A study on the efficient production of lactic acid with metabolically engineered *Saccharomyces cerevisiae*

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Chapter 1

General Introduction

1.1. Plant- and crop-based renewable plastics

A worldwide environmental problem is mass consumption, and large-scale reduction of resources is gradually becoming serious. An important goal in the 21st century is to form a sustainable society that is in harmony with the earth. To achieve such a new society, it is necessary to create an original technology that harmonizes the global environment with maintenance of the social system.

Plant- and crop-based renewable plastics, bio plastics, are being paid attention as environmentally friendly plastics that do not need petroleum as a raw material. A typical plant-based renewable plastic is a polymer material made from a biomass resource, such as starch (corn, sugar cane, rice, wheat and garbage), sucrose (molasses), cellulose (wood and old paper), xylose (wood), by means of a biological or chemical process. A key distinguishing feature of such a polymer is that it is resolved into only H_2O and CO_2 on hydrolysis and bio degradation, because this renewable plastic is made from a biomass resource. H_2O and CO_2 produced on this degradation can be reused by plants through photosynthesis, and there is the advantage of not increasing the CO_2 density in the atmospheric any further. This effect is called “Carbon neutral”. Poly lactic acid (PLA), poly hydroxyalkane (PHA), starch-based plastics, and soybean-based polyol polyuretan are known as typical bio plastics.

Some renewable plastics have already been developed for practical use. In particular, the poly lactic acid excels in both transparency and tolerance to water and oil. In addition, its physical properties and mechanical strength, such as luster, transparency, etc., are also excellent. PLA is being paid attention as the most promising material for plant- and crop-based renewable plastic (Fig. 1. 1). Although PLA has already been put to practical use, its use is not so widespread because the production cost is too high in comparison with that for petroleum-based plastics currently in use. Its thermostability and resistance to impact are also lower, so there are a lot of problems to be solved before its use as a general plastic. Therefore, to develop PLA as a general-purpose plastic,

improvement of its physical properties and a decrease in the production cost are indispensable.

Fig. 1. 1. Poly lactic acid (PLA), a plant- and crop-based renewable plastic. A, PLA pellets. B, PLA samples.

1.2. Poly lactic acid (PLA)

Poly lactic acid (PLA) is a polymer synthesized by Carothars of Du Pont Co. for the first time in around 1930. PLA was put into practical use in the 1970s with the advancement and refinement of polymerization technology for lactic acid, although it was expensive. At first, the research on it as a medical material, including suture thread, a scaffold for tissue regeneration, and the matrix of drug delivery systems, advanced because PLA had the property of biodegradation. Later, PLA came to be paid attention as a "plant- and crop-based renewable plastic" with the increase in consideration of the environmental problem.

The production of PLA

The production of PLA from a biomass resource can be roughly divided into the following three steps (Fig. 1. 2).

1: The process of producing lactic acid from a biomass resource (Fig. 1. 3).
(Saccharification → Lactic acid fermentation → Purification)

2: The process of synthesizing PLA through polymerization (Fig. 1. 4).

3: The process of improving the properties of PLA
(Thermostability, impact resistance, etc.).

"The process of producing lactic acid" accounts for the largest percentage of the manufacturing cost of PLA, in particular, it has been pointed out that the fermentation and purification of lactic acid are expensive. It is known that the price of PLA is 3-4 times higher than that of polyethylene, which is a major petroleum-based plastic. The development of efficient means of lactic acid fermentation and its purification are important to reduce the production cost of PLA. In this study, I particularly concentrate on the development of a highly effective means of production of lactic acid. First of all, I summarize the current technology for the lactic acid fermentation process as a background to this study.

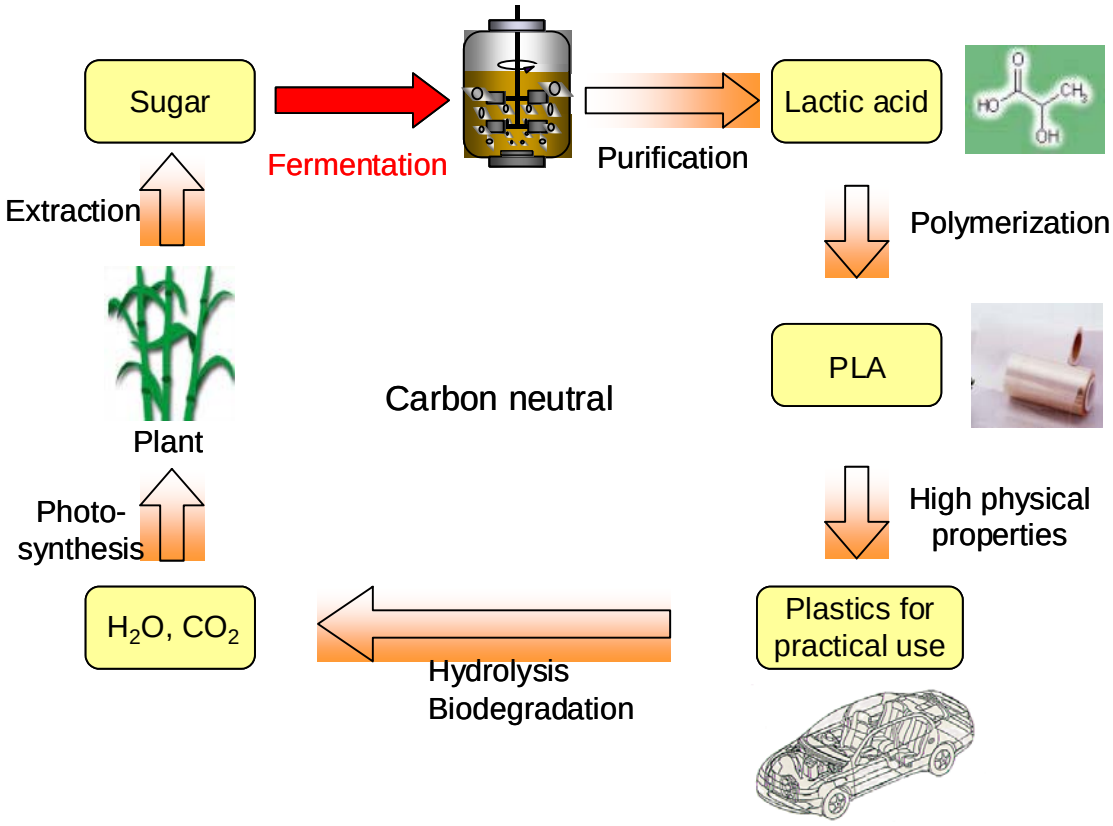

Fig. 1. 2. Process of poly lactic acid (PLA) production and the concept of carbon neutral. Firstly, lactic acid is produced from extracted sugar. Secondly, PLA is synthesized through polymerization of lactic acid, and lastly PLA is endowed with high physical properties. PLA is resolved into only H_2O and CO_2 on hydrolysis and bio-degradation. These substances can be reused by plants through photosynthesis, and the CO_2 density is not increased any further (carbon neutral). In this study, I particularly concentrate on lactic acid fermentation (red arrow).

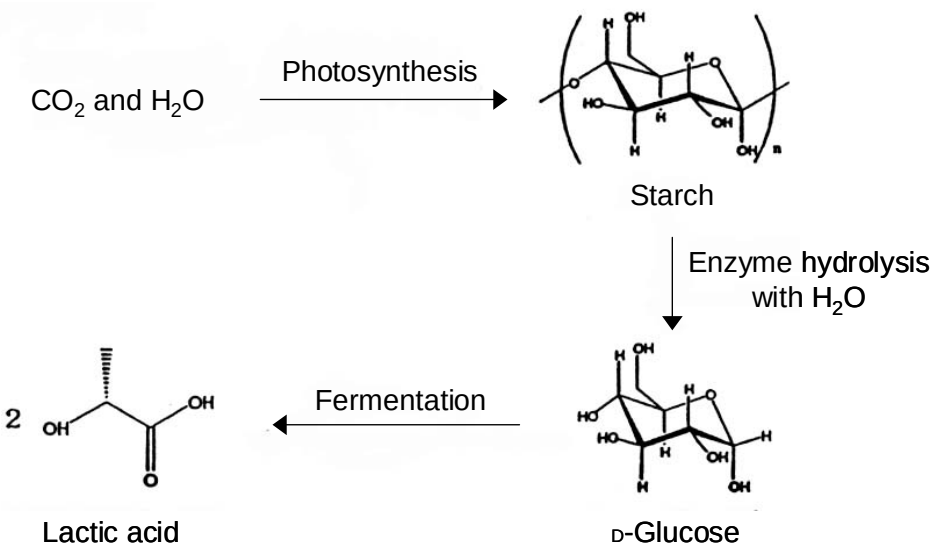

Fig. 1. 3. The process of producing lactic acid from a biomass resource. Generally, lactic acid is produced with lactic acid bacteria.

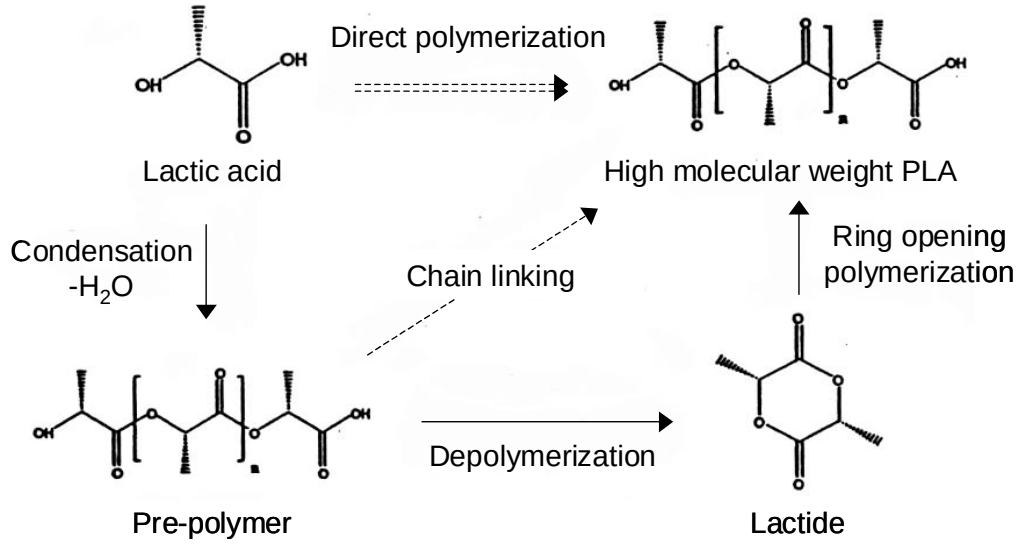

Fig. 1. 4. The process of synthesizing PLA through polymerization. Three polymerization methods are known. Solid line: ring opening polymerization of lactide. Dashed line: chain linking of pre-polymer. Double dashed line: direct polymerization of lactic acid.

1.3. Lactic acid production with lactic acid bacteria

Lactic acid is generally produced by lactic acid bacteria (Marshall, 1987), such as *Lactobacillus, Streptococcus, Lactococcus, Leuconostoc,* and *Pediococcus* species. The lactic acid fermentation by these species can be mainly divided into two types, homothallic and heterothallic fermentation (Fig. 1. 5). Lactic acid bacteria exhibit high auxotrophy, and demand many kinds of amino acids and vitamins besides sugars. In general, lactic acid fermentation takes place under neutralizing conditions, because a low pH has inhibitory effects on cell growth and lactic acid production.

Studies on carbon sources for lactic acid fermentation

Biomass resources such as lactose (milk sugar), sucrose (molasses), starch (wheat, corn, rice and garbage), cellulose (wood and old paper), and xylose (wood) have been examined to their suitability as carbon sources for lactic acid fermentation. In the case of starch, saccharification by amylase is necessary, and the lactic acid bacteria that exhibit amylase activity are known (*L. fermentum*, *L. amylophilus*, and *L. amylovorus*). In the case of cellulose and xylose, either acid or cellulase treatment is necessary. It has been understood that the use of a refined sugar contributes to effective lactic acid production. However, a refined sugar is expensive, and it is not suitable for industrial production. Therefore, lactic acid production from a waste biomass resource has been attempted. Some reports of studies on carbon sources for lactic acid fermentation are summarized in Table 1.1.

Studies on nitrogen sources for lactic acid fermentation

Lactic acid bacteria exhibit high auxotrophy, and abundant resources could lead to the efficient production of lactic acid. Nitrogen sources such as urea, peptone, yeast extract, and corn steep liquor have been examined. Although yeast extract is the most suitable nitrogen source, it is too expensive. Therefore, the

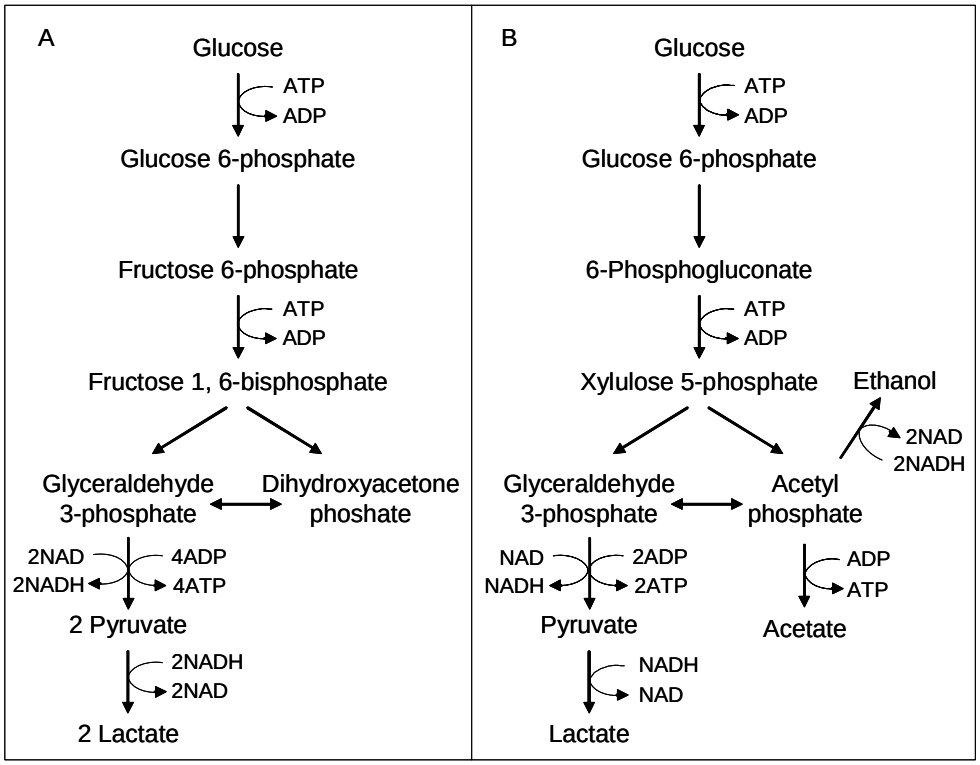

Fig. 1. 5. Metabolic pathway of lactate fermentation with lactic acid bacteria. A, homothallic fermentation. B, heterothallic fermentation.

Table 1. 1. Study on lactic acid fermentation from a waste biomass resource.

Biomass	Species	Pre-treatment	Nitrogen source	Lactic acid concentration	Yield	Reference
Molasses	*L. rhamnosus* ATCC10863	invertase	yeast extract	16 g/liter	81%	Aksu et al. 1986
Molasses	*L. salivalius* ATCC11742	invertase	yeast extract	5.5 g/liter	85%	Montelongo *et al*. 1993
Potato peeling	*L. delbrueckii* ATCC12315	amylase	corn steep liquor	93 g/liter	78%	Tsai *et al*. 1994
Paper mill sludge	*L. pentosus* B227	hydrolysis	yeast extract peptone	58 g/liter	74%	Mccaskey *et al*. 1994
Old paper	*L. delbrueckii* B445	cellulase	yeast extract	31 g/liter	84%	Schmidt *et al*. 1997
Milk serum	*L. casei* SU.No.22	cellulase	yeast extract peptone	40 g/liter	83%	Roukas *et al*. 1998
Garbage	*L. plantarum*	amylase	yeast extract	45 g/liter	45%	Sakai *et al*. 2000
Pulp mill solid	*L. delbrueckii* B445	cellulase	yeast extract	62 g/liter	86%	Thomas, 2000

most important means of cost reduction is to find a cheap nitrogen source to take place of the yeast extract.

Tejayadi and Cheryan reported about soybean peptone and five kinds of vitamin B. For L-lactate fermentation with *L. caseisubsp. rhamnosus*, it was found to be possible to decrease the yeast extract concentration to 5 g/liter by adding 25 mg/liter of vitamin B and 3.3 g/liter of soybean peptone (Tejayadi and Cheryan, 1995). Hujanen and Linko reported lactic production with 11 nitrogen sources (yeast extract, malt, peptone, grass extract, corn steep liquor, NZ-case plus, NZ-amine YT, casein hydrolysate, distiller refuse, ammonium phosphate, and urea). As a result, a decrease in the concentration of yeast extract from 22 g/liter to 4 g/liter was achieved, when 100 g/liter of malt was added, and L-lactate production reached 70-90 g/liter (Hujanen and Linko, 1996).

Borgardts *et al*. isolated a new *Lactococcus lactis* strain that can use milk serum as a nitrogen source. This strain exhibited lactate accumulation of 41 g/liter with dairy factory sewage as a nitrogen source, and low cost production (Borgardts *et al*. 1998). Kwon *et al*. reported lactate fermentation with *Lactobacillus rhamnosus* using vitamin-supplemented soybean hydrolysate as the only nitrogen source. As a result of optimization, they succeeded in the complete replacement of 15 g/liter of yeast extract with 19.3 g/liter of soybean hydrolysate. Under these conditions, lactic acid accumulation reached 125 g/liter from 150 g/liter of D-glucose, and the production yield of lactic acid was 92 % (Kwon *et al*. 2000).

Studies on transgenic lactic acid bacteria

A few attempts have been made to improve L-lactic acid production through metabolic engineering of lactic acid bacteria. Ferain *et al*. reported the cloning of the gene encoding the L-lactate dehydrogenase (L-*LDH*) from *L. plustanm* DG301. The nucleotide sequence of the L-*LDH* gene predicted a protein of 320 amino acids closely related to that of *Lactobacillus pentosus*. The introduction of multiple copies of this gene into *L. plantarum* resulted in a 13-fold increase in L-LDH activity. However, they failed to increase the production of L-lactate (Ferain *et al*. 1994).

Similarly in *Lactococcus lactis*, when the copy number of the lac operon in which the L-*LDH* gene was present was increased, only a slight increase in lactic acid production resulted (Davidson *et al*. 1995).

Lapierre *et al.* developed a transgenic *Lactobacillus johnsonii* that exhibits D-LDH inactivation. The mutant, completely lacking the D-*LDH* gene, showed rerouting of pyruvate to L-lactate with a minimum increase in secondary end products acetaldehyde, acetoin and diacetyl (Lapierre *et al.* 1999). Kyla-Nikkila *et al.* also reported that D-LDH negative *L. helveticus* CNRZ32 mutant strain only produced L-lactic acid in amounts equal to the total lactate produced by the wild type. The maximum L-LDH activity in the mutants was found to be 93% higher than that in the wild-type strain (Kyla-Nikkila *et al*. 2000).

1.4. Lactic acid production with fungi

It is also known that *Rhizopus oryzae,* which is a fungus, produces L-lactic acid under aerobic conditions. *R. oryzae* has the distinguishing feature that fermentation is possible with only the addition of a small amount of mineral salt to sugar, i. e., without the addition of expensive yeast extract, because this species shows low auxotrophy in comparison with lactic acid bacteria. It is known that *R. oryzae* exhibits various forms. In particular, in the case of the pellet form, the separation and recovery of this fungus can be easily performed. In addition, the L-lactic acid that is produced by *R. oryzae* shows high optical purity.

Studies on L-lactic acid production

There have been some reports on lactic acid fermentation with *R. oryzae*. For instance, Yin *et al*. reported that *R. oryzae* produced a final concentration of 102 g/liter of L-lactic acid from corn starch. The yield of lactic acid was 85% (Yin *et al*. 1997 and 1998). Zhou *et al.* also investigated optimization of L-lactic acid production from glucose by *R. oryzae* ATCC 52311. Results showed a maximum lactic acid production rate of 2.58 g/liter/h was obtained with an initial glucose concentration of 94 g/liter. The final lactic acid concentration of 83 g/liter was achieved after 32 h of fermentation with a weight of 0.88 g lactic acid/g glucose consumed (Zhou *et al.,* 1999). Miura *et al*. isolated a mutant of the *R. oryzae* MK96 strain that showed ammonia tolerance on NTG (N-Methyl-N'-Nitro-N-Nitrosoguanidine) mutagenesis. The yield of lactic acid with this mutant was almost twice with half the incubation time compared with the parent strain (Miura *et al*. 2003). With in an airlift bioreactor, which involves ammonia as a neutralizer, this isolated strain gave a final concentration of lactic acid of 90 g/liter, the yield being 87% (Miura *et al*. 2004).

In general, *R. oryzae* is known to be in a pellet form during fermentation, and the pellet form changes into the line form on the addition of an inorganic substance and polyethylene oxide. Park *et al*. reported that it is more efficient to use the line

form than the pellet form for lactic acid fermentation with an airlift bioreactor. The culture solution flows efficiently with the line form of *R. oryzae*, and this phenomenon allows lactic acid production with a final concentration of 104.6 g/liter and a yield of 87% (Park *et al*. 1998).

Studies on carbon sources for lactic acid fermentation

Regarding lactic acid production with *R. oryzae*, some studies on carbon sources have been reported. Bulut *et al*. compared the lactic acid production with glucose, sucrose, beat molasses, and wheat bran as substrates for lactic fermentation. As a result, it was found that the lactic acid concentration was the highest, 60 g/liter, when only glucose was used as a carbon source, and the production yield was 60%. On the other hand, in the cases of other carbon sources, it has been confirmed that sucrose, beat molasses, and wheat bran are unsuitable as substrates for lactate fermentation with *R. oryzae*. Only when the supernatant of a bean extract was used, it observed 58 g/liter of lactic acid production (Bulut *et al*. 2004). Park *et al*. examined the effect on lactate fermentation using an enzymatic hydrolysate office paper was used as the carbon source. In 4 days culture, 82.8 g/liter glucose, 7.0 g/liter xylose, 3.4 g/liter cellobiose contained in the hydrolysate were consumed to produce 49.1 g/liter of lactic acid. It was considered that the production rate may be inhibited by xylose derived from hemicellulose in paper pulp (Park *et al*. 2004). Ruengruglikit and Hang demonstrated lactate fermentation with corncobs as the carbon source, and this yield exhibited 30 % (Ruengruglikit and Hang, 2003).

1.5. Lactic acid production with a transgenic *Escherichia coli*

It is known that *E. coli* produces both organic acids (D-lactic acid, acetate, formic acid, and succinic acid) and ethanol through the metabolism of five or six monosaccharides with only mineral salt as a nutrient. Recently, an attempt at efficient production of lactic acid was made using genetic engineering techniques. The advantage of a transgenic *E. coli* is as follows. Firstly, the main resource needs only to be a mineral one and the growth rate is high too. Secondly, many kinds of sugar can be used as carbon sources. Lastly, the production of lactic acid of high optical purity is possible without a byproduct with a metabolically engineered *E. coli* (Chang *et al*. 1999).

Studies on L-lactic acid production

Dien *et al.* achieved efficient production of L-lactic acid with a transgenic *E. coli* that expresses the L-*LDH* gene from *Streptococcus bovis*. This recombinant exhibited lactate productivity of 2.2 g/liter/h, the yield being 93% (Dien *et al.* 2001). Zhou *et al*. also developed a metabolically engineered *E. coli* containing five chromosomal deletions (*pflB*, *ackA*, *adhE*, *ldhA*, and *frdBC,* Fig. 1. 7) and a chromosomally integrated L-(+)-*LDH* gene from *Pediococcus acidilactici*. This recombinant produced a high yield of optically pure L-lactic acid from glucose and xylose in a mineral salts medium (Zhou *et al*. 2003-a).

Studies on D-lactic acid production

There have been some reports on D-lactic acid production with a transgenic *E. coli* in addition to L-lactic acid production. *E.coli* originally produces a mixture of organic acids (D-lactate, acetate, succinate, and formate) and ethanol to accommodate reducing equivalents generated during glycolysis. Therefore, four gene deletions (*pflB*, *ackA*, *adhE*, and *frdBC,* Fig. 1. 7) in *E. coli* led to D-lactic acid production. Zhou *et al*. developed 13 kinds of transgenic *E.coli*, and analyzed

D-lactate production with them. In particular, the SZ58 (*pflB, frdBC, adhE,* and *ackA*) and SZ63 (*pflB, frdBC,* and *adhE*) recombinant strains showed high yields of D-lactate (80-90%) in a mineral salts medium including 5% (wt/vol.) of glucose (Zhou *et al.* 2003-b). Additionally, Shukla *et al*. reported the examination of carbon sources. They developed a transgenic *E. coli* SZ63 strain that expresses sucrose utilization genes (*scrKYAB* and *scrR*), and demonstrated the production of optically pure D-lactate from sucrose and molasses (Shukla *et al.* 2004).

Although effective production has been achieved using a recombinant *E. coli*, a low pH inhibits cell growth and lactic acid production under non-neutralizing conditions, because *E. coli* can not tolerate a low pH. Therefore, it is necessary to produce free lactic acid under neutralizing conditions.

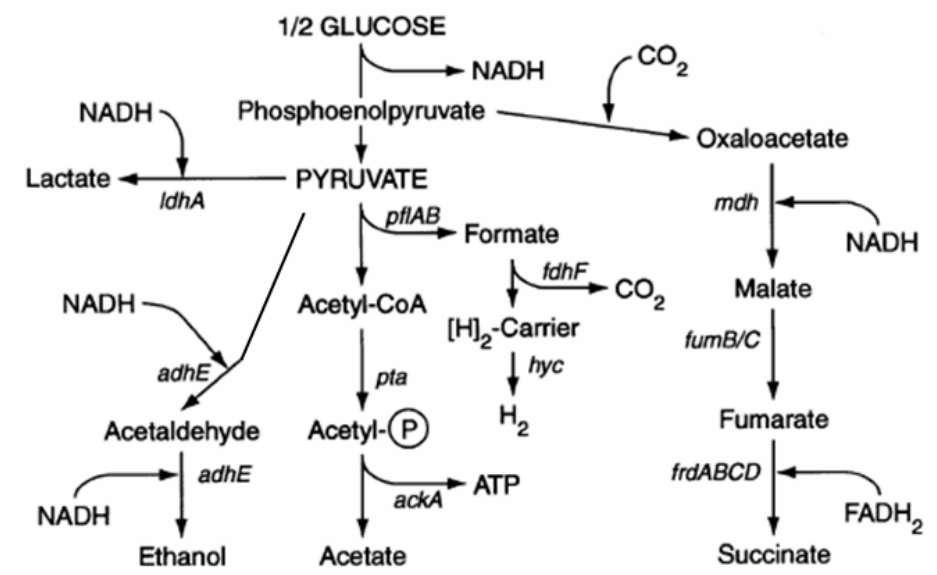

Fig. 1. 7. Fermentation pathways of *E. coli* reactions represented by the names of the corresponding genes (Jiang *et al*., 2001): *ldhA*, lactate dehydrogenase; *pflAB*, pyruvate formate lyase; *adhE*, alcohol dehydrogenase; *pta*, phospho-transacetylase; *ackA*, acetate kinase; *fdhF*, formate dehydrogenase; *hyc*, hydrogenase; *mdh*, malate dehydrogenase; *fumBIC*, fumarate hydratase; *frdABCD*, fumarate reductase.

1.6. Lactic acid production with transgenic yeast

Generally, yeast, including *Saccharomyces cerevisiae*, hardly produce either L- or D-lactic acid. However, *S. cerevisiae* is more tolerant to low pH than lactic acid bacteria, *R. oryzae*, and *E. coli*. Therefore, if a genetically engineered *S. cerevisiae* is used, free lactic acid production can be expected without pH control. Also, a transgenic yeast producing lactic acid is hoped for high optical purity, which is an effective factor for high physical properties of poly lactic acid. Recently, a new method for producing lactic acid with a genetically engineered *S. cerevisiae* was developed and applied to large-scale production on a trial base.

Studies on L-lactic acid production

In 1994, Dequin and Barre first developed an L-lactic acid-producing *S. cerevisiae* by expressing the heterologous L-*LDH* gene from *Lactobacillus casei*. However, the yield of the lactic acid was low, approximately 20%, and the major fermentation product was ethanol (Dequin *et al.* 1994). Porro *et al*. reported L-lactic fermentation with *S. cerevisiae* into which the bovine L-*LDH* gene with a multi-copy plasmid was introduced. This recombinant strain accumulated lactic acid to a final concentration of 20 g/liter, the productivity being 11 g/liter/h. However, this recombinant also produced a large amount of ethanol besides L-lactic acid (Porro *et al.* 1995). The by-product ethanol has become a major problem for lactic acid fermentation with a transgenic yeast.

In *S. cerevisiae*, there are three structural pyruvate decarboxylase genes, *PDC1*, *PDC5* (Schmitt *et al*. 1983; Schaalf *et al*. 1989), and *PDC6* (Hohmann, 1991) for ethanol production. The PDC activity in yeast is mainly due to *PDC1* and *5* genes (Flikweert *et al*. 1996 and 1999-a). The expression of these genes is controlled by auto-regulatied system, that is to say, *PDC1* deletion leads to a great increase in *PDC5* promoter-driven mRNA expression (Hohmann and Cederberg, 1990). A *PDC*-negative mutant lacking all three genes exhibited a three-fold lower growth rate in a complex medium containing glucose than the wild type strain

(Flikweert *et al*. 1996). Additionally, Flikweert *et al*. proposed that the reduction in the growth rate depended on the regeneration of NADH in the absence of PDC activity (Flikweert *et al.* 1999-b). In lactic acid-producing yeast, Brambilla *et al*. tried to improve the regeneration of NADH on glycolysis by using a recombinant strain expressing *Lactobacillus casei* L-*LDH*. But, it was found that NADH reoxidation does not control glycolytic flux during exposure of respiring *S. cerevisiae* cultures to an excess of glucose (Brambilla *et al*. 1999).

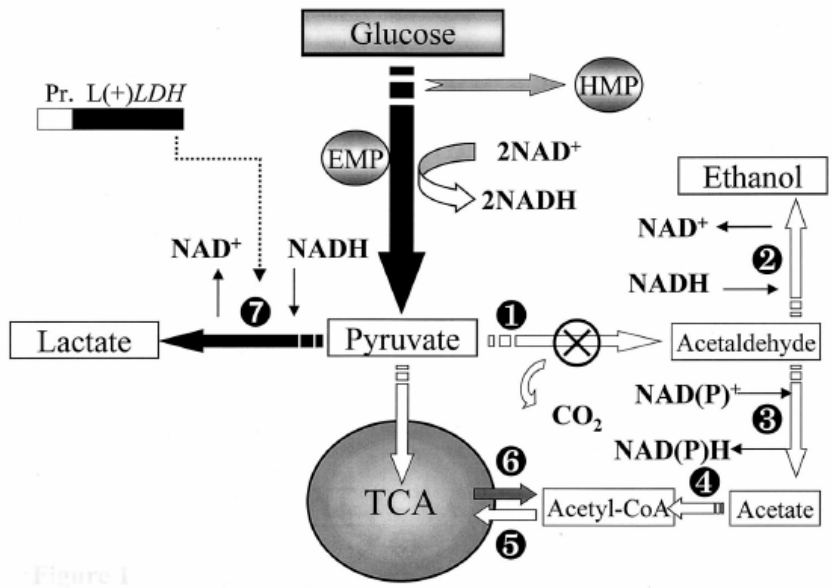

Fig. 1. 8. Schematic representation of the main pyruvate metabolism pathways in Yeast (Porro et al. 1999). EMP, Embden-Meyerhof pathway; HMP, hexose-monophosphate pathway; TCA, tricarboxylic acid cycle. Key enzymatic reactions at the pyruvate branch point are catalyzed by the following enzymes. ①Pyruvate decarboxylase. ②Alcohol dehydrogenase. ③ Acetaldehyde dehydrogenase. ④ Acetyl coenzyme A (CoA) synthetase. ⑤ Acetyl CoA shuttled from the cytosol to mitochondria. ⑥Acetyl CoA shuttled from mitochondria to the cytosol. ⑦ L-lactate dehydrogenase. Black arrows indicate the metabolic pathway leading to the production of lactic acid from glucose.

On the other hand, to increase the metabolic flow from pyruvic acid to lactic acid, a mutant strain, such as *pdc* and *adh*, has already been reported to have the genetic background for obtaining an L-*LDH* gene-expressing yeast. Adachi *et al*. tried the destruction of *PDC1*, *PDC5*, and *PDC6* gene. But only a *PDC1* knockout strain was obtained (Adachi *et al*. 1998). Skory reported the isolation and expression of the L-*LDH* gene from *R. oryzae*. This recombinant accumulated L-lactic acid to a final concentration of 38 g/liter, the yield being 0.44 g lactic acid/g glucose. The optimal pH for lactic acid production by the diploid strain was pH 5. Next, *S. cerevisiae* mutants with diminished PDC1 and ADH1 activities showed decreases in ethanol production. However, the efficiency of lactic acid production was also decreased (Skory, 2003).

Another approach for lactic acid production involves the use of a crabtree-negative yeast, such as *Kluyveromyces lactis* (Porro *et al*. 1999). *K. lactis* has a single *PDC1* gene, *KlPDC1*, expressing PDC activity (Bianchi *et al*. 1996). Bianchi *et al.* used a *K. lactis* strain lacking either PDC activity or PDC and PDH activities, and transformed with the L-*LDH* gene expressed under the control of the *KlPDC1* promoter using a stable multicopy vector. This transgenic strain led to further improvement of lactic acid production. The concentrations, productivities, and yields of lactic acid as high as 109 g/liter, 0.91 g/liter/h and 1.19 mol/mole of glucose consumed, respectively, were obtained under fed-batch conditions (Bianchi *et al.* 2001). Colombie´ *et al.* also developed a transgenic *S. cerevisiae* into which the L-*LDH* gene from *Lactobacillus plantarum* had been introduced. The lactate production with this strain reached 50 g/liter, the productivity being 0.3 g/liter/h (Colombie´ *et al.* 2003). Above all, a key distinguishing feature of this study is the integration of the L-*LDH* gene into the chromosome DNA.

1.7. Purpose

The final goals of this study are to develop an efficient means of production of lactic acid, and thereby establish a low-cost process for poly lactic acid production. Previously, I described various approaches for lactic acid production with fungi, a transgenic *E. coli*, and a transgenic yeast, besides the general method involving lactic acid bacteria. The merits and the demerits of each microorganism are summarized in Table 1. 2.

The greatest problem associated with lactic acid fermentation has been the nutritional requirements of a host organism, which can accounted for up to 35% of the production cost (Oh *et al*. 2003) and end product inhibition (Hofvendahl and Hahn-Hagerdal, 2000). To prevent end product inhibition, efforts have been made to develop processes for removal of lactic acid from the fermentation broth in the case of lactic acid bacteria. Various methods have been reported, which include cross flow filteration with cell recycling (Taniguchi *et al*. 1987; Bibal *et al*. 1991; Shimizu *et al*. 1994), electrodialysis (Boyaval *et al*. 1987; Nomura, 1992; Vonktaveesuk *et al.* 1994), ion exchange resins (Vaccari *et al*. 1993; Srivastava *et al*. 1992) and extraction from the fermentation broth (Honda *et al*. 1995; Ye *et al*. 1996). Additionally, the US patent 6,475,759 covers a process for producing lactic acid that involves the incubation of acid tolerant bacteria in nutrient medium to produce a fermentation broth with a high level of free lactic acid (Carlson *et al*. 2002). However, no method has led to a drastic decrease in the production cost.

The next problem is the optical purity of the lactic acid. It has been pointed out that a general poly lactic acid is weak as to heat and impact in comparison with the petroleum-based plastics currently in use. Since it has been reported that the optical purity of lactic acid affects the physical characteristics, such as crystallization, thermostability, the biodegradation rate and performance (Tsuji, 2002), it is important to establish a processing technology for L-lactic acid of high optical purity. The optical purity of L-lactic acid derived with lactic acid bacteria is approximately 95% (Hofvendahl and Hahn-Hagerdal, 2000), but this purity is not

suitable for high physical properties of PLA. To improve the purification of lactic acid, the separation of optical isomers through crystallization has been reported (Benthin and Villadsen, 1995; van Breugel *et al*. 2002). But this step leads to a high production cost.

To solve these problems, I believe that the use of a lactic acid-producing yeast is the most effective approach compared with that with other organisms. Although the productivity reported here is low, lactic acid production with a recombinant *S. cerevisiae* has a lot of advantages (Table 1. 2). In particular, its acid tolerance is higher than that of other organisms, and this great feature is strongly related to the possibility of free lactic acid production without a neutralization process. In this study, to achieve mass production of L- and D-lactic acid using *S. cerevisiae*, I developed a recombinant strain that produces lactic acid efficiently.

Table 1. 2. Merits and demerits of each organism for lactic acid fermentation.

	Merits	Demerits
Lactic acid bacteria	・The lactic acid production is higher than that with other microorganisms. ・On homolactic fermentation, the generation of CO_2 does not occur.	・Neutralization is necessary. ・The optical purity of lactic acid is low. ・The media are too expensive because the auxotrophy is complex. ・Hard to cultivate at high density.
Rhizopus oryzae	・The optical purity of lactic acid is high. ・Easy to collect as the pellet form. ・Yeast extract is not needed for fermentation (low auxotrophy). ・An ammonium-tolerant strain is known.	・Neutralization is necessary. ・Aeration is necessary on fermentation.
Recombinant *E. coli*	・The optical purity of lactic acid is high. ・Yeast extract is not needed for fermentation (low auxotrophy). ・Genetic engineering is easy. ・ Industrialization is easy.	・Neutralization is necessary. ・Highly initial glucose case inhibition of growth and fermentation.
Recombinant Yeast	・The acid tolerance is good. ・Yeast extract is not needed for fermentation (low auxotrophy). ・Easy to collect because the cell size is large. ・Ethanol fermentation technology is already established. Industrialization is easy. ・High density cultivation is easy. ・Genome data are maintained.	・The lactic acid production is low. A large amount of ethanol is produced.

1.8. Outline

This study focused on the efficient production of L- and D-lactic acid using a metabolically engineered *S. cerevisiae* into which the heterogous lactate dehydrogenase gene had been introduced. *S. cerevisiae* is more tolerant to low pH than lactic acid bacteria, *R. oryzae*, and transgenic *E. coli*. Generally, yeast hardly produces either L- or D-lactic acid. But, if a genetically engineered *S. cerevisiae* is used, free lactic acid production with high optical purity can be expected without pH control. After 1994, some attempts at lactic acid production involving transgenic yeast were reported. However, remarkable improvement in the production of this organic acid was not observed, because the by-product ethanol in the fermentation process is a major problem.

In Chapter 2, I described the development of a metabolically engineered *S. cerevisiae* that produces L-lactic acid efficiently. In this recombinant, the coding region for *PDC1* on chromosome XII is substituted for that of 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, while *PDC1* is completely disrupted. Yeast cells expressing L-*LDH* were observed to convert glucose to both lactate (55.6 g/liter) and ethanol (16.9 g/liter), with up to 62.2% of the glucose being transformed into L-lactic acid under neutralizing conditions. This strain, which expresses bovine L-*LDH* under the control of the *PDC1* promoter, also showed high lactic acid production (50.2 g/liter) under non-neutralizing conditions. The differences in lactic acid production was compared among four different recombinants expressing a heterologous L-*LDH* gene (i.e., either the bovine or *Bifidobacterium longum* one): two transgenic strains with 2μm plasmid-based vectors and two genome-integrated strains (Ishida *et al*., 2005).

In Chapter 3, I described the effect of *pdc1 pdc5* double knockout in *S. cerevisiae* on L-lactic acid production. As for *S. cerevisiae*, it is known that the

auto-regulation system, that is to say, *PDC1* deletion leads to a great increase of *PDC5* expression. The ethanol production was still confirmed in the recombinant strain in previous Chapter 2, although it showed effective lactic acid production. Then, a transgenic strain with the *PDC1* and *PDC5* genes completely disrupted was constructed. With this recombinant, the yield of lactate was 82.3 g/liter, up to 81.5% of the glucose being transformed into L-lactic acid on neutralizing cultivation. This new strain showed lactate productivity improvement of as much as 1.5 times higher compared with the previous strain in Chapter 2, this production yield being the highest value for L-lactic acid producing yeast reported so far. However, disruption of both the *PDC 1* and *PDC5* genes led to insignificant decreases in cell growth and the fermentation speed. Analysis of two kinds of L-*LDH* (i.e., either the bovine or *Bifidobacterium longum* one)-expressing recombinant revealed the speed improvement effect was greater for the bovine L-*LDH* integration strain. It was considered that high LDH activity contributed to improvement of the intracellular redox balance (Ishida *et al*., 2006-c).

In Chapter 4, I described effect of increasing copy number of the L-*LDH* gene on the *S. cerevisiae* genome for L-lactic acid production. In the previous chapter, although the efficient production of lactic acid has been achieved, the new problem of inhibition of cell growth was observed. Then, the effect on the L-lactic acid productivity was examined, not only both *PDC1* and *PDC5* deletion but also an increasing copy number of the L-*LDH* gene on the genome. The newly developed recombinant *S. cerevisiae* has six copies of the bovine L-*LDH* genes on the genome under the control of the *PDC1* promoter, and it was confirmed that the level of the lactic acid production rose in proportion to the copy number of the L-*LDH* gene. Next, to confirm optically pure lactate production in a low-cost medium, a cane juice-based medium was used for fermentation under neutralizing conditions. As a result, L-lactate production reached 122 g/liter, with 61% of the 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,

i.e., 99.9% or over (Ishida *et al*., 2006-b).

In Chapter 5, I described the construction of a recombinant *S. cerevisiae* that produces D-lactic acid efficiently. Poly D-lactic acid is also an important polymer because it improves the thermostability of poly L-lactic acid through stereo complex formation. In the developed recombinant yeast, the coding region of *PDC1* was completely deleted, and two copies of the D-*LDH* gene from *Leuconostoc mesenteroides* subsp. *mesenteroides* strain NBRC3426 were introduced into the genome. The final D-lactate production reached 61.5 g/liter, 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 was obtained. These findings revealed 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 *E. coli* (Ishida *et al*., 2006-a).

In the final Chapter 6, I have summarized this study and mentioned the future of lactic acid-producing yeast. The development of a new lactic acid production approach that differs from a production method involving lactic acid bacteria was described. It is considered that the low-cost production of L- and D-lactic acid become possible due to these studies, because the problem pointed out for lactic acid bacteria has been overcome.

Chapter 2

Genome-integrated L-*LDH* gene leads to efficient production of lactic acid with *S. cerevisiae*

2.1. Introduction

Plant- and crop-based renewable plastics (McLaren and Faulkner, 1999), including poly lactic acid, are polymeric materials that could be produced with a fermentation process and would be biodegraded to H_2O and CO_2 finally (Ozeki, 1996). In the sustainable society to come, renewable plastics should be available at a lower price than petroleum-based plastics currently in use. Lactic acid, which is used as a monomer for polymerization into poly lactic acid, has a global market in excess of 100,000 tons per year (Hester, 2000), and further increased demand is predicted. Lactic acid is generally produced with lactic acid bacteria (Marshall, 1987), such as *Lactobacillus* species, which are hard to cultivate at high density and show high auxotrophy regarding growth (Hofvendahl and Hahn-Hagerdal, 2000). During lactic acid fermentation, a low pH has inhibitory effects on cell growth and lactic acid production. 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. With the potential demand for an increase in lactic acid production comes a greater interest in finding another approach to producing lactic acid.

Yeasts, such as *S. cerevisiae*, are more tolerant to low pHs than lactic acid bacteria. Recently, new methods for producing lactic acid with genetically engineered yeast have been developed and applied for large-scale production on a trial basis. In ethanol fermentation, pyruvic acid is converted into acetaldehyde by pyruvate decarboxylase (PDC; EC 4.1.1.1), and then the acetaldehyde is converted into ethanol by alcohol dehydrogenase (EC 1.1.1.1) (Pronk *et al*., 1996). A transgenic yeast expressing exogenous L-lactate dehydrogenase (L-LDH; EC 1.1.1.27) could produce lactic acid from pyruvic acid. Such metabolically engineered yeasts were first reported by Dequin and Barre and Porro *et al*., who showed that the recombinants yielded about 10 to 20 g of lactate/liter (Dequin and Barre, 1994; Porro *et al*., 1995). In both cases, a considerable amount of ethanol was produced concurrently because *S. cerevisiae* predominantly produces ethanol under

anaerobic conditions. The by-product ethanol has become a problem in lactic acid fermentation with a transgenic yeast.

There are three structural *PDC* genes, *PDC1*, *PDC5* (Schmitt *et al*., 1983; Schaalf *et al*., 1989), and *PDC6* (Hohmann, 1991), in the *S. cerevisiae* genome. To increase the metabolic flow from pyruvic acid to lactic acid, a mutant strain, such as the *pdc1*, *pdc5* (Adachi *et al*., 1998), or *adh1* (Skory, 2003) mutant strain, was utilized as the genetic background for obtaining an L-*LDH* gene-expressing yeast. However, a remarkable improvement in L-lactic acid production has not been observed. In addition to the fact that the PDC activity in yeast is due mainly to PDC1, PDC5 was observed to compensate for a PDC1 deficit, because *PDC1* deletion led to a great increase in *PDC5* promoter driven mRNA expression (Hohmann and Cederberg, 1990). A double knockout strain with *pdc1 pdc5* exhibited significantly impaired growth on glucose medium (Flikweert *et al*., 1996 and 1999). The other approach for lactic acid production involved the use of a crabtree-negative yeast, such as *Kluyveromyces lactis* (Porro *et al*., 1999). *K. lactis* has a single *PDC1* gene, *KlPDC1*, expressing PDC activity (Bianchi *et al.,* 1996). Bianchi *et al*. used *K. lactis* strains lacking either PDC activity or PDC and PDH activities, transformed with the *LDH* gene placed under the control of the promoter of *KlPDC1* gene and cloned into stable multicopy vector. Transgenic *K. lactis* strains showed remarkable improvement under the fed-batch condition (Bianchi *et al*., 2001).

To achieve mass production of lactic acid by using *S. cerevisiae*, a new recombinant strain that produces lactic acid efficiently has been developed. This recombinant has two characteristics. Firstly, PDC1 was completely inactive, and secondly, genome integrated *LDH* was regulated under the control of the *PDC1* promoter.

2.2. Materials and methods

Strains and media

The *E. coli* strain used for molecular cloning was JM109 (Toyobo, Osaka). *E. coli* cultivation and medium were as described previously (Sambrook *et al*., 1989). The *S. cerevisiae* OC-2T (a/α *trp1/trp1*) was derived from the wine yeast strain NBRC2260 (Saitoh *et al.,* 1996). The culture medium used for *S. cerevisiae* was YPD medium (1% Bacto yeast extract, 2% Bacto peptone, 2% glucose, wt/vol).

Synthesis of an L-*LDH* gene

Bovine (Gene Bank accession number D90141) (Ishiguro *et al.*, 1990) and *B. longum* (Gene Bank accession number M33585) (Minowa *et al.,* 1989) L-*LDH* sequences were designed based on major codon usage according to the database on *S. cerevisiae* (Codon Usage Database [http://www.kazusa.or.jp/codon/]). The amino acids sequences encoded by these sequences were the same as those in the database. The experimental method was that of Horton *et al*. (1989). Each oligonucleotide primer was synthesized at 100-mer intervals (QIAGEN GmbH, Hilden, Germany) and then fused with the overlapping region by PCR. In the PCR, KOD DNA polymerase (Toyobo) was used for amplification. Each fragment was subcloned into the pBluescript II SK+ vector (Stratagene, La Jolla, CA, USA) according to a previous report (Sambrook *et al*., 1989). 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 the subcloning of the vector, the nucleotide sequence was determined with an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA). The resulting fragments were named LDHKCB (bovine *LDH*) and LDHKCL (*B. longum LDH*).

Construction of plasmid vectors

Maps of the plasmids used in this study are shown in Fig. 2. 1, and the vectors in which the L-*LDH* gene was replaced were pBTRP-PDC1P-LDHKCB and pBTRP-PDC1P-LDHKCL.

2μm based multicopy plasmid vectors, including pESC-LDHKCB (Fig. 2. 1A) and pESC-LDHKCY, consisted of the *PDC1* promoter and *LDH* within the pESC-TRP vector (Stratagene). Also, the pBBLE-Δpdc1 vector consisted of the *PDC1* promoter, the Tn*5 BLE* gene cassette, and a *PDC1* downstream fragment. The phleomycin resistance gene was the bleomycin gene (Tn*5 BLE*) of bacterial transposon Tn*5* (Gatignol *et al.*, 1987), which was fused downstream from the *S. cerevisiae TDH3* (glyceraldehyde 3-phosphate dehydrogenase 3) promoter.

Integration vectors, including pBTRPPDC1-LDHKCB (Fig. 2. 1B) and pBTRP-PDC1-LDHKCY, consisted of the *PDC1* promoter, *LDH*, *TRP1* (tryptophan requiring 1), and the *PDC1* downstream fragment. *TRP1* was obtained by treating pRS404 (Stratagene) with *Aat* II and *Ssp* I and ligating it to the pBluescript II SK+ vector treatment with T4 DNA polymerase. 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 Fast DNA kit (Qbiogene, Carlsbad, CA, USA), and the concentration was determined with an Ultrospec 300 spectral photometer (Pharmacia Biotech, Uppsala, Sweden). KOD DNA polymerase was used for PCR amplification, and the primers and their oligonucleotide sequences were as follows. For the *PDC1* promoter fragment, PDC1P-U had the sequence 5'-ATATATGGATCC-GCGTTTATTTACCTATCTC-3', containing a *Bam*H I restriction site (underlined), and PDC1P-D had the sequence 5'-ATATATGAATTCTTTGATTGATTTGACTGTG-3', containing an *Eco*R I restriction site (underlined). For the *PDC1* downstream fragment, PDC1D-U had the sequence 5'-ATATATCTCGAGGCCAGCTAACTTCTTG-GTCGAC-3',containing a *Xho* I restriction site (underlined). This fragment was upstream -705 bp from the *PDC1* open reading frame (ORF) start codon. PDC1D-D had the sequence 5'-ATATATGGGCCCCTCGTCAGCAATAGTGGTCAAC-3', containing an *Apa* I restriction site (underlined). For the PDC1 3' end, the *PDC1*

downstream fragment of 518 bp in length was between +501 and +1,018 from the *PDC1* ORF start codon. The amplification fragments were treated with each restriction enzyme (Takara Bio, Otsu) and were ligated to a vector, and all plasmids constructed in this work were obtained by using standard techniques (Sambrook *et al*., 1989).

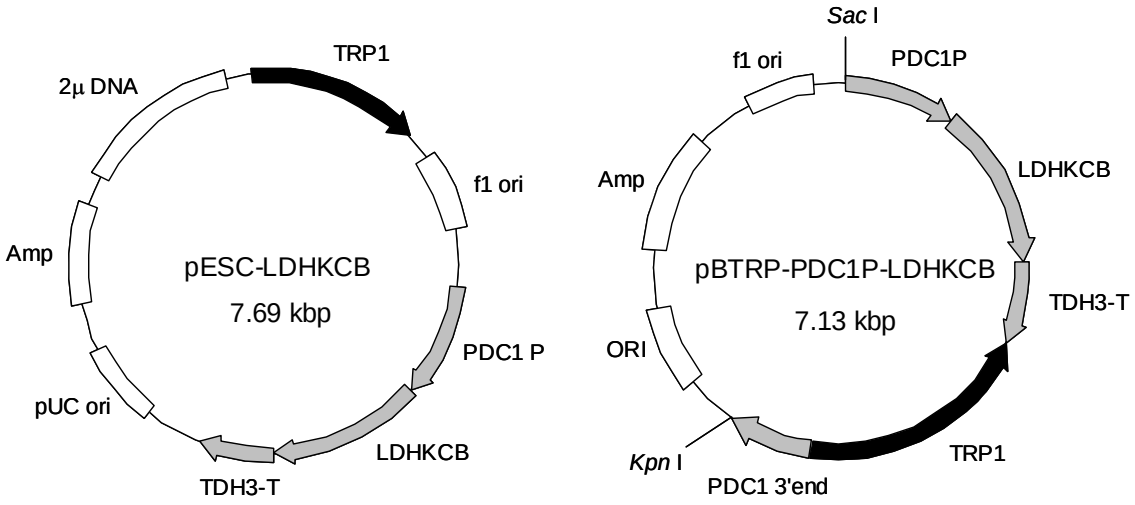

Fig. 2. 1. Maps of the plasmid vectors used for *LDH* expression. PDC1 P, *PDC1* promoter from *S. cerevisiae* OC-2T. This fragment was -705 bp upstream from the *PDC1* ORF start codon. TDH3-T, *TDH3* terminator fragment. LDHKCB, bovine *LDH*. PDC1 3' end, *PDC1* downstream fragment of 518 bp in length was between +501 and +1,018 from the *PDC1* ORF start codon. Two other vectors (pESC-LDHKCY and pBTRPPDC1P-LDHKCY) were obtained by substituting the LDHKCB in these vectors for LDHKCL (*B. longum LDH*).

Yeast transformation

S. cerevisiae transformation was performed by the lithium acetate procedure (Ito *et al*., 1983). Host strain OC-2T is a diploid and homothallic strain (Saitoh *et al*., 1996). 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) and a micro-manipulator (Narishige Science, Tokyo). After the colonies had been isolated, the target gene introduction was confirmed by PCR.

Construction of YEp multicopy control strains

Recombinant strains, such as YEBL-8A and YEBO-1B, were constructed in the following way. By use of the pBBLE-Δpdc1 vector, a *PDC1* deletion strain was constructed. After the colonies had been isolated, the *PDC1* deletion was confirmed by PCR. Next, the pESC-LDHKCY and pESC-LDHKCB vectors were introduced into these strains, and the recombinants were named YEBL-8A and YEBO-1B, respectively.

Construction of *PDC1* integration strains

Recombinant strains, such as YIBL-2D and YEBO-7A, were constructed in the following way (Fig. 2. 2A). By use of the pBTRP-PDC1-LDHKCB vector, the heterozygous integrated strain was constructed. But the *PDC1* gene in this transformant was not completely deleted, because the OC-2T strain is a homothallic yeast. The *LDH* gene on one side of the chromosome could be amplified, and the *PDC1* gene was completely deleted upon spore formation (Fig. 2. 2B).

Enzyme-specific activity in cell extracts

Cell extracts were prepared with a SONIFIER 250 (Branson, Danbury, CT, USA) as described previously (Pronk *et al*., 1996). PDC-specific activity in freshly prepared extracts, as described by Pronk *et al.* (1996), was determined by using

Ubest-55 spectrophotometer at 340 nm (Japan Spectroscopic, Tokyo). LDH-specific activity was determined in freshly prepared extracts as described by Minowa *et al.* (1989). Protein concentrations in cell extracts were determined with a DC protein assay kit (Bio-Rad, Richmond, CA, USA) by using bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) as a standard.

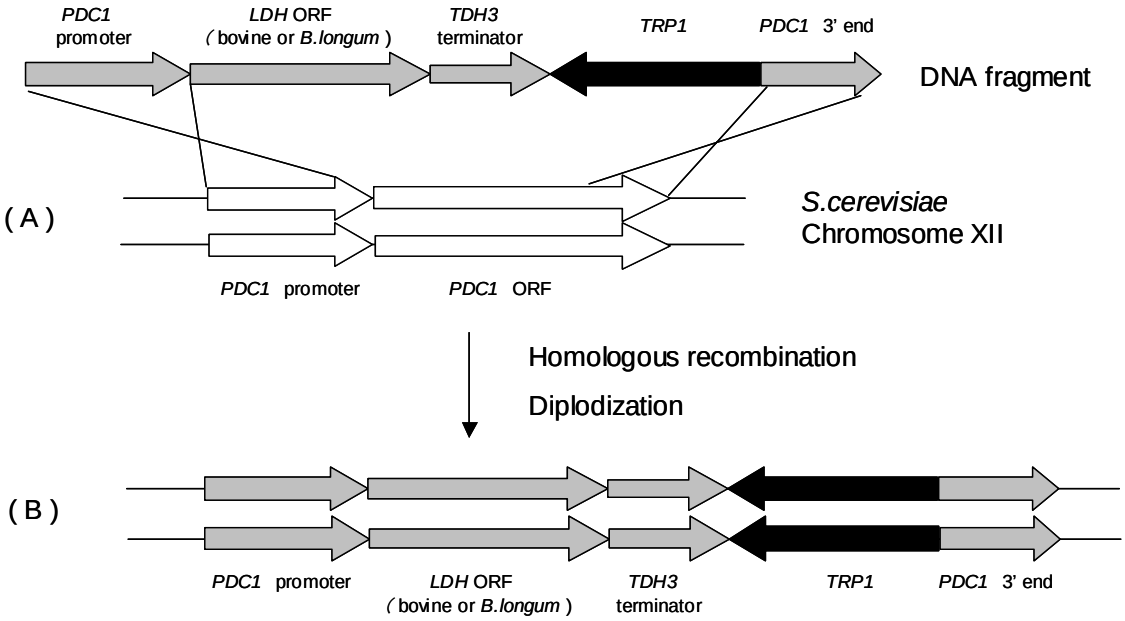

Fig. 2. 2. The improved method for *LDH* gene expression. (A) The constructed vector was integrated into the *PDC1* locus of *S. cerevisiae* chromosome XII through homologous recombination. (B) Firstly, one copy of the target *LDH* was introduced into the host, the OC-2T strain. Secondly, diploidization occurred through spore formation. The introduced *LDH* was amplified into two copies, and the *PDC1* coding region was completely disrupted.

Fermentation

Fermentation experiments were performed at 30°C in 100 ml flasks with working volumes of 40 ml in YPD10 medium (1% Bacto yeast extract, 2% bacto peptone, 10% D-glucose) containing 3% sterilized calcium carbonate. The inoculum was 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 on a shaker and then transferred to the fermentation medium of 0.1% packed cell volume at the inoculum size. The glucose, lactic acid, and ethanol concentrations were measured with a biosensor BF-4S instrument (Oji Keisoku Kiki, Amagasaki).

2.3. Results

Lactate production with multicopy plasmids

I constructed two kinds of recombinant yeasts expressing a heterologous *LDH* gene, bovine or *B. longum* (Table 2. 1), under the control of the *PDC1* promoter using a 2μm plasmid based vector (Fig. 2. 1A). The recombinants with the *PDC1* deletion showed no significant differences in either PDC or LDH activities, and the level of PDC activity was one-third of that in the case in which OC-2T was used as the host strain (Table 2. 2). The YEBO-1B strain, which expresses bovine *LDH*, was cultivated for 72 h, and the yields of lactate and ethanol were 16.3 g/liter and 34.3 g/liter, respectively (Fig. 2. 3A). The yield of lactate for YEBL-2D, which expresses *B. longum LDH*, was almost the same as that for YEBO-1B (Fig. 2. 3B), with no difference in yield being seen between the two kinds of LDH.

Lactate production with *PDC1* integration

I also constructed two other lactate-producing yeast recombinants, YIBL-2D (*pdc1*::P_{pdc1}- *B. longum* L-*LDH*) and YIBO-7A (*pdc1*::P_{pdc1}-bovine L-*LDH*), with integration in the *PDC1* locus (Table 2. 1). For the initial physiological characterization, the growth rates of these *PDC1*-disrupted recombinants were determined in shake flask cultures on YPD medium. The growth rate of the YIBL-2D strain was 0.535 h^{-1}, while that of parent strain OC-2T was 0.648 h^{-1}. As compared to the host strain, extreme suppression of the growth rate was not observed, although the *PDC1* was completely disrupted. The LDH-specific activities of both YIBL-2D and YIBO-7A were clearly higher than those of the transgenic strains including a multicopy plasmid, although these recombinants have only two copies of the *LDH* genes (Table 2. 2). The PDC-specific activities of the four recombinant strains were one-third of that of the OC-2T host strain and seemed to be the same. The YIBO-7A strain expressing bovine *LDH* under the control of the *PDC1* promoter was observed to produce both lactate (55.6 g/liter) and ethanol (16.9 g/liter), with up to 62.2% of the glucose being transformed into

lactic acid (Fig. 2. 4B). YIBL-2D, the other strain which expresses the *B. longum* L-*LDH* gene, produced 25.7 g of L-lactate/liter and 31.1 g of ethanol/liter (Fig. 2. 4A).

Table 2. 1. Yeast strains used in this study.

Strain	Relevant genotype	*LDH* expression cassette	Description	Reference
OC-2T	a/α, *trp1/trp1*	——	Host strain	Saitoh *et al.,* 1996
YEBL-8A	a/α, *pdc1/pdc1,* phl^r	P_{PDC1}- *B.longum LDH*	YEp (2μm DNA)	This study
YEBO-1B	a/α, *pdc1/pdc1,* phl^r	P_{PDC1}- Bovine *LDH*	YEp (2μm DNA)	This study
YIBL-2D	a/α, *pdc1/pdc1*	P_{PDC1}- *B.longum LDH*	YIp (*PDC1* locus)	This study
YIBO-7A	a/α, *pdc1/pdc1*	P_{PDC1}- Bovine *LDH*	YIp (*PDC1* locus)	This study

Table 2. 2. Specific activities of PDC and LDH.

Strain	PDC activity [a] (mU/mg of protein)	LDH activity [a] (mU/mg of protein)
OC-2T	225.0 ± 2.5	N.D
YEBL-8A	87.0 ± 1.2	4.6 ± 3.3
YEBO-1B	88.2 ± 3.9	5.1 ± 4.8
YIBL-2D	84.0 ± 3.1	12.7 ± 2.2
YIBO-7A	89.7 ± 2.5	26.7 ± 3.1

[a]These strains were cultivated for 24 h. Enzyme-specific activity in freshly prepared cell extracts was determined. The average and deviation were determined for three independent experiments. N.D, not determined.

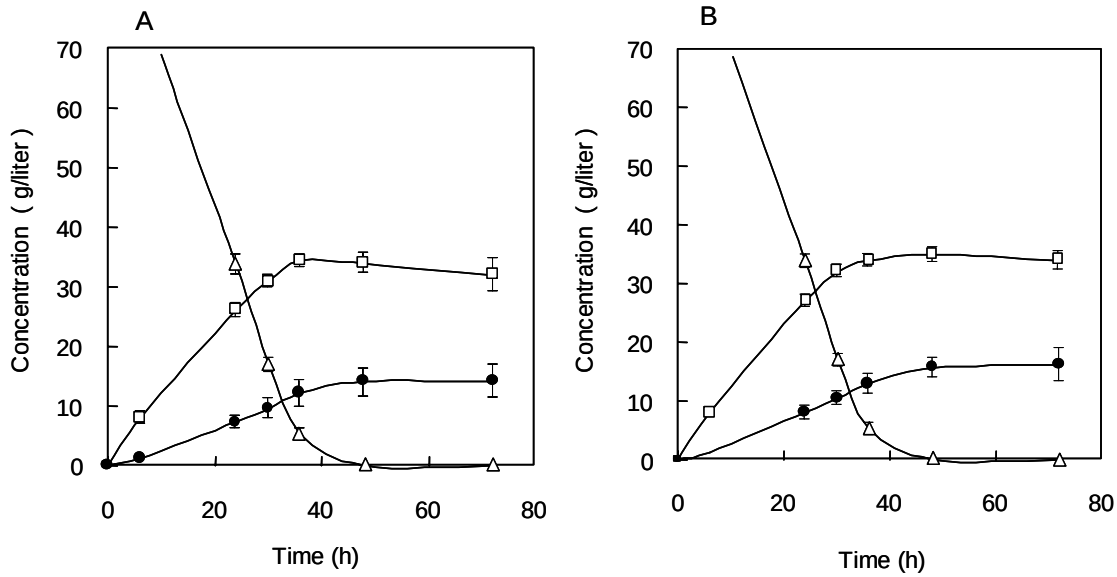

Fig. 2. 3. Comparison of fermentation with the *S. cerevisiae* YEBL-8A (A) and YEBO-1B (B) strains in YPD medium containing 100 g of glucose/liter and 50 g of $CaCO_3$/liter. ●, lactate; □, ethanol; △, glucose. Each strain was cultivated for 72 h under micro-anaerobic conditions at 30°C. The averages and standard deviations (error bars) for three independent experiments are presented.

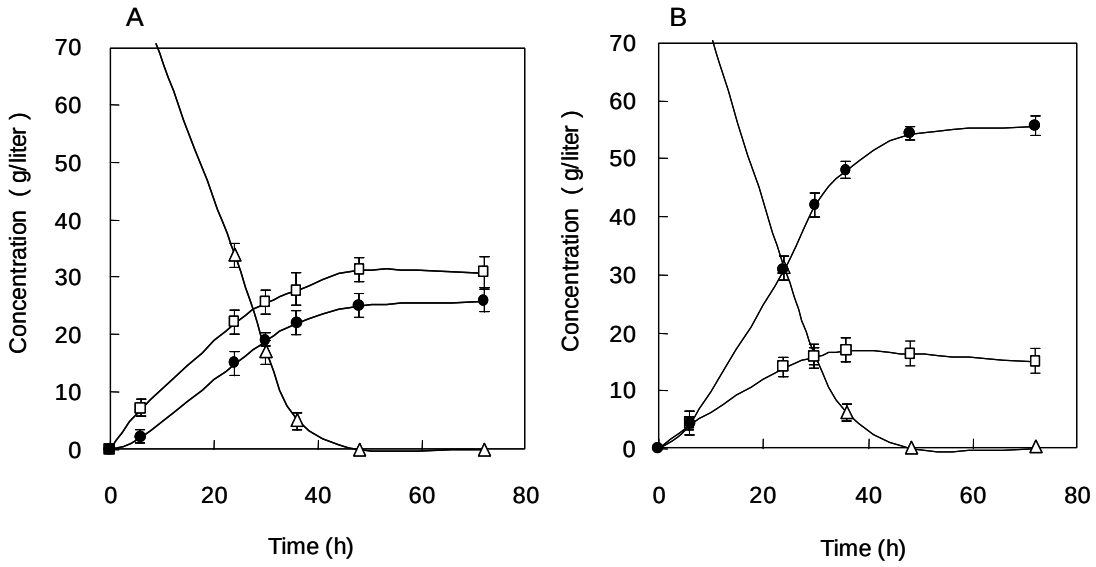

Fig. 2. 4 Comparison of fermentation with the *S. cerevisiae* YIBL-2D (A) and YIBO-7A (B) strains in YPD medium containing 100 g of glucose/liter and 50 g of $CaCO_3$/liter. ●, lactate; □, ethanol; △, glucose. Each strain was cultivated for 72 h under micro-anaerobic conditions at 30°C. The averages and standard deviations (error bars) for three independent experiments are presented.

Two genome integration recombinants, YIBL-2D and YIBO-7A, exhibited high levels of production of lactate compared with those of the YEBO-1B and YEBL-8A strains including a 2μm DNA multicopy plasmid, although the genome-integrated strains maintained only two *LDH* genes. However, the yield of lactate with YEBO-7A expressing bovine *LDH* was almost twice that with YEBO-1B, and an effect of bovine *LDH* and *B. longum LDH* was observed clearly for the two genome integrated strains. This difference in production on account of the different *LDH* genes was not observed for the multicopy strain.

Lactic acid production under non-neutralizing conditions

The production of lactic acid was examined by cultivation under non-neutralizing conditions without $CaCO_3$. The accumulations of lactic acid and ethanol are shown in Fig. 2. 5. The YIBO-7A strain, which had integrated the bovine *LDH* gene, gave yields of 50.2 g of lactic acid/liter and 16.7 g of ethanol/liter. The glucose was completely consumed in 72 h in the case of neutralizing conditions. The final pH was 2.8. The effect of changing the initial glucose concentration from 100 to 150 g/liter on the yield of the lactic acid production was examined. However, as judged by fermentation analysis, under the non-neutralizing conditions, an improvement of the lactic acid production was not observed; it did not reach 50 g/liter or more (data not shown).

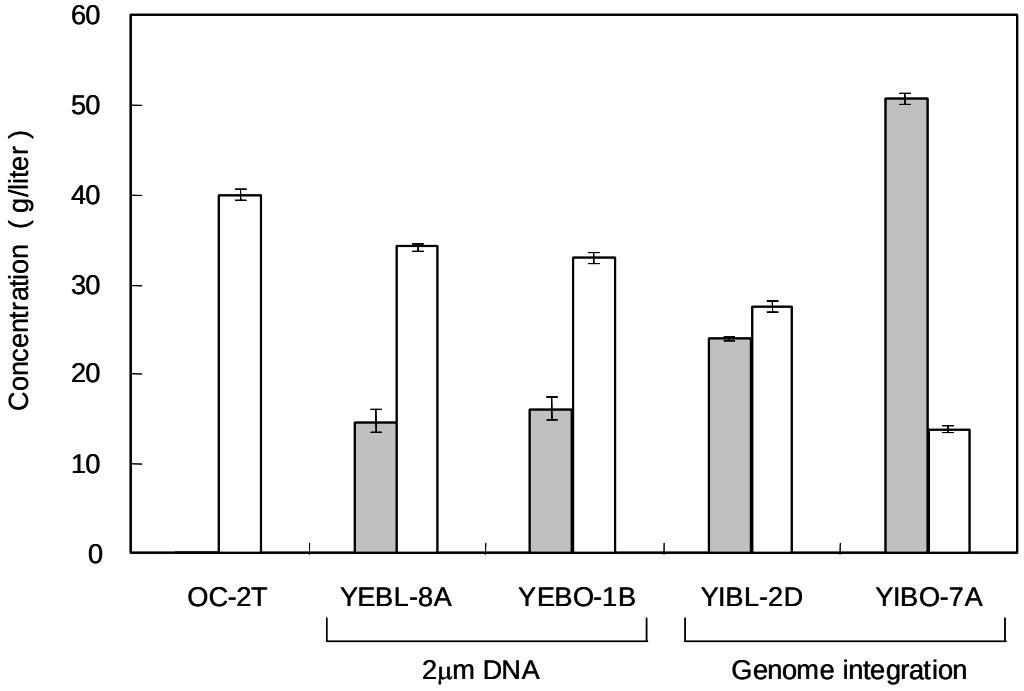

Fig. 2. 5. Accumulations of lactic acid (gray bars) and ethanol (white bars) by *S. cerevisiae* recombinants in YPD medium containing 100 g of glucose/liter but without $CaCO_3$. Each strain was cultivated for 72 h under micro-anaerobic conditions at 30°C. The glucose had been completely consumed after 72 h. The averages and standard deviations (error bars) for three independent experiments are presented.

2.4. Discussion

In this chapter, a recombinant yeast exhibiting efficient production of lactic acid has been developed by substituting the coding region of *PDC1* on chromosome XII for that of *LDH* through homologous recombination (Fig. 2. 2). This method allowed robust gene expression under the control of the native *PDC1* promoter. Steady lactate production was also maintained with the recombinant strain that was subcultured two times in nonselective medium (data not shown). For *LDH* expression in yeast, a YEp multicopy plasmid is generally used, but remarkable improvement of lactate production was not observed in this study (Table 2. 3). In the case of multicopy strains, it was expected that unstable *LDH* expression would decrease the number of intracellular plasmids in nonselective medium. However, genome integration does not affect the number of *LDH* genes during fermentation; therefore, steady expression according to the promoter occurs. The comparison with transgenic yeast is shown in Table 2. 4. In the batch culture condition, it can be confirmed that the strain of this report shows a high yield of lactic acid. A transgenic strain in which the *Lactobacillus plantarum LDH* gene was integrated into the genome was developed recently (Colombie *et al*., 2003); this recombinant yields about 58 g of lactate/liter. The *ADH1* promoter was used for the expression of the heterogeneous gene. In this study, the native *PDC1* promoter on chromosome XII was used. PDC1 converts pyruvic acid to acetaldehyde and plays an important role in the production of ethanol. In general, host strain OC-2T was able to produce a high level of ethanol, even with a high glucose concentration; besides, only *PDC1* usually worked for the production of ethanol. The choice to use this promoter was based on the fact that the expression of *PDC1* is strongly induced by glucose (Kellermann *et al.*, 1988), and glucose-responding elements in *S. cerevisiae* have already been reported (Butler *et al.*, 1988; Liesen *et al.,* 1996). It is advantageous to use the *PDC1* promoter for fermentation with a high initial glucose level. Similarly, Bianchi *et al*. used *KlPDC1* promoter for bovine *LDH* expression. Transgenic *K. lactis* strains showed 60 g of lactic acid/liter under the fed-batch condition, but it

expended 500 h (Bianchi *et al.,* 2001). In this chapter, *S. cerevisiae* strain disrupted only *PDC1* and not *PDC5*, and genome-integrated *LDH* was regulated under the control of the native *PDC1* promoter.

Table 2. 3. L-Lactic acid and ethanol productions from wild type and transgenic strains during batch growth on 10% (wt/vol.) glucose base YPD medium.

Strain	Vector	*LDH* gene	Neutralization		Yield of Lactic acid [a] (%)	Non-neutralization		Yield of Lactic acid [a] (%)
			Lactate (g/liter)	EtOH (g/liter)		Lactic acid (g/liter)	EtOH (g/liter)	
OC-2T	-	-	-	40.8	-	-	40.5	-
YEBL-8A	YEp	*B.longum*	14.1	33.9	17.2	14.7	34.8	17.6
YEO-1B	YEp	Bovine	16.3	34.3	19.2	16.1	33.1	19.6
YIBL-2D	YIp	*B.longum*	25.7	31.1	29.5	24.0	27.6	30.3
YIBO-7A	YIp	Bovine	55.6	16.9	62.2	50.6	13.8	64.7

[a]Yields are expressed as grams of lactate produced per liter divided by grams of glucose consumed per liter.

In the experiment using *B. longum* and bovine LDH, an improvement in lactate production was observed in the case of the integrated bovine LDH recombinant; however, a remarkable change was not observed in the case of a YEp-based multicopy plasmid (Table 2. 4). The two LDHs were found to differ in amino acid level (40.3% identity; the calculation was performed with genetic analysis software GENETYX version 7.0.2). In order to determine the substrate affinity for pyruvic acid, bovine LDH was examined (K_m, 0.13 mM; V_{max}, 3,333 U/mg of protein) (data not shown). Also, the K_m value for pyruvate of *B. longum* LDH has already been reported (K_m, 1.05 mM; V_{max}, 1,670 U/mg of protein) (Iwata *et al*., 1989). The two LDHs are different in substrate affinities and enzyme activity levels. Because steady gene expression can be expected in the genome-integrated strain compared with a recombinant constructed with the YEp vector, it is considered that the differences in

enzyme characteristics between the two LDHs were reflected by the lactic acid production. The combination of bovine *LDH* and the native *PDC1* promoter on the genome is important for the efficient production of lactic acid.

The maximal concentration of lactate with YIBO-7A, which expresses bovine *LDH* under the control of the *PDC1* promoter, was 58.8 g/liter under neutralizing conditions (Table 2. 4). This recombinant, including two copies of the *LDH* gene on the genome, showed higher production than the transgenic strain, which had integrated one copy of the *LDH* gene on the genome (data not shown). It will be possible to improve the lactate production by increasing the number of *LDH* genes through genome integration. An analysis of a recombinant with an increased copy number of *LDH* is described elsewhere (Chapter 4). On the other hand, if lactic acid is obtained directly under non-neutralizing conditions without a desalination process, lowering the cost on a manufacturing scale is possible. It has been pointed out that for a conventional method involving lactic acid bacteria, the process of desalination of lactate is a factor that increases the cost. In the case of lactic acid bacteria, it has been reported that the use of the genome-shuffling method improves the low pH tolerance (Patnaik *et al*., 2002). *S. cerevisiae* is well known to grow and survive at low pHs compared with lactic acid bacteria. Lactic acid production under non-neutralizing conditions was attempted by using a transgenic yeast (Adachi *et al.,* 1998; Dequin *et al.*, 1994; Porro *et al*., 1999; Skory, 2003), but this production has not been reported so far. The present strain, which expresses bovine *LDH* under the control of the *PDC1* promoter, exhibited high lactic acid production (50.2 g/liter) without pH control (Table 2. 4). This result might lead to lactic acid fermentation that does not need the desalination process. But the level of lactic acid production did not become 50 g/liter or more. The final pH was 2.8, and it was expected that the low pH suppressed the lactic acid production. To improve the production under non-neutralizing conditions, it is important to make more progress regarding the low pH tolerance of intracellular LDH or host cells. *S. cerevisiae* is a suitable microorganism for an industrial scale. This strategy could be useful for applications for lactic acid production other than the use of lactic acid bacteria.

Table 2. 4. The study of L-lactic acid production by transgenic yeast.

Host	Vector	Promoter	*LDH* gene type	Maximum production (g/liter)	Yield of lactate (%)	Reference
Batch culture						
S.cerevisiae	YEp	*ADH1*	*Lactobacillus casei*	12.0	24.0	Dequin *et al.*, 1994
S.cerevisiae (pdc1,pdc5,pdc6)	YEp	*ADH1*	*Bovine*	7.9	26.3	Adachi *et al.*, 1989
S.cerevisiae (wine yeast)	YEp	*ADH1*	*Lactobacillus casei*	8.6	4.3	Dequin *et al.,* 1999
S.cerevisiae (pdc1, adh1)	YEp	*ADH1*	*Rhizopus oryzae*	38.0	41.3	Skory, 2003
S.cerevisiae	YIp	*ADH1*	*L. plantarum*	58.0	29.0	Colombie *et al.,* 2003
S.cerevisiae (wine yeast)	YIp	*PDC1*	Bovine	55.6	62.2	This study
Fed-batch culture						
S.cerevisiae	YEp	*ADH1*	Bovine	20.0	-	Porro *et al.*, 1995
K.lactis (*klpdc1*)	YRp	*KLPDC1*	Bovine	26.7	-	Bianchi *et al*., 2001
K.lactis (*klpdc1,klpda1*)	YRp	*KLPDC1*	Bovine	60.0	-	Porro *et al.*, 1999

[a]In batch culture, yield is expressed of lactate produced per liter divided by glucose consumed per liter.

Chapter 3

The effect of *pdc1 pdc5* double knockout in *S. cerevisiae* on L-lactic acid production

3.1. Introduction

PLA has been receiving increasing attention due to its biodegradation into H_2O and CO_2, in comparison with petroleum-based plastics (Ozeki, 1996). 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 (Marshall, 1987), 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, and Porro *et al*., who showed that the recombinants yielded approximately 10 to 20 g of lactate/liter in the end (Dequin and Barre, 1994; Porro, *et al*.1995). In both cases, a considerable amount of ethanol was produced concurrently because *S. cerevisiae* predominantly produces ethanol under anaerobic conditions. The by-product ethanol has become a problem in lactic acid fermentation with a transgenic yeast (Fig. 3. 1).

In *S. cerevisiae*, there are three structural genes, *PDC1*, *PDC5*, and *PDC6*, that contribute directly to ethanol production (Schmitt *et al*., 1983; Hohmann, 1991). PDC activity in yeast is due mainly to the PDC1 and PDC5 (Flikweert *et al*., 1996 and 1999-a), 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 (Hohmann and Cederberg, 1990). 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 (Flikweert *et al*., 1996). Additionally, in the absence of PDC activity, it was proposed that the reduction in the growth rate depended on the regeneration of NADH (Flikweert *et al.* 1999-b). 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-LDH. But it was found that NADH reoxidation does not control glycolytic flux during exposure of respiring *S. cerevisiae* cultures to glucose excess

(Brambilla *et al*., 1999). 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 (Adachi *et al.,* 1998) or the *adh1* mutant strain (Skory, 2003), 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 Chapter 2, I attained efficient production of L-lactic acid with a recombinant wine yeast. Three key distinguishing features of 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 inactive. 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 chapter, I explored a transgenic strain with *pdc1 pdc5* double 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*.

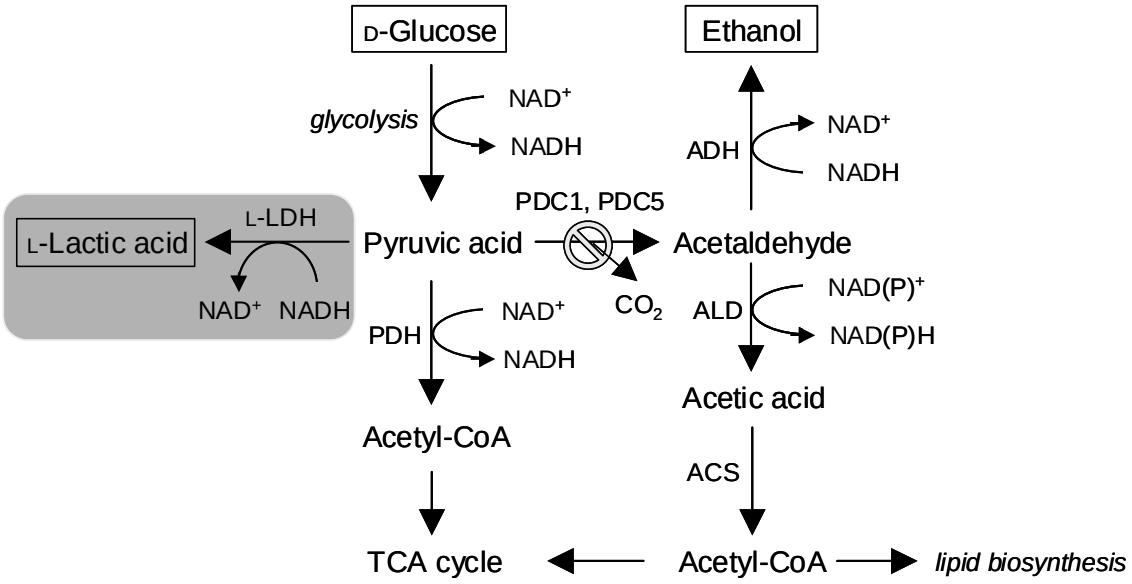

Fig. 3. 1. Scheme of pyruvate metabolism in *S. cerevisiae* expressing the L-*LDH* gene. Enzymes: ACS, acetyl-CoA synthetase; ADH, alcohol dehydrogenase; ALD, acetaldehyde dehydrogenase; L-LDH, L-lactate dehydrogenase; PDC, pyruvate decarboxylase; PDH, pyruvate dehydrogenase complex. The gray area indicates a new metabolite pathway in the transgenic strain.

3.2. Materials and Methods

Strains and media

The *E. coli* strain used for plasmid vector construction was JM109 (Toyobo, Osaka). *E. coli* cultivation and the medium were described previously (Sambrook *et al*., 1989). The *S. cerevisiae* OC-2T strain (a/α, *trp1*/*trp1*) was derived from the wine yeast NBRC2260 strain (Saitoh *et al.,* 1996), 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 Chapter 2. The culture medium used for *S. cerevisiae* was YPD medium (1% bacto yeast extract, 2% bacto peptone, and 2% D-glucose, wt/vol.).

Plasmid construction

Genome integration vector, pBG418-PDC5 (Fig. 3. 2), was constructed using the pBluescript II SK+ vector (Stratagene, La Jolla, CA, USA). This vector consisted of two *PDC5* fragments for homologous recombination and the *G418* gene cassette. The G418 (kanamycin) resistance gene is the aminoglycoside phosphotransferase (*APT*) gene (Hadfild *et al.,* 1990), which confers geneticin resistance on yeasts, fused downstream from the *S. cerevisiae* glyceraldehyde-3-phosphate dehydrogenase 3 (*TDH3*) promoter. Two *PDC5* 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'-ATATATGGATCCTCTTGCAACACAT-TTTCTGATATA-3', containing a *Bam*H I site (underlined). And for the PDC5 downstream fragment, PDC5D, PDC5D-F: 5'- ATATATGTCGACCGTCCAAGTCTTG-

TGGGGTTC-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 pre-incubation at 96°C for 1 min, and 25 cycles of 95°C for 30 sec, 53°C for 30 sec and 72°C for 30 sec. 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 (Ito *et al*., 1983) and each transformant was selected on YPD medium containing 150 μg/ml G418 (Calbiochem, San Diego, CA, USA). The pBG418-PDC5 vector fragment (Fig. 3. 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 Chapter 2. The *PDC5* gene was completely disrupted through spore formation, because the host strain, OC-2T, is a diploid, homothallic strain (Saitoh *et al*., 1996). 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 micro-manipulator (Narishige Science, Tokyo). After colonies were isolated, target gene integration was confirmed by PCR. The primer sequences used were as follows. PDC5CH-F: 5'-ACTGCCATCACTAG-AGAAG-3', PDC5CH-R: 5'-CTAAAACTATATC TATGCC-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.

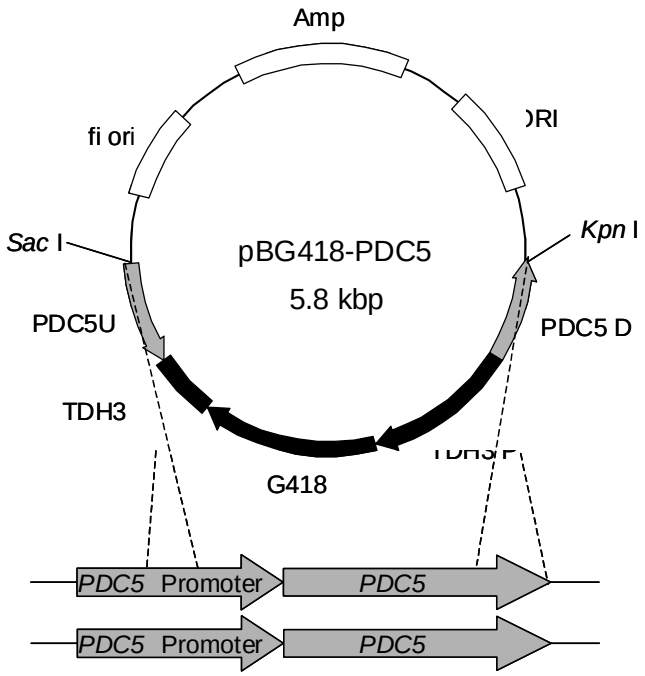

Fig. 3. 2. Map of the plasmid vector and breeding of transgenic *S. cerevisiae*. The constructed DNA fragment, which was obtained by digesting the pBG418-PDC5 vector with *Sac* I and *Kpn* I, was integrated into the *PDC5* ORF region of the YIBL-2D strain (*pdc1*::P_{pdc1}-*B. longum* L-*LDH*) and the YIBO-7A strain (*pdc1*::P_{pdc1}-bovine L-*LDH*).

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 day. 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-4 (Oji Keisoku Kiki, Amagasaki).

3.3. Results and discussion

Growth of the *pdc1 pdc5* double knockout strain

According to the Chapter 2, two transgenic strains, YIBL-*pdc1/5*Δ and YIBO-*pdc1/5*Δ, that expressed L-*LDH* in the genetic background of pd*c1 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. However 2 clones could grow, but slowly. Whereas for the YIBO-*pdc1/5*Δ strain expressing the 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. 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 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* (Chapter 2). The decrease in the growth rate of the YIBL-*pdc1/5*Δ strain expressing *B. longum* L-*LDH* appeared to be proportional to the difference in LDH activity.

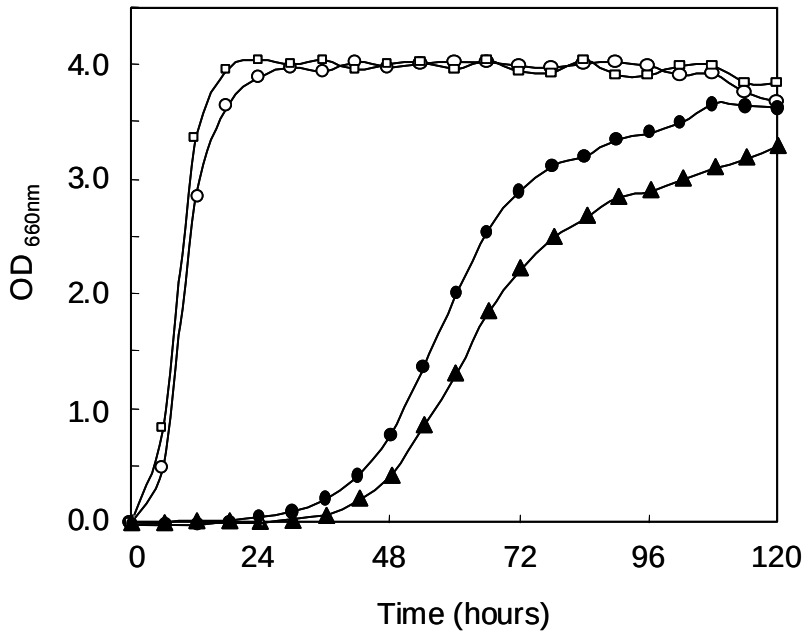

Fig. 3. 3. Growth curves for *pdc1* mutant and *pdc1 pdc5* double mutant strains with the genetic background to obtain an L-*LDH* gene-expressing yeast. One percent of precultured cells was inoculated into 2% of YPD medium. The strains were cultivated at 30°C, and OD_{660nm} was monitored. □, OC-2T (host strain); ○, YIBO-7A; ●, YIBO-*pdc1*/5Δ; ▲, YIBL-*pdc1*/5Δ. The averages and deviations for three independent experiments are presented.

Fermentation analysis

L-Lactate and ethanol production were examined under neutralizing micro-anaerobic conditions in YPD medium containing 100 g/liter D-glucose as a carbon source. In my preliminary experiments, it was clarified that this micro-anaerobic condition is optimum for lactic acid-producing yeast (unpublished results). The YIBO-*pdc1/5*Δ 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. 3. 4-A). In comparison with parent strain YIBO-7A, in which the *PDC5* gene was not deleted, the YIBO-*pdc1/5*Δ 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-*pdc1/5*Δ strain produced 2.8 g/liter of ethanol (Fig. 3. 4-B). 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 (Hohmann and Cederberg, 1990), and 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-*pdc1/5*Δ strain expressing *B. longum* L-*LDH* was also observed, and the final concentration of lactic acid was 80.7g/liter, but it took 216 h until the plateau phase was reached (Fig.3. 5). In comparison with the case of bovine L-LDH, a further decrease in the fermentation speed was observed. This finding indicates the close relationship with the growth rate, which was lower than that of the YIBO-*pdc1/5*Δ strain that expresses bovine L-*LDH*. In addition, high LDH activity also has a profound effect on fermentation.

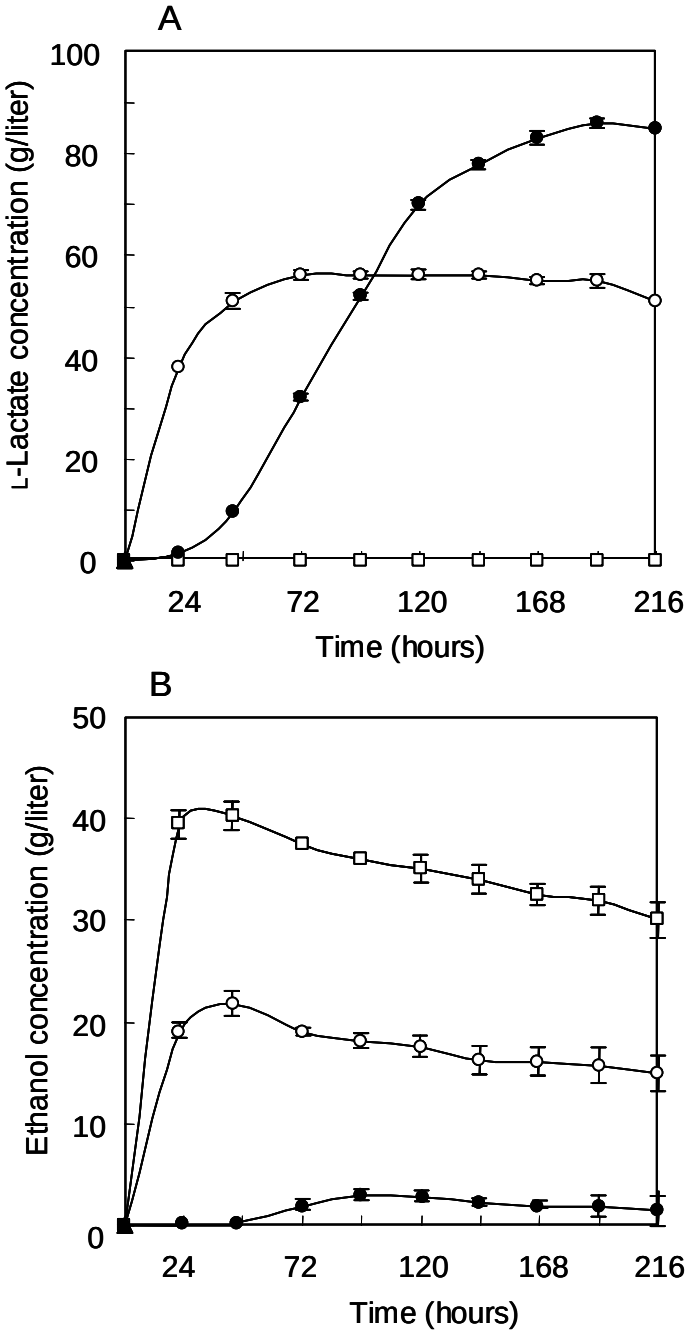

Fig. 3. 4. Time courses of cultivation of YIBO-7A and YIBO-*pdc1/5*Δ strains on a flask scale. (A) L-Lactate concentration. (B) Ethanol concentration. □, OC-2T (host strain); ○, YIBO-7A; ●, YIBO-*pdc1/5*Δ. The averages and deviations for three independent experiments are presented.

Adachi *et al*. also tried to develop a *pdc1 pdc5* recombinant expressing bovine L-LDH using 2μm plasmid (Adachi *et al*., 1998), but the fermentation data for the *pdc1 pdc5* mutant strain was not presented in detail. In this research, I took a different approach to integrating a heterologous gene into the genome, not using 2μm plasmid. Genome integration into the downstream of the *PDC1* promoter led to five times higher LDH activity than with the use of 2μm plasmid (Chapter 2). 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 the further detailed analysis of the *pdc1 pdc5* double mutant introducing L-*LDH* genes with the use of 2μm plasmid is necessary. Also, I constructed recombinant yeast exhibiting higher LDH activity by increasing the copy number of the *LDH* gene on the genome (Chapter 4). An interesting effect is expected on deletion of *PDC1* and *PDC5* in lactic acid-producing yeast exhibiting higher LDH activity.

The effect of the amount of initial inoculum at the fermentation stage of YIBO-*pdc1/5*Δ 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, 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. 3. 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* showed

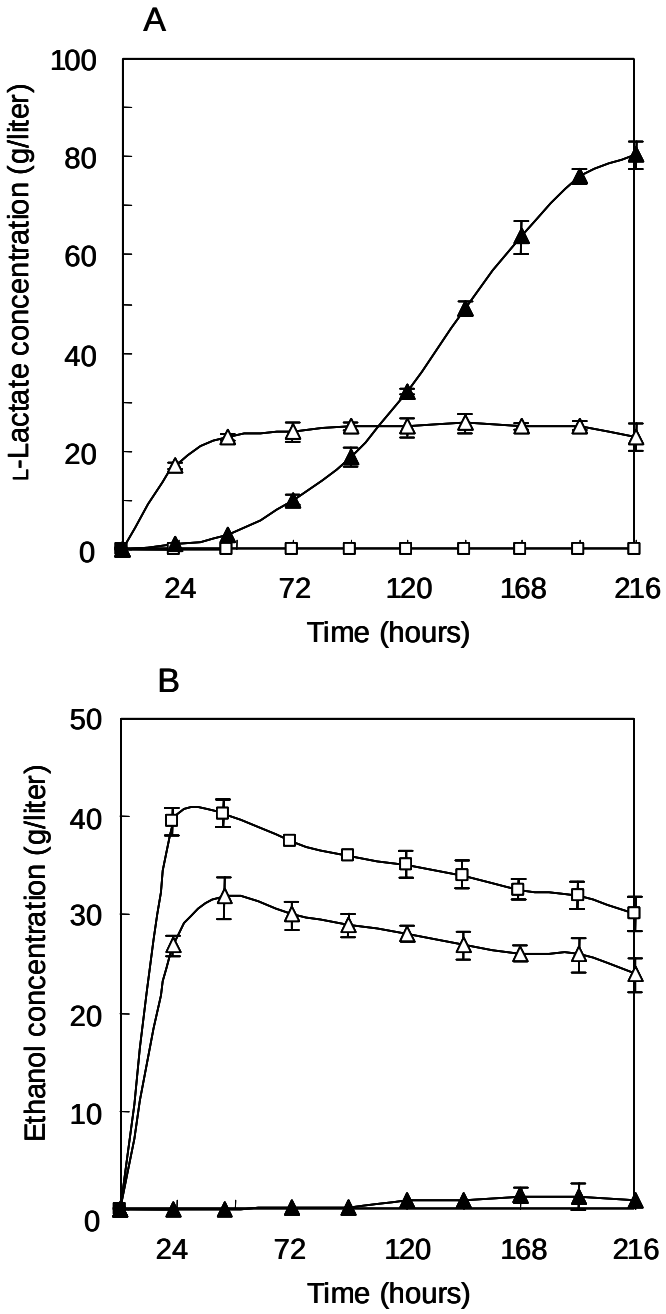

Fig. 3. 5. Time courses of cultivation of YIBL-2D and YIBL-*pdc1/5*Δ strains on a flask scale. (A) L-Lactate concentration. (B) Ethanol concentration. □, OC-2T (host strain);△, YIBL-2D;▲, YIBL-*pdc1/5*Δ. The averages and deviations for three independent experiments are presented.

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 (van Maris *et al.,* 2004). 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.

Chapter 4

Increasing copy number of the L-*LDH* gene on *S. cerevisiae* genome

4.1. Introduction

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 approximately 58°C. The problem that PLA is weak as to heat has been receiving increasing attention for the expanded use of this renewable plastic. Since it has been reported that optical purity affects the physical characteristics, such as crystallization, thermostability, the biodegradation rate and performance (Ozeki, 1996), 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* species. The optical purity of this monomer is approximately 95% (Hofvendahl and Hahn-Hagerdal, 2000), 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 (Benthin, 1995; van Breugel *et al*., 2002). But it is important to obtain L-lactic acid of extremely high optical purity during the fermentation stage. *Lactobacillus* species are not able to produce lactic acid of extremely high optical purity, because some of them have both *L*- and *D-LDH* genes. Even *Lactobacillus sakei*, which has only an L-*LDH*, also produces D-lactic acid due to its lactate racemase activity (Hiyama *et al*., 1968). Recently, improvement of its optical purity with *Lactobacillus* species was reported (Malleret *et al*., 1998; Lapierre *et al*., 1999; Kyla-Nikkila *et al*., 2000), but it was not enough for industrial use.

On the other hand, yeasts, including *S. 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 and Porro *et al*., who showed that the recombinants yielded about 10 to 20 g of lactate/liter (Dequin and Barre, 1994; Porro *et al*., 1995). To increase the metabolic flow from pyruvic acid to lactic acid, a mutant strain, such as the *pdc1*, *pdc5* (Adachi *et al*., 1998), or *adh1* (Skory, 2003) 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 previous Chapter 2, the efficient production of L-lactic acid with a recombinant wine yeast was attained. To achieve mass production of L-lactic acid of extremely high optical purity by using *S. cerevisiae*, I 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. I also 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 (Fontana *et al*., 1996; Narita *et al*., 2004), corn steep liquor (Ohara *et al*., 2001), hydrolyzed sago starch (Hipolto *et al*., 2002), and biological pretreatment corncob (Miura *et al*., 2004-b) has been reported. However, the reported production involving the use of a transgenic yeast involved YPD medium rather than an inexpensive medium. In this chapter, I also examined the lactic acid productivity under the cane juice-based medium. It is expected that the achievement of the efficient production of the lactic acid in an inexpensive medium contributes to low-cost production of PLA.

4.2. 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 carried out by standard procedures (Sambrook *et al*., 1989). The *S. cerevisiae* OC-2T strain (a/α, *trp1*/*trp1*) was derived from the wine yeast NBRC2260 strain (Saitoh *et al*., 1996). The culture medium used for *S. cerevisiae* was YPD medium (1% bacto yeast extract, 2% bacto peptone, 2% glucose, wt/vol.).

Plasmid construction

The plasmid maps used in this study are shown in Fig. 4. 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 Chapter 2. Two genome integration vectors, pBBLE-LDHKCB (Fig. 4. 1A) and pBG418-LDHKCB (Fig. 4. 1B), were constructed using the pBluescript II SK+ vector (Stratagene, La Jolla, CA, USA).

In the pBBLE-LDHKCB vector, the phleomycin resistance gene cassette was *Tn5 BLE* of bacterial transposon *Tn5* (Gatignol *et al*., 1987), which was fused downstream from the *S. cerevisiae* cytochrome C (*CYC1*) promoter. The *PDC1* promoter, *PDC5* and *SLX4* 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 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 4. 1 (see Chapter 2 for details of the primer sequence of the *PDC1* promoter fragment). The 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, Foster City, CA, USA).

In the pBG418-LDHKCB vector, the kanamycin (G418) resistance gene is the aminoglycoside phosphotransferase (*APT*) gene (Hadfild *et al*., 1990), which confers geneticin resistance on yeasts, fused downstream from the *S. cerevisiae* glyceraldehyde-3-phosphate dehydrogenase 3 (*TDH3*) promoter. The *PDC6* and *CTT1* 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 4. 1. The vector was constructed by a similar technique to that described above.

Table 4. 1. Primers used for constructing pBBLE-PDC1P-LDHKCB and pBG418G- PDC1P-LDHKCB.

Primer	Sequence (5'-3') [a]	Restriction 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] Restriction sites in the primer sequences are underlined.

Breeding of yeasts

S. cerevisiae transformation was performed by the lithium acetate procedure (Ito *et al*. 1983), and each transformant was selected on YPD medium containing 7.5 μg/ml phleomycin (Sigma-Aldrich, St. Louis, MO, USA) or 150 μg/ml G418 (Calbiochem, San Diego, CA, USA).

Table 4. 2 shows the transgenic strains that were constructed in this study. Firstly, the pBBLE-LDHKCB vector fragment, which had been digested with *Sac*I and *Apa*I, was transformed into the YIBO-7A recombinant strain (Fig. 4. 1A), which showed high lactic acid production in Chapter 2. Host strain OC-2T is a diploid and homothallic strain (Saitoh *et al.,* 1996). After transformation, the L-*LDH* cassette 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% 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 micro-manipulator (Narishige Science, Tokyo). After colonies had been isolated the target gene integration was confirmed by PCR. The primer sequences used were as follows; F2, 5'-ACCAGCCCATCTCA-ATCCATCT-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.

Secondly, the pBG418-LDHKCB vector fragment, which had been digested with similar restriction enzymes, was transformed into the T157 strain (Fig. 4. 1B). The transgenic strain was constructed by a similar method to that described above. Target gene integration was confirmed by PCR. The primer sequences used were as follows; F3, 5'-TCATTGGTGACGGTTCTCTACA-3'; R3, 5'-CGATAGCAAGTAGA-TCAAGACA-3'. The resulting recombinant yeast was named the T165 strain, which included six copies of L-*LDH* gene on the genome.

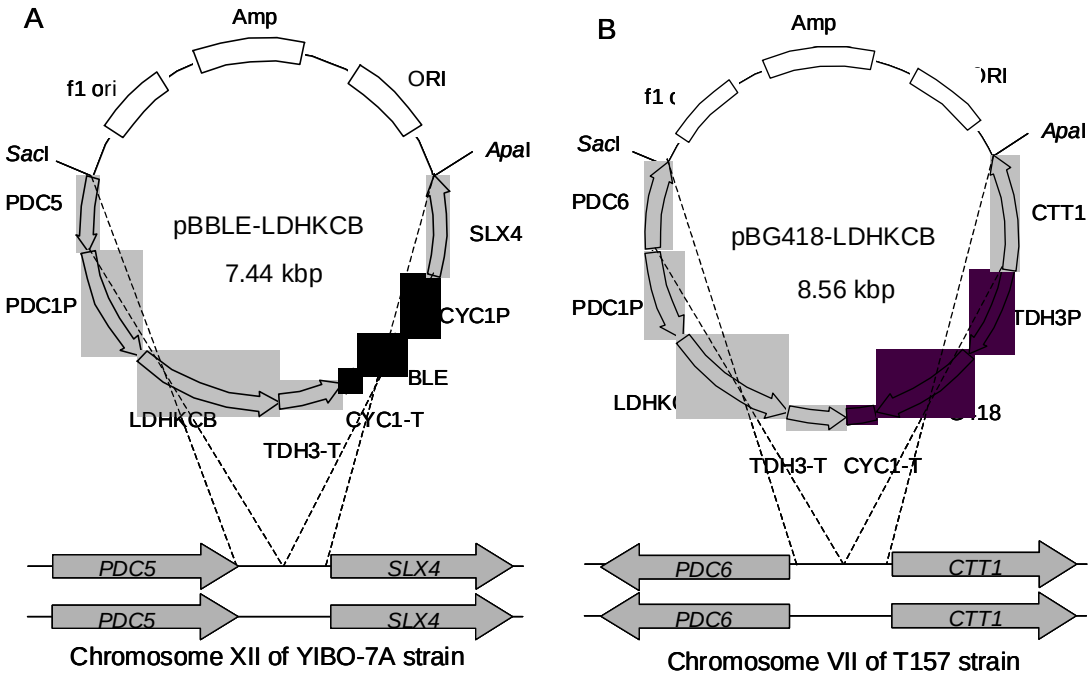

Fig. 4. 1. Maps of the plasmid vectors and breeding of transgenic *S. cerevisiae*. (A) Construction of the T157 strain. The constructed DNA fragment, which was obtained by digesting the pBBLE-LDHKCB vector with *Sac* I and *Apa* I, was integrated into the *PDC5* downstream locus of chromosome XII in 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.

Table 4. 2. Transgenic strains used in this study.

Strain	Relevant genotype	Copy number of *LDH* gene	Reference
OC-2T	*trp1*/*trp1*	0 copies	Saitoh *et al.*, 1996
YIBO-7A	*pdc1*/*pdc1*	2 copies	Chapter 2
T157	*pdc1*/*pdc1*, *phleomycin*^{r}	4 copies	This study
T165	*pdc1*/*pdc1*, *phleomycin*^{r}, *kanamycin*^{r}	6 copies	This study
T165R	*pdc1*/*pdc1*, *phleomycin*^{r}, *kanamycin*^{r}, *EMS mutagenized*	6 copies	This study

Ethylmethane sulfonate (EMS) mutagenesis

Stationary phase cells were harvested from independent cultures grown in YPD. The cells were resuspended in 200 mM sodium phosphate (pH7.0) containing 50 μl EMS. The mixtures were vortexed, then agitated gently on a rotary shaker for 1 h at room temperature. The cells were promptly washed in 10ml sterile water. The final suspensions, which contained approximately 10^6 to 10^7 viable cells/ml, were stored at 4°C for 4 days on YPD medium.

LDH specific activity

Cell extracts were prepared with a SONIFIER 250 (Branson, Danbury, CT, USA) as described previously (Pronk *et al*., 1996). LDH-specific activity was determined in freshly prepared extracts as described (Minowa *et al*., 1989). Protein concentrations in cell extracts were determined with a DC protein assay kit (Bio-Rad, Richmond, CA, USA), using bovine serum albumin (Sigma-Aldrich) 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% bacto peptone, 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% PCV (packed cell volume). Cells were inoculated into a 1 liter jar-fermenter (Biotto, Tokyo). The jar conditions were kept at 32°C and pH 5.2, with aeration at 0.15 liter/min. Its agitation rate was controlled at 60 rpm, and NaOH was used for neutralization. The medium consisted of cane juice (sugar concentration, approximately 20%) containing 0.3% yeast extract (wt/vol.). Glucose, lactic acid, and ethanol concentrations were measured with a biosensor BF-4S (Oji Keisoku Kiki, Amagasaki). 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.

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

4.3. Results

Construction of strain containing multi-copies of L-*LDH* gene

A transgenic *S. cerevisiae* with an increased L-*LDH* copy number was constructed based on the YIBO-7A strain, which was constructed in Chapter 2. In the YIBO-7A strain (*pdc1*::P_{pdc1}-bovine L-*LDH*), 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, while *PDC1* is completely disrupted. Firstly, T157 strain, which has four copies of L-*LDH* gene, was constructed. The heterologous L-*LDH* was integrated between *PDC5* and *SLX4* (Fig. 4. 1-A), but these two coding regions were not disrupted on homologous recombination, because it was reported that *pdc1 pdc5* double mutant strain shows suppression of growth rate (Adachi *et al*., 1998). Secondly, the T165 strain, which has six copies of L-*LDH*, was constructed (Fig. 4. 1-B). 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* gene) was used as a control. As shown in Fig. 4. 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 (Kellermann and Hollenberg, 1988), and glucose responding elements in *S. cerevisiae* have already been reported (Kellermann and Hollenberg, 1988; Butler and Mc Connell, 1988). 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 approximately 2.8 times increase compared with for the YIBO-7A strain, which had

two copies of the L-*LDH*.

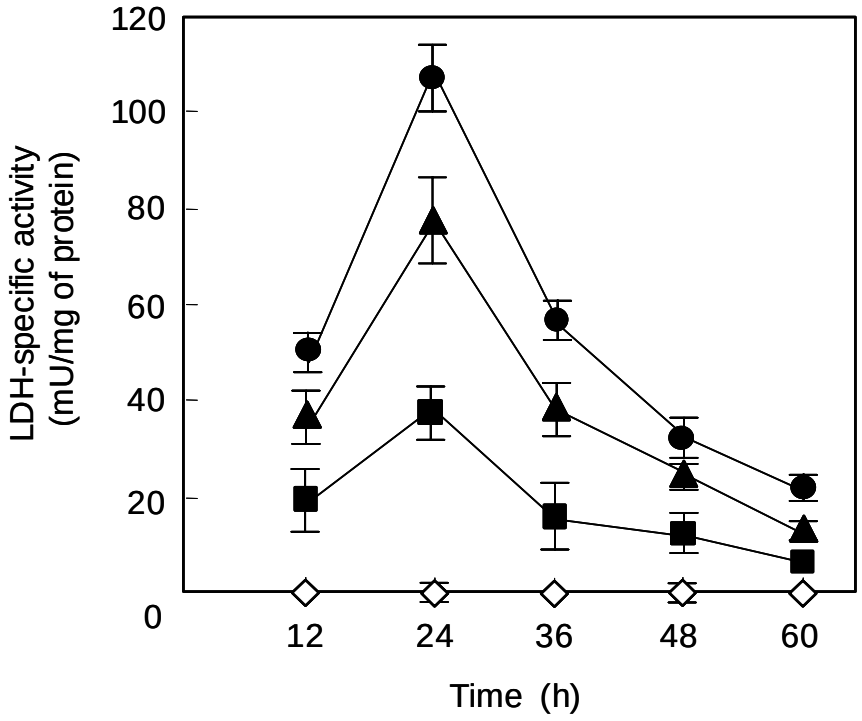

Fig. 4. 2. Comparison of LDH specific activity with increasing gene copy number. The control strain OC-2T (◇). YIBO-7A strain with two copies (■), T157 strain with four copies (▲), and T165 strain with six copies of the L-*LDH* gene (●). The average and deviation for three independent experiments are presented.

EMS mutagenesis of T165 recombinant strain

In spite of high LDH activity, the T165 strain (L-*LDH*; six copies) exhibited remarkably suppressed of the growth rate (data not shown). Furthermore, this strain could not completely consume the glucose in fermentation, and lactate productivity decreased (Fig. 4. 3). Following this, the T165 recovered strain (T165R) was selected 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. 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 glucose 100 g/liter. The T165R strain, with six copies of L-*LDH* gene being expressed from *PDC1* promoter, was observed to produce both L-lactate (68.0 g/liter) and ethanol (9.8 g/liter), with up to 68.0% of the glucose being transformed into L-lactic acid (Fig. 4. 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 Chapter 2. 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. 4. 2).

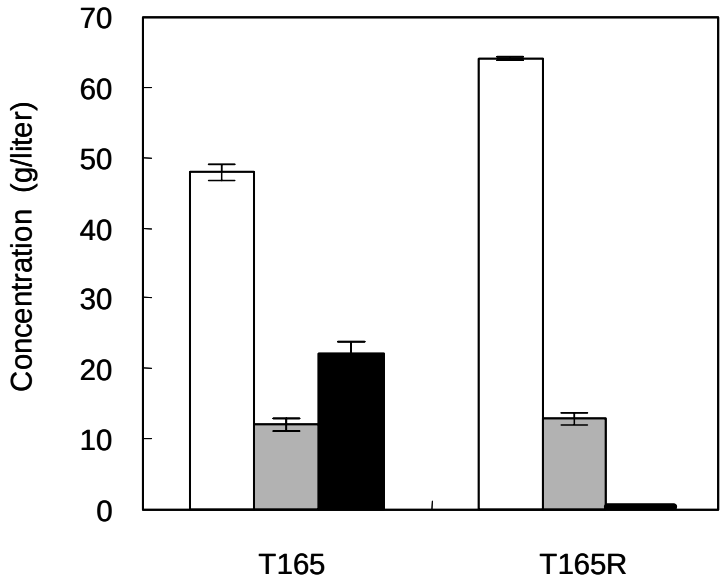

Fig. 4. 3. Comparison of fermentation with the *S. cerevisiae* T165 strain and T165R strain (EMS mutagenesis) in YPD medium containing 100 g/liter of glucose and 50 g/liter of $CaCO_3$. L-Lactate (white bars), ethanol (gray bars), and glucose (black bars). Each strain was cultivated for 72 h under micro-anaerobic conditions at 30°C. The average and deviation for two independent experiments are presented.

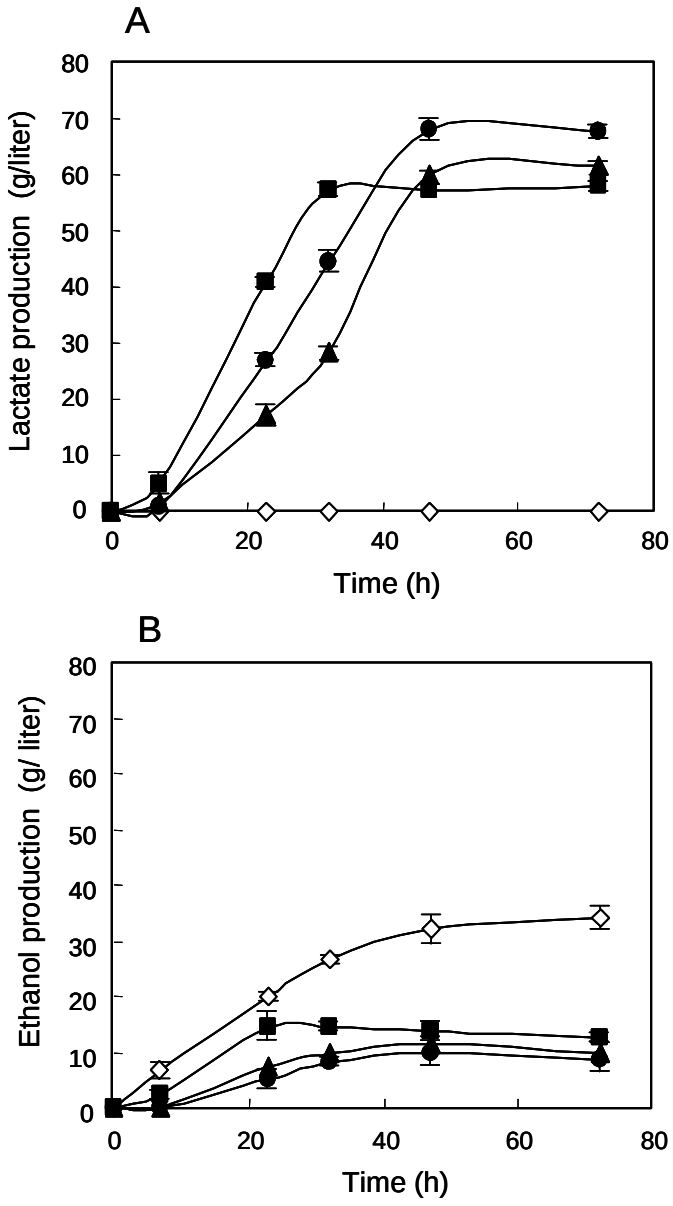

Fig. 4. 4. Time courses of cultivation of four transgenic *S. cerivisiae* strains on a flask scale. (A) L-Lactate, (B) Ethanol. The control strain OC-2T (◇). YIBO-7A strain with two copies (■), T157 strain with four copies (▲), and T165R strain with six copies of the L-*LDH* gene (●). The average and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

Fermentation of T165R strain in cane juice-based medium

To confirm lactate production in low-cost medium, I examined other media except for YPD medium. 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 liter jar-fermenter with pH control, L-lactate production of T165R strain reached 122 g/liter, with up to 61.0% of the sugar being transformed into lactic acid (Fig. 4. 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 medium (68.0%, Fig. 4. 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 4. 3).

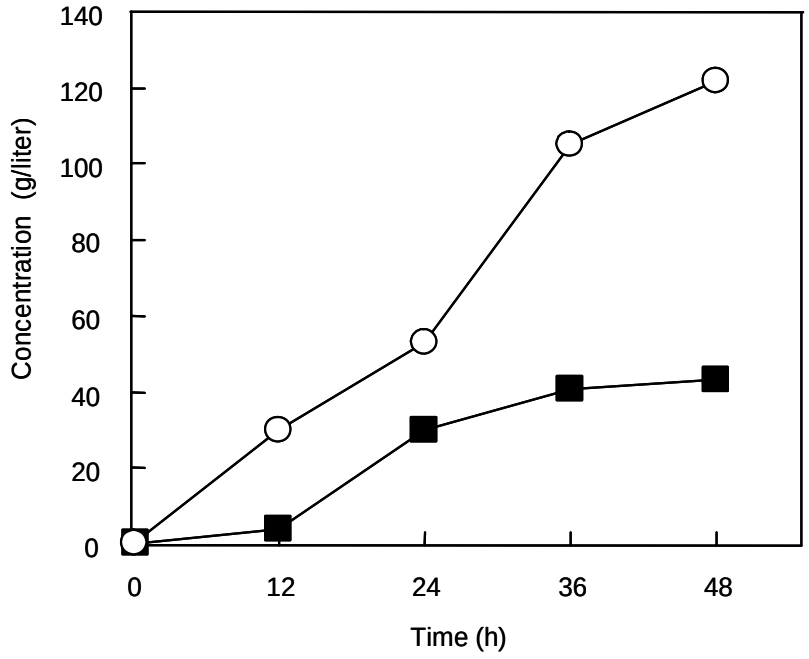

Fig. 4. 5. Fermentation analysis with the *S. cerevisiae* T165R strain in the cane juice-based medium (sugar concentration, approximately 20%). The pH was controlled at pH 5.2 with 1N NaOH. ○, L-Lactate; ■, Ethanol. Each strain was cultivated for 72 h under micro-anaerobic conditions at 30°C.

Table 4. 3. Comparison of optical purity of L-lactic acid between the transgenic yeast and lactic acid bacteria.

Strain	Optical purity	Reference
Saccharomyces cerevisiae OC2T T165R	> 99.9%	This study
Lactobacillus amylophilus ATCC49845	93.0%	Yumoto *et al*., 1995
Lactobacillus delbrueckii sp. *bulgaricus* ATCC11842	91.0 - 95.0%	Hofvendahl *et al.*, 1997
Lactobacillus delbrueckii sp. *delbrueckii* ATCC9649	94.0 - 95.0%	Hofvendahl *et al.*, 1997
Lactobacillus rhamnosus ATCC10863	95.0%	Olmos-Dichara *et al*., 1997
Lactobacillus salivarius sp. *salivarius* ATCC11742	86.0 - 90.0%	Siebold *et al.*, 1995
Streptococcus bovis 148	95.6%	Narita *et al.*, 2004

4.4. Discussion

The final goal of this study is to establish a mass production system of L-lactic acid monomer, and to obtain L-lactic acid of high optical purity with a transgenic wine yeast. In a previous Chapter 2, I constructed a 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. For mass production of this monomer, new recombinant strains with increasing copy numbers of the L-*LDH* gene on the genome were constructed. 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, reduced glucose consumption, and finally decreased lactate productivity, although the LDH-specific activity was increased. It was pointed out by van Maris *et al*. 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 (van Maris *et al*., 2004). To relieve intracellular stress, transgenic yeasts excrete intracellular lactic acid extracellularly, for which a lot of 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. 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/liter of L-lactate was produced from 10% YPD medium in 72 h finally. In the case of the fermentation in a jar-fermenter, the micro-aerobic conditions（aeration at 0.15 liter/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 million tons of it being generated in Latin America and Tropical Asia (Fontana *et al*., 1996). Cane juice, which contains a lot of mineral salts and sucrose, is a low-cost medium compared with YPD medium. As shown in Fig. 4. 5, the T165R strain showed high lactic acid production even in cane juice-based medium (more than 120 g/liter 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 NBRC2260 strain, which has been used for producing wine on an industrial scale. Lactic acid is generally produced with lactic acid bacteria, such as *Lactobacillus* species, which are hard to cultivate at high density and show high auxotrophy as to growth (Ohara *et al*., 2001). 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 (Ozeki, 1996). 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 (van Breugel *et al*., 2002), however, this process affects the production cost. Lactic acid bacteria have a racemase or D-LDH, and so attempts to delete these genes have been made. On the other hand, other hosts, *i.e.* fungi (Miura *et al*., 2004; Skory, 2004), genetically engineered *E. coli* (Chang *et al*., 1999; Dien *et al.*, 2001; Zhou *et al.*, 2003), and genetically engineered yeasts (Dequin *et al*., 1994; Porro *et al*., 1995; Adachi *et al*., 1998; Skory *et al*., 2003), have been investigated as to the production of L-lactic acid. Although several organisms producing lactic acid have been discussed about optical purity (Zhou *et al*., 2003-a, -b), genetically engineered yeasts have been not analyzed about it yet. In this chapter, I confirmed that a recombinant wine yeast was able to produce L-lactate of high optical purity (99.9%, Table 4. 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 as to the use of this recombinant yeast. Firstly, L-lactic acid of high optical purity can be produced. Secondly, lactic acid can be produced even if one uses an inexpensive media, such as one based on cane juice. Lastly, because yeasts exhibit a low pH tolerance, free lactic acid production can be expected without neutralization (Adachi *et al*., 1998). As to 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. It is expected this research will lead to further use of transgenic yeasts.

Chapter 5

The efficient production of D-lactic acid using the genome-integration technique

5.1. Introduction

Plant- and crop-based plastics, including PLA, are being developed as renewable alternatives to conventional petroleum-based plastics (Ohara *et al*., 2001). 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 (Ozeki *et al*., 1996). 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. The stereo-complex type of blending polymer is characterized by its high melting temperature, approximately 50°C higher than that of the PLA (Ikada *et al*., 1987; Tuji *et al.*, 1991). 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 (Yanez *et al*., 2003) or rice starch (Fukushima *et al*., 2004) 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 *E. coli* has also been developed (Chang *et al.*, 1999; Zhou *et al*., 2003), because lactic acid bacteria are hard to cultivate at high density and show high auxotrophy regarding growth (Hofvendahl and Hahn-Hagerdal, 2000). 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 (Shukla e*t al*., 2004) or metabolic flux analysis for some mutant strains (Zhu *et al.*, 2004 and 2005). 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 *S. cerevisiae,* are more tolerant to low pH than *E. coli* (Porro *et al*., 1995; Adachi *et al*., 1998), and thus high density cultivation is possible. *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 (Porro *et al*., 1995; Adachi *et al*., 1998; Dequin *et al.*, 1994). I also attained efficient production of L-lactic acid with a recombinant wine yeast in previous Chapter 2. In this chapter, I isolated the D-*LDH* gene from *Leuconostoc mesenteroides* subsp. *mesenteroides* strain NBRC3426, and tried to integrate this heterologous gene into the *PDC1* coding region using 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. These 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.

5.2. 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 (Sambrook *et al.*, 1989). *S. cerevisiae* OC-2T (a/α, *trp1*/*trp1*) was derived from the wine yeast NBRC2260 strain (Saitoh *et al*., 1996). 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 (Man *et al.,* 1960).

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 number L29327 (Dartois *et al*., 1995). The oligonucleotide sequences of these primers were as follows (Qiagen K.K., Tokyo). DLDHLM-U; 5'-ATATATGATATCATGAAGAT-TTTTGCTTACGGC-3', and DLDHLM-D; 5'-ATATATGATATCTTAATATTCAACAGCA-ATAGC-3'. Both primers contained an *Eco*R V 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 sec, 53°C for 30 sec and 72°C for 90 sec. The amplified DNA fragment was subcloned into the pBluescript II SK+ vector (Stratagene, La Jolla, CA, USA) *Eco*R V site according to the previously described method (Sambrook *et al*., 1989). 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 Genetyx-Win (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 number AB233384.

Plasmid construction

A map of the plasmid vector used in this study is presented in Fig. 5. 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'-ATATATGGATCCGCGTTTATTTACCTATCTC-3', containing a *Bam*H I restriction site (underlined); and PDC1P-D, 5'-ATATATGAATT-CTTTGATTGATTTGACTGTG-3', containing an *Eco*R I restriction site (underlined). This fragment was -705 bp upstream from the *PDC1* ORF start codon. *PDC1* 3'end fragment: PDC1D-U, 5'-ATATATCTCGAGGCCAGCTAACTTCTTGGTCGAC-3', containing a *Xho* I restriction site (underlined); and PDC1D-D, 5'-ATATATGGGCCC-CTCGTCAGCAATAGTGGTCAAC-3', containing an *Apa* I restriction site (underlined). The *PDC1* downstream fragment of 518 bp in length was between +501 ~ +1,018 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 (Sambrook *et al*., 1989).

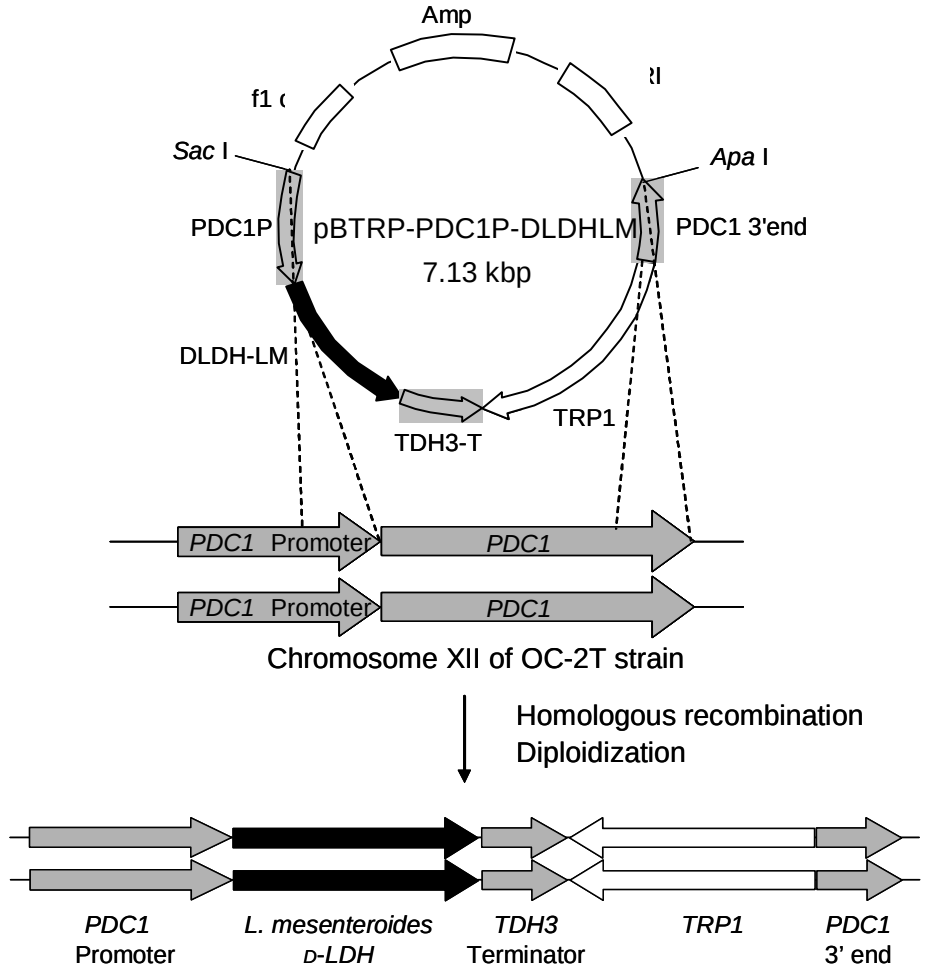

Fig. 5. 1. Map of the plasmid vector and breeding of transgenic *S. cerevisiae*. The constructed DNA fragment, which was obtained by digesting the pBTRP-PDC1P-DLDHLM vector with *Sac* I and *Apa* I, was integrated into the *PDC1* ORF region of the wine yeast OC-2T strain.

Breeding of the yeast

S. cerevisiae transformation was performed by the lithium acetate procedure (Ito *et al*. 1983). 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 (Saitoh *et al*., 1996). 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 (*pdc1*::P_{pdc1}-*L. mesenteroides* D-*LDH*).

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 day. 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 (Pronk *et al.*, 1996). PDC-specific activity in freshly prepared extracts 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*. (Kochhar *et al*. 1992). 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 for 72 h at 30°C in 100 ml flasks with a working volume of 40 ml, in 10% YPD medium (1% bacto yeast extract, 2% bacto-peptone, and 10% D-glucose) containing 3% of sterilized calcium carbonate under the anaerobic condition. 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 anaerobic 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 biosensor 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.

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

5.3. Results

Cloning and sequence analysis of the D-*LDH* gene homolog

A 1.0-kbp DNA fragment, which was amplified from the *L. mesenteroides* subsp. *mesenteroides* strain NBRC3426, was subcloned into the pBluescript II SK+ vector, and the nucleotide sequence (999 bp) was determined. The deduced amino acid sequence showed significant similarity to the D-LDH sequence that had already been reported (Dartois *et al.*,1995). The amino acid identity was 94.3% in comparison with the previously reported sequence, but a clear distinction was seen, especially in the amino acid residues from Gly-266 to Phe-277. In the DNA sequence of the *L. mesenteroides* subsp. *mesenteroides* strain NBRC3426, this clearly distinct area was caused by a frameshift (Fig. 5. 2a). The *Lactobacillus johnsonii* (Gene Bank accession number AF071558, Lapierre *et al.*, 1999) and the *Lactobacillus plantarum* (Gene Bank accession number D90339, Taguchi *et al.*, 1991) D-LDHs have also been reported to have high amino acid identity to the *L. mesenteroides* subsp. *cremoris*; 65.0% and 54.2% respectively. On comparison of this frameshift region between four sequences, it was found that of *L. mesenteroides* subsp. *mesenteroides* in this study was similar to these two D-LDHs rather than that of *cremoris* (Fig. 5. 2b).

Growth rate and enzyme activity of the YILM-2B strain

I constructed a recombinant yeast, YILM-2B strain, in which the *L. mesenteroides* D-*LDH* was integrated into the *PDC1* locus on the genome. For initial physiological characterization, the growth rate of this *PDC1*-disrupted recombinant was determined under aerobic conditions. The growth rate of the YILM-2B strain was 0.537 h^{-1}, while that of parent strain OC-2T was 0.642 h^{-1} (data not shown). As compared to the host strain, extreme suppression of the growth rate was not observed, although the *PDC1* was completely disrupted. This tendency was the same as in the case of L-*LDH* integrated strain in previous Chapter 2, and provided further support. The PDC- and D-LDH-specific activity of the recombinant strain was

A

B

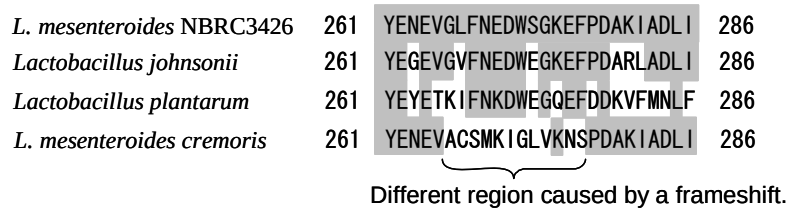

Fig. 5. 2. (A) Alignment of the DNA sequences of *L. mesenteroides* from NBRC3426 (this study) and *L. mesenteroides* subsp. *cremoris* (Dartois *et al.*, 1995) from 781 to 839 bp. The amino acid sequence is shown at the edge of both DNA sequences. Identical sequences are shadowed. (B) Alignment of the amino acid sequence of D-LDH from residues 261 to 286. *L. mesenteroides* subsp. *mesenteroides* (this study), *Lactobacillus johnsonii* (Lapierre *et al.,* 1999), *Lactobacillus plantarum* (Taguchi *et al*., 1991), and *L. mesenteroides* subsp. *cremoris* (Dartois *et al.*, 1995). Identical amino acid residues are shadowed.

also confirmed, respectively. The PDC activity of YILM-2B strain was 80.7 mU/mg of protein, and this value decreased to one-third in comparison with OC-2T host strain (221.3 mU/mg of protein). The PDC activity in *S. cerevisiae* is due mainly to the PDC1 and PDC5, and it was reported that *PDC1* deletion led to a great increase in *PDC5* promoter-driven mRNA expression (Hohmann and Cederberg, 1990). Therefore, it appeared that this activity was related to PDC5. On analysis of the D-LDH activity, that of the YILM-2B strain was 30.1 mU/mg of protein, but that of host strain OC-2T was not detected. This result showed that the D-*LDH* gene from prokaryote cells introduced in yeast, a eukaryote, functioned precisely. I also tried to develop two other kinds of D-*LDH* integration, i.e., with either *E. coli* (Gene Bank accession number U36928, Bunch *et al.*, 1997) or *Octopus vulgaris* (Gene Bank accession munber AB050737). Each amino acid identity showed 40.0% and 30.1% in comparison with the D-LDH from *L. mesenteroides* subsp. *mesenteroides*, and three D-LDHs are greatly different in the amino acid sequence. However, these two recombinants did not exhibit D-LDH activity (data not shown).

D-Lactate production under neutralizing conditions

On fermentation analysis in the YPD medium containing 100 g/liter glucose including $CaCO_3$ as a corrective, the YILM-2B strain expressing *L. mesenteroides* D-*LDH* was observed to produce both 61.5 g/liter D-lactate and 17.3 g/liter ethanol (Fig. 5. 3). The glucose was rapidly converted into D-lactate in 72 h with a yield of 61.2%. In this culture medium, L-lactate was not produced, as measured with a bio-sensor, and the optical purity of D-lactic acid from YILM-2B strain was at least 99.9%. Also, the nonexistence of L- and D- lactic acid in the media was confirmed in advance. This purity was obviously high compared with that of D-lactic acid produced by lactic acid bacteria. Additionally, host strain OC-2T produced neither L- nor D-lactate. This finding showed a similar tendency to in the case of L-lactate production on genome introduction in previous Chapter 2. As in the case of D-LDH activity measurement, the other two recombinants into which the D-*LDH* from *E. coli* or *O. vulgaris* was introduced did not produce this target substance.

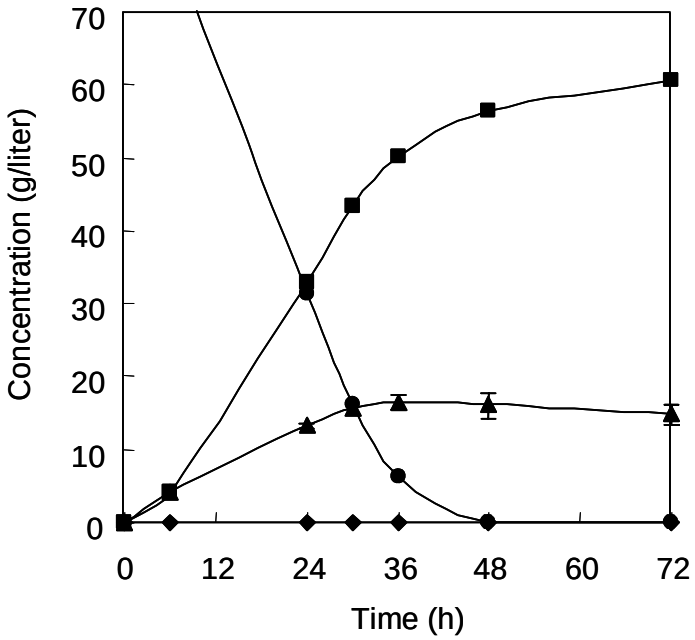

Fig. 5. 3. Time courses of cultivation of the transgenic *S. cerevisiae* YILM-2B strain on a flask scale under the neutralizing condition. Symbols: ■, D-lactate; ◆, L-lactate; ▲, ethanol; ●, glucose. The averages and standard deviations (error bars) for three independent experiments are presented.

D-Lactic acid production under non-neutralizing conditions

S. cerevisiae is more tolerant to low pH than lactic acid bacteria and *E. coli* (Porro *et al*., 1995). D-Lactic acid production by the YILM-2B strain was confirmed under non-neutralizing conditions. As for the culture conditions, they were the same as for the neutralizing ones except for $CaCO_3$. The accumulation and the yield of free D-lactic acid in 72 h are shown in Fig. 5. 4. For the YILM-2B strain, which had integrated the *L. mesenteroides* D-*LDH*, the maximal concentrations of D-lactic acid and ethanol reached 53.2 g/liter and 20.7 g/liter, respectively. The glucose was completely consumed in 72 h, and L-lactic acid was not observed as under the non-neutralizing conditions. The yield of D-lactic acid reached 53.0%, which was a little decreased compared with in the case of the neutralizing conditions. The final pH of this culture solution was 2.8. The effect on the yield of lactic acid on changing of the initial glucose concentration from 100 to 150 g/liter was examined. However, as judged on fermentation analysis under non-neutralizing conditions, improvement of the lactic acid production was not observed (54.2 g/liter), and it did not reach 60 g/liter or more. This YILM-2B strain could not consume glucose completely, and the yield of D-lactic acid was decreased extremely compared with in the case of 100 g/liter of the initial glucose concentration.

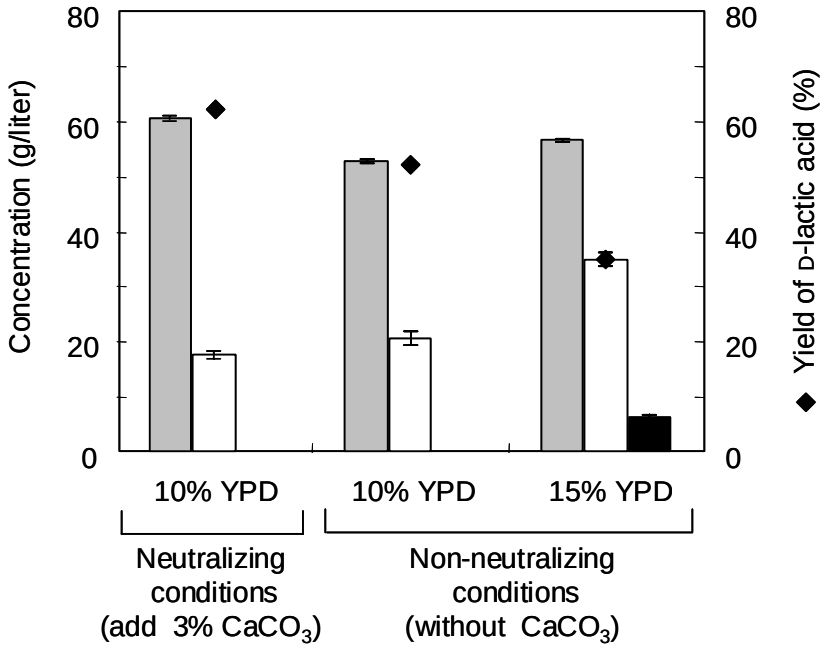

Fig. 5. 4. Accumulation of D-lactic acid (gray bars), ethanol (white bars), and glucose (black bars) by the YILM-2B strain under different culture conditions. Closed diamond shows the yield of D-lactic acid. Each strain was cultivated for 72 h under the anaerobic conditions at 30°C. The averages and standard divisions (error bars) for three independent experiments are presented.

5.4. Discussion

Poly-D-lactic cacid is an important polymer because it improves the thermostability of poly-L-lactic acid through stereo complex formation (Ikeda *et al*., 1987; Tsuji *et al*., 1991). However, fermentation of D-lactic acid monomer has been little studied in comparison with L-lactic acid. In this chapter, a metabolically engineered *S. cerevisiae* exhibiting efficient D-lactic acid production have been developed by substituting the coding region of *PDC1* on chromosome XII through homologous recombination (Fig. 5. 1). It has already been established by previous study that genome integration of the L-*LDH* using the *PDC1* promoter leads to efficient production of L-lactic acid (Chapter 2). This present approach is almost the same except for the introduction of another heterologous gene, D-*LDH* from *L. mesenteroides* subsp. *mesenteroides*. Thus, these findings can be considered to indicate that this method is effective for the production of other organic acids.

For the mass production of an optically pure target substance, I examined D-lactate production using three recombinants, expressing different D-*LDH* genes (*i.e.*, *L. mesenteroides, E. coli,* and *O. vulgaris*). It was expected that the effect of the D-lactic acid production would be proved by different D-LDHs. However significant production has been observed only in the case of *L. mesenteroides* D-*LDH*. The affinity for pyruvic acid of each D-LDH was reported to be as follows. *L. mesenteroides* subsp. *cremoris*, K_m=0.3 mM (Dartois *et al*., 1995); *E. coli*, K_m=4.4 mM (Tarmy *et al.*, 1967); *O. vulgaris*, K_m=1.1 mM (our laboratory data using recombinant *E. coli*). In previous examination of L-lactic acid production, bovine L-LDH with high affinity for pyruvic acid was more effective than another L-LDH showing low substrate affinity. Thus, the performance of D-LDH has a profound effect on D-lactic acid production. Since specific enzyme activity could not be confirmed for either enzyme in this attempt, it is necessary to both examine the gene expression and protein analysis. Compared to D-LDH from *L. mesenteroides* subsp. *cremoris*, that has already been reported, it was shown that a clear difference in the amino acid residues from Gly-266 to Phe-277 was caused by a

frameshift. This interesting finding might be useful as to evolution of the D-*LDH*. The effect on both amino acid sequences will be demonstrated by paying attention to the frameshift site in the near future.

The final goal of this study was to establish an efficient production system for D-lactic acid of high optical purity involving metabolically engineered yeast. The maximal concentration of this target with the YILM-2B strain, which expresses *L. mesenteroides* subsp. *mesenteroides* D-*LDH* under the control of the *PDC1* promoter, was 61.5 g/liter under neutralizing conditions (Fig. 5. 3), and the yield was approximately 61.2%. In my previous study concerning L-lactate production, *pdc1 pdc5* double knockout (Chapter 3) and increasing copy numbers of the *LDH* (Chapter 4) lead to improvement of this organic acid production. In the case of D-lactic acid, the YILM-2B strain, having two copies of the D-*LDH* on the genome, showed higher production than the recombinant that had integrated one copy of this gene (data not shown). Therefore, it will also be possible to increase the D-lactate production by further improvement of this strain.

For stereo complex formation from poly L- and D-lactic acid, high optical purity of the polymers is necessary, because a stereo-complex is not well formed, when both polymers are of low optical purity. And as a result, the thermostability of poly-lactic acid cannot be improved (Ikeda *et al*., 1987; Tsuji *et al*., 1991). This model makes it clear that metabolically engineered *S. cerevisiae* can produce optically pure D-lactic acid. Especially, as for wine yeast, fermentation in an inexpensive medium, such one containing as cane juice, is also possible (Chapter 4). Attempts at the production of D-lactate using lactic acid bacteria and *E. coli* are summarized in Table 5. 1. Actually, efficient production, the maximal yield of D-lactate being over 80%, has already been achieved with *E. coli* recombinants (Hofvendahl *et al.*, 2000; Shukla *et al*., 2004), and productivity improvement by means of flux analysis has been reported (Zhu and Shimizu, 2004 and 2005). For a conventional method, it has been pointed out that the desalination step of lactate for free lactic acid is the main factor that increases the cost. This present strain, which expresses D-*LDH* from *L. mesenteroides* under the control of the *PDC1*

promoter, produced D-lactic acid (53.2 g/liter) of high optical purity without $CaCO_3$ (Fig. 5. 4). Although the yield of D-lactic acid is still low compared with previous results (Table 5. 1), a key distinguishing feature of this approach is the use of *S. cerevisiae* for free D-lactic acid production without neutralization, because yeast is well known to grow and survive at low pH compared with lactic acid bacteria and *E. coli*. If lactic acid is obtained directly under non-neutralizing conditions without a desalination process in an inexpensive medium, lowering of the cost on a manufacturing scale is possible. To improve the production under non-neutralizing conditions, it is important to make more progress as to low pH tolerance of intracellular LDH or host cells. Future study will focus on the stress response mechanism under low pH conditions. *S. cerevisiae* is a suitable microorganism for an industrial scale. This approach could be a powerful method for free D-lactic acid production other than the use of a transgenic *E. coli* or lactic acid bacteria in the future.

Table 5. 1. Study of D-lactate production.

Host strain	Relevant genotype	Carbon source (wt/vol.)	D-lactate production (g/l)	Yield of D-lactate	Time (h)	Optical purity	Reference
Lactobacillus delbrueckii	——	10% rice saccharificate	62.6	70%	50	98.4%	Fukushima *et al.* , 2004
E. coli RR1	*pta, ppc*	10% glucose	62.2	72%	60	——	Chang *et al.* , 1999
E. coli W3110	*pflB, frdBC, adhE*	5% glucose	50.8	95%	24 (aeration)	> 99.8%	Zhou *et al* ., 2003
E. coli W3110	*pflB, frdBC, adhE, ackA, CSCR* , *CSCA* , *CSCKB*	5% sucrose 5.4% molasses	51.2 48.7	97% 90%	36 96	> 99.8%	Shukla *et al.* , 2004
E. coli BW25113	*pflA* or *pflB*	1% glucouse	6.6	70%	10	——	Zhu *et al.* , 2004
S. cerevisiae OC2	*pdc1*::*Ppdc1- D-LDH* (*L. mesenteroides D-LDH*)	10% glucose	61.5 54.2	61% (neutralizing) 53.0% (non-nutralizing)	72 72	99.9% 99.9%	This study

Gene designations: PTA, phospho transacetylase; PPC, phosphoenol pyruvate carboxylase; PFL, pyruvate-formate lyase; FRD, fumarate reductase; ADH, alcohol dehydrogenase; ACK, acetate kinase; CSCR, sucrose gene cluster consists of an operon encoding a repressor protein; CSCA, operon encoding invertase; CSCKB, operon encoding fructokinase and an anion symport for sucrose.

Chapter 6

Conclusion

This study described the achievement of efficient production of L- and D-lactic acid with a metabolically engineered *S. cerevisiae* into which the heterogous *LDH* gene was introduced. In the final Chapter 6, I summarize this study and discuss the future of lactic acid-producing yeast.

6.1. Summary

Generally, a method involving lactic acid bacteria has been the main means of lactic acid production. However, the following problems cause a high cost of poly lactic acid production. (1) Neutralization with $CaCO_3$ or NH_4 is necessary, because produced lactic acid inhibits fermentation. Therefore, the desalination of lactate becomes complex. (2) The auxotrophy is complex, and the media are too expensive. (3) Cultivation at high density is hard. (4) The optical purity of the lactic acid is low. To solve these problems, other approaches to use different hosts such as *R. oryzae* and recombinant *E. coli* have been developed. Also, recombinant yeast have been paid attention as to lactic acid production. *S. cerevisiae* hardly produces any L- or D-lactic acid. However, yeast is more tolerant to low pH than lactic acid bacteria, *R. oryzae*, and *E. coli*. This unique feature allows the possibility of free lactic acid production under the non-neutralizing conditions without corrective. Additionally, a transgenic *S. cerevisiae* producing lactic acid of high optical purity can be expected which is an effective factor for high physical properties of poly lactic acid.

At the beginning, I explained that the genome integrated L-*LDH* gene in *S. cerevisiae* leads to efficient production of L-lactic acid. Although by-product ethanol production still produced, this recombinant could produce L-lactic acid under neutralizing and non-neutralizing conditions, respectively (Chapter 2). Secondly, control of the metabolic pathway to ethanol production (Chapter 3) and L-LDH activity (Chapter 4) led to more efficient production of L-lactic acid. Another key

distinguishing feature of this transgenic *S. cerevisiae* is the production of lactic acid of high optical purity (99.9% or higher) under the cane juice-based medium. Finally, the genome integration technique also contributed to the efficient production not only L-lactic acid, but also D-lactic acid (Chapter 5). Throughout the course of this study, I have attempted to develop a new lactic acid production approach that differs from those involving lactic acid bacteria. However, highly effective production under non-neutralizing conditions is not possible yet. Therefore, further studies focusing on the mechanism of acid tolerance of *S. cerevisiae* are necessary. By using a lactic acid-producing yeast, it is expected that the low-cost production of L-and D-lactic acid will become possible in the future, because the problem pointed out for lactic acid bacteria could be overcome.

6.2. Development for the future

The development of various researches can be expected with the use of a lactic acid-producing yeast. In this section, I described the possibilities of the present transgenic yeast.

1. The tolerance response mechanism for free lactic acid

The lactic acid tolerance of yeast is known to be higher than that of lactic acid bacteria and *E. coli.* However, lactic acid fermentation with the use of lactic acid-producing yeast ceases under non-neutralizing conditions when free lactic acid accumulates in the culture medium although high production has been demonstrated under neutralizing conditions. Elucidation of the mechanism underlying free lactic acid tolerance of yeast is essential for further improvement of free lactic acid production. A study concerning lactic acid tolerance has not been

performed, because yeast does not usually produce lactic acid. However, with analysis involving gene deletion library and DNA microarray, attempts at improvement of lactic acid tolerance will increase.

2. The yeast cell surface display

Further development of lactic acid-producing yeast can be expected with the application of the yeast cell surface display technology (arming-yeast). This technology was developed by Ueda *et al*., and is characteristic in that functionality enzymes are displayed on the cell surface and cells are given a new function (Ueda *et al*. 2000). It was developed to produce ethanol directly from starch without the addition of enzymes using a recombinant *S. cerevisiae* displaying cell-surface amylase (Kondo *et al*. 2002). Furthermore, not only production from starch but also technology concerning for direct production from cellulose and xylose has been reported (Fujita *et al.* 2002, and Katahira *et al*. 2004). By applying the arming-yeast technology to lactic acid-producing yeast, the possibility of conversion of starch or cellulose directly into lactic acid can be expected.

3. Solvent extraction of free lactic acid

Although efficient fermentation processes have been developed for lactic acid production, an economical method for lactic acid recovery from the fermentation broth is still needed. Yeast shows tolerance to solvents in comparison with lactic acid bacteria, because yeast maintains its cell wall, of which the principal ingredient is glucomannan. It has been reported that alamine 336 is the optimum solvent for lactic acid extraction (Wasewar *et al.* 2002). Additionally, a hollow-fiber membrane extraction process for the recovery and separation of lactic acid from an aqueous solution using alamine 336 in 2-octanol has also been developed (Huang *et al.* 2004). A contribution to low-cost production of lactic acid can be expected with the combination of recombinant *S. cerevisiae* in this study and a purification system involving solvent extraction.

4. Immobilized yeast cell

The immobilization of yeast cells is considerable interest with regard to the efficient production of ethanol. The types of immobilization carriers used include ceramics (Salter, *et al*. 1990), ion-exchange resins (Yoshioka and Shimmura, 1986), polyurethane (Endo *et al*. 1988), chitosan (Shinonaga *et al*. 1992), calcium-alginate gel (McGhee *et al.* 1982), and cellulose (Sakurai *et al*. 2000). Using immobilized yeast, an attempts at the improvement of ethanol productivity have been reported. Additionally, an attempt to make saccharification and fermentation proceed at the same time with two immobilized microorganisms has been reported (Tanaka *et al*. 1986). Further efficient production of lactic acid can be expected by examining the optimal immobilization carriers in the present lactic acid-producing yeast.

6.3. Finally

This study described the achievement of efficient production of L- and D-lactic acid using a metabolically engineered *S. cerevisiae* into which a heterogous lactate dehydrogenase gene had been introduced. Yeast is an indispensable microorganism in the fermentation industry for alcohol production, and it is being focused on as a model organism for genetic research. The progress of basic findings is also more remarkable because of whole-genome decipherment, enhancement of gene deletion library, etc. In this study, a diploid yeast, the OC-2 strain, was used as a host cell. The OC-2 strain is a famous wine yeast isolated by Dr. Kinichiro Sakaguchi, emeritus professor of The University of Tokyo, and it is widely known as a yeast that has a fast growth rate and is suitable for fermentation. A basic technology for producing lactic acid with yeast could be established through this study. However, further detailed work is necessary for low-cost production of poly L- and D-lactic acid. I want to extend the study on lactic acid production from the basic and applied sides further in the future.

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Abstract

Abstract

This study described the efficient production of L- and D-lactic acid using a metabolically engineered *S. cerevisiae* into which a heterogous *LDH* gene had been introduced. *S. cerevisiae* hardly produces either L- or D- lactic acid. However, *S. cerevisiae* is more tolerant to low pH than lactic acid bacteria, *R. oryzae*, and *E. coli*. Therefore, if a genetically engineered *S. cerevisiae* is used, free lactic acid production can be expected without pH control. Also, a transgenic yeast is hoped for producing lactic acid of high optical purity, which is an effective factor for high physical properties of poly lactic acid.

Firstly, I explained that a genome integrated L-lactate dehydrogenase (L-*LDH*) gene from bovine leads to efficient production of L-lactic acid. Although by-product ethanol still produced, this recombinant could produce L-lactic acid efficiently under neutralizing (55.6 g/liter) and non-neutralizing (50.2 g/liter) conditions, respectively. The production yield being the highest vale for transgenic yeast reported so far. Secondly, control of the metabolic pathway to ethanol and increasing the copy number of L-*LDH* gene on the genome led to more efficient production. The final concentration of L-lactate reached 122 g/liter in a cane juice-based medium. Another key distinguishing feature of this transgenic *S. cerevisiae* is the production of lactic acid of high optical purity (99.9% or higher). On the other hand, poly D-lactic acid is also important polymer because it improves the thermostability of poly L-lactic acid through stereo complex formation. Finally, I developed recombinant *S. cerevisiae* into which a D-*LDH* gene from *Leuconostoc mesenteroides* had been introduced. At this result, the genome integration technique also contributed to the efficient production of D-lactic acid (61.5 g/liter), not only L-lactic acid.

A new lactic acid production approach that differed from that with lactic acid bacteria was developed in this study. *S. cerevisiae* is an indispensable microorganism for alcohol production in the fermentation industry and more over it is focused on as a model organism for genetic research. It can be expected that the low-cost production of L- and D-lactic acid will become possible in the future, and this study could solve the problem with lactic acid bacteria pointed out.

論 文 の 内 容 の 要 旨

論文題目　A study on the efficient production of lactic acid with metabolically engineered *Saccharomyces cerevisiae*
（遺伝子組換え酵母による乳酸の効率生産に関する研究）

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近年の地球温暖化問題により、化石燃料に依存したCO_2排出型社会からCO_2循環型（カーボンニュートラル）社会への転換が、21 世紀の重要課題として取り上げられている。特にポリ乳酸に代表される植物由来プラスチックは、循環型社会に貢献できる材料として大きく注目されている。

ポリ乳酸は、植物由来のでんぷん・糖などを原料に、乳酸発酵を経て生産される L−乳酸を重合して得られるポリマーであり、農業用シートや家電の一部に採用されるなどして実用化されている。しかし、耐熱性および耐衝撃性が低く、製造コストも高いことから、あまり普及していないのが現状である。そのため、ポリ乳酸を汎用プラスチック並みに展開するためには、耐熱性、耐衝撃性の向上とコストの低減が不可欠であると指摘されている。一般的に、原料である L−乳酸の生産技術としては、乳酸菌を用いた手法が主流である。しかし乳酸菌の場合、高い栄養要求性や高密度培養が難しいといった課題がある。さらに、ポリ乳酸の高性能化に必須であるといわれている L−乳酸の光学純度が低い（約 95%程度）ことも指摘されており、これらの問題点がポリ乳酸のコストを上げる要因となっている。

一方で、*Saccharomyces cerevisiae* に代表される酵母は、高密度培養が可能で、ケーンジュースなどの低コスト培地からでも発酵が行えることから、エタノールを中心とする発酵産業に欠かせない真核生物として知られている。一般的に酵母は、乳酸脱水素酵素遺伝子（lactate dehydrogenase; 以下 *LDH* と称す）を持たないことから、乳酸は生産されない。しかし、*LDH* 遺伝

子を発現させることにより、酵母での乳酸生産は可能である(図 1)。この場合、発酵生産される乳酸の光学純度は高いことが予想されると同時に、酵母は低 pH にも比較的耐性を示すことから、非中和条件下でフリー乳酸を生産できる可能性もある。さらに酵母は、高密度培養が可能であり、培養における栄養要求性も乳酸菌ほど高くない。このような期待から、*S. cerevisiae* で L-*LDH* 遺伝子を発現させ L-乳酸を発酵生産させる試みが、これまでに数件報告されている。しかし、いずれの報告においても10～20g/liter程度のL-乳酸しか得られず(グルコースからの変換効率として20%前後)、高い生産効率は認められなかった。元来 *S. cerevisiae* は強いエタノール生産能を有することから、酵母で効率的な乳酸生産を実現するためには、エタノール生産に関わるピルビン酸脱炭酸酵素遺伝子 (pyruvate decarboxylase; 以下 *PDC* と称す)の発現を制御することが指摘されていた。そこで、高光学純度の L-および D-乳酸を高効率で生産させることを目的に、代謝工学的手法を基盤とした乳酸生産酵母構築に関する以下の研究に取り組んだ。

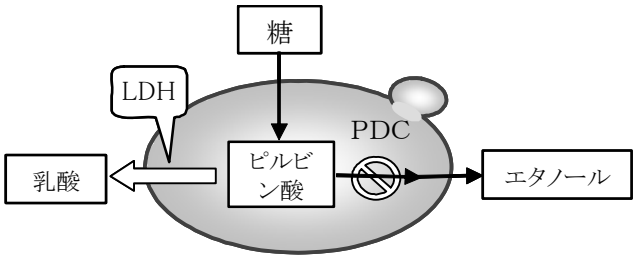

図1 遺伝子組換え酵母による乳酸生産

1、 酵母染色体の*PDC1* 部位への*L-LDH*遺伝子導入は、効率的なL-乳酸生産をもたらす

遺伝子組換え酵母によるL-乳酸の生産は、従来、2μm DNAを含んだマルチコピー型ベクターを利用した試みが主流であった。しかしその場合、高い乳酸生産は確認されていない。そこで、*S. cerevisiae* 第 12 番染色体上に位置する *PDC1* プロモーター下流に L-*LDH* 遺伝子を導入し、*PDC1* ORF遺伝子のみが破壊される新しい遺伝子発現系を試みた。本手法で作製した組換え酵母は、導入された L-*LDH* 遺伝子が内在性の *PDC1* プロモーター制御下で発現される特徴をもつ。作製した組換え酵母と、マルチコピー型ベクターを使って作製した従来株とを比較した結果、*PDC1* 遺伝子部位への染色体導入株では、L-*LDH* 遺伝子が染色体中に 2 コピーしか挿入されていないにも関わらず、高い LDH 活性を示した。さらに、100g/liter グルコース含有 YPD 培養液による発酵試験の結果、58g/liter の高い L-乳酸生産を確認することができた(図 2)。

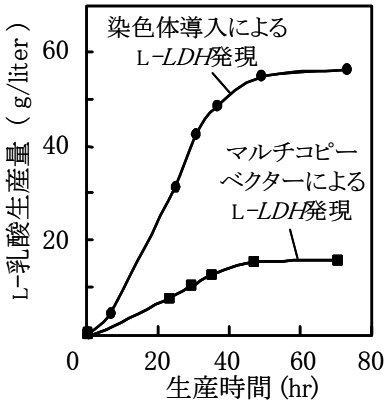

図2 染色体導入による
L-乳酸生産の効果

2、 *pdc1 pdc5* 二重破壊株において、高いLDH発現が発酵速度改善に寄与する

*S. cerevisiae*は、エタノール生産に関与する*PDC1*の破壊に応答して、相同性の高い*PDC5*が高発現するオートレギュレーション機構が存在している。上記1での組換え酵母は、乳酸生産収率 60%と高い生産効率を示しているものの、依然としてエタノールの生産は確認されており、これが PDC5 に起因することが予想された。そこで、*PDC1*および*PDC5*遺伝子を破壊した組換え酵母を作製し、乳酸生産酵母における*pdc1 pdc5* 二重破壊の効果を検証した。その結果、対糖収率 80%以上と、これまでの報告をはるかに上回る高い乳酸生産効率の向上効果を確認した(図 3)。しかし同時に、極端な増殖、発酵速度の低下が観察された。2 種類の L-*LDH*(ウシ由来、乳酸菌由来)を個々に導入した*pdc1 pdc5*破壊株での検証の結果、ウシ由来 L-*LDH* 導入株において、速度改善効果があったことから、高い LDH 活性が細胞内レドックスバランスの改善に寄与していることが考察された。

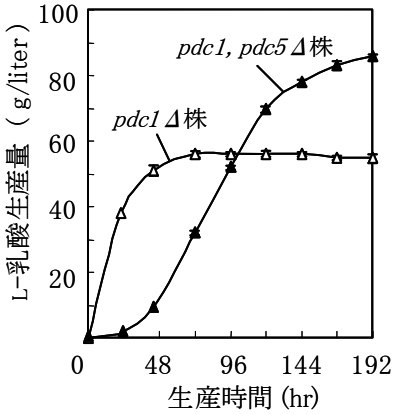

図3 *pdc1 pdc5*二重破壊株におけるL-乳酸生産の効果

3、 *L-LDH*遺伝子を染色体中に複数コピー導入することでL-乳酸生産能は向上する

上記 2 では、効率的な乳酸生産収率を達成したものの、菌体増殖速度が低下するという新たな課題が発生した。そこで *PDC5* 破壊ではなく、ウシ由来 L-*LDH* 遺伝子を酵母染色体中に複数コピー導入し、乳酸生産量への影響を調べた。L-*LDH* 遺伝子を染色体中に 6 コピーまで導入した組換え酵母での解析の結果、導入コピー数の向上とともに、LDH 活性の向上が観察された。同時に、乳酸生産量もコピー数に比例して上昇することが確認された（図 4)。L-*LDH* 遺伝子を 6 コピー導入した株は、ケーンジュースより作成した低コスト培地であっても 120g/liter 以上の L-乳酸生産能を示し、生産される L-乳酸の光学純度は、99.9%以上を示していた。これらの結果から、乳酸菌での L-乳酸生産において課題となっていた、低コスト培地の利用や光学純度に関する問題が、乳酸生産酵母によって、解決できることが明らかになった。

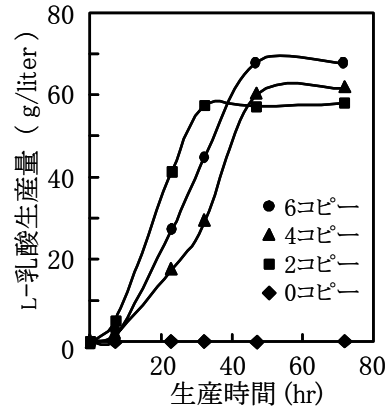

図4 L-*LDH* 遺伝子導入コピー数の検討

4、 本技術は、D-乳酸の高効率生産へも利用可能である

ポリ L-乳酸は、製造コストが高いという問題のほかに耐熱性が低いという点も指摘されている。近年、L-乳酸の光学異性体として知られるD-乳酸のポリマーをポリL-乳酸と混合させると、ポリL-乳酸の耐熱性が 58℃から 120℃にまで向上することが報告されている。しかし一方で、純粋な D-乳酸生産に関する報告例は少ない。そこで L-乳酸生産で行った技術を利用して、D-乳酸を高生産する酵母の構築を試みた。D-乳酸を生産する乳酸菌 *Leuconostoc mesenteroides* よりD-*LDH*遺伝子を取得し、これを染色体中の *PDC1* プロモーター下流に導入したところ、L-乳酸の場合と同様に、高い D-乳酸生産を確認することができた。また中和条件下のみならず、非中和条件下でもフリー乳酸を生産していることも確認された(図 5)。

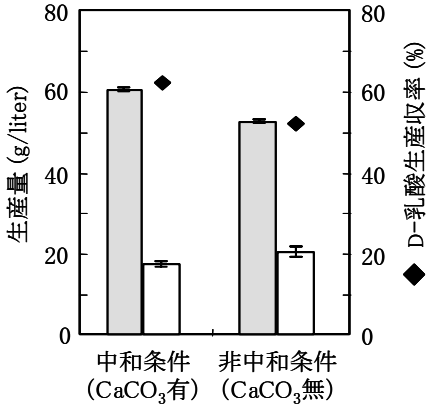

図5 中和および非中和条件下におけるD-乳酸生産

□ D-乳酸、□ エタノール

5、 まとめ

ポリ乳酸の低コスト生産を目指し、高光学純度を有する L-および D-乳酸を効率的に生産可能な遺伝子組換え酵母の研究に取り組んだ。その結果、染色体の *PDC1* プロモーター下流領域に L-*LDH* 遺伝子を導入することによって、高効率で L-乳酸を生産できることを証明した(本文第 2 章)。さらに、エタノール生産への代謝経路の制御(本文第 3 章)や、L-LDH 活性の増強(本文第 4 章)などの手段により、L-乳酸の生産効率を飛躍的に高めることに成功した。また本手法は、L-乳酸だけに限らず、D-乳酸の高生産においても利用可能であり、ポリ乳酸のもう一つの課題である耐熱性向上への糸口を見出した(本文第 5 章)。これらの研究成果により、乳酸菌を用いた従来の生産法とは異なる新しい乳酸生産手法を開発することができた。乳酸菌で指摘されていた問題点が本アプローチによって解決され、低コストでの乳酸生産が可能になると考えられる。

なお本研究には、宿主として 2 倍体酵母 *S. cerevisiae* OC-2 株を利用した。*S. cerevisiae* OC-2 株は、東京大学名誉教授 坂口謹一郎博士によって単離されたワイン酵母であり、生育速度が速く、培養適性に優れた酵母として広く知られている。*S. cerevisiae* は、アルコール製造を中心とする発酵産業に欠かせない微生物であると同時に、古くより遺伝学のモデル生物としても扱われている。さらにゲノム解読や遺伝子破壊ライブラリーなどの充実により、基礎的な知見の進展も目覚しい。今後は、本成果をさらに発展させ、基礎的並びに応用的な側面から、乳酸生産酵母に関する研究を深めていきたいと考えている。

List of publications

Nobuhiro Ishida, Satoshi Saitoh, Kenro Tokuhiro, Eiji Nagamori, Takashi Matsuyama, Katsuhiko Kitamoto and Haruo Takahashi.

"Efficient production of L-lactic acid by metabolically engineered *Saccharomyces cerevisiae* with a genome-integrated L-lactate dehydrogenase gene."

Applied and Environmental Microbiology Vol. 71, No. 4, p1964-1970 (2005).

Nobuhiro Ishida, Tomiko Suzuki, Kenro Tokuhiro, Eiji Nagamori, Toru Onishi, Satoshi Saitoh, Katsuhiko Kitamoto and Haruo Takahashi.

"D-Lactic acid production by metabolically engineered *Saccharomyces cerevisiae.*"

Journal of Bioscience and Bioengineering Vol.101, No. 2, p172-177 (2006).

Nobuhiro Ishida, Satoshi Saitoh, Toru Onishi, Kenro Tokuhiro, Eiji Nagamori, Katsuhiko Kitamoto and Haruo Takahashi.

"Metabolic engineering of *Saccharomyces cerevisiae* for efficient production of pure L-(+)-lactic acid."

Applied Biochemistry and Biotechnology Vol.131, Issue.1-3, p795-807 (2006).

Nobuhiro Ishida, Satoshi Saitoh, Toru Onishi, Kenro Tokuhiro, Eiji Nagamori, Katsuhiko Kitamoto and Haruo Takahashi.

"The effect of pyruvate decarboxylase gene knockout in *Saccharomyces cerevisiae* on L-lactic acid production."

Bioscience, Biotechnology, and Biochemistry (2006) in press.

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