Research Report

Development of a Metabolically Engineered Yeast for Efficient Production of Pure D-Lactic Acid

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ABSTRACT Poly D-lactic acid (PDLA) has attracted attention as valuable polymer for improving the thermostability of polylactic acid through stereo-complex formation. However, methods for the mass production of D-lactic acid monomer have been studied little in comparison with those for L-lactic acid. In this study, we attempted to develop a metabolically engineered yeast that was able to efficiently produce D-lactic acid monomer with high optical purity. To generate this recombinant strain, the pyruvate decarboxylase 1 (*PDC1*) gene, which plays a key role in the major metabolic pathway for ethanol fermentation, was completely deleted, and six copies of the D-lactate dehydrogenase (D-*LDH*) gene from lactic acid bacteria were introduced under control of two types of robust promoters. Using inexpensive cane juice-based medium as a carbon source, D-lactate production by the recombinant strain reached 80.8 g/l, with 79.4% of sugar being transformed into D-lactate. Notably, the optical purity of this monomer was extremely high, reaching 99.9%. Our approach may be a powerful method for the efficient production of D-lactic acid with high optical purity on an industrial scale.

KEYWORDS Poly lactic acid, D-Lactic acid production, Optical purity, Metabolically engineered yeast, *Saccharomyces cerevisiae*

1. Introduction

Poly lactic acid (PLA) is being developed as a renewable alternative to conventional petroleum-based plastics. The advancement of a sustainable society has created an urgent need for large-scale production of optically pure lactic acid, which is used as a monomer for polymerization of PLA. Although PLA is suitable for several practical applications, its use is not particularly widespread because of its lower thermostability and impact resistance than petroleum-based plastics.[1] In particular, the susceptibility of PLA to heat is a major limitation that is preventing the expanded use of this renewable plastic.

It was reported that a polymer blend of poly L-lactic acid (PLLA) and poly D-lactic acid (PDLA) yields a racemic crystal, termed a stereo-complex (**Fig. 1**). This stereo-complex type of blended polymer is characterized by its high melting temperature, which is approximately 50°C higher than that of PLA.[2,3] The finding that D-lactic acid significantly improves the thermostability of PLA has prompted investigation for effective methods for the production of D-lactic acid.

D-Lactic acid is typically produced using lactic acid bacteria, such as *Lactobacillus* species, and a few trials of the bacterial production of this organic acid from rice and cellulose substrates have been reported.[4,5] However, it was noted that lactic acid bacteria are difficult to culture at high density and required complicated media because most species are highly auxotrophic. In addition, a number of *Lactobacillus* species are unable to produce lactic acid with extremely high optical purity, as they possess both L- and D-lactate dehydrogenase (L-, D-*LDH)* genes.[6] Although the optical purity of the D-lactic acid monomer generated using bacterial systems can reach 95%, this purity is not suitable to achieve the superior physical properties exhibited by PDLA.[1] To improve

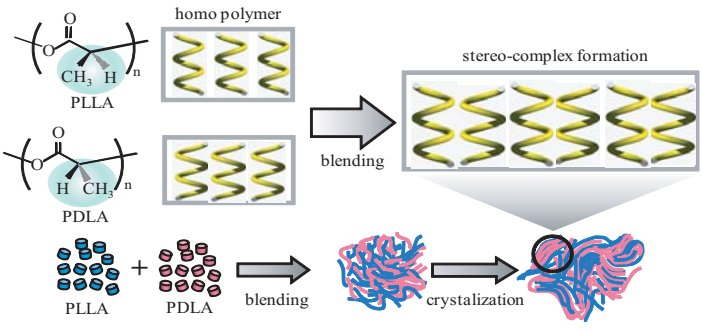

Fig. 1 Stereo-complex formation from PLLA and PDLA. Racemic crystallization resulting from the blending of the two types of polymer leads to improved thermostability of poly lactic acid (PLA).

the purification of D-lactic acid, the separation of optical isomers through crystallization has been reported,[7] but this process significantly increases the production cost of lactic acid. Hence, improved methods for the production and purification of D-lactic acid are needed.

Yeasts, such as *Saccharomyces cerevisiae*, which is commonly used in the production of alcoholic beverages, are more tolerant to low pH and reach higher cell densities than lactic acid bacteria. Generally, *S. cerevisiae* doesn't produce L- and D-lactic acid because of a lack of L- and D-*LDH*. However, if a genetically engineered *S. cerevisiae* with an introduced heterologous D-*LDH* gene is used, D-lactic acid production with high purity can be expected through the conversion of pyruvic acid to D-lactic acid by the action of D-*LDH*. In our previous research, we established an efficient system for the production of L-lactic acid with high optical purity using a metabolically engineered *S. cerevisiae* strain that expressed a heterologous L-*LDH* gene under control of the pyruvate decarboxylase 1 (*PDC1*) promoter.[8-10]

Here, we attempted to develop a metabolically engineered *S. cerevisiae* strain that produces D-lactic acid with high efficiency and optical purity. In addition, to achieve low-cost production of this organic acid, we also examined D-lactic acid production using an inexpensive cane juice-based medium. It is expected that this novel approach for the production of D-lactic acid will supplant conventional methods using lactic acid bacteria.

2. Materials and methods

2. 1 Microbial strains and culture media

Escherichia coli JM109 strain (Toyobo, Osaka) was used for molecular cloning, and was routinely cultured in medium described previously.[11] *S. cerevisiae* OC-2T strain was derived from the wine yeast NBRC 2260,[12] and was routinely cultured using YPD medium (1% yeast extract, 2% peptone, and 2% D-glucose, wt/vol).

2. 2 Construction of plasmid vectors for transgenic yeast

For the generation of transgenic yeast expressing multiple copies of the D-*LDH* gene from *Leuconostoc mesenteroides* strain NBRC 3426,[13] three integration vectors were constructed (**Figs. 2** and **3**). The first integration vector, pBTRP-PDC1P-DLDHLM (Fig. 2), was constructed with the *PDC1* promoter, D-*LDH*, tryptophan requiring 1 (*TRP1*), and a downstream fragment of *PDC1*. The second vector, pBCAT-DLDHLM (Fig. 3(a)), was constructed with the hyper osmolarity responsive 7 (*HOR7*) promoter, D-*LDH*, chloramphenicol resistance gene (*CAT*) from *E. coli*, and two glycerol-3-phosphate dehydrogenase 1 (*GPD1*) fragments. The third vector, pBHPH-DLDHLM (Fig. 3(b)), was constructed with the *HOR7* promoter, D-*LDH*, hygromycin resistance gene (*HPH*) from *E. coli*, and two *GPD2* fragments. All DNA fragments, except for D-*LDH* and the antibiotic resistance genes, were isolated by PCR using the genomic DNA of *S. cerevisiae* OC-2T as a template. Amplified fragments were treated with appropriate restriction enzymes (Takara Bio, Otsu) prior to ligation

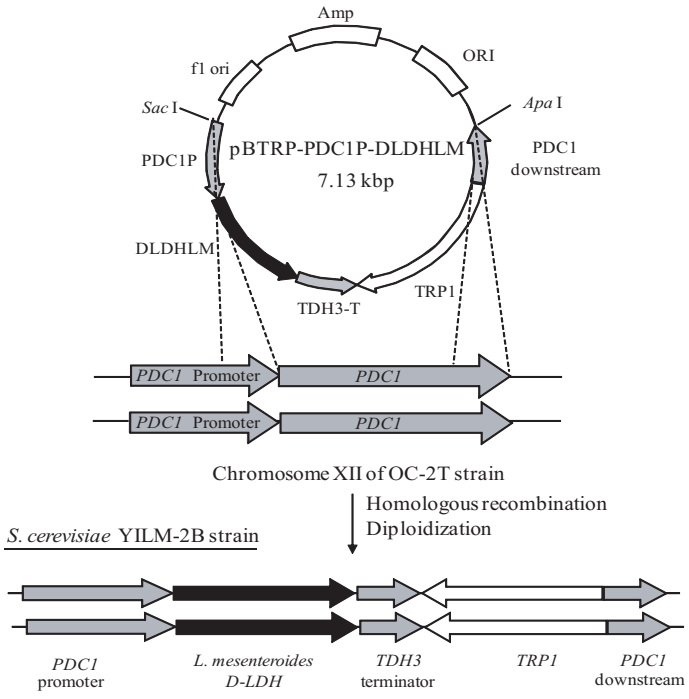

Fig. 2 Map of the first integration vector used for the breeding of transgenic *S. cerevisiae* expressing the D-*LDH* gene from *Leuconostoc mesenteroides*.[13] The constructed DNA fragment for integration, which contained D-*LDH* under control of the *PDC1* promoter and was obtained by digestion of the pBTRP-PDC1P-DLDHLM vector, was integrated into the *PDC1* ORF region of *S. cerevisiae* OC-2T strain by homologous recombination. YILM-2B strain (D-*LDH* 2 copies) was subsequently generated through spore formation.

into vectors, and all plasmids constructed in this study were obtained using standard techniques.[11]

2. 3 Breeding of D-lactic acid-producing yeasts

S. cerevisiae transformation was performed by the lithium acetate procedure.[14] The pBTRP-PDC1P-DLDHLM vector fragment was transformed into the host *S. cerevisiae* OC-2T strain (Fig. 2), which is diploid and homothallic.[12] After transformation, the D-*LDH* cassette was typically located on a single chromosome, and the heterologous gene on the chromosome pair could be duplicated through spore formation, which was performed on sporulation plates (1% acetate, 0.05% D-glucose, 0.1% yeast extract, and 2% agar, wt/vol) as previously described.[12] Diploid formation was performed utilizing the homothallic property of OC-2T, and tetrad cells were isolated under an optical microscope (Olympus, Tokyo) with a micro-manipulator (Narishige Science, Tokyo). After colonies were isolated, target gene integration was confirmed by PCR, and the resulting transgenic yeast, which contained two copies of *L. mesenteroides* D-*LDH*, was named YILM-2B (Fig. 2). Next, YILM-4A strain, which included four copies of D-*LDH*, was constructed using the pBCAT-DLDHLM vector by a similar method as described above (Fig. 3(a)). Subsequently, YILM-6B strain, which included six copies of D-*LDH*, was constructed using the pBHPH-DLDHLM vector, as above (Fig. 3(b)).

2. 4 D-Lactic acid fermentation

Fermentation was performed at 30°C in a 100-ml flask with a working volume of 40 ml YPD10 medium (1% yeast extract, 2% peptone, and 10% D-glucose) containing 3% sterilized calcium carbonate (wt/vol). The initial cell inoculum was prepared by transferring a strain from a stock culture to a flask containing 5 ml YPD medium. The pre-culture was performed for 72 h at 30°C with shaking at 120 rpm, followed by transfer to fermentation medium using an inoculum ratio of 0.1% packed cell volume (PCV). In the experiment using a fermentation volume of 500 ml, the medium consisted of cane juice (approximately 10% sugar) containing 0.3% yeast extract (wt/vol), and was added to a 1-l jar fermenter (Biotto Co., Tokyo). The fermentation conditions were maintained at 30°C and pH 5.0 (NaOH was used for neutralization), with aeration at 0.15 l/min and an agitation rate of 80 rpm.

The D-lactic acid concentration in medium was measured with a model BF-4 biosensor (Oji Keisoku Kiki, Amagasaki), and the optical purity of D-lactic acid was calculated as follows. Optical purity (%,

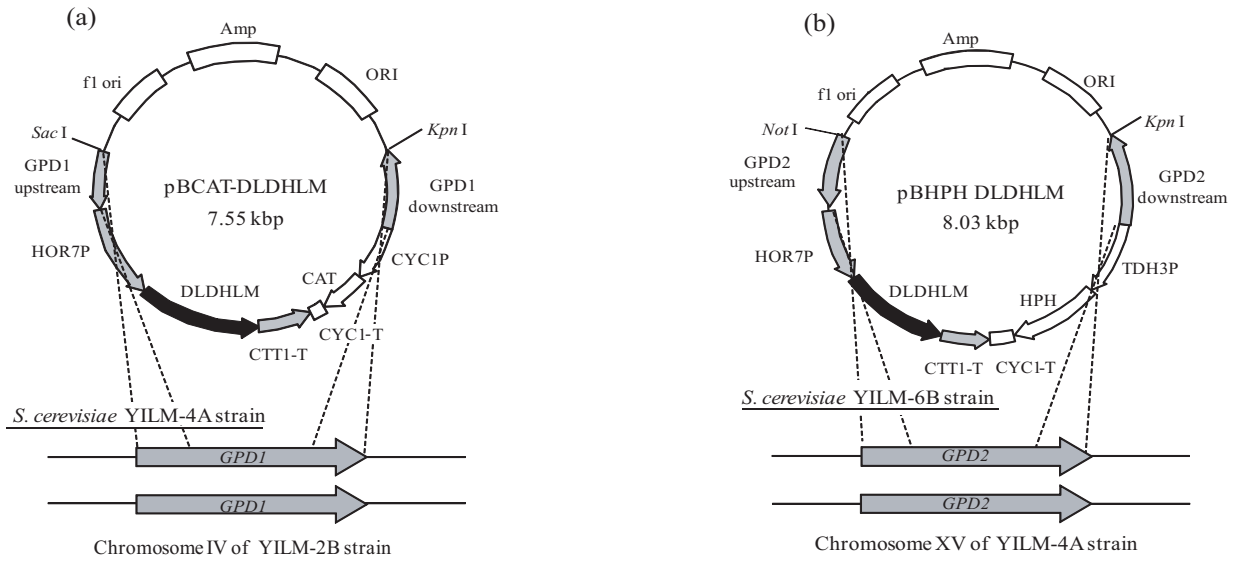

Fig. 3 Maps of the second and third integration vectors and breeding of transgenic *S. cerevisiae*. (a) The pBCAT-DLDHLM vector was integrated into the *GPD1* locus of chromosome IV in YILM-2B strain by homologous recombination to generate *S. cerevisiae* YILM-4A strain (D-*LDH*, 4 copies). (b) The pBHPH-DLDHLM vector was integrated into the *GPD2* locus of chromosome XV in YILM-4A strain by homologous recombination to generate *S. cerevisiae* YILM-6B strain (D-*LDH*, 6 copies). *GPD1* and *GPD2* were disrupted through these integration steps.

wt/vol) = (D-lactic acid quantity - L-lactic acid quantity) / (D-lactic acid quantity + L-lactic acid quantity). The absence of L- and D-lactic acid in prepared media was confirmed prior to the initiation of experiments.

3. Results

3. 1 D-Lactic acid production under neutralizing and non-neutralizing conditions

In the fermentation analysis using YPD medium supplemented with 100 g/l glucose and $CaCO_3$ as a buffer to main neutral conditions, YILM-2B strain expressing two copies of *L. mesenteroides* D-*LDH* produced 61.5 g/l D-lactate and 17.3 g/l ethanol after 72 h of fermentation (**Fig. 4**).[13] L-Lactate was not detected in the culture medium, and the optical purity of D-lactic acid obtained from YILM-2B strain was at least 99.9%.

As *S. cerevisiae* is more tolerant to low pH than lactic acid bacteria,[15] the production of D-lactic acid by YILM-2B strain was also examined under non-neutralizing conditions. The culture conditions were identical as those used for the pH neutral cultures, except for the lack of $CaCO_3$ in the YPD medium. The accumulation and yield of free D-lactic acid after 72 h are shown in Fig. 4. For YILM-2B strain, the maximal yield of D-lactic acid reached 53.0%, which was slightly lower than the yield of 61.2% under neutralizing conditions. Further, the effect of changing the initial glucose concentration from 100 to 150 g/l on the yield of lactic acid was examined under non-neutralizing conditions. However, as judged by the fermentation analysis, improvement of lactic acid production was not observed in the presence of additional glucose, as the final yield (35.9%) was significantly lower than that using YPD supplemented with 100 g/l glucose under either neutralizing or non-neutralizing conditions. YILM-2B strain could not consume glucose completely, and the final pH of the culture solution was 2.8.

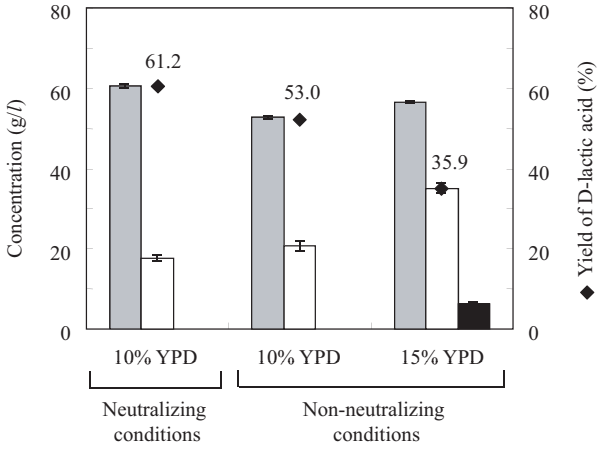

Fig. 4 Accumulation of D-lactic acid (gray bars), ethanol (white bars), and glucose (black bars) by YILM-2B strain (D-*LDH*, 2 copies) under neutralizing and non-neutralizing culture conditions.[13] YILM-2B strain was cultivated for 72 h under anaerobic conditions at 30°C. Closed diamonds and adjacent values show the yield of D-lactic acid, which represents the conversion ratio from total glucose in YPD medium. The averages and standard deviations (error bars) for three independent experiments are presented.

3. 2 Increasing copy number of the D-*LDH* gene in the genome

Based on our previous study of L-lactic acid production,[9] it was expected that D-lactate production would be improved by increasing the number of D-*LDH* genes in the genome through integration. For efficient fermentation of optically pure D-lactate monomers, two recombinant strains with increased copy numbers of D-*LDH* in the genome were constructed, and D-lactate production was then examined in YPD10 medium. As shown in **Fig. 5**, the strain containing the most copies of D-*LDH*, YILM-6B strain (D-*LDH*, 6 copies), produced the highest concentration of D-lactate after 72 h (76.7 g/l), with up to 79.6% of the total glucose being transformed into lactic acid. Notably, the yield was improved by greater than 1.2 fold compared with that of YILM-2B strain.

YILM-6B strain was designed to express D-*LDH* under the control of two different promoters, *PDC1* and *HOR7*. It was previously confirmed that the *HOR7* promoter is activated in response to lactic acid.[16] In a metabolically engineered *S. cerevisiae* YILM-6B strain, increasing the copy number of D-*LDH* and using a dual promoter led to a remarkable increase of D-lactate productivity.

Finally, to examine the production of D-lactate using an inexpensive medium, lactate fermentation by YILM-6B strain using a cane juice-based medium was also examined. Cane juice represents one of the largest sources of available biomass, and can be prepared at a significantly reduced cost compared with YPD. Using

jar fermenters, D-lactate production reached 80.8 g/l after 72 h, with up to 79.4% of total sugar being transformed into D-lactate (**Fig. 6**). These results demonstrated that D-lactate can be produced in inexpensive cane juice-based medium with equal productivity as that using YPD medium.

4. Discussion

PDLA is an important polymer because it significantly improves the thermostability of PLLA through stereo-complex formation.[2,3] However, approaches for the efficient fermentation of D-lactic acid monomer have been studied relatively little in comparison with those for L-lactic acid. In this study, we developed a metabolically engineered *S. cerevisiae* strain capable of efficient production of D-lactic acid by introducing six copies of the D-*LDH* gene from *L. mesenteroides* in the genome. Using a similar approach, we previously established that genomic integration of the L-*LDH* gene under control of the *PDC1* promoter leads to efficient production of L-lactic acid.[8-10] However, the additional use of the *HOR7* promoter for D-*LDH* gene expression had a significant effect on lactic acid production in comparison with use of the *PDC1* promoter alone.[9,16] Our findings also suggest that this method may be effective for the production of other organic acids. However, ethanol was still produced as a by-product, which was likely due to the overexpression of *PDC5* mRNA resulting from the *PDC1* gene deletion.[17] Hence, modification of the metabolic pathway for ethanol production, such as the deletion of *PDC5*[18] and *ADH1*,[19] are expected to result in further improvement of D-lactic acid productivity.

For stereo-complex formation from PLLA and PLDA, high optical purity of the polymers is necessary. Our present findings demonstrate that metabolically engineered *S. cerevisiae* can produce optically pure D-lactic acid at levels exceeding 99.9%, which is necessary to improve the thermostability of PLA.[2,3] Notably, we have also shown that fermentation in an inexpensive medium, such as cane juice, also yields high concentrations of D-lactic acid monomers (Fig. 6).

For conventional methods of generating D-lactic acid using lactic acid bacteria, it has been noted that the desalination step of free lactic acid is the main factor that increases production costs. The present engineered strains, which express D-*LDH* from *L. mesenteroides*, produce free D-lactic acid with high optical purity under non-neutralizing conditions (Fig. 4), because yeast is well known to grow and survive at low pH compared with lactic acid bacteria. If lactic acid is obtained directly under non-neutralizing conditions

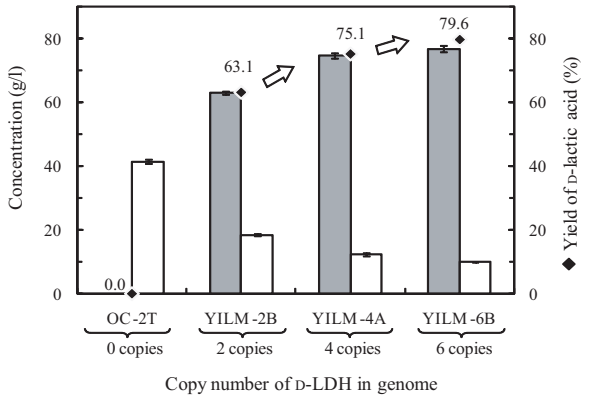

Fig. 5 Comparison of D-lactate production with increasing gene copy number of D-*LDH* gene. Gray and white bars indicate D-lactate and ethanol, respectively. Strains YILM-2B, YILM-4A, and YILM-6B, containing two, four, and six copies of the D-*LDH* gene, were individually cultivated for 72 h under anaerobic conditions at 30°C. Closed diamonds and adjacent values show the yield of D-lactic acid, which represents the conversion ratio from total glucose in YPD medium. The averages and standard deviations (error bars) for three independent experiments are presented.

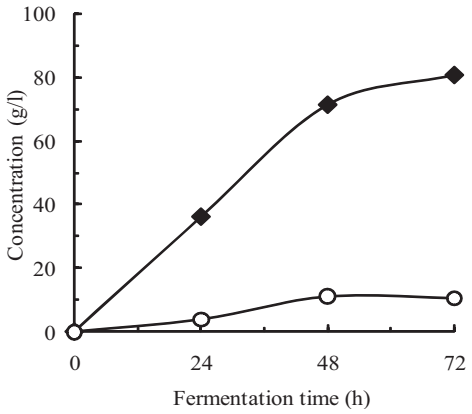

Fig. 6 Time courses of cultivation of *S. cerevisiae* YILM-6B strain (D-*LDH*, 6 copies) in cane juice-based medium as a carbon source using a jar fermenter. Closed diamonds and open circles indicate the concentration of produced D-lactate and ethanol, respectively.

without a desalination step in an inexpensive medium, the cost of large-scale manufacturing would be significantly reduced. However, to further improve the yields of D-lactic acid under non-neutralizing conditions, it is important to increase the low pH tolerance of intracellular LDH and host cells. To this end, the stress response mechanism of *S. cerevisiae* under low pH conditions will be examined in future studies.

S. cerevisiae is a suitable microorganism for industrial-scale applications, such as ethanol fermentation in the food industry. The strain developed here has the following three advantages for the production of D-lactic acid. First, D-lactic acid of extremely high optical purity can be produced. Second, lactic acid can be produced even if an inexpensive medium, such as one based on cane juice, is used. Finally, because *S. cerevisiae* exhibits low pH tolerance, free lactic acid production can be achieved without the need for medium neutralization. In conclusion, our approach may be a powerful method for the efficient production of D-lactic acid with high optical purity on an industrial scale in the future.

Acknowledgments

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References

(1) Ozeki, E., "Characteristics of Poly (L-Lactide) as Biodegradable Plastics", *Shimadzu Rev.*, Vol.53 (1996), pp.1-8.
(2) Ikada, Y., et al., "Stereocomplex Formation between Enantiomeric Poly (Lactic Acid)s", *Macromolecules*, Vol.20 (1987), pp.904-906.
(3) Tsuji, H., et al., "Stereocomplex Formation between Enantiomeric Poly (Lactic Acid)., 3. Calorimetric Studies on Blend Films Cast from Dilute Solution", *Macromolecules*, Vol.24 (1991), pp.5651-5656.
(4) Yanez, R., et al., "Production of D(-)-Lactic Acid from Cellulose by Simultaneous Saccharification and Fermentation Using *Lactobacillus coryniformis* subsp. *torquens*", *Biotechnol. Lett.*, Vol.25 (2003), pp.1161-1164.
(5) Fukushima, K., et al., "Production of D-Lactic Acid by Bacterial Fermentation of Rice Starch", *Macromol. Biosci.*, Vol.4 (2004), pp.1021-1027.
(6) Hofvendahl, K. and Hahn-Hagerdal, B., "Factors Affecting the Fermentative Lactic Acid Production from Renewable Resources (1)", *Enzyme Microb. Technol.*, Vol.26 (2000), pp.87-107.
(7) van Breugel, J., et al., "Method of Industrial-scale Purification of Lactic Acid", United States Patent No.6,630,603.
(8) Ishida, N., et al., "Efficient Production of L-Lactic Acid by Metabolically Engineered *Saccharomyces cerevisiae* with a Genome-integrated L-Lactate Dehydrogenase Gene", *Appl. Environ. Microbiol.*, Vol.71 (2005), pp.1964-1970.
(9) Ishida, N., et al., "Metabolic Engineering of *Saccharomyces cerevisiae* for Efficient Production of Pure L-(+)-Lactic Acid", *Appl. Biochem. Biotechnol.*, Vol.131 (2006), pp.795-807.
(10) Ishida, N., "Efficient Production of Lactic Acid with High Optical Purity by Transgenic Yeast (Japanese Review)", *Bio-Industry,* Vol.27 (2010), pp.45-50.
(11) Sambrook, J., et al., *Molecular Cloning*: *A Laboratory Manual, 3rd ed.* (2001), Cold Spring Harbor Laboratory Press, NY.
(12) Saitoh, S., et al., "Breeding of a New Type of Baker's Yeast by δ-integration for Overproduction of Glucoamylase Using a Homothallic Yeast", *J. Ferment. Bioeng.*, Vol.81 (1996), pp.98-103.
(13) Ishida, N., et al., "D-Lactic Acid Production by Metabolically Engineered *Saccharomyces cerevisiae*", *J. Biosci. Bioengin.*, Vol.101 (2006), p.172-177.
(14) Ito, H., et al., "Transformation of Intact Yeast Cells with Treated with Alkali Cations", *J. Bacteriol.,* Vol.153 (1983), pp.163-168.
(15) Porro, D., et al., "Development of Metabolically Engineered *Saccharomyces cerevisiae* Cells for the Production of Lactic Acid", *Biotechnol. Prog.*, Vol.11 (1995), pp.294-298.
(16) Ishida, N., et al., "Promoter in the Presence of Organic Acid and Utilization Thereof", United States Patent No.4,460,876.
(17) Hohmann, S. and Cederberg, H., "Autoregulation may Control the Expression of Yeast Pyruvate Decarboxylase Structural Genes *PDC1* and *PDC5*", *Eur. J. Biochem*., Vol.188 (1990), pp.615-621.
(18) Ishida, N., et al., "The Effect of Pyruvate Decarboxylase Gene Knockout in *Saccharomyces cerevisiae* on L-Lactic Acid Production", *Biosci. Biotechnol. Biochem.*, Vol.70 (2006), pp.1148-1153.
(19) Tokuhiro, K., et al., "Double Mutation of the *PDC1* and *ADH1* Genes Improves Lactate Production in the Yeast *Saccharomyces cerevisiae* Expressing the Bovine Lactate Dehydrogenase Gene", *Appl. Microbiol. Biotech.*, Vol.82 (2009), pp.883-890

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