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Highly accumulative production of L(+)-lactate from glucose by crystallization fermentation with immobilized *Rhizopus oryzae*

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In order to produce microbiologically large amount of L(+)-lactic acid (LA) from glucose, batch and fed-batch (intermittent addition of sterilized glucose powder aseptically) cultures of *Rhizopus oryzae* NBRC 5384 (identical to NRRL 395 and ATCC 9363) whose mycelia were immobilized *in situ* within sponge-like cubic particles (3.5 mm edge long) were carried out at 37°C in a three baffled shake flask. Appropriately calculated amount of fine powdery calcium carbonate ($CaCO_3$) was added initially or intermittently to control pH of the culture liquids. High accumulations of LA (145 g/L and 231 g/L, in reality 176 g/L and 280 g/L as anhydrous calcium lactate) were achieved by a batch (glucose concentration = 150 g/L) and a fed-batch cultures (the initial glucose concentration = 150 g/L and the intermittent addition of glucose equivalent to 100 g/L). In these cultures the yields and productivities of LA were, 95.0%, 1.42 g/L·h and 92.5%, 1.83 g/L·h, respectively. Existence of considerable amounts of calcium lactate ($Ca(LA)_2$) as crystals in the culture broth was experimentally proved by two evidences: (i) heating up (70°C) followed by quick low centrifugal force to remove remaining $CaCO_3$ solids from culture broth and then cooling down (37°C) followed by incubation of the culture supernatant at 37°C to observe recrystallization of $Ca(LA)_2$, and (ii) the measurement of solubility of $Ca(LA)_2$ in the culture media. It was conceptually discussed to be able to avoid the product inhibition by means of crystallization fermentation for the high accumulation of LA by *R. oryzae*.

[**Key words:** Lactic acid; Crystallization fermentation; Fed-batch culture; *Rhizopus oryzae*; Sponge-like cubic particle; Calcium lactate; Immobilized cells]

Lactic acid and its derivatives represent important category of bulk chemical for industries producing food, chemicals and pharmaceutical products. It finds use in topical preparations and cosmetics to adjust acidity and for its disinfectant and keratolytic properties. It is found primarily in sour milk products such as koumiss, laban, yogurt, kefir, and some cottage cheeses. The casein in fermented milk is coagulated (curdled) by lactic acid. Lactic acid is also responsible for sour flavor of sourdough breads. It is used in beer brewing to lower the pH and increase the body of beer.

Highly purified preferably 100% optically pure L(+)-lactic acid (LA) anhydrous monomer is required for the production of bio-based polymer, poly lactic acid, which is environmentally friendly replacement of plastics derived from petrochemical materials.

In medicine, lactate is one of the main components of lactated Ringer's solution and Hartmann's solution. These intravenous fluids consist of sodium and potassium cations along with lactate and chloride anions in solution with distilled water, generally in concentrations isotonic with human blood. It is most commonly used for fluid resuscitation after blood loss due to trauma, surgery, or burn injury.

Calcium lactate ($Ca(LA)_2$) is the soluble calcium salt of LA. Calcium lactate is used in calcium fortification of juices and juice drinks, nectars, non-clear beverages, acidified dairy/soy drinks, powdered drinks, infant food and is formulated into capsules, tablets and liquids as therapeutic or nutritional supplement.

LA can be produced either by homolactic acid bacteria, or a filamentous fungus *Rhizopus oryzae* or recombinant yeast and *Escherichia coli*. Among those microorganisms, *R. oryzae* has following advantages over other microorganisms. It produces optically pure LA, and requires only a simple synthetic medium composition containing some inorganic minerals and ammonium salt as sole nitrogen source, whereas homolactic bacteria require complex supplements such as various kind of amino acids and vitamins as well as some minerals, which adds to the costs of LA production and complicates purification of LA. *R. oryzae* produces mainly LA from glucose with weight yield of 70–90% depending on culture conditions, and does usually very small amount of byproducts (glycerol, ethanol, malic acid and fumaric acid). *R. oryzae*, even its wild strain, utilizes not only glucose but also xylose as carbon sources to yield LA.

However, LA fermentation by the fungus has some disadvantages. *R. oryzae* fermentation is aerobic, which means the need of cost of aeration (air supply and/or agitation of bioreactor). *R. oryzae* shows different morphology, i.e., filamentous cells, clump, pulpy, floc and pellets, depending on various factors such as agitation rate,

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medium composition, pH, temperature and so on. Fermentation with filamentous fungi is complicated by increased broth viscosity due to mycelial growth, wall growth, and reduced oxygen transfer, which is critical to LA production. Various methods have been studied to control cell morphology and to achieve higher production rate, cell density, product yield and productivity. These methods until the year of 2002 were summarized by Tay and Yang (see Table I in Ref. 1). In these studies, cell immobilization was achieved either by cell entrapment within polymeric matrix, by cell attachment via adsorption to a surface, or self-immobilization by forming pellets. By the immobilization, it is easier to operate and control the bioreactor and separate the culture broth from the fungal cells. It also facilitates the reuse of fungal cells for long-term LA production. After the publication of the study of Tay and Yang (1), a number of research groups still attempted to enhance efficiency of LA production by *R. oryzae* in terms of strain mutation and new bioreactor (2), cell morphology (3), carbon source variation (4–6), screening of thermotolerant strain (7), novel immobilization (8,9), culture optimization technique (10), oxygen transfer (11–13), scale-up of bioreactor (12), operational mode (14), etc.

The aim of this study was to raise the final lactate concentration in batch and fed-batch fermentations of *R. oryzae*, which would be beneficial to reduce the costs of fermentation and of downstream processing. Filamentous cells of *R. oryzae* were immobilized *in situ* within sponge-like particles, which had been proved quite effective for not only making cultures macroscopically homogeneous but also easy separation of cells from the culture broth (Japanese patent 2009-045262, 2009; Yamane, T., Sakakibara, H., Saiki, T., and Asami, N., Abstr. 61st Annu. Meeting. Soc. Biotechnol., Jpn., p. 178, 2009).

MATERIALS AND METHODS

Microorganism *R. oryzae* NBRC 5384, which is identical to the strain NRRL 395 and ATCC 9363, was used throughout the study. It was obtained from NITE Biological Resource Center, Department of Biotechnology, National Institute of Technology and Evaluation, Kazusa, Kisarazu City, Chiba, Japan. The strain was first cultured on potato-dextrose-agar (PDA) plate (Dainippon Seiyaku Co., Ltd., Osaka) at 30°C for 7 days. Its fresh spores were suspended in a sterile solution of 20% (v/v) glycerol and 0.05% (v/v) Tween 80. The glycerol stock suspension thus prepared was distributed into many microtubes and they were stored at −80°C. One microtube was taken out and thawed only once for each experiment.

Chemicals All the chemicals, unless otherwise indicated, were of reagent-grades purchased from Kanto Chemicals Co., Ltd. (Tokyo). Calcium carbonate ($CaCO_3$) powder was a gift (the trade name 'Korokaruso-EX') from Shiraishi Calcium Co., Ltd. (Tokyo). It is precipitated calcium carbonate (purity >99%, density 2.7 g/cm^3). In the powder state, it's average diameter is 5–7 μm due to coagulation, but the first precipitated particles during production are semi-colloidal fine powders whose average diameter is less than 0.5 μm. Calcium L(+)-lactate pentahydrate ($Ca(LA)_2 \cdot 5H_2O$) was purchased from Sigma–Aldrich, Japan (Tokyo) (Product No. C8356-250G, purity >98%).

Sponge-like cubic particle The sponge-like particle was cubic and made of polyurethane foam (type AQ-10, gifted by INOAC Co., Ltd., Nagoya, Aichi). It was 3.5 mm edge long, of 95–96% porosity, and autoclavable. The particles were effective for *in situ* immobilization of *R. oryzae* mycelia because no free mycelial fragments were observed in the culture liquid through a microscopic observation.

TABLE 1. The amounts of 5 components added to 160 mL culture medium.

Initial glucose concentration (g/L)	100	150	200
1. Glucose solution			
Water (mL)	16.0	24.0	32.0
Glucose (g)	16.0	24.0	32.0
2. Water (mL) to prepare the major mineral solution[a]	128.0	115.2	100.8
3. Trace mineral solution[b] (mL)	4.8	4.8	4.8
4. $CaCO_3$ powder (g)	9.0	13.3	18.0
5. Sponge-like cubic particles (pieces)	960	960	960

[a] Contained 352 mg $(NH_4)_2SO_4$, 288 mg KH_2PO_4, 96 mg Na_2HPO_4, and 240 mg $MgSO_4 \cdot 7H_2O$.
[b] The composition is described in the text.

Culture medium The composition of the culture medium was per liter: 100, 150, or 200 g (the initial concentration when in the fed-batch culture) glucose, 2.2 g $(NH_4)_2SO_4$, 2.0 g KH_2PO_4, 0.6 g Na_2HPO_4, 1.5 g MgSO4·7H_2O, 30 mL trace mineral solution; 56, 83 or 112 g $CaCO_3$ powder, and 6000 pieces (=12.86 g) of the sponge-like cubic particles. The composition of the trace mineral solution was per liter: 4.0 g $ZnSO_4 \cdot 7H_2O$, 2.0 g $FeSO_4 \cdot 7H_2O$, 0.2 g $CuSO_4 \cdot 5H_2O$, 0.2 g $MnCl_2 \cdot 4H_2O$, 0.2 g $CoCl_2 \cdot 6H_2O$, and 0.05 g $Na_2MoO_4 \cdot 2H_2O$. The concentrations of the major minerals were 3 times higher than the ones usually adopted in the previous works done by other people, and trace minerals were added although they were not usually added in the other reported works.

The medium (160 mL) was cultured in a 1-L three-baffled shake flask (Pyrex-Iwaki, AGC Co., Ltd., Tokyo). The culture volume (160 mL) was decided after preliminary experiments to know effect of the oxygen transfer on the LA production in the 1-L three-baffled sake flask culture because dissolved oxygen or oxygen transfer rate affect much in the LA production by *R. oryzae* (1,2,11–13). The preliminary experiments had been carried out by changing the culture volumes ranging from 50 mL to 450 mL. A result had been obtained as LA production rates (g-LA/L·h) vs. the culture volumes (mL), which had shown that the rates were the highest and unchanged below 200 mL, and then gradually decreased.

The culture medium composition was divided into five components: glucose solution, the major mineral solution, the trace mineral solution, $CaCO_3$ powder, and the sponge-like cubic particles. The amounts of these components for 160 mL medium depending on the initial glucose concentration are listed in Table 1. The theoretical amount of $CaCO_3$ powder to be added, x (g/L), was calculated by the following equation on an assumption that all the LA produced is converted to anhydrous $Ca(LA)_2$:

$$x = \{100.04/(2 \times 90.08)\} \times Y \times C \quad (1)$$

where 100.04 and 90.08 are molecular weights of $CaCO_3$ and LA, respectively, Y is yield of LA from glucose (g LA produced/g glucose consumed) and was assumed to be 0.90 and C is the initial glucose concentration (g/L). In practice a little excess amount of $CaCO_3$ was added to avoid its shortage anxiety during later stage of the culture. The glucose solution, the major mineral solution plus the sponge-like cubic particles, the trace mineral solution and $CaCO_3$ powder were autoclaved separately. Since the sponge-like cubic particles contained air and floated on the surface of the culture medium, they were degassed in advance by pressing them with a punching metal plate in a mess cylinder containing the major mineral solution, and the major mineral solution plus the degassed sponge-like cubic particles were autoclaved together in the shake flask. Finally, the sterilized glucose solution, the trace mineral solution and $CaCO_3$ powder were added into the major mineral solution plus the sponge-like cubic particles in the flask in a clean bench.

Cultivations (batch and fed-batch cultures) To begin cultivations spores of *R. oryzae* were inoculated with the size of 2×10^5 or 2×10^6/mL. Four-five divided portions of the spores were inoculated to distribute them as uniformly as possible while the medium was being shaken. The cultivations were carried out at 37°C, 120 rpm, and 22 mm amplitude on a rotary shaker (Bio Shaker, model BR-22UM, Taitec Co. Ltd., Tokyo) which was installed next to the clean bench.

For fed-batch cultures both 1–5 g glucose and 0.5–2.5 g $CaCO_3$ powders were weighed and put each separately into a 20-mL vials, then all the vials were very tightly closed with screwed caps to avoid moisture adsorption from outside, and were autoclaved at 121°C for 15 min. Fed batch cultures were performed by adding the sterilized glucose and $CaCO_3$ powders intermittently according to the traced glucose concentration. During the fed-batch cultures, 300 μL was sampled out at 6 h intervals in the clean bench. Then the sake flask was returned quickly to the shaker. The sampled culture broth was centrifuged, and the glucose concentration of the supernatant was determined enzymatically (for details refer to the analytical methods). Both the amounts of glucose to keep its concentration around 40–60 g/L and the corresponding $CaCO_3$ powders to be added were estimated according to Table 2. They were aseptically added into the shake flask in the clean bench, and then the culture was continued. It took about 25 min from sampling the culture broth till supplementing both glucose and $CaCO_3$ powders. A little amount of sterilized antifoam (Adekanol LG-126, Adeka Corporation, Tokyo) was added usually at the time of the additions of glucose and $CaCO_3$ powders to the culture broth to prevent foaming during the fermentation if necessary.

TABLE 2. The estimated increase in the glucose concentration and the corresponding amount of $CaCO_3$ powder to be added (culture broth volume = 160 mL).

Amount of glucose powder added (g)	Estimated increase in glucose concentration (g/L)	Amount of CaCO3 powder added[a] (g)
1.0	6.25	0.50
2.0	12.50	1.00
3.0	18.75	1.50
4.0	25.00	2.00
5.0	31.25	2.50

[a] Estimated similarly to Eq. 1.