J. Microbiol. Biotechnol. (2014), 24(5), 661–666
http://dx.doi.org/10.4014/jmb.1402.02017

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Cell-Recycle Continuous Fermentation of *Enterococcus faecalis* RKY1 for Economical Production of Lactic Acid by Reduction of Yeast Extract Supplementation

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Received: February 11, 2014
Revised: February 20, 2014
Accepted: February 21, 2014

First published online
February 25, 2014

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pISSN 1017-7825, eISSN 1738-8872

Both lactic acid productivity and cell growth were linearly correlated with yeast extract supplementation in batch fermentation. During conventional continuous operation, although fresh feed was introduced into the bioreactor with a significantly low dilution rate (0.04 h^{-1}), the amount of yeast extract employed was not enough to maintain the growth of microorganism. However, when the fresh feed contained 100 g/l glucose and 2 g/l yeast extract during cell-recycle continuous operation at a dilution rate of 0.04 h^{-1}, more than 90 g/l lactic acid was continuously produced, with the average productivity of 3.72 g/l·h. In this experiment, 82 g of yeast extract (77% of reduction yield) could be reduced for the production of 1 kg of lactic acid compared with batch fermentation of a similar volumetric productivity.

Keywords: Cell-recycle, continuous fermentation, *Enterococcus faecalis*, lactic acid, yeast extract

Introduction

Lactic acid is one of the important building-block organic acids obtained from renewable resources. Owing to the presence of two reactive functional groups like –OH and –COOH, lactic acid can be converted into a wide variety of industrial compounds through simple chemical reactions [19]. This chemical property enables lactic acid to participate in application in a wide range of industries, such as the food, pharmaceutical, cosmetic, textile, and other chemical sectors [21]. Among a variety of applications of lactic acid, polylactic acid (PLA) has gained worldwide interest owing to its biodegradable and biocompatible characteristics [14, 17]. Although there are two routes to production of lactic acid, such as chemical synthesis and microbial fermentation, the fermentative production of lactic acid has gained much attention owing to finite petrochemical resources and environmental pollution [21]. In addition, microbial fermentation with suitable microorganisms is always favored to produce high optical purity of lactic acid, which can be used to produce pure polymers of poly(L-lactic acid) and poly(D-lactic acid) [3, 9]. The optical purity of lactic acid influences the physical properties and the biodegradability of the resulting PLA, and D-lactic acid is usually harmful to human metabolism [4, 22]. Therefore, fermentative production of L-lactic acid has received much interest from researchers in the recent years [8, 11, 16, 20, 22, 24].

On an industrial scale, the manufacturing cost of fermentative lactic acid is targeted to be less than 0.8 US$/kg, because the selling price of PLA should decrease significantly to compete with synthetic polymers [21]. The highest cost in lactic acid production by fermentation corresponds to the raw materials as well as the recovery processes. González and co-workers [6] proposed the economical evaluation for the production of 50% food-grade lactic acid from ultrafiltered whey and yeast extract as major raw materials. They reported that the cost of yeast extract is the major contributor to the operating costs, corresponding to 25% of total annual costs. Åkerberg and Zacchi [1] also reported that the raw material cost for lactic acid production

by fermentation was evaluated to be 34% of the total production cost. Therefore, there is a need to develop an industrially attractive process that considers raw material cost and productivity. The continuous operation in a membrane bioreactor coupled to fermentation and ultrafiltration processes would lead to a reduced raw material cost without a decrease of productivity [6].

The present study aimed to reduce the nitrogen source supplement to the fermentation medium for economical production of lactic acid. The effect of yeast extract supplement to the fermentation medium on lactic acid production by batch culture of *Enterococcus faecalis* RKY1 was investigated, and then the continuous fermentations were carried out by coupling a membrane bioreactor to reduce the yeast extract supplement. In addition, the cell-recycle continuous process coupling to the membrane bioreactor was compared with the traditional continuous process.

Materials and Methods

Bacterial Strain and Medium

Enterococcus faecalis RKY1 KCTC 8890P [10, 23, 24] as a homofermentative L(+)-lactic acid producer was used throughout this study. The stock cultures were maintained in a 5 ml vial containing the broth and 50% (v/v) glycerol at −20°C until used. The growth medium was composed of 30 g/l glucose, 10 g/l yeast extract, and 6 g/l K_2HPO_4. The production medium for batch and continuous fermentation was supplemented with 100 g/l glucose, 0−40 g/l yeast extract, and 6 g/l K_2HPO_4. Solutions with all chemical constituents were sterilized at 121°C for 15 min prior to use in fermentation. Glucose and K_2HPO_4 were purchased from Yakuri Pure Chemicals Co. (Kyoto, Japan), and yeast extract was obtained from Difco Laboratories (Detroit, USA).

Inoculum Preparation

Cells from stock culture were transferred to a 20 ml vial containing 15 ml of growth medium, and then it was incubated at 38°C for 12 h on a rotary shaker (KMC-8480SF; Vision Scientific Co., Korea). After three consecutive transfers of the culture to the 20 ml vial, 2 ml of final culture was transferred to a 50 ml vial containing 40 ml of growth medium, which was cultivated at 38°C for 6 h on a rotary shaker. Then, the resultant culture was inoculated into a 2.5 L bioreactor containing 1 L of production medium. The amount of final inoculation was 4% (v/v) based on the working volume of the bioreactor.

Experimental Equipment

The 2.5 L bioreactor was used for cell-recycle fermentation, and the working volume was 1 L. Fig. 1 shows the schematic illustration of the overall experimental set-up used in this study. The temperature was maintained at 38°C, whereas the agitation speed was set at 200 rpm. The bioreactor was integrated with a cross-flow ultrafiltration membrane module (SKUF-103-0830, 300 × 25 mm; SK Chemical, Suwon, Korea) having a 0.06 m^2 surface area. Cross-flow ultrafiltration was carried out with polysulfone membranes, and the molecular mass cut-off of the membranes was 30 kDa. Two pressure gauges were attached to the inlet and outlet of the module for monitoring transmembrane pressure. The fermentation broth was circulated through the UF membrane module by a diaphragm pump (Cole-Parmer Instrument, Vernon Hills, IL, USA). A 2 L product vessel was assembled with the system to collect permeate. A 3-channel peristaltic pump (Teledyne Isco, Lincoln, NE, USA) was used to provide fresh medium and to remove the fermentation broth. After each run, cleaning and sterilization of ultrafiltration membranes were performed with 0.1 M NaOH and 200 ppm NaOCl. Sterilized deionized water was used for flushing the module to remove residual NaOH and NaOCl solutions. Before each experiment, the bioreactor, feed vessel, and product vessel were autoclaved at 121°C for 15 min.

Fermentations

Fermentation was performed with 1 L of medium with 4% (v/v) inoculum in a 2.5 L bioreactor system. Fermentation was started in a batch process at 38°C and 200 rpm, and the culture pH was automatically controlled at 7.0 by addition of 10 M NaOH solution.

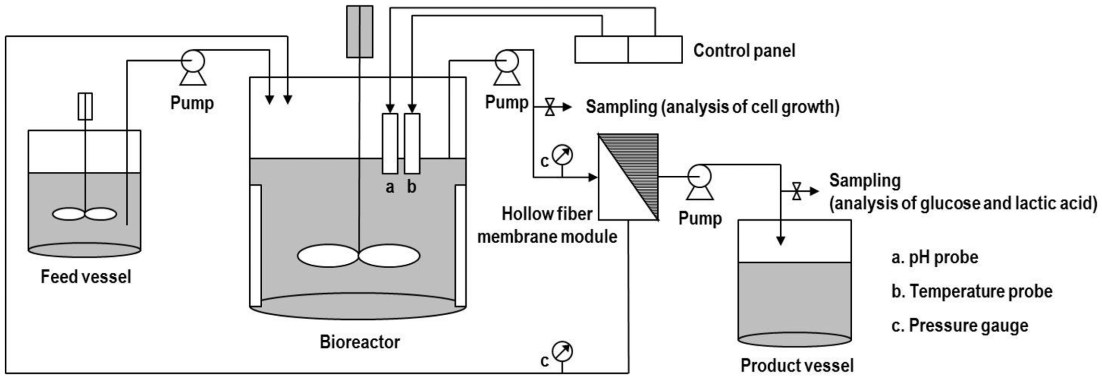

Fig. 1. Schematic illustration of membrane-integrated bioreactor system for cell-recycle continuous production of lactic acid.

For continuous fermentation, the batch process continued up to 6 h, and then ultrafiltration with cell-recycle was started. This time period culture grown inside of the bioreactor was in the exponential phase. The working volume of the bioreactor was maintained at 1 L by supplying fermentation medium in balance with permeate from the ultrafiltration module and by pumping out the excess volume of the bioreactor. The permeate flow rate from the ultrafiltration unit was maintained in such a way that it was equal to the fresh feed flow rate to the bioreactor. The broth was tested at regular intervals in order to predict the time when solution inside the bioreactor reached the steady-state condition.

Analytical Methods

The samples were taken out at predetermined time intervals, and the optical density of those samples was measured by a UV spectrophotometer (UV-1700; Shimadzu, Japan) at 660 nm for measuring cell growth. Samples were then centrifuged at 13,000 ×*g* for 20 min by using a bench-top centrifuge (Vision Scientific Co.) and supernatants were collected for the analysis of lactic acid and glucose. Lactic acid concentration was measured using an HPLC (Young Lin Instruments, Korea) equipped with an Aminex HPX-87H ion-exclusion column (300 × 7.8 mm; Bio-Rad, Hercules, CA, USA) and a UV detector set at 210 nm. The mobile phase was 5 mM H_2SO_4 solution at the flow rate of 0.6 ml/min. The injected sample volume was 20 µl after filtration with a 0.2 µm syringe filter paper. The column temperature was fixed at 35°C. The glucose concentration was enzymatically analyzed by using an assay kit (Asan Pharmaceutical, Korea). The red dye developed from two serial reactions catalyzed by glucose oxidase and peroxidase was determined by absorbance measurements at 505 nm. Each assay was carried out in triplicate and the mean values were presented. Dilution rate was defined as the ratio of the feed flow rate to the working volume of the bioreactor. Lactic acid productivity was calculated using the following equation.

$$\text{Productivity } (g/l \cdot h^{-1}) = \text{Lactic acid concentration } (g/l) \times \text{Dilution rate } (h^{-1})$$

$$\text{Dilution rate } (h^{-1}) = \frac{\text{Feed flow rate } (l/h)}{\text{Working volume of the bioreactor } (L)}$$

Results and Discussion

Effect of Yeast Extract Concentrations on Batch Production of Lactic Acid

The effect of yeast extract concentrations on batch lactic acid fermentation was investigated by using culture medium containing 100 g/l glucose and 0–40 g/l yeast extract, and the results are shown in Table 1. Both cell growth and lactic acid productivity were linearly increased up to 25 g/l yeast extract supplementation, but these were rarely influenced by yeast extract supplementation beyond 25 g/l. The similar improvement of lactic acid fermentation by yeast extract supplementation was described by Kulozik and Wilde [12], who reported that cell growth and lactic acid productivity were linearly increased by supplementation of yeast extract up to 10 or 15 g/l when 60 g/l lactose was used as a carbon source for lactic acid production by *Lactobacillus helveticus*. However, the raw material cost for lactic acid production should be significantly increased when the fermentation requires a high level of yeast extract supplementation. Therefore, it is necessary to develop alternative raw materials and/or processes for the economical production of lactic acid. The cell-recycle systems using membranes are efficient for lactic acid fermentation, as lactic acid production is typically associated with cell growth [5, 13, 22].

Effect of Yeast Extract Concentrations on Continuous Production of Lactic Acid

Before continuous operation, fermentation was started with the batch mode and it was operated at 38°C and

Table 1. Effect of yeast extract concentrations on batch lactic acid fermentation by *E. faecalis* RKY1.

Yeast extract (g/l)	Fermentation time (h)	Lactic acid (g/l)	Max. dry cell weight (g/l)	Productivity ($g/l \cdot h^{-1}$)
0	66[a]	6.9 ± 0.1	0.41 ± 0.01	0.10 ± 0.01
2	66[a]	59.2 ± 0.3	2.27 ± 0.09	0.90 ± 0.00
5	42[b]	95.2 ± 0.8	5.52 ± 0.23	2.27 ± 0.02
10	24[b]	94.5 ± 1.1	7.47 ± 0.39	3.94 ± 0.04
15	16[b]	93.8 ± 1.6	10.92 ± 0.59	5.86 ± 0.10
20	12[b]	94.6 ± 1.2	14.95 ± 0.79	7.88 ± 0.14
25	10[b]	95.1 ± 1.7	17.94 ± 0.95	9.51 ± 0.17
30	10[b]	95.3 ± 1.8	18.93 ± 0.95	9.53 ± 0.18
40	10[b]	95.9 ± 1.0	18.74 ± 0.96	9.59 ± 0.10

[a]The fermentation was not finished owing to a lack of yeast extract supplementation.

[b]The fermentation was completely finished.

200 rpm in a 2.5 L bioreactor using 1 L of its working volume up to 6 h. Batch mode of fermentation was conducted using 100 g/l glucose, 15 g/l yeast extract, and 6 g/l K_2HPO_4. For continuous operation, fresh feed containing 100 g/l glucose and 0–4 g/l yeast extract was introduced into the bioreactor with a dilution rate of 0.04 h^{-1}. Lactic acid concentration and cell growth during the experiment are indicated in Fig. 2.

During the initial continuous cultivation stage, lactic acid concentration and dry cell weight reached higher than 95 and 13 g/l, respectively, when the fresh feed containing 4 g/l yeast extract was introduced into the bioreactor. Overall, lactic acid concentration and cell mass concentration started to decrease within several hours of continuous fermentation with the introduction of fresh medium containing less than 5 g/l yeast extract. Although fresh feed was introduced into the bioreactor with a significantly low dilution rate, the amount of yeast extract used in this experiment was not enough to maintain or to stimulate the growth of the microorganism. The increase of yeast extract supplement to fresh feed may result in stimulating cell growth and lactic acid production, but this is economically unfavorable because the cost of nitrogen source is one of the main bottlenecks for the competitive biological production of lactic acid [15].

Effect of Yeast Extract Concentrations on Cell-Recycle Continuous Production of Lactic Acid

Cell-recycle continuous fermentation of *E. facalis* RKY1 was carried out with a membrane filtration device in order to prevent reduction of cell mass concentration in the bioreactor and to enhance substrate conversion to lactic acid. To this end, fermentation was initially started with the batch mode, which was operated at 38°C and 200 rpm in a 2.5 L bioreactor using 1 L of its working volume. Batch mode of fermentation was performed using 100 g/l glucose, 15 g/l yeast extract, and 6 g/l K_2HPO_4. For the cell-recycle continuous operation, fresh feed containing 100 g/l glucose and 0–4 g/l yeast extract was introduced

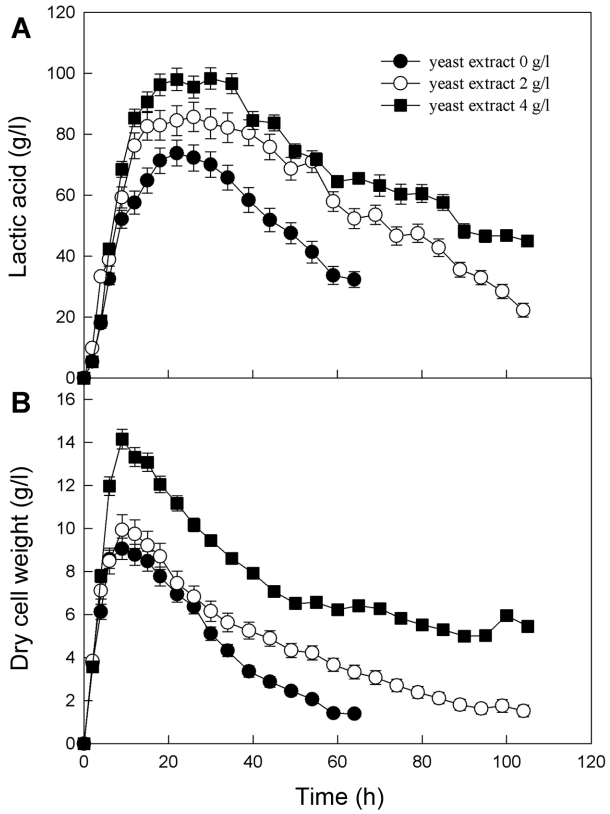

Fig. 2. Effect of yeast extract concentrations on (**A**) lactic acid production and (**B**) cell growth during continuous fermentation by *E. faecalis* RKY1.
The dilution rate for continuous operation was set at 0.04 h^{-1}.

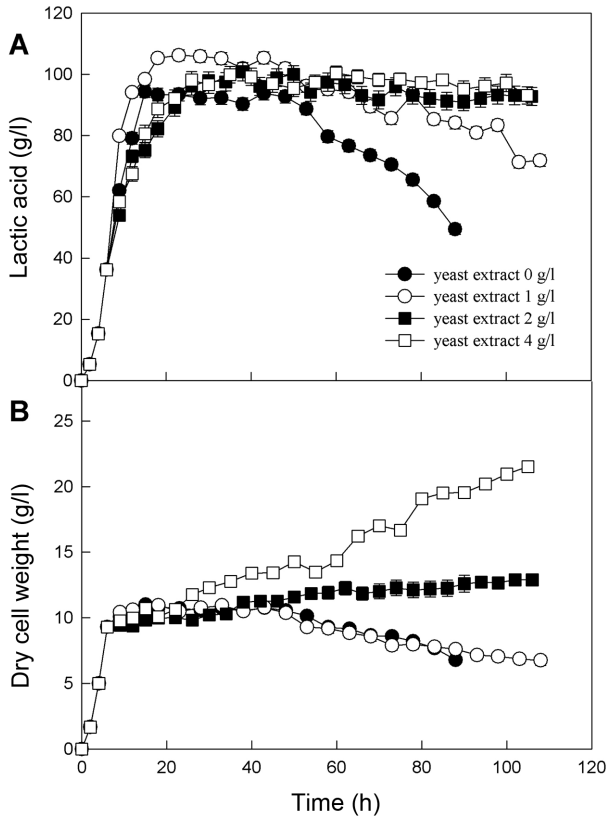

Fig. 3. Effect of yeast extract concentrations on (**A**) lactic acid production and (**B**) cell growth during cell-recycle continuous fermentation by *E. faecalis* RKY1.
The dilution rate for continuous operation was set at 0.04 h^{-1}.

into the bioreactor with a dilution rate of 0.04 h^{-1}, after 6 h of batch mode operation. Lactic acid concentration and cell growth during this experiment are shown in Fig. 3.

When the fresh feed was composed of 100 g/l glucose and 2 g/l yeast extract, more than 90 g/l lactic acid was continuously produced, with the average productivity of 3.72 g/l·h. Under this experimental condition, residual glucose concentration in the bioreactor was maintained below 5 g/l (data not shown). As shown in Table 1, this degree of lactic acid productivity (3.72 g/l·h) obtained from cell-recycle fermentation corresponds to lactic acid productivity achieved when the batch fermentation was carried out using 100 g/l glucose and 10 g/l yeast extract. If the lactic acid yield is 0.95 g/g, it should be necessary to supply 105 g of yeast extract for the production of 1 kg of lactic acid by batch fermentation. On the other hand, cell-recycle fermentation under the dilution rate of 0.04 h^{-1} needs only 23 g of yeast extract for the production of 1 kg of lactic acid.

Effect of Dilution Rates on Cell-Recycle Continuous Production of Lactic Acid

Cell-recycle continuous operation was performed at dilution rates between 0.04 and 0.1 h^{-1} to evaluate the effect of the dilution rate on lactic acid production by *E. faecalis* RKY1. The fermentation was started with the batch mode of operation at 38°C and 200 rpm in a 2.5 L bioreactor using 1 L of its working volume. The initial batch fermentation was carried out using 100 g/l glucose, 15 g/l yeast extract, and 6 g/l K_2HPO_4, and fresh feed containing 100 g/l glucose and 2 g/l yeast extract was introduced into the bioreactor with dilution rates of 0.04–0.1 h^{-1} after 6 h of batch mode operation. Lactic acid concentration and cell mass concentration during this experiment are presented in Fig. 4.

As shown in Fig. 4, the cell mass concentration was gradually increased with increases in dilution rate, and the maximum dry cell weight (24 g/l) was obtained at the dilution rate of 0.1 h^{-1}. However, the residual glucose concentration was increased up to 57 g/l at the dilution rate of 0.1 h^{-1}, and at the same time the lactic acid concentration was decreased. At higher dilution rates, the fermentation broth may have insufficient time to convert glucose to lactic acid, because the residence time of the medium is increased with increases in dilution rate. González-Pajuelo *et al*. [7] and Shene and Bravo [18] previously reported similar results in their studies using *Clostridium butyricum* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, respectively.

Bibal *et al*. [2] reported that high cell mass concentration (88 g/l) and lactic acid productivity (51.5 g/l·h) were

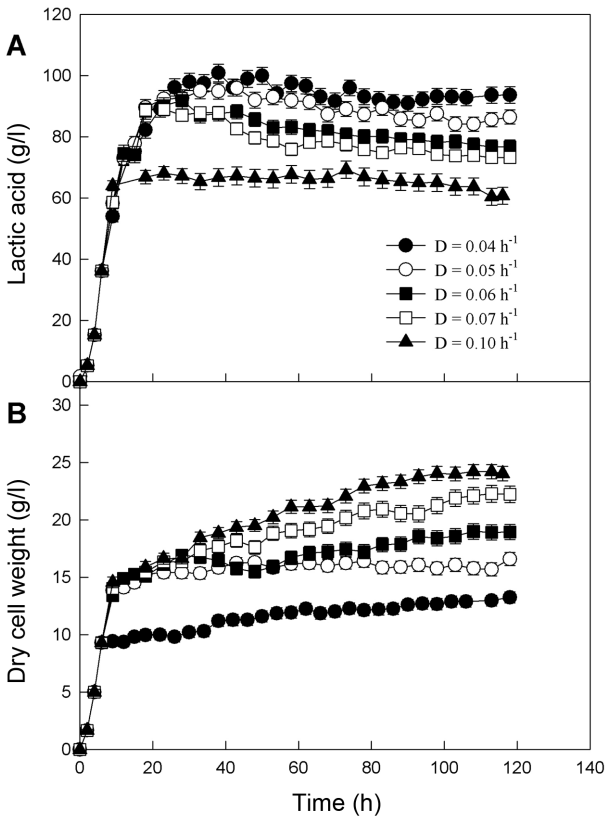

Fig. 4. Effect of dilution rates on (**A**) lactic acid production and (**B**) cell growth during cell-recycle continuous fermentation by *E. faecalis* RKY1.

The yeast extract concentration of the fresh feed solution for continuous operation was maintained at 2 g/l.

obtained when *Lactococcus cremoris* was continuously cultivated in a cell-recycle bioreactor without cell bleeding under the dilution rate of 1.05 h^{-1}. However, in their study, the substrate conversion ratio to lactic acid was significantly low (approximately 65% based on initial carbon source concentration), and 240 g of yeast extract and/or peptone should be necessary to produce 1 kg of lactic acid. In our current study, when *E. faecalis* RKY1 was cultivated in cell-recycle continuous bioreactor under the dilution rate of 0.04 h^{-1}, 82 g of yeast extract could be reduced for the production of 1 kg of lactic acid compared with the batch fermentation of a similar volumetric productivity. In the cell-recycle continuous operation, the reduction yield of yeast extract supplementation is estimated to be 77% compared with the batch fermentation. If the price of yeast extract is assumed to be 6.5 US$/kg, our cell-recycle continuous fermentation result shows the reduction of raw material cost with 0.53 US$ for the production of 1 kg of lactic acid.

Therefore, it can be concluded that the cost of the fermentation step could be reduced by operating in a continuous mode, and recycling the ultrafiltration concentrate containing unused proteins and cells to the bioreactor. This kind of operation would reduce microorganisms and yeast extract consumption and would increase the volumetric productivity of the bioreactor. Thus, continuous production of lactic acid through a membrane-integrated bioreactor system is an economically quite viable and attractive option.

References

1. Åkerberg C, Zacchi G. 2000. An economic evaluation of the fermentative production of lactic acid from wheat flour. *Bioresour. Technol.* **75:** 119-126.
2. Bibal B, Vayssier Y, Goma G, Pareilleux. 1990. High-concentration cultivation of *Lactococcus cremoris* in a cell-recycle reactor. *Biotechnol. Bioeng.* **37:** 746-754.
3. Datta R, Henry M. 2006. Lactic acid: recent advances in products, processes and technologies – a review. *J. Chem. Technol. Biotechnol.* **81:** 1119-1129.
4. Datta R, Tsai SP, Bonsignore P, Moon SH, Frank JR. 1995. Technological and economic potential of poly(lactic acid) and lactic acid derivatives. *FEMS Microbiol. Rev.* **16:** 221-231.
5. Gao MT, Michiteru K, Rie G, Hirokazu T, Makoto H, Tadashi H. 2005. Development of a continuous electrodialysis fermentation system for production of lactic acid by *Lactobacillus rhamnosus*. *Process Biochem.* **40:** 1033-1036.
6. González MI, Álvarez S, Riera F, Álvarez R. 2007. Economic evaluation of an integrated process for lactic acid production from ultrafiltered whey. *J. Food Eng.* **80:** 553-561.
7. González-Pajuelo M, Adrade JC, Vasconcelos I. 2005. Production of 1,3-propanediol by *Clostridium butyricum* VPI 3266 in continuous cultures with high yield and productivity. *J. Ind. Microbiol. Biotechnol.* **32:** 391-396.
8. John RP, Nampoothiri KM, Pandey A. 2006. Simultaneous saccharification and L(+)-lactic acid fermentation of protease-treated wheat bran using mixed culture of Lactobacilli. *Biotechnol. Lett.* **28:** 1823-1826.
9. John RP, Nampoothiri KM, Pandey A. 2007. Fermentative production of lactic acid from biomass: an overview on process developments and future perspectives. *Appl. Microbiol. Biotechnol.* **74:** 524-534.
10. Kang KH, Yun JS, Ryu HW. 2000. Effect of culture conditions on the production of succinate by *Enterococcus faecalis* RKY1. *J. Microbiol. Biotechnol.* **10:** 1-7.
11. Karp SG, Igashiyama AH, Siqueira PF, Carvalho JC, Vandenberghe LPS, Thomaz-Soccol V, *et al*. 2011. Application of the biorefinery concept to produce L-lactic acid from the soybean vinasse at laboratory and pilot scale. *Bioresour. Technol.* **102:** 1765-1772.
12. Kulozik U, Wilde J. 1999. Rapid lactic acid production at high cell concentrations in whey ultrafiltrate by *Lactobacillus helveticus*. *Enzyme Microb. Technol.* **24:** 297-302.
13. Kwon S, Yoo IK, Lee WG, Chang HN, Chang YK. 2001. High rate continuous production of lactic acid by *Lactobacillus rhamnosus* in a two-stage membrane cell-recycle bioreactor. *Biotechnol. Bioeng.* **73:** 225-234.
14. Lunt J. 1998. Large-scale production, properties and commercial applications of polylactic acid polymers. *Polym. Degrad. Stabil.* **59:** 145-152.
15. Martinez FAC, Balciunas EM, Salgado JM, González JMD, Converti A, Oliveira RPS. 2013. Lactic acid properties, applications and production: a review. *Trends Food Sci. Technol.* **30:** 70-83.
16. Quyang J, Ma R, Zheng Z, Cai C, Zhang M, Jiang T. 2013. Open fermentative production of L-lactic acid by *Bacillus* sp. strain NL01 using lignocellulosic hydrolyzates as low-cost raw material. *Bioresour. Technol.* **135:** 475-480.
17. Sädergård A, Stolt M. 2002. Properties of lactic acid based polymers and their correlation with composition. *Prog. Polym. Sci.* **27:** 1123-1163.
18. Shene C, Bravo S. 2007. Whey fermentation by *Lactobacillus delbrueckii* subsp. *bulgaricus* for expolysaccharide production in continuous culture. *Enzyme Microb. Technol.* **40:** 1578-1584.
19. Varadarajan S, Miller DJ. 1999. Catalytic upgrading of fermentation-derived organic acids. *Biotechnol. Prog.* **15:** 845-854.
20. Watanabe M, Makino M, Kaku N, Koyama M, Nakamura K, Sasano K. 2013. Fermentative L(+)-lactic acid production from non-sterilized rice washing drainage containing rice bran by a newly isolated lactic acid bacterial without any additions of nutrients. *J. Biosci. Bioeng.* **115:** 449-452.
21. Wee YJ, Kim JN, Ryu HW. 2006. Biotechnological production of lactic acid and its recent applications. *Food Technol. Biotechnol.* **44:** 163-172.
22. Wee YJ, Ryu HW. 2009. Lactic acid production by *Lactobacillus* sp. RKY2 in a cell-recycle continuous fermentation using lignocellulosic hydrolyzates as inexpensive raw materials. *Bioresour. Technol.* **100:** 4262-4270.
23. Wee YJ, Yun JS, Kim D, Ryu HW. 2006. Batch and repeated batch production of L(+)-lactic acid by *Enterococcus faecalis* RKY1 using wood hydrolyzate and corn steep liquor. *J. Ind. Microbiol. Biotechnol.* **33:** 431-435.
24. Wee YJ, Yun JS, Park DH, Ryu HW. 2004. Biotechnological production of L(+)-lactic acid from wood hydrolyzate by batch fermentation of *Enterococcus faecalis*. *Biotechnol. Lett.* **26:** 71-74.