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Continuous Cultivation of *Lactobacillus rhamnosus* with Cell Recycling Using an Acoustic Cell Settler

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Abstract Continuous production of lactic acid from glucose by *Lactobacillus rhamnosus* with cell recycling using an acoustic cell settler was carried out. The performance of the system, such as the concentration of cell and product were compared with the control experiment without recycling. The acoustic settler showed cell separation efficiency of 67% during the continuous operation and the cell concentration in the fermentor with recycle exceeded that of the control by 29%. Compared with the control, lactic acid production was increased by 40%, while glucose consumption was only increased by 8%. The higher value of lactic acid production to substrate consumption ($Y_{P/S}$, product yield coefficient) achieved by cell recycling is interpreted to indicate that the recycled cell mass consumes less substrate to produce the same amount of product than the control. Within system environmental changes due to the longer mean cell residence time induced the cells maintaining the metabolic pathways to produce less by-product but more product, lactic acid.

Keywords: cell filtration system, acoustic cell settler, separation efficiency, lactic acid

INTRODUCTION

In continuous culture of microbial cells, the cell concentration in a fermentor is directly related to the product concentration, and the productivity of the system [1,2]. To increase the cell density in a fermentor a certain kind of a filter, centrifuge or type of separating equipment is needed in the effluent stream to filter or concentrate and then return the cell mass to the fermentor.

Recycling facilities of this type, such as microfilters or centrifuges have some restrictions in terms of industrial scale applications. Membrane fouling during long term operation results in reducing membrane capacity, and requires replacement membranes and the high electrical power consumption of the centrifuge and low recovery efficiencies, limits applications, especially in the fermentation industry [3-5].

Acoustic cell settlers have several advantages over the conventional filters for filtering microbial cells from culture broth i.e., there is no need to change filter media, and cells are easily returned to the fermentor [6-8]. Acoustic cell settler devices were applied to various kinds of high density cell culture system, a plant [9], insect [10], animal [11] and hybridoma cell [12].

Lactic acid is an important chemical feedstock, which has many industrial applications, such as in foods, cosmetics, pharmaceuticals, and in biodegradable polymers [13]. To enlarge the lactic acid market and compete with substitute chemicals, production costs should be lowered by novel lactic acid fermentation technologies. In order to improve productivity and product yield in continuous lactic acid fermentation, the microbial density should be increased by recycling the cell mass obtained by separating the effluent stream.

In the present study, the feasibility of using acoustic cell settler for cell-recycled continuous lactic acid fermentation by *Lactobacillus rhamnosus* for its improved performance was examined.

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MATERIALS AND METHODS

Microorganisms

Lactobacillus rhamnosus ATCC 10863 (American Type Culture Collection, Rockville, MD, USA) was used throughout this study. The strain was maintained at 70°C in modified MRS (deMan, Rogosa and Sharpe) media [14] with 25% glycerol.

Media and Inoculum Preparation

The composition of the modified MRS media used for culture maintenance and for the inoculum was as follows; yeast extract 15 g/L, KH_2PO_4 1 g/L, sodium acetate · $3H_2O$ 1 g/L, $MgSO_4$ 0.2 g/L, $MnSO_4$ · H_2O 0.03 g/L, $FeSO_4$ · $7H_2O$ 0.03 g/L and glucose 30 g/L. $CaCO_3$ (4 g/L) was added to the inoculum media to control pH. The medium was sterilized at 121°C for 30 min. The inoculum broth was cultivated at 41°C, for 16 h in a shaking incubator at 100 rpm. For the fermentor culture

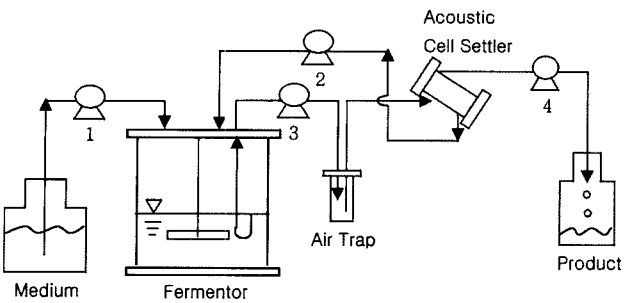


Fig. 1. Schematic diagram of continuous fermentation with cell recycling using an acoustic cell settler.

100 mL of the shake flask-cultured cells was transferred to the fermentor. The same medium composition was used for the continuous fermentation except that the glucose concentration was increased to 60 g/L.

Continuous Fermentation Equipments and Procedures

Fermentation was done in a 2.5-L stirred jar fermentor (KoBiotech Co., Incheon, Korea). The experimental system of the continuous culture with cell recycle using acoustic cell settler is shown in Fig. 1. As a cell recycle system, the fermentation broth was pumped to an acoustic cell settler by via an air trap. To control the 4 different flows used, 4 sets of peristaltic pumps ((1) MS-4 Reglo, Ismatec, (2) Micro-tube pump MP-3, Eyela, (3) Digital-pump Model 7518-00, Masterflex, (4) 101U pump, Watson-Marlow) were used.

Fermentation culture conditions were controlled at 41°C, 100 rpm and pH 5.2. NaOH solution (5 N) was used as a pH controlling agent.

At first, a batch culture was done for 15 h, then the continuous culture medium was pumped to the fermentor at the constant dilution rate of 0.0686 h^{-1}. After about 190 h, a steady state was reached and continuous fermentation with cell recycling was carried out for about 100 h. The recycle rate of the stream 2 (liquid flow from an acoustic cell settler to a fermentor) was set at 150% of the feed rate of the stream 1 (flow from a medium bottle to a fermentor).

Analytical Techniques

The cell concentration was monitored by measuring the optical density (OD) at 600 nm using a spectrophotometer (Genesys 5, Spectronic) and by determining the dry cell weight. The dry cell weight (g/L) was found to be equivalent to an OD_{600} of 0.357. Glucose and lactic acid concentrations were determined using a glucose analyzer (2700 SELECT, Biochemistry Analyzer, YSI). The metabolites such as pyruvic, succinic, acetic, propionic and lactic acid were analyzed using HPLC (LC-10AD, Shimadzu) under the following conditions; MetaCarb 87H Ion exchange column (Aminex, Bio-Rad), mobile phase: 0.008 N H_2SO_4, flow rate: 0.5 mL/min, temperature: 45°C, Waters 486 tunable absorbance detector.

Cell Recycling System with Acoustic Cell Settler

Separation using ultrasound was performed by using an ultrasonic standing wave that was generated by a transducer (Shinjin Ultra Co., Korea, 3 MHz, ceramic). An ultrasonic standing wave is the combination of two oppositely directed traveling waves. These traveling waves are generated from two independent transducers or from a transducer and a reflector. An ultrasonic standing wave has pressure amplitude with maximum (antinodal) and zero (nodal) values. Due to the discontinuity in the propagating phase of particle and cell in the ultrasonic resonance field, a position-dependent acoustic potential energy is formed. Based on these phenomena particles and cells are moved to positions of minimum acoustic potential energy and become entrapped into the ultrasonic standing wave [15].

The cells entrapped in the ultrasonic standing wave tend to form aggregates, which are settled by gravity. Consequently, the cell suspension is separated into two-phases namely a relatively clear top phase and a dense bottom phase consisting of cells [16,17]. The bottom phase of dense microbial cells is recycled to the fermentor and the top phase of broth forwarded to a product storage tank.

The acoustic cell settler consists of an ultrasonic generator (Shinjin Ultra Co., Korea), a 3 MHz transducer, acoustic chamber and reflector. The acoustic chamber was made of acryl pipe [18]. A glass reflector was located parallel to the transducer plate. The dimensions of the acoustic settler were 25 mm diameter, and 15 mm depth, which was the distance between the transducer and the glass reflector. To ease collection and increase the cell density of the bottom cell, the settler was tilted.

Separation efficiency (E) was defined by

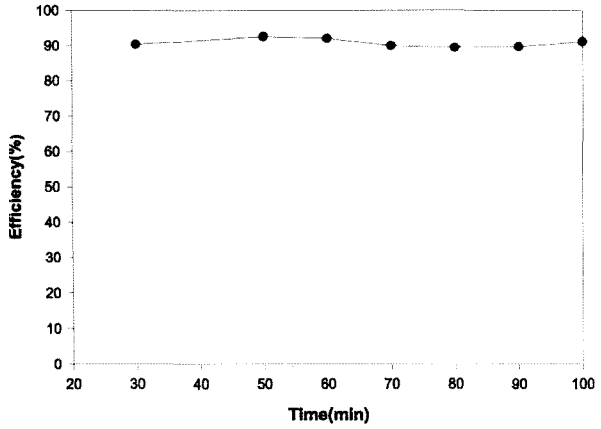
$$E = (1 - C_o / C_i) \times 100$$

Where C_o is the top phase cell concentration collected in the product tank, C_i is the cell concentration of the feed stream fed into the acoustic chamber.

RESULTS AND DISCUSSION

Separation Efficiency of Acoustic Cell Settler

Lactobacillus rhamnosus was cultured for 24 h in batch to measure separation efficiency in the tilted acoustic cell settler. As shown in Fig. 2, the separation efficiency was determined to be about 90%, which was a high level of separation efficiency. In the case of *Saccharomyces cerevisiae* the separation efficiency was reported to be 94% [18]. Perhaps because this microbial yeast has a higher gravity setting velocity than *L. rhamnosus*, result-



Fi g. 2. Separation efficiency of *L. rhamnosus* in an acoustic cell se tler using 3 MHz ultrasound.

ir g in a better separation efficiency.

n the early phase of the continuous fermentation w th cell recycling, the appearance of the broth in the ti ted acoustic cell settler started to change from a ho- m ogeneous suspension (Fig. 3(a)) to a solution with la ge aggregates or cell floc. These settled as time passed. F ially at the steady state the broth in the settler was se parated into two zones as shown in Fig. 3(b). The u per clear supernatant zone showed a lower cell con- c ntration of 2.49 g/L and the bottom dense zone had tl e higher value of 11.07 g/L. The feed cell concentrati n, that is the fermentor cell concentration, was m aintained at 7.64 g/L. The separation efficiency was m easured to be about 67%. These results showed that tl e cell recycle system incorporating an acoustic cell se ttler could be used to increase the cell concentrations ir continuous fermentation.

C ontinuous Cultivation with Cell Recycling

\fter the batch culturing for 15 h, the culture medium w as pumped continuously into the fermentor. The st eady state was reached after about 190 h of continuo us feeding. Continuous fermentation with cell recyc ng was carried out for a further 100 h.

The whole process of cultivation, including batch, c ntinuous without recycle, and continuous with recyc e is shown in Fig. 4. The important parameters of conti uous fermentation, namely, the yield coefficient and p oductivity were obtained after the cell concentration, a d glucose and lactic acid concentrations after the st eady state had been reached [19-21].

The cell concentration in the fermentor with cell recyc ng was higher than the control by 29%. The residual g ucose concentration was decreased from 8.9 g/L to 4.9 g L and the accumulated product lactic acid concentrat n increased from 38 g/L to 53.4 g/L. Lactic acid prod uction was increased by 40%, while glucose consumpt n was increased by 8% compared with the control e periment without recycling.

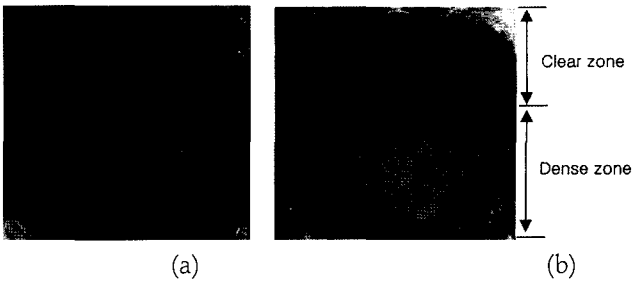


Fig. 3. Internal state of acoustic cell settler. (a) initial state, (b) steady state.

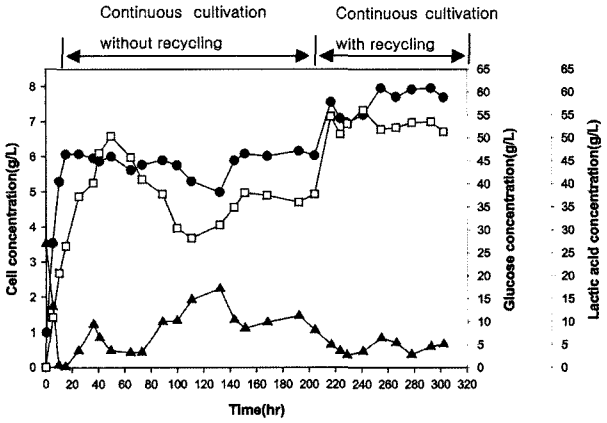


Fig. 4. Continuous cultivation of *L. rhamnosus* carried out with an acoustic cell settler from 15 h to 204 h without recycle, and from 204 h to 302 h with recycle. ●: cell concentration, □: lactic acid concentration, ▲: glucose concentration.

Table 1. Comparisons of lactic acid yield for continuous fermentation without recycling and continuous fermentation with recycling

Fermentation	$Y_{P/S}$	$Y_{X/S}$
Continuous fermentation without recycling	0.74	0.116
Continuous fermentation with recycling	0.97	0.045

$Y_{P/S}$: Yield Coefficient (g lactic acid/g glucose)
$Y_{X/S}$: Yield Coefficient (g cell mass/g glucose)

The yield coefficients were calculated and are shown in Table 1. With the cell recycling, the $Y_{P/S}$ value was higher than the control by 30%. In the control experiment, 38 g/L of lactic acid was produced with 5.92 g/L of cell mass and 8.9 g/L of residual glucose concentration. The yield coefficient for product, $Y_{P/S}$ was found to be lower than the reported reference values by other workers, 0.9-0.98 [19,20]. However, the recycled system had a $Y_{P/S}$ value of 0.97.

The cell residence time in the continuous culture with recycling was 53.2 h, which was 3.6 times longer than in the control system. Furthermore, the recycled cells accumulated in the fermentor consumed less substrate

Table 2. Comparison of organic acid concentrations for continuous fermentation without recycling and continuous fermentation with recycling

Organic acid	Continuous fermentatin without recycling	Continuous fermentation with recycling
Pyruvic acid (g/L)	0.12	0.02
Succinic acid (g/L)	4.38	1.68
Acetic acid (g/L)	0.46	0.32
Propionic acid (g/L)	2.69	2.66
Lactic acid (g/L)	38.1	53.4
Glucose (g/L)	8.9	4.9

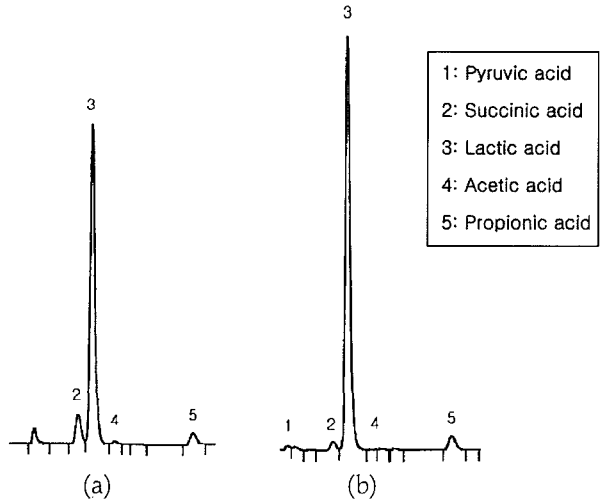


Fig. 5. HPLC results of fermentation broth of *L. rhamnosus*. (a) continuous fermentation without recycling, (b) continuous fermentation with recycling.

for a given cell mass than the cells without recycling.

The major metabolic acids in the fermentation broth, formed during the fermentation of *Lactobacillus rhamnosus,* namely, pyruvic, succinic, acetic, propionic and lactic acids were analyzed by HPLC, as shown in Table 2 and Fig. 5. The recycled cells produced more lactic acid and less of the other metabolic acids, especially of succinic acid.

Environmental changes caused by longer mean cell residence time (cell age) allowed cells to produce more product, lactic acid, and less by-product.

CONCLUSION

The acoustic cell settler was used in this study to concentrate microorganisms as a part of recycling equipment. Cell concentrations with recycling were increased by 29% versus the control. The recycle system allowed operation at a productivity of 3.6 g of lactic acid L^{-1} hr^{-1}, which was 40% higher than the control.

The tilted acoustic cell settler showed a cell separation efficiency of 67% during continuous fermentation. No significant malfunction due to overheating of the ultrasonic generator and transducer was experienced during continuous culture.

We conclude that the acoustic cell settler system can be successfully applied to the continuous fermentation of lactic acid. The effect of longer cell residence times on continuous fermentation with recycling and the effects of acoustic ultrasound energy on microbial cells need further study in terms of altered metabolic pathways, affecting glucose consumption and organic acid formation.

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NOMENCLATURE

C_i	the cell concentration of feed stream into the acoustic chamber
C_o	the top phase cell concentration collecting to product tank
E	separation efficiency
$Y_{P/S}$	yield coefficient (g lactic acid/g glucose)
$Y_{X/S}$	yield coefficient (g cell mass/g glucose)

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