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Production of D-lactic acid in a continuous membrane integrated fermentation reactor by genetically modified *Saccharomyces cerevisiae*: Enhancement in D-lactic acid carbon yield

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Poly D-lactic acid is an important polymer because it improves the thermostability of poly L-lactic acid by stereo complex formation. To demonstrate potency of continuous fermentation using a membrane-integrated fermentation reactor (MFR) system, continuous fermentation using genetically modified *Saccharomyces cerevisiae* which produces D-lactic acid was performed at the low pH and microaerobic conditions. D-Lactic acid continuous fermentation using the MFR system by genetically modified yeast increased production rate by 11-fold compared with batch fermentation. In addition, the carbon yield of D-lactic acid in continuous fermentation was improved to 74.6 ± 2.3% compared to 39.0 ± 1.7% with batch fermentation. This dramatic improvement in carbon yield could not be explained by a reduction in carbon consumption to form cells compared to batch fermentation. Further detailed analysis at batch fermentation revealed that the carbon yield increased to 76.8% at late stationary phase. *S. cerevisiae*, which exhibits the Crabtree-positive effect, demonstrated significant changes in metabolic activities at low sugar concentrations (Rossignol et al., Yeast, 20, 1369–1385, 2003). Moreover, lactate-producing *S. cerevisiae* requires ATP supplied not only from the glycolytic pathway but also from the TCA cycle (van Maris et al., Appl. Environ. Microbiol., 70, 2898–2905, 2004). Our finding was revealed that continuous fermentation, which can maintain the conditions of both a low sugar concentration and air supply, results in Crabtree-positive and lactate-producing *S. cerevisiae* for suitable conditions of D-lactic acid production with respect to redox balance and ATP generation because of releasing the yeast from the Crabtree effect.

[**Key words:** Lactic acid; Continuous fermentation; Membrane bioreactor; *Saccharomyces cerevisiae*; Crabtree effect]

Lactic acid (2-hydroxypropionic acid) is a widely used chemical in the food and pharmaceutical industries. It has been recently highlighted as a raw material for polylactic acid (PLA). PLA consists of lactic acid derived from biomass and was developed as an environmentally friendly biodegradable plastic that can substitute for synthetic plastics derived from petroleum feedstock (1,2). However, lactic acid must be produced at a lower cost if synthetic plastics are to be replaced by PLA in the future.

PLA can be divided into poly (L-lactic acid) (PLLA), poly (D-lactic acid) (PDLA), and poly (DL-lactic acid) (PDLLA), polymers of L-lactic acid, D-lactic acid, and DL-lactic acid. Both of the homopolymers, PLLA and PDLA, are crystalline and have the same melting temperature of approximately 170°C. However, more commonly used PLA plastics require melting temperatures in excess of 185–190°C (3). It was recently discovered that the enantiomers of lactic acid form stable stereocomplexes. Stereocomplex-type PLA (sc-PLA) is characterized by a high melting temperature, which is approximately 50°C higher than homopolymers (4). This finding supported the importance of D-lactic acid production. Before sc-PLA can be considered for industrial production, a process must be developed where lactic acid can be manufactured at a high optical purity and high chemical purity at a low cost.

We developed a membrane-integrated fermentation reactor (MFR) system (5) to achieve high productivity using maximum membrane performance via continuous fermentation. The concept of a critical flux was applied to the continuous fermentation process as an operational strategy to reduce the flux decline caused by membrane fouling. D-Lactic acid continuous fermentation using the MFR system with *Sporolactobacillus laevolacticus* demonstrated improved optical purity (6) and reduction in the amount of supplied sub-raw materials (7). In the case of D-lactic acid production by *S. laevolacticus*, a low pH demonstrated inhibitory effects on cell growth. Thus, chemicals ($CaCO_3$, $Ca(OH)_2$, NaOH, or NH_4OH) were added to neutralize the lactic acid. To produce free lactic acid from raw polymer materials, a purification process needs to be developed to perform acidification or ion exchange.

However, yeasts, such as *Saccharomyces cerevisiae*, are more tolerant to low pH compared to lactic acid bacteria. Recently, new

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methods for producing lactic acid using genetically engineered yeast have been developed and applied in large-scale production on a trial basis. In ethanol fermentation, pyruvic acid is converted into acetaldehyde and CO_2 by pyruvate decarboxylase (PDC; EC 4.1.1.1), and the acetaldehyde is subsequently converted into ethanol by alcohol dehydrogenase (ADH; EC 1.1.1.1). Transgenic yeast expressing exogenous L-lactate dehydrogenase (L-LDH; EC 1.1.1.27) could produce lactic acid from pyruvic acid. Such metabolically engineered yeast were first reported by Dequin and Barre (8), Porro et al. (9), and Adachi et al. (10), who showed that the recombinants yielded approximately 10–20 g/l lactate. 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 using transgenic yeast. In *S. cerevisiae*, there are three pyruvate decarboxylase (PDC) genes, namely *PDC1*, *PDC5*, and *PDC6*, which directly contribute to ethanol production (11,12). PDC activity in yeast is due mainly to the *PDC1* and *PDC5* genes (13,14), and the expression of these genes is controlled by an auto-regulation system; that is, *PDC1* deletion resulted in a large increase in *PDC5* promoter-driven mRNA expression (15). The genetically modified yeast, which integrated L-LDH (bovine or *Bifidobacterium longum*) into the *PDC1* coding region, demonstrated a deletion of *PDC1*. The genetically modified yeast was observed to convert glucose to L-lactate (55.6 g/l) and ethanol (16.9 g/l), with up to 62.2% of the glucose being transformed into L-lactic acid under neutralizing conditions. In addition, this transgenic yeast showed high L-lactic acid production (50.2 g/l) under non-neutralizing conditions (16).

The D-lactate-producing yeast, D-LDH, obtained from the *Leuconostoc mesenteroides* subsp. *mesenteroides* strain NBRC3426, was isolated, and this *LDH* was introduced into the coding region of *PDC1* on chromosome XII using homologous recombination (17). Ishida et al. (17) reported that high affinity for pyruvic acid (K_m = 0.3 mM) in *L. mesenteroides* D-LDH produced D-lactic acid more effectively. Sawai et al. successfully cloned the D-LDH gene of *Limulus polyphemus* which had the highest affinity for pyruvic acid (K_m = 0.07 mM) (18), and genetically modified yeast in which D-LDH gene of *L. polyphemus* was introduced into the coding region of *PDC1* on chromosome XII using homologous recombination were constructed SW092-2D (pdc1::Lp.D-LDH-TRP1) (19).

This study is the first report of D-lactic acid continuous fermentation using genetically modified *S. cerevisiae* to reduce the amount of neutralizing agent. Additionally, the potency of continuous fermentation using genetically modified *S. cerevisiae* was examined by analyzing the carbon yield of fermentation products in comparison to batch fermentation.

MATERIALS AND METHODS

Strain and culture mediums The genetically modified yeast which parental strain is *S. cerevisiae* NBRC 10505 used in this study was SW092-2D (pdc1::Lp.D-LDH-TRP1) (*MATa ura3 leu2 trp1 his3 ade2 LYS2 ARG pdc1::D-ldh-TRP1*) (19). The fermentation medium and seed medium used for D-lactic acid production contained, per liter, 75 g of raw cane sugar (Muso, Osaka, Japan), 1.5 g of ammonium sulfate, 152 mg of uracil, 760 mg of L-leucine, 152 mg of L-histidine, and 4.3 g of adenine. The pre-seed medium contained, per liter, 10 g of yeast extract (Becton, Dickinson and Company, NJ, USA), 20 g of peptone (Becton, Dickinson and Company), and 20 g of glucose. The medium was sterilized at 121°C for 20 min. The fermentation medium for continuous fermentation was sterilized at 121°C for 240 min using a 20 L tank (Nalge Nunc International Corporation, NY, USA).

Preparation of seed broth The pre-seed broth was prepared by inoculating 5 ml of seed medium with a few colonies from plate. The pre-seed broth in the test tube was incubated at 30°C for 24 h in a shaking incubator at 120 rpm. Next, the seed broth was prepared by inoculating 50 ml of seed medium with 5 ml of pre-seed broth. The seed broth in the Erlenmeyer flask was incubated at 30°C for 24 h in a shaking incubator at 120 rpm.

Batch fermentation The batch fermentor experiments were performed in a 2-L fermentor (Able, Tokyo, Japan). An initial media broth volume of 1.5 L was used at a temperature of 30°C and an agitation speed of 400 rpm, and air was introduced into the broth at 50 ml/min to maintain microaerobic conditions. The neutralizer, a $Ca(OH)_2$ slurry (equivalent to 5 M), was automatically added to maintain a pH of 4.5 referring to Adachi et al. (10) regarding of fermentation study using lactate-producing *S. cerevisiae*. Samples were withdrawn for analysis of organic acids, ethanol, residual sugar, and cell concentration. The yield was calculated using the following equation:

$$\text{Carbon yield [\%]} = \text{Carbon weight of metabolic product [g]/Carbon weight of consumed sugars [g]} \times 100 \quad (1)$$

Continuous fermentation using the MFR system Continuous fermentation by retaining cells was performed using the MFR system, which consisted of a 2-L fermentor (working volume 1.5 L) and microfilter element as previously reported (5). The fermentor containing the microfilter element was sterilized at 121°C for 20 min prior to cultivation. A separation membrane was used in continuous fermentation using the MFR system (20). Continuous fermentation using the MFR system was maintained at 30°C and an agitation speed of 400 rpm, and air was introduced at 50 ml/min to maintain microaerobic conditions. The neutralizer, a $Ca(OH)_2$ slurry (equivalent to 5 M), was automatically added to maintain a pH of 4.5. The filtration and medium supply began at 24 h after inoculation. The permeate flow from the membrane was controlled using a peristaltic pump. The filtration condition was set at a flux below the critical flux, and intermittent filtration was performed in which the filtration was stopped for 1-min intervals over a period of 9 min.

The critical flux of the SW092-2D (pdc1::Lp.D-LDH-TRP1) batch cultivated broth was determined using the stepwise flux method (5) as 0.550 $m^3/m^2/d$. Thus, the filtration conditions consisted of constant dilution rates: 0.17 h^{-1} and flux: 0.500 $m^3/m^2/d$. The permeation rate was equal to the rate at which a medium plus an alkali was added to maintain a constant working volume (1.5 L) in the fermentor.

Continuous fermentation without retaining cells Continuous fermentation without retaining cells experiments were performed in a 2-L fermentor (working volume 1.5 L). The cultures were maintained at a temperature of 30°C and an agitation speed of 400 rpm, and air was introduced at 50 ml/min to maintain microaerobic conditions. The neutralizer, a $Ca(OH)_2$ slurry (equivalent to 5 M), was automatically added to maintain a pH of 4.5. The feed and removal of the fermentation broth began at 46 h after inoculation. The feed rate of the continuous fermentation was 6 ml/h constant (Dilution rate: 0.004 h^{-1}). The edge of the pipe that removed the fermentation broth was set at the surface of the fermentation broth in the 2-L fermentor. The pump that removed the fermentation broth was always run at a higher rate compared to the feed rate.

Analysis Organic acid and sugar concentrations were determined using a high-performance liquid chromatograph system (HPLC) (SIL10A Series, Shimadzu, Kyoto, Japan) equipped with an electro-conductivity detector. A shim-pack SPR-H column was used with 5 mM *p*-toluenesulfonic acid as the mobile phase at a flow rate of 0.8 ml/min, and 5 mM *p*-toluenesulfonic acid, 20 mM Bis–Tris, and 0.1 mM EDTA·2Na as the reaction phase at a flow rate of 0.8 ml/min. The column temperature was maintained at 45°C. Optical isomers of D- and L-lactic acids were analyzed using HPLC equipped with an electro-conductivity detector. A TSK-gel Enantio L1 column (Tosoh, Tokyo, Japan) was used with 1 mM $CuSO_4$ as the mobile phase at a flow rate of 1 ml/min, and the column temperature was maintained at 37°C. Samples were filtered through a membrane (pore size, 0.22 μm). The ethanol concentration was measured using gas chromatography (GC2010, Shimadzu) equipped with a flame ionization detector under the following conditions: capillary column TC-1 (0.53 mm i.d. by 15 m) (GL Science, Tokyo, Japan); temperatures of the column, injector and detector were 45°C, 200°C, and 250°C, respectively; flow rate of helium carrier gas, 3 ml/min. The cell concentration was measured as the optical density at $OD_{600\ nm}$. The conversion coefficient of dry cell weight (DCW)/l/$OD_{600\ nm}$ was measured using Infrared moisture determination balance FD-720 (Kett Electric Laboratory, Tokyo, Japan).

RESULTS

Batch fermentation Raw sugar from sugar cane was used as the substrate because sugar cane is an inexpensive feedstock, which enables the economic production of lactic acid (21). The D-lactic acid batch fermentation was examined using genetically modified yeast (SW092-2D (pdc1::Lp.D-LDH-TRP1)). These results are shown as the time course of the concentration of organic acids, ethanol, glycerol, sugars, and $OD_{600\ nm}$ (Fig. 1). The D-lactic acid accumulations were 30.8 ± 1.29 g/l at 45 h. The production rates were 0.684 g/l/h. For the main by-product, the ethanol produced by *PDC5* accumulation was 20.4 ± 0.55 g/l. The