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D-Lactic Acid Production by Metabolically Engineered *Saccharomyces cerevisiae*

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Poly D-lactic acid is an important polymer because it improves the thermostability of poly L-lactic acid by the stereo complex formation. We constructed a metabolically engineered *Saccharomyces cerevisiae* that produces D-lactic acid efficiently. In this recombinant, the coding region of pyruvate decarboxylase 1 (*PDC1*) was completely deleted, and two copies of the D-lactate dehydrogenase (D-*LDH*) gene from *Leuconostoc mesenteroides* subsp. *mesenteroides* strain NBRC3426 were introduced into the genome. The D-lactate production reached 61.5 g/l, the amount of glucose being transformed into D-lactic acid being 61.2% under neutralizing conditions. Additionally, the yield of free D-lactic acid was also shown to be 53.0% under non-neutralizing conditions. It was confirmed that D-lactic acid of extremely high optical purity of 99.9% or higher. Our finding obtained the possibility of a new approach for pure D-lactic acid production without a neutralizing process compared with other techniques involving lactic acid bacteria and transgenic *Escherichia coli*.

[**Key words:** D-lactic acid production, D-lactate dehydrogenase, *Leuconostoc mesenteroides*, *Saccharomyces cerevisiae*, non-neutralization]

Plant- and crop-based plastics, including poly L-lactic acid (PLA), are being developed as renewable alternatives to conventional petroleum-based plastics (1). 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 approximately 58°C (2). The problem that PLA is weak as to heat has been receiving increasing attention for the expanded use of this renewable plastic. It was reported that a polymer blend of poly L-lactic acid and poly D-lactic acid gives a racemic crystal called stereo-complex. This stereo-complex type of blending polymer is characterized by its high melting temperature, approximately 50°C higher than that of the PLA (3, 4). This finding has attached an increased importance to the production of D-lactic acid.

D-Lactic acid is generally produced with lactic acid bacteria, such as *Lactobacillus* species, and a few trials at the production of D-lactic acid from cellulose (5) or rice starch (6) have been reported. However, the production of D-lactic acid by lactic acid bacteria has been little studied in comparison with L-lactic acid. On the other hand, another approach involving transgenic *Escherichia coli* has also been developed (7, 8), because lactic acid bacteria are hard to cultivate at high density and show high auxotrophy regarding growth (9). The use of recombinant *E. coli* led to efficient production of this target monomer, and further improvements have been reported, such as through sucrose utilization gene expression (10) or metabolic flux analysis for some mutant strains (11, 12). Although effective production has been achieved by using recombinant *E. coli*, a low pH inhibits cell growth and D-lactic acid production under non-neutralizing conditions, because *E. coli* can not tolerate a low pH. Therefore, it is necessary to produce D-lactic acid under neutralizing conditions. 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.

On the other hand, yeasts, such as *Saccharomyces cerevisiae*, are more tolerant to low pH than *E. coli* (13, 14), and thus high density cultivation is possible. Generally, *S. cerevisiae* hardly produces either L- or D-lactic acid. But, if a genetically engineered yeast with an introduced heterologous D-lactate dehydrogenase gene (D-*LDH*) is used, free D-lactic acid production of high purity can be expected without pH control. Attempts to produce the D-lactic acid monomer using a recombinant yeast has been few, although trials concerning L-lactic acid production have been reported (13–15). We also attained efficient production of L-lactic acid with a recombinant wine yeast previously (16). In this research, we isolated the D-*LDH* gene from *Leuconostoc me-*

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senteroides subsp. *mesenteroides* strain NBRC3426, and tried to integrate this heterologous gene into the pyruvate decarboxylase 1 (*PDC1*) coding region by means of our previous approach. Additionally, the possibility of D-lactic acid production by a transgenic wine yeast was examined by analyzing the fermentation products under neutralizing and non-neutralizing conditions. Our findings in this study can be thought of as a new approach for producing free D-lactic acid of extremely high optical purity without a neutralizing process.

MATERIALS AND METHODS

Strains and media The *E. coli* strain used for molecular cloning was JM109 (Toyobo, Osaka). *E. coli* cultivation and the medium were described previously (17). *S. cerevisiae* OC-2T (a/α, Δ*trp1*/Δ*trp1*) was derived from the wine yeast NBRC2260 strain (18). The culture medium used for *S. cerevisiae* was YPD medium (1% bacto yeast extract, 2% bacto peptone, and 2% D-glucose, wt/vol). The *L. mesenteroides* subsp. *mesenteroides* strain NBRC3426 used for cloning the D-*LDH* gene was grown on MRS medium (19).

Amplification, cloning and sequence analysis of the D-*LDH* homolog Genomic DNA from the *L. mesenteroides* subsp. *mesenteroides* strain NBRC3426 was prepared using a Fast DNA kit (Q-Biogene, Carlsbad, CA, USA), and the concentration was determined with an Ultro-Spec 300 spectral photometer (Pharmacia Biotech, Uppsala, Sweden). PCR primer pairs for the amplification for cloning of the D-*LDH* ORF region were designed based on the D-*LDH* DNA sequence of *L. mesenteroides* subsp. *cremoris*; Gene Bank accession no. L29327 (20). The oligonucleotide sequences of these primers were as follows (Qiagen K.K., Tokyo). DLDHLM-U; 5′-ATATATGATATCATGAAGATTTTGCTTACGGC-3′, and DLDHLM-D; 5′-ATATATGATATCTTAATATTCAACAGCAATA GC-3′. Both primers contained an *Eco*RV restriction site (underlined). In the PCR reaction, KOD DNA polymerase (Toyobo) was used for amplification. Reactions were carried out using a Gene Amp PCR system 9600 (Applied Biosystems, Foster City, CA, USA) with preincubation at 96°C for 5 min, and 25 cycles of 95°C for 30 s, 53°C for 30 s and 72°C for 90 s. The amplified DNA fragment was subcloned into the pBluescript II SK+ vector (Stratagene, La Jolla, CA, USA) *Eco*RV site according to the previously described method (17). 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. To confirm subcloning of this fragment in this vector, D-*LDH* nucleotide sequence from three independent clones was determined with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The DNA sequence was analyzed with the GenetyxWin (ver. 5.1) software package (Software Development, Tokyo), and the D-*LDH* homolog sequence in the *L. mesenteroides* subsp. *mesenteroides* strain NBRC3426 was deposited in the DDBJ database (DNA Data Bank of Japan, http://www.ddbj.nig.ac.jp/) under accession no. AB233384.

Plasmid construction A map of the plasmid vector used in this study is presented in Fig. 1. Integration vectors, pBTRP-PDC1P-DLDHLM, consisted of the *PDC1* promoter, D-*LDH* from *L. mesenteroides* subsp. *mesenteroides*, *TRP1*, and the *PDC1* downstream fragment. *TRP1* was obtained by treating pRS404 (Stratagene) with *Aat*II and *Ssp*I, and then ligated to the pBluescript II SK+ vector *Hinc*II site after treatment with T4 DNA polymerase (Takara Bio, Otsu). 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 Kit, Dr. GenTLE for yeast (Takara Bio), and the concentration was determined with an Ultro-Spec 300 spectral photometer (Pharmacia Biotech). KOD DNA polymerase was used for PCR amplification, and the oligonucleotide sequences of the primers were as follows (Qiagen K.K.). *PDC1* promoter fragment: PDC1P-U, 5′-ATATATGGATCCGCGTTTAT TTACCTATCTC-3′, containing a *Bam*HI restriction site (underlined); and PDC1P-D, 5′-ATATATGAATTCTTTGATTGATTTGA CTGTG-3′, containing an *Eco*RI restriction site (underlined). This fragment was −705 bp upstream from the *PDC1* ORF start codon. *PDC1* 3′ end fragment: PDC1D-U, 5′-ATATATCTCGAGGCCAG CTAACTTCTTGGTCGAC-3′, containing a *Xho*I restriction site (underlined); and PDC1D-D, 5′-ATATATGGGCCCCTCGTCAGC AATAGTGGTCAAC-3′, containing an *Apa*I restriction site (underlined). The *PDC1* downstream fragment of 518 bp in length was between +501~+1018 from the *PDC1* ORF start codon. The amplification fragments were treated with each restriction enzyme (Takara Bio), and then ligated to a vector, and all plasmids constructed in this work were obtained using standard techniques (17).

Breeding of the yeast *S. cerevisiae* transformation was performed by the lithium acetate procedure (21). The pBTRP-PDC1P-DLDHLM vector fragment, which had been digested with *Sac*I and *Apa*I, was transformed into the *S. cerevisiae* OC-2T strain (18). The host strain OC-2T is a diploid and homothallic strain. 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 tetrad cells were dissected under an optical microscope (Olympus, Tokyo) with a micro-manipulator (Narishige Science, Tokyo). After colonies had been isolated, the target gene integration was confirmed by PCR. The resulting recombinant yeast in this study was named the YILM-2B strain.

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 in a 20 ml test tube containing 2 ml of YPD medium, followed by shaking (100 rpm) at 30°C. Optical density measurements were performed with a Bio-photo recorder (Advantec, Tokyo).

Specific activity of PDC and D-LDH Cell extracts were prepared with a SONIFIER 250 (Branson, Danbury, CT, USA) as described previously (22). PDC-specific activity in freshly prepared extracts, as described by Pronk *et al*. (22), was determined by using an Ubest-55 spectrophotometer at 340 nm (Japan Spectroscopic, Tokyo). D-LDH specific activity was determined in freshly prepared extracts as described by Kochhar *et al*. (23). Protein concentrations in cell extracts were determined with a DC protein assay kit (Bio-Rad, Richmond, CA, USA), using bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) as a standard.

Batch cultivation for fermentation Fermentation experiments were performed at 30°C under anaerobic conditions with a working volume of 40 ml, in 10% YPD medium (1% bacto yeast extract, 2% bactopeptone, and 10% D-glucose) containing 3% of sterilized calcium carbonate. The inoculums were prepared by transferring strains from stock cultures to flasks containing 5 ml of YPD medium. Each culture was incubated for 18 h at 30°C under the aerobic condition, and transferred to the fermentation medium at 0.1% PCV (packed cell volume) in inoculum size. The D-glucose, L- or D-lactic acid, and ethanol concentrations were measured with a bio-sensor BF-4S (Oji Keisoku Kiki, Amagasaki). The optical purity of D-lactic acid was calculated as follows. Each value of the following expression shows the quantity (%, wt/vol) of lactic acid.

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