bovine *L-LDH* gene on the genome.

Second, the pBG418-LDHKCB vector fragment, which had been digested with similar restriction enzymes, was transformed into the T157 strain (Fig. 1B). The transgenic strain was constructed by a similar method to that described earlier. Target gene integration was confirmed by PCR. The primer sequences used were as follows; F3,5′-TCATTGGTGACGGTTCTCTACA-3′;R3,5′-CGATAGCAAGTAGATCAAGACA-3′. The resulting recombinant yeast was named the T165 strain, which included six copies of *L-LDH* gene on the genome.

Ethylmethane Sulfonate Mutagenesis

Stationary phase cells were harvested from independent cultures grown in YPD. The cells were resuspended in 200 m*M* sodium phosphate (pH 7.0) containing 50 μL ethylmethane sulfonate (EMS). The mixtures were vortexed, and then agitated gently on a rotary shaker for 1 h at room

temperature. The cells were promptly washed in 10 mL sterile water. The final suspensions, which contained approx 10^6–10^7 viable cells/mL, were incubated at 30°C on YPD medium.

LDH Specific Activity

Cell extracts were prepared with a SONIFIER 250 (Branson, Danbury, CT) as described previously *(25)*. LDH-specific activity was determined in freshly prepared extracts as described by Minowa et al. *(26)*. Protein concentrations in cell extracts were determined with a DC protein assay kit (Bio-Rad, Richmond, CA), using bovine serum albumin (Sigma, St. Louis, MO) as a standard.

Fermentation

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% bactopeptone, 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 24 h at 30°C on a shaker, followed by transfer to the fermentation medium at an inoculum size of 0.1% packed cell volume (PCV). Cells were inoculated into a 1-L jar-fermenter made by Biotto Corporation (Tokyo, Japan). The jar conditions were kept at 32°C and pH 5.2, with aeration at 0.15 L/min. Its agitation rate was controlled at 60 rpm, and NaOH was used for neutralization. The medium consisted of cane juice (sugar concentration, approx 20%) containing 0.3% yeast extract (wt/vol). Glucose, lactic acid, and ethanol concentrations were measured with a Biosensor BF-4 (Oji Keisoku Kiki, Amagasaki, Japan). The optical purity of L-lactic acid was calculated as follows. Each value of the following expression shows the quantity (%, wt/vol) of lactic acid.

$$\text{Optical purity of L-lactic acid (\%)} = \frac{\text{L-lactic acid} - \text{D-lactic acid}}{\text{Total lactic acid}} \times 100$$

Results

Construction of Strain Containing Multi-Copies of L-LDH *Gene*

A transgenic *S. cerevisiae* with an increased *L-LDH* gene copy number was constructed based on the YIBO-7A strain, which was constructed in previous study *(14)*. In the YIBO-7A strain, the coding region for *PDC1* on chromosome XII is substituted for that of the *L-LDH* through homologous recombination. The expression of mRNA for the genome-integrated *L-LDH* is regulated under the control of the native *PDC1* promoter, whereas *PDC1* is completely disrupted *(14)*. First, T157 strain, which has four copies of *L-LDH* gene, was constructed. The heterologous *L-LDH* was integrated

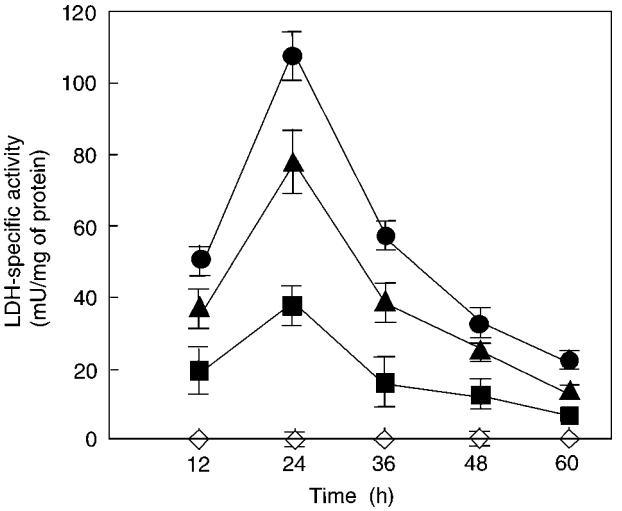

Fig. 2. Comparison of LDH specific activity with increasing gene copy number. The control strain OC-2T (□). YIBO-7A strain with two copies (■), T157 strain with four copies (▲), and T165 strain with six copies of the *L-LDH* gene (•). The average and deviation for three independent experiments are presented.

between *PDC5* and *SLX4* (Fig. 1A), but these two coding regions were not disrupted on homologous recombination, because it was reported that *pdc1* and *pdc5* double mutant strain shows suppression of growth rate *(12)*. Second, the T165 strain, which has six copies of *L-LDH*, was constructed (Fig. 1B). The *PDC6* and *CTT1* genes were also not disrupted during the integration.

LDH-Specific Activity

LDH-specific activity of these three recombinant strains, YIBO-7A (*L-LDH*; two copies), T157 (four copies), and T165 (six copies), were measured. Host strain OC-2T (no *L-LDH*) was used as a control. As shown in Fig. 2, in every strain, the highest activity was observed at 24 h, and the activity decreased significantly after 36 h. The expression of *PDC1* is strongly induced by glucose *(27)*, and glucose responding elements in *S. cerevisiae* have already been reported *(28)*. It was supposed that the time-course of the activity was correlated closely with the glucose concentration in the medium. Improvement of the LDH-specific activity was observed with an increasing *L-LDH* copy number on the genome. The T165 strain showed the highest LDH activity, 108.2 mU/mg of protein, at 24 h. This is an approx 2.8 times increase compared with for the YIBO-7A strain, which had two copies of the *L-LDH*.

EMS Mutagenesis of T165 Recombinant Strain

Despite high LDH activity, the T165 strain (*L-LDH*; six copies) exhibited remarkably suppressed of the growth rate (data not shown).

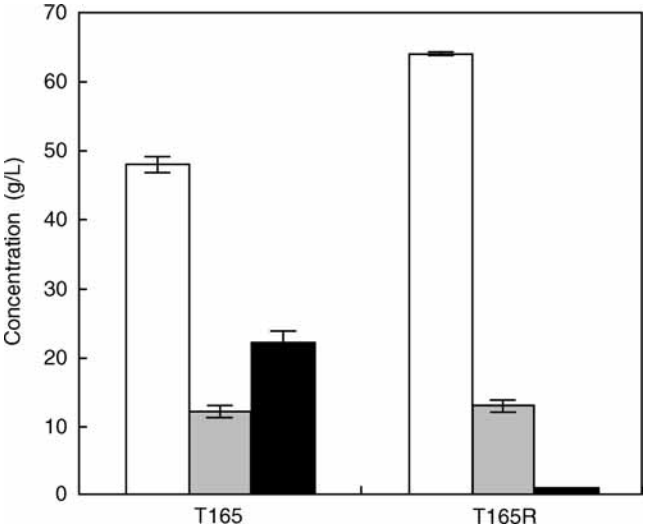

Fig. 3. Comparison of fermentation with the *S. cerevisiae* T165 strain and T165R strain (EMS mutagenesis) in YPD medium containing 100 g/L of glucose and 50 g/L of $CaCO_3$. L-Lactate (white bars), ethanol (gray bars), and glucose (black bars). Each strain was cultivated for 72 h under micro-anaerobic conditions at 30°C. The average and deviation for two independent experiments are presented.

Furthermore, this strain could not completely consume the glucose in fermentation, and lactate productivity decreased (Fig. 3). Following this, we selected the T165 recovered strain (T165R) by EMS mutagenesis. The growth of the T165R strain was recovered to the same level as for the T157 strain (four copies) and this strain completely consumed the glucose on fermentation (Fig. 3). In fermentation analysis, it was clarified that the micro-aerobic condition is optimum for the T165R strains (data not shown), whereas the efficient production of lactate was not confirmed in the anaerobic condition.

Fermentation of Recombinant Strain in YPD Medium

The lactate productivity of these recombinants was examined by cultivation in YPD medium containing 100 g/L of glucose. The T165R strain, with six copies of *L-LDH* gene being expressed from *PDC1* promoter, was observed to produce both L-lactate (68 g/L) and ethanol (9.8 g/L), with up to 68% of the glucose being transformed into L-lactic acid (Fig. 4). However, the T157 and T165R strains exhibited glucose consumption ability. The lactate productivity of T167R was improved by more than 1.28 times compared with that of the recombinant strain YIBO-7A in a previous study (*14*). Improvement of the productivity was observed with increasing *L-LDH* copy number on the genome, as well as from the LDH-specific activity results (Fig. 2).

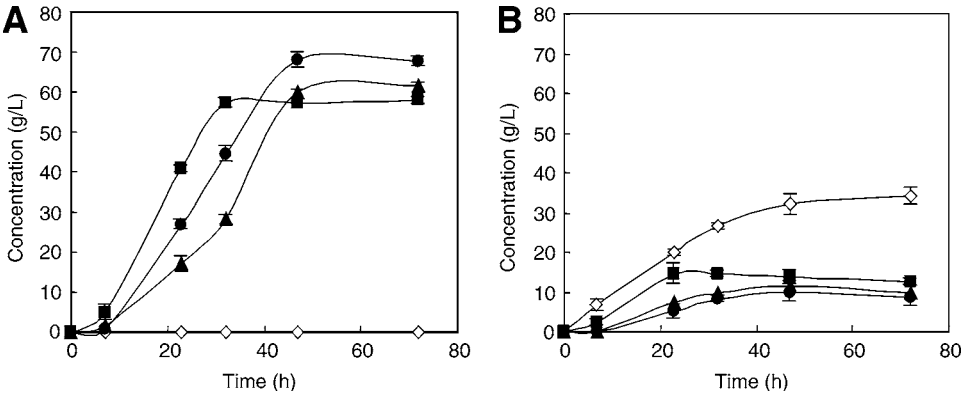

Fig. 4. Time courses of cultivation of four transgenic *S. cerivisiae* strains on a flask scale. **(A)** L-Lactate, **(B)** Ethanol. The control strain OC-2T (□). YIBO-7A strain with two copies (■), T157 strain with four copies (▲), and T165R strain with six copies of the *L-LDH* gene (•). The average and deviation for three independent experiments are presented.

Fermentation of T165R Strain in Cane Juice-Based Medium

To confirm lactate production in low-cost medium, we examined media other than YPD. Cane juice is obtained by squeezed sugar cane, and contains high concentration of glucose, sucrose, and many vitamins and minerals. To prepare cane-based medium, the cane juice was diluted into 20% of sugar concentration, and 0.3% yeast extract (wt/vol) was added. Using a 1-L jar-fermenter with pH control, L-lactate production of T165R strain reached 122 g/L, with up to 61% of the sugar being transformed into lactic acid (Fig. 5). The T165R strain showed production of high concentration of L-lactate, although yield on sugar was decreased compared with in the case of 10% YPD (68%, Fig. 4).

Optical Purity

The optical purity of L-lactic acid produced by the T165R strain was measured. The purity of L-lactate was at least 99.9% in both YPD and cane juice-based medium. This purity was obviously high compared with that of lactic acid produced by other lactic acid bacteria (Table 3).

Discussion

Our goals are to establish a means of mass production of l-lactic acid monomer, and to obtain L-lactic acid of high optical purity with transgenic wine yeast. In a previous study, we constructed recombinant yeast that expressed bovine *L-LDH* under the control of the *PDC1* promoter on the genome. Also, genome integration led to efficient lactic acid production compared with the YEp-multicopy method *(14)*. For mass production of lactic acid, we newly constructed recombinants with increased copy

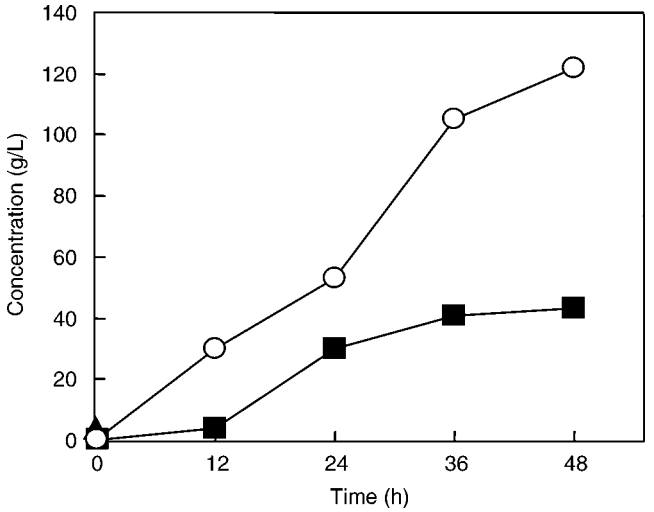

Fig. 5. Fermentation analysis with the *S. cerevisiae* T165R strain in the cane juice-based medium (sugar concentration, approx 20%). The pH was controlled at pH 5.2 with 1 *N* NaOH. o, L-lactate; ■, ethanol. Each strain was cultivated for 72 h under micro-anaerobic conditions at 30°C.

Table 3
Comparison of Optical Purity of L-Lactic Acid Between the Transgenic Yeast and Lactic Acid Bacteria

Strain	Optical purity %	Reference
Saccharomyces cericisiae OC2T T165R	>99.9 (D-lactic acid < 0.01)	This study
Lactobacillus amylophilus ATCC49845	93.0	*35*
Lactobacillus delbrueckii spp. *bulgaricus* ATCC11842	91.0–95.0	*36*
Lactobacillus delbrueckii spp. *delbrueckii* ATCC9649	94.0–95.0	*36*
Lactobacillus rhamnosus ATCC10863	95.0	*37*
Lactobacillus salivarius spp. *salivarius* ATCC11742	86.0–90.0	*38*
Streptococcus bovis 148	95.6	*16*

numbers of the *L-LDH* gene on the genome. However, a decrease in cell growth rate was observed with increasing copies of the *L-LDH*. In particular, transgenic strain T165 (*L-LDH*; six copies) exhibited remarkable suppression of the growth, reducted glucose consumption, and finally decreased lactate productivity, although the LDH-specific activity was increased. It was pointed out by Maris et al. *(29)* that intercellular ATP regeneration and the redox balance were important for increasing lactic acid productivity, and oxygen-limited chemostat cultures showed that lactic acid-producing *S. cerevisiae* strains require oxygen for the generation

of ATP. To relieve intracellular stress, transgenic yeasts excrete intracellular lactic acid extracellularly, for which more energy is needed. The intracellular ATP decrease caused remarkable suppression of the growth. The T165R strain selected through EMS mutagenesis exhibited growth, and showed complete glucose consumption compared with the T165 unmutagenized strain. As shown in Fig. 4, the improved L-lactate productivity of the T165R strain was explained by the increased number of copies of the *L-LDH*, and 68.0 g/L of L-lactate was produced from 10% YPD medium in 72 h finally. In the case of the fermentation in a jar-fermenter, the microaerobic conditions (aeration at 0.15 L/min) led to higher proliferation than under anaerobic conditions. These results show that the improved L-lactic acid productivity of the T165R strain would be the cause of the recovery of ATP generation through the TCA cycle.

For industrial scale production of L-lactic acid, it is also important to use inexpensive media. Cane juice is one of the largest biomass sources available, more than 100×10^6 t of it being generated in Latin America and Tropical Asia *(15)*. Cane juice, which contains more of mineral salts and sucrose, is a low-cost medium compared with YPD. As shown in Fig. 5, the T165R strain showed high lactic acid production even in cane juice-based medium (more than 120 g/L of lactate was produced from this medium in 48 h with a yield of 60% based on the initial sugar concentration). This yield was not greatly decreased compared with in the case of YPD medium. The host strain, OC-2T, was derived from the wine yeast IFO2260 strain, which has been used for producing wine on an industrial scale. Lactic acid is generally produced with lactic acid bacteria, such as *Lactobacillus* spp., which are hard to cultivate at high density and show high auxotrophy concerning growth *(3)*. Considering fermentative production in inexpensive media, it is appropriate to use an industrial yeast, such as a wine yeast, for low cost production of PLA.

The optical purity of L-lactic acid affects the physical properties of poly L-lactic acid, such as the biodegradation rate, crystallization, and thermostability *(2)*. The optical purity of L-lactic acid produced with lactic acid bacteria is generally about 95%, but this purity is insufficient for the high physical properties of PLA. To improve the optical purity, the separation of optical isomers through crystallization has been reported *(5)*, however, this process increases the production cost. Lactic acid bacteria have D-LDH, and attempt to delete this gene have been reported *(8)*. On the other hand, other hosts, i.e., fungi *(19,30)*, genetically engineered *E. coli* *(31–33)*, and genetically engineered yeasts *(10–13)*, have been investigated regarding the production of L-lactic acid. Although several organisms producing lactic acid have been discussed about optical purity *(33,34)*, genetically engineered yeasts have been not analyzed about it yet. In this study, we confirmed that a recombinant wine yeast was able to produce L-lactate of high optical purity (99.9%; Table 3). With recombinant strain T165R, the yield of lactic acid was low compared with those with lactic acid bacteria,

because ethanol was still produced. However, it can be said that there are the following three advantages regarding the use of this recombinant yeast. First, L-lactic acid of high optical purity can be produced. Second, lactic acid can be produced even if one uses an inexpensive media, such as one based on cane juice. Last, because yeasts exhibit a low pH tolerance, free lactic acid production can be expected without neutralization *(12)*. Regarding the mass production of L-lactic acid of high optical purity, these results indicate that the use of this transgenic wine yeast has several advantages. We expect this research will lead to further use of transgenic yeasts.

Acknowledgments

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References

1 Ozeki, E. (1996), *Shimadzu Rev.* **53,** 1–8.
2 Ohara, H. (1998), *Nippon Kagaku Kaishi* **6,** 323–331.
3 Hofvendahl, K. and Hahn-Hagerdal, B. (2000), *Enzyme Microb. Technol.* **26,** 87–107.
4 Benthin, S. (1995), *Appl. Microbiol. Biotechnol.* **42,** 826–829.
5 van Breugel, J., Van Krieken, J., Cerda Baro, A., Vidal Lancis, J. M., and Camprubi Vila, M. (2002), US Patent No. 6,630,603.
6 Hiyama,T., Fukui, S., and Kitahara, K. (1968), *J. Biochem.* **64,** 100–107.
7 Malleret, C., Lauret, R., Ehrlich, S. D., Morel-Deville, F., and Zagorec, M. (1998), *Microbiology* **144,** 3327–3333.
8 Lapierre, L., Germond, J. E., Ott, A., Delley, M., and Mollet, B. (1999), *Appl. Environ. Microbiol.* **65,** 4002–4007.
9 Kyla-Nikkila, K., Hujanen, M., Leisola, M., and Palva, A. (2000), *Appl. Environ. Microbiol.* **66,** 3835–3841.
10 Dequin, S. and Barre, P. (1994), Bio/Technology **12,** 173–177.
11 Porro, D., Barmbilla, L., Ranzi, B. M., Martegani, E., and Alberghina, L. (1995), *Biotechnol. Prog.* **11,** 294–298.
12 Adachi, E., Torigoe, M., Sugiyama, S., Nikawa, J., and Shimizu, K. (1998), *J. Ferment. Bioeng.* **86,** 284–289.
13 Skory, C. D. (2003), *J. Ind. Microbiol. Biotehnol.* **67,** 22–27.
14 Ishida, N., Saitoh, S., Tokuhiro, K., et al. (2005), *Appl. Environ. Microbiol.* **71,** 1964–1970.
15 Fontana, J. D., Guimaraes, M. F., Martins, N. T., Fontana, C. A., and Baron. M. (1996), *Appl. Biochem. Biotechnol.* **58,** 413–422.
16 Narita, J., Nakahara, S., Fukuda, H., and Kondo, A. (2004), *J. Biosci. Bioeng.* **97,** 423–425.
17 Ohara, H., Doi, U., Otsuka, H., Okuyama, H., and Okada, S. (2001), *Nippon Seibutukougaku Kaishi* **79,** 142–148.
18 Hipolto, C. N., Matsunaka, T., Kobayashi, G., Sonomoto, K., and Ishizaki, A. (2002), *J. Biosci. Bioeng.* **93,** 281–287.
19 Miura, S., Arimura, T., Itoda, N., et al. (2004), *J. Biosci. Bioeng.* **97,** 153–157.
20 Sambrook, J., Fritsh, E. F., and Maniatis, T. (1989), *Molecular Cloning: A Laboratory Manual*. 2nd ed., Cold Spring Harbor Laboratory Press, NY.
21 Saitoh, S., Mieno, Y., Nagashima, T., Kumagai, C., and Kitamoto, K. (1996), *J. Ferment. Bioeng.* **81,** 98–103.
22 Gatignol, A., Baron, M., and Tiraby, G. (1987), *Mol. Gen. Genet.* **207,** 342–348.

23 Hadfild, C., Jordan, B. E., Mount, R. C., Pretorius, G. H. J., and Burak, E. (1990), *Curr. Genet.* **18,** 303–313.
24 Ito, H., Fukuda, Y., Murata, K., and Kimura, H. (1983), *J. Bacteriol.* **153,** 163–168.
25 Pronk, T. J., De Steensma, H. Y., and van Dijiken, J. P. (1996), *Yeast* **12,** 1607–1633.
26 Minowa, T., Iwata, S., Sakai, H., and Ohata, T. (1989), *Gene* **85,** 161–168.
27 Kellermann, E. and Hollenberg, C. P. (1988), *Curr. Genet.* **14,** 337–344.
28 Butler, G. and Mc Connell, D. J. (1988), *Curr. Gent.* **14,** 405–412.
29 van Maris, A. J. A., Winkler, A. A., Porro, D., van Dijken, J. P., and Pronk, J. T. (2004), *Appl. Environ. Microbiol.* **70,** 2898–2905.
30 Skory, C. D. (2004), *Appl. Microbiol. Biotechnol.* **64,** 237–242.
31 Chang, D.E., Shin, S., Rhee, J., and Pan, J. (1999), *Appl. Environ. Microbiol.* **65,** 1384–1389.
32 Dien, B. S., Nichols, N. N., and Bothast, R. J. (2001), *J. Ind. Microbiol. Biotechnol.* **27,** 259–264.
33 Zhou, S., Causey, T. B., Hasona, A., Shanmugam, K. T., and Ingram, L. O. (2003), *Appl. Environ. Microbiol.* **69,** 399–407.
34 Zhou, S., Shanmugam, K. T., and Ingram, L. O. (2003), *Appl. Environ. Microbiol.* **69,** 2237–2244.
35 Yumoto, I. and Ikeda, K. (1995), *Biotechnol. Lett.* **17,** 543–546.
36 Hofvendahl, K. and Hahn-Hagerdal, B. (1997), *Enzyme Microb. Technol.* **20,** 301–307.
37 Olmos-Dichara, A., Ampe, F., Uribelarrea, J. L., Pareilleux, A., and Goma, G. (1997), *Biotechnol. Lett.* **19,** 709–714.
38 Siebold, M., von Frieling, P., Joppien, R., Rindfleisch, D., Schugerl, K., and Roper, H. (1995) *Process Biochem.* **30,** 81–95.