Genetically Engineered Wine Yeast Produces a High Concentration of 
L-Lactic Acid of Extremely High Optical Purity

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For mass production of lactic acid, we newly constructed a transgenic wine yeast strain that included six copies of the bovine L-lactate dehydrogenase gene on the genome. On fermentation in inexpensive cane juice-based medium, L-lactate production of this recombinant reached 122 g/liter and the optical purity was 99.9% or higher.

Polylactic acid is being developed as a renewable alternative for conventional petroleum-based plastics. Since it has been reported that optical purity of L-lactic acid affects physical characteristics such as crystallization, thermostability, biodegradation rate, and performance (4, 14, 25), it is important to establish a processing technology for high purity.

L-lactic acid is generally produced using lactic acid bacteria such as Lactobacillus species. The optical purity of this monomer is not high (approximately 95%), because some lactic acid bacteria have both L-lactate dehydrogenase (L-LDH) and D-lactate dehydrogenase (D-LDH) genes (9). This purity is not suitable for the high physical properties of polylactic acid (25). To improve the optical purity, the separation of optical isomers through crystallization has been reported (2, 26), and genetically engineered lactic acid bacteria were also developed (12, 13, 15).

Other hosts, i.e., fungi (17, 24), genetically engineered Escherichia coli (3, 5, 30), and genetically engineered yeasts (1, 20, 23), have also been investigated as to the production of L-lactic acid. Although several organisms producing lactic acid have been discussed in terms of optical purity (29, 30), genetically engineered yeasts have not yet been analyzed. In this study, we developed a more recombinant Saccharomyces cerevisiae strain following previous research (10) and examined the optical purity of L-lactic acid. 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. To achieve efficient production in an inexpensive medium, we examined the lactic acid productivity with a cane juice-based medium.

Construction of plasmids. Maps of the plasmids are shown in Fig. 1, and these vectors were constructed using pBluescript SKII+ (Stratagen, La Jolla, Calif.). The pBBLE-LDHKCB vector (Fig. 1A) consisted of the PDC1 promoter, L-LDH gene, and Tn5 bleomycin resistance (Tn5 BLE) gene cassette, PDC5 gene fragment, and SLX4 gene fragment. The phleomycin resistance gene cassette was Tn5 BLE of bacterial transposon Tn5 (6), which was fused downstream from the S. cerevisiae cytochrome c gene (CYC1) promoter. The pBG418-LDHKCB vector (Fig. 1B) consisted of the PDC1 promoter, L-LDH gene, APT1 gene cassette, PDC6 gene fragment, and cytosolic catalase T1 (CTT1) gene fragment. The katamycin (G418) resistance gene is the aminoglycoside phosphotransferase gene (7), which confers Geneticin resistance, fused downstream from the S. cerevisiae glyceraldehyde-3-phosphate dehydrogenase 3 (TDH3) promoter. Each DNA fragment, PDC5, SLX4, PDC6, and CTT1, was isolated by PCR using the genomic DNA of the S. cerevisiae OC-2T strain as a template.

Breeding of yeasts. In a previous study, we constructed a recombinant, YIBO-7A strain, including two copies of the bovine LDH gene on the genome (10). For mass production of lactic acid, we newly constructed recombinants with increased copy numbers of the LDH gene. S. cerevisiae transformation was performed by the LiAc procedure (11), and each transformant was selected on YPD medium containing 7.5 μg/ml phleomycin (Sigma, St. Louis, Mo.) or 150 μg/ml G418 (Calbiochem).

We constructed the T157 (LDH gene, four copies) strain by using the pBBLE-LDHKCB vector (Fig. 1A). The LDH gene cassette on one side of a chromosome could be duplicated through spore formation, because host strain OC-2T is a diploid and homothallic strain (21). Next, the T165 strain (LDH gene, six copies) was constructed by using the pBG418-LDHKCB vector (Fig. 1B). However, strain T165 could not completely consume the glucose on fermentation analysis. Following this, we selected the T165 recovered strain (T166R) by ethyl methanesulfonate mutagenesis and this strain completely consumed the glucose (data not shown).

Effect of increasing copies of the LDH gene. Three recombinants, YIBO-7A (LDH gene, two copies), T157 (LDH gene, four copies), and T165R (LDH gene, six copies), were examined for LDH specific activity. LDH specific activity was determined in freshly prepared extracts as described by Minowa et al. (16). Protein concentrations in cell extracts were deter-
mined with a DC protein assay kit (Bio-Rad, Richmond, Calif.). As shown in Fig. 2, the highest activity was observed at 24 h and improvement of the specific activity was observed with an increasing LDH gene copy number on the genome. The T165R (LDH gene, six copies) strain showed high activity (108.2 mU/mg of protein) at 24 h. This is an increase of approximately 2.8 times compared with the YIBO-7A (LDH gene, two copies) strain.

The lactate production of these recombinants was examined in YPD medium (1% yeast extract, 2% Bacto Peptone, 10% \(\alpha\)-glucose) containing 3% of sterilized calcium carbonate (wt/vol.). The culture was performed for 72 h at 30°C, and an inoculum size was 0.1% PCV (packed cell volume). L-Lactate concentrations were measured with a Biosensor BF-4 (Oji Keikoku Kiki, Hyogo, Japan). The T165R (LDH gene, six copies) strain was observed to produce 68.0 g/liter of L-lactate (Fig. 3).
This production was improved by 1.28 times or more compared with that of the YIBO-7A (LDH gene, two copies) strain. Improvement of production was observed with increasing LDH gene copy number on the genome, as well as from the LDH specific activity results.

**Fermentation analysis in cane juice-based medium.** To confirm lactate production in inexpensive media, we examined cane juice-based medium. Cane juice is obtained by squeezing sugar cane and contains glucose, sucrose, and many vitamins and minerals. This juice was diluted until the sugar level became 20%, and then 0.3% yeast extract (wt/vol.) was added. Using a 1-liter jar-fermenter (Biottol, Tokyo, Japan) with pH control, we examined the lactate production of the 16SR strain. As shown in Fig. 4, lactate production reached 122 g/liter, with up to 61.0% of the sugar being transformed into lactic acid. This production was obviously high compared with that of the YIBO-7A (LDH gene, two copies) strain (data not shown). Additionally, it was pointed out 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 (27). In the case of T165R strain fermentation in a jar-fermenter, the microaerobic conditions (aeration at 0.15 liters/min) also led to higher proliferation than under anaerobic conditions (data not shown).

**Optical purity.** The optical purity of L-lactic acid produced by the T165R strain was measured by two independent methods. One involved a Biosensor BF-4 (Oji Keisoku Kiki), and the other involved a Diagnostic kit (Roche Diagnostics, Japan; detection limit, 0.01%), and purity was calculated as follows: optical purity = (1-lactic acid quantity − D-lactic acid quantity) / (1-lactic acid quantity + D-lactic acid quantity). Also, the nonexistence of L- and D-lactic acid in the media was confirmed in advance. As a result, the optical purity was at least 99.9% in cane juice-based medium and this purity was obviously high compared with that of lactic acid produced by other lactic acid bacteria (Table 1).

**Conclusion.** We confirmed that a recombinant wine yeast was able to produce 1-lactate of high optical purity. With the T165R (LDH gene, six copies) strain, the yield of lactic acid was low compared with those of lactic acid bacteria, because ethanol was still produced. However, it can be said that there are the following three advantages as to the use of this recombinant. First, L-lactic acid of extremely high optical purity can be produced. Second, lactic acid can be produced even if one uses an inexpensive medium, such as one based on cane juice. Yeast can also be cultivated at high density as to growth. And last, because yeasts exhibit a low pH tolerance, free lactic acid production can be expected without neutralization (10). As to the mass production of L-lactic acid of high optical purity, these results indicate that the use of this transgenic yeast has several advantages and we expect this research will lead to further use of transgenic yeasts.

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