Lactic Acid Production by Coimmobilized Cells of *Lactococcus lactis* TISTR 1401 and *Lactobacillus casei* TISTR 1341 Using Whey as Substrate

Sukjai Choojun[1] and Rutairat Suttisuwan[2*]

[1]Department of Applied Biology, Faculty of Science, King Mongkut's Institute of Technology Lardkrabang, Thailand 10520
[2]Department of Biology, Faculty of Science and Technology, Rajamangala University of Technology Krungthep, Thailand 10120

**Corresponding author, E-mail: rutairat.s@rmutk.ac.th*

ABSTRACT

Lactic acid production from whey by batch fermentation of coimmobilized cells of Lactococcus lactis TISTR 1401 and Lactobacillus casei TISTR 1341 was investigated in order to decrease the manufacturing cost of lactic acid. The fermentation was conducted in a two liters fermentor at 37°C and pH 6.5 with an agitation rate of 100 rpm. The maximum lactic acid concentration was obtained with a value of 29.89 $g.l^{-1}$ and the coimmobilized cells had consistent potential to recycle two rounds of fermentation by producing 17.38 and 12.51 $g.l^{-1}$ lactic acid in 24 h, for each batch of the first and the second cycle, respectively, while lactic acid produced by free cells in mixed cultures of the two species of the bacteria was 16.63 $g.l^{-1}$ in 48 h. These results suggested that coimmobilized cell cultures were more effective than free cell mixed cultures in improving lactic acid production.

Key words: Whey, Lactic acid, Mixed cultures, Coimmobilized cell cultures

INTRODUCTION

Lactic acid is used as a biopreservative in food as lactic acid is effective in adding flavor and taste to food, controlling pH and inhibiting growth of microorganisms and germination of spores (Sachin et al., 2006). In addition, lactic acid is useful in biodegradable plastic production (Nabil et al., 2001). Lactic acid can be produced from chemical production or biological fermentation but the cost of chemical production is high and the product is difficult to be purified; therefore, biological fermentation by lactic acid bacteria (LAB) is made use of (Senthuran et al., 1999).

Synergistic effect of LAB has been reported recently regarding enhanced lactic acid production. KiBeom (2005) observed that mixed cultures of LAB might be more effective than single culture for improving lactic acid production. Moreover, immobilized cell technology can be established, leading to improved productivity (Sheng-Tsiung and Sheng-Tsiung, 1991).

Nowadays, research efforts are focused on looking for new and effective nutritional sources and new progressive fermentation techniques of both high substrate conversion and high production yields. Whey is the predominant substrate and usually contains about 5% lactose, 1% protein and 1% salts (Roukas and kotzekidou, 1998), therefore, whey is used for lactic acid production because it is a relatively rich medium having high lactose and salt content, including some minerals (Pauli and Fitzpatrick, 2002 ; Fitzpatrick et al., 2003).

The aim of this study was to improve the production of lactic acid from biological fermentation of the mixed cultures of *L. lactis* TISTR 1401 and L. casei TISTR 1341 by using cell immobilization technique and using whey which is a by-product from the manufacturing of cheese and casein (Marshall, 1982) as the substrate.

MATERIALS AND METHODS

Media

Whey was received freshly from Minor Cheese Limited, a cheese plant in Bangkok. The whey was supplemented with 5 $g.l^{-1}$ yeast extract, 10 $g.l^{-1}$ peptone, 0.25 $g.l^{-1}$ K2HPO4, 0.03 $g.l^{-1}$ MnSO4, 0.10 $g.l^{-1}$ $MgSO_4$ and 20 $g.l^{-1}$ $CaCO_3$ (Mostafa, 1995; Youseef et al. 2000).and its pH was adjusted to 6.5 before being sterilized at 121°C and 15 $l.b/inch^2$ for 15 min.

The Cultures

L. lactis TISTR 1401 and *L. casei* TISTR 1341 used in the lactic acid fermentation were from TISTR Culture Collection, Bangkok Mircen, Thailand.

Inoculum preparation

Inocula of L. lactis TISTR 1401 and L. casei TISTR 1341, a homofermentative L(+)-Lactic acid producer, were propagated separately in 150 ml MRS broth in 250 ml Erlenmeyer flasks. The cultures were incubated at 37°C for 2 days and each species of the bacteria was used when the optical density (OD_{660}) of the culture reached 0.5, with a total poplation of 7.50 x 106 cfu ml^{-1}. The cultures were used for inoculum 5, 7.5 and 10%.

Immobilization of cells

Each inoculum of mixed culture of the 5% *L. lactis* and 10% *L. casei* (Senthuran et al., 1999) was centrifuged at 3,000 g for 20 min and the spun broth was decanted. The pellet cells were resuspended in steriled 0.85% NaCl solution and again centrifuged. After the NaCl solution was decanted, the pellet cells were mixed with steriled 0.85% NaCl solution and steriled 2% sodium alginate at a volumetric ratio of 10 : 3 : 2. The mixtures were then extruded by a peristaltic pump through the tube into 0.1 M $CaCl_2$ solution to form beads. The distance from the end of the tube to the surface of the $CaCl_2$ solution was 15 cm and the flow rate was 7 ml/min. The beads were suspended in $CaCl_2$ solution at 4°C for 2 h and washed thoroughly twice with sterile distilled water before being used.

Fermentation conditions and cell recycling

The fermentation was performed in a two liters glass fermentor (B.Braun Biotech International Gmb H,D - 34212 Melhungen, Germany) with a working volume of 1.4 liters.The fermentor was sterilized at 121°C, 15 l.b/inch^2 for 30 min. After cooling, the fermentor was inoculated with immobilized cells of *L. lactis* and *L. casei*. The fermentor was incubated at 37°C with an agitation rate of 100 rpm and the pH was maintained at 6.5 by automatic addition of sterile 5M NaOH. The sample was centrifuged at 10,000 g for 20 min and the supernatant was stored at 0°C for high performance liquid chromatography (HPLC) analysis.

For cells recycling, when the concentration of lactic acid was stabilized after first fermentation (Batch 1), the medium was then drained off from the fermentor and the fresh medium was added to the beads before a repeated batch (Batch 2) fermentation was started.

Assay Methods

The number of viable cells was determined by plate counting on MRS agar (A.O.A.C. 2000). The amount of lactose was detected by the protocol of Dubois et al.(1956). L(+)-Lactic acid concentration was measured by HPLC analysis. The HPLC system (SHIMADZU Co., Tokyo, Japan) was equipped with an Inertsil C8 - 3 column and was operated at room temperature using 20 mM KH_2PO_4 (pH 3) as the mobile phase. The flow rate was maintained at 1 ml /min. Lactic acid was detected by the UV detector at 210 nm. The concentrations of lactic acid were calculated by comparing the peak areas with the standard graph.

Data analyses

Data of the triplicate concentrations of lactic acid were used for statistical analyses by Duncan's New Multiple Range test.

RESULTS AND DISCUSSION

Effects of inoculum sizes for lactic acid production by mixed cultures of free cells of *L. lactis* and *L. casei* in a two liters flask

The fermentation was studied with free cells pure cultures of 5% and 10% *L. lactis* and the maximum concentrations of lactic acid, 6.81g.l^{-1} and 8.48 g.l^{-1}, respectively, were obtained in 24 h when the cultures were incubated at 37°C in a stationary flask. The fermentations by free cells in pure cultures of 5% and 10% *L. casei* gave the maximum concentrations of lactic acid with the values of 7.36 g.l^{-1} and 9.04 g.l^{-1}, respectively, in 84 h. It was found that 10% pure culture gave higher lactic acid than 5% pure culture while *L. casei* gave the highest lactic acid but needed much longer fermentation time.

For fermentation in mixed cultures of 5% each of *L. lactis* and *L. casei*, the maximum concentration of lactic acid obtained was 7.64 g.l^{-1} within 60 h. It was found from this study that mixed cultures produced higher lactic acid than did the pure culture.

Fermentation in mixed cultures with 10% *L. lactis* and 10% *L. casei*, 5%

L. lactis and 10% *L. casei*, 10% *L. lactis* and 5% *L. casei*, 7.5% *L. lactis* and 7.5% *L. casei* gave the maximum concentration of lactic acid with the values of 10.71 $g.l^{-1}$, 11.40 $g.l^{-1}$, 10.70 $g.l^{-1}$ and 9.14 $g.l^{-1}$, in 60 h, respectively (Table 1).

When lactose residues in mixed cultures and in pure cultures were compared, it was found that residued lactose in mixed cultures was less than that in the pure cultures, resulting in having higher lactic acid in the mixed cultures with the reason that more lactose was changed into lactic acid.

Table 1. Comparisons of various inoculum sizes of *Lactococcus lactis* and *Lactobacillus casei* for lactic acid production.

Inoculum size	Fermentation time (h)	Concentration of lactic acid ($g.l^{-1}$)	Yield (g/g)	Productivity ($g.l^{-1}.h$)	Residued lactose ($g.l^{-1}$)
5% *L. lactis*	24	6.81 ± 0.163^{f}	0.401±0.009	0.284±0.007	21
10% *L. lactis*	24	8.48 ± 0.245^{d}	0.339±0.010	0.353±0.010	14
5% *L. casei*	84	7.36 ± 0.163^{e}	0.526±0.012	0.088±0.012	20
10% *L. casei*	84	9.04 ± 0.163^{c}	0.362±0.007	0.108±0.002	13
5% *L. lactis* and 5% *L. casei*	60	7.64 ± 0.081^{e}	0.294±0.003	0.127±0.012	11
5% *L. lactis* and 10% *L. casei*	60	11.40 ± 0.326^{a}	0.317±0.009	0.190±0.008	3
10% *L. lactis* and 5% *L. casei*	60	10.70 ± 0.163^{b}	0.324±0.005	0.178±0.002	7
10% *L. lactis* and 10% *L. casei*	60	10.71 ± 0.161^{b}	0.346±0.045	0.179±0.003	8
7.5% *L. lactis* and 7.5% *L. casei*	60	9.14 ± 0.245^{c}	0.315±0.009	0.152±0.004	10

*a,b,c,d,e,f means significantly different at 95% confidence level

Yun et al. (2003) studied the effects of carbon sources for lactic acid production of *Enterococcus faecalis* RYK 1 by using glucose, fructose, maltose, galactose, glycerol, xylose, whey and starch. It was found that using glucose, fructose and maltose produced 18.18 $g.l^{-1}$, 17.95 $g.l^{-1}$ and 16.80 $g.l^{-1}$ lactic acid, respectively while galactose, lactose, glycerol, xylose, whey and starch produced low concentrations of lactic acid in the values of 2.70 $g.l^{-1}$, 1.26 $g.l^{-1}$, 2.24 $g.l^{-1}$, 1.68 $g.l^{-1}$, 1.83 $g.l^{-1}$ and 1.19 $g.l^{-1}$, respectively.

Effects of agitation rate for lactic acid production by free cells in mixed cultures of *L. lactis* and *L. casei* in a two liters fermentor

Agitation rates at 0, 100 and 200 rpm gave the maximum Concentrations of lactic acid, i.e., 13.90 $g.l^{-1}$ in 60 h, 16.63 $g.l^{-1}$ in 48 h and 7.03 $g.l^{-1}$ in 48 h, respectively. At the 200 rpm agitation rate, the lowest amount of lactic acid was produced because the high agitation resulted in a higher shear rate and injured the cells. The fermentation of lactic acid by free cells in mixed cultures of 5% *L. lactis* and 10% *L. casei* in the fermentor was better than that in the flask

because fermentation in the fermentor required shorter fermentation time and had higher concentration of lactic acid than that in the flask.

By using statistical analysis, it was found that the concentration of lactic acid produced at the agitation rate of 100 rpm was significantly higher (95% confidence level) than those at 0 and 200 rpm (Table 2).

Table 2. Comparisons of various agitation rates in a two liters fermentor by mixed cultures of 5% Lactococcus lactis and 10% Lactobacillus casei for lactic acid production.

Agitation rate (rpm)	Fermentation time (h)	Concentration of lactic acid ($g.l^{-1}$)	Yield (g/g)	Productivity ($g.l^{-1}.h$)	Residued lactose ($g.l^{-1}$)
0	60	13.90 ± 0.24^{b}	0.366±0.006	0.232±0.004	2
100	48	16.63 ± 0.25^{a}	0.414±0.020	0.348±0.017	0
200	48	7.03 ± 0.21^{c}	0.412±0.012	0.146±0.004	24

*a,b,c,d,e,f means significantly different at 95% confidence level

Effects of whey for lactic acid production by coimmobilized and free cells in mixed cultures of *L. lactis* and *L. casei* in a two liters fermentor

Free cells fermentation by mixed cultures of 5% *L. lactis* and 10% *L. casei* in two liters fermentor produced 16.63 $g.l^{-1}$ lactic acid in 48 h. In an initial experiment, the concentration of lactose was increasing to be 40 $g.l^{-1}$ while the concentration of residued lactose was decreasing. The total amount of lactose, 40 $g.l^{-1}$, was produced in 80 h and a number of viable cells increased from $8.00x10^{6}$ cfu/ml to $2.00x10^{8}$ cfu/ml as shown in Figure 1.

The fermentation by coimmobilized cells gave the maximum concentration of lactic acid, 17.38 $g.l^{-1}$, in 24 h. Residued lactose decreased from 40 $g.l^{-1}$ to 0 $g.l^{-1}$ in 24 h as shown in Figure 2. The production of lactic acid by free cells when compared the fermentation between using free cell and coimmobilized cells, the shorter fermentation time and higher lactic acid production were found when using coimmobilized cells.

Chromopoulos et al., (2002) reported lactic acid fermentation by *L. casei* in free cells and in immobilized cells on gluten pellets. They were successful in immobilizing cells on gluten pellets, in fermenting glucose and sucrose in a shorter time (18 h), and in increasing the lactic acid production, 42 $g.l^{-1}$ and 41 $g.l^{-1}$, from glucose and sucrose, respectively.

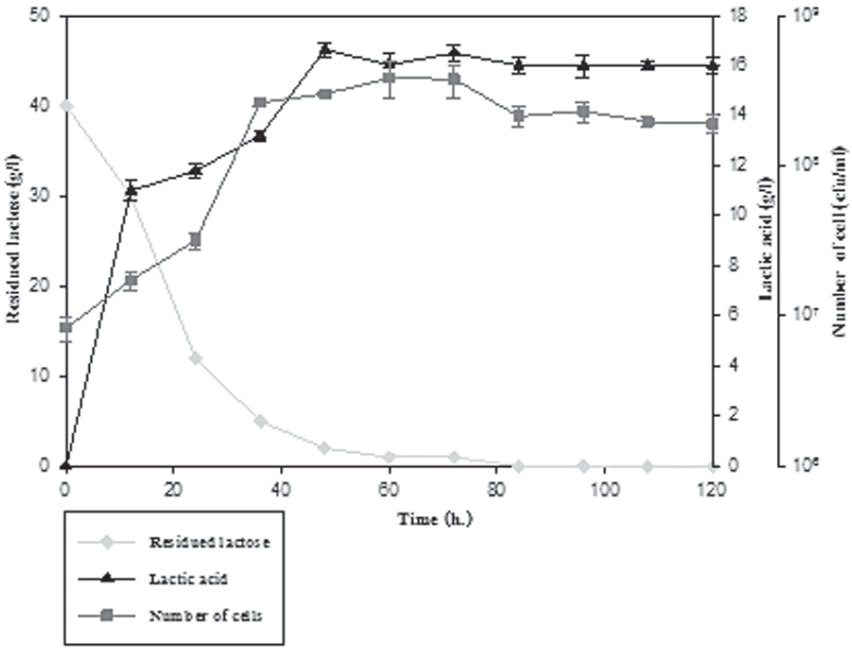

Figure 1. Lactic acid production and number of viable cells in mixed cultures of 5% *Lactococcus lactis* and 10% *Lactobacillus casei* in a two liters fermentor.

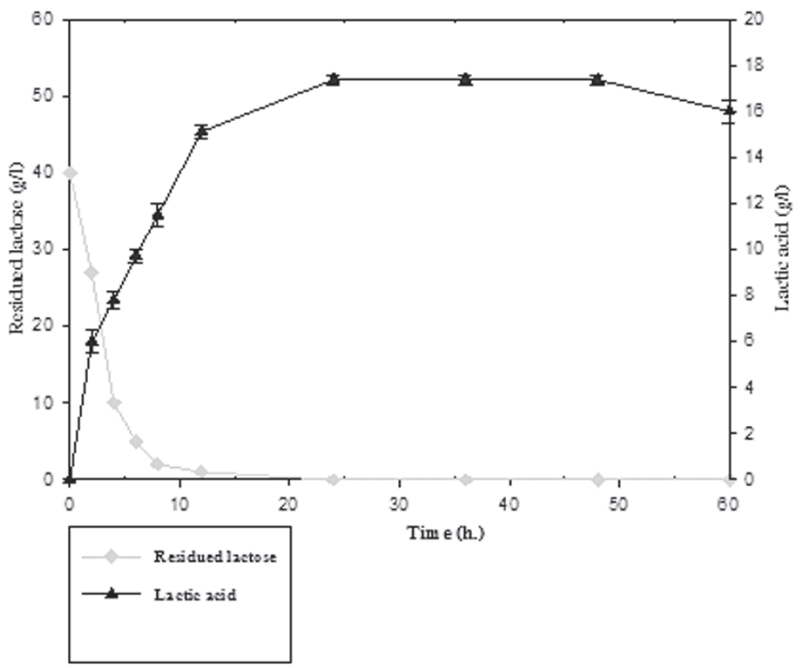

Figure 2. Lactic acid production by coimmobilized cells of 5% *Lactococcus lactis* and 10% *Lactobacillus casei* in a two liters fermentor.

Data of the lactic acid used for statistical analyses between free and coimmobilized cells in mixed cultures are shown in Table 3. Concentrations and productivities of lactic acid of the two treatments were significantly different (95% confidence level) but the yields of lactic acid of the two treatments were not significantly different. Concentration, yield and productivity by the coimmobilized cells were higher than those by the free cells in mixed cultures.

Table 3. Comparisons of lactic acid production by free and coimmobilized cells in mixed cultures of 5% *Lactococcus lactis* and 10% *Lactobacillus casei*

Form of cell	Fermentation time (h)	Concentration of lactic acid ($g.l^{-1}$)	Yield (g/g)	Productivity ($g.l^{-1}.h$)	Residued lactose ($g.l^{-1}$)
free cells mixed cultures	48	16.63±0.25	0.414±0.020	0.348±0.017	0
coimmobilized cells	24	17.38±0.16	0.434±0.004	0.724±0.005	0
p-value		0.001*	0.921^{ns}	0.001*	

ns, p-value > 0.05 means not significantly different at 99% confidence level
*, p-value 0.0001 < p-value < 0.005 means significantly different at 95% confidence level

Effects of fermentation by recycling coimmobilized cells of *L. lactis* and *L. casei* in a two liters fermentor

The fermentation by coimmobilized cells of 5% *L. lactis* and 10% *L. casei* in Batch 1 produced maximum lactic acid, 17.38 $g.l^{-1}$, in 24 h while in Batch 2, the maximum lactic acid produced was 12.51 $g.l^{-1}$ in 24 h. However, after Batch 2, the coimmobilized cells could not be reused for the next cycle because when the drain medium from the fermentor was removed before pulting the new medium, it was found that the amount of the immobilized gels was decreased and therefore, fermentation could not occur in the next cycle with the reason that pH in the fermentor was controlled by NaOH which dissolved the gels. Figure 3 shows the concentration of lactic acid from Batch 1 and Batch 2. When concentrations of lactic acid from both batches were compared, Batch 1 gave higher lactic acid than Batch 2. The residued lactose of Batch 1 and Batch 2 was 0 $g.l^{-1}$ and 10 $g.l^{-1}$, respectively.

The comparisons of lactic acid production from fermentations in both batches are shown in Table 4. Concentrations and productivity of lactic acid from both batches were significantly different (95% confidence level) while the yield of lactic acid the two treatment were not significantly different.

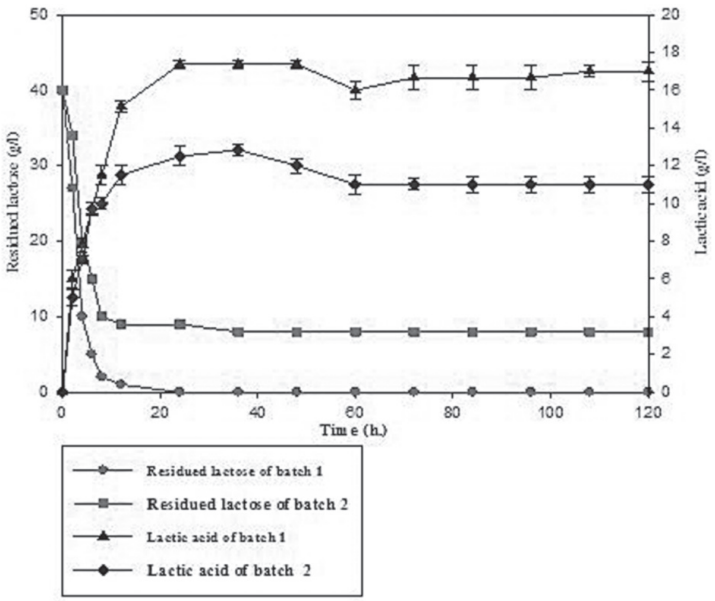

Figure 3. The comparisons of lactic acid production by coimmobilized cells of Batch 1 and Batch 2 in a two liters fermentor.

Table 4. Comparisons of lactic acid production by immobilized cells of 5% *Lactococcus lactis* and 10% *Lactobacillus casei* in Batch 1 and Batch 2.

Batch	Fermentation time (h)	Concentration of lactic acid ($g.l^{-1}$)	Yield (g/g)	Productivity ($g.l^{-1}.h$)	Residued lactose ($g.l^{-1}$)
1	24	17.38±0.16	0.434±0.004	0.724±0.005	0
2	24	12.51±0.41	0.403±0.013	0.521±0.017	10
p-value		0.001*	0.057^{ns}	0.001*	

ns, p-value > 0.05 means not significantly different at 99% confidence level
*, p-value 0.0001 < p-value < 0.005 means significantly different at 95% confidence level

CONCLUSION

Lactic acid can be produced efficiently from whey by mixed cultures and coimmobilized cells of *L. lactis* TISTR 1401 and *L. casei* TISTR 1341. It was found in this experiment that the mixed culture of 5% *L. lactis* and 10% *L. casei* was the optimal Initial inoculum size for lactic acid production. Lactic acid production by the mixed culture in a two liters fermentor using the agitation rate at 100 rpm produced higher lactic acid than those produced at 0 and 200 rpm. For fermentation in a two liters fermentor, the coimmobilized cells produced higher lactic acid than the free cells of the mixed culture and reduced the fermentation time. Coimmobilized cells had consistent potential and could recycle only two rounds of fermentation. Batch 1 produced 17.38 $g.l^{-1}$ lactic acid while that produced by Batch 2 was 12.51 $g.l^{-1}$ and the fermentation times in both batches were 24 h.

REFERENCES

A.O.A.C. Official Method of Analysis of A.O.A.C. 2000.International. 17th ed. A.O.A.C. International. The United States of America.

Chromopoulos, G., A. Bekatorou, E. Bezirtzoglou, A. Kaliafas, A.A. Koutinas, R. Marchant, and I.M. Banat. 2002. Lactic acid fermentation by *Lactobacillus casei* in free cell form and immobilised on gluten pellets. Biotechnology Letters 24: 1233-1236.

Dubois, M., K.A. Gijjes, J.K. Hamilton, P.A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substrate. Analytical Chemistry 28: 350-356.

Fitzpatrick J.J., C. Murphy, F.M. Mota, and T. Pauli. 2003. Impurity and cost considerations for nutrient supplementation of whey permeate fermentation to produce lactic acid for biodegradable plastics. International Dairy Journal 13: 575-580.

KiBeom, L. 2005. Comparison of fermentative capacities of lactobacilli in single and mixed culture in industrial media. Process Biochemistry 40: 1559-1564.

Marshall, K.R. 1982. Industrial isolation in developments in dairy chemistry. New York: Applied Science Publish.

Mostafa, N.A. 1995. Production of lactic acid from whey with agar immobilized cells in a continuous packed tubular reactor. Energy Convers 37: 253-260.

Nabil, N., N. Aicha, B. Amel, B. Chouki, B. Fabrice, and J. Boudrant. 2001. The effect of supplementation by Different nitrogen sources on the production of lactic acid from date juice by *Lactobacillus casei* subsp. *rhamnosus*. Bioresource Technology 78: 149-153.

Pauli T., and J.J. Fitzpatrick. 2002. Malt combining nuts as a nutrient supplement to whey permeate for producing lactic acid by fermentation with *Lactobacillus* casei. Process Biochemistry 38: 1-6.

Roukas, T., and P. Kotzekidou. 1998. Lactic acid production from deproteinized whey by mixed cultures of free and coimmobilized *Lactobacillus casei* and *Lactococcus lactis* cells using fedbatch culture. Enzyme and Microbial Technology 22: 199-204.

Sachin, R.K., S.P. Sudarshan, B.B. Kulbhushan, M. K. Jayant, and V.G. Digambar. 2006. Strain improvement of *Lactobacillus delbrueckii* NCIM 235 for lactic acid production. Process Biochemistry 41: 120-126.

Senthuran, A., V. Senthuran, R.H. Kaul, and B. Mattiasson. 1999. Lactic acid production by immobilized *Lactobacillus casei* in recycle batch reactor:a step towards optimization. Journal of Biotechnology 73: 61-70.

Sheng-Tsiung, H., and Y. Sheng-Tsiung. 1991. Propionic acid fermentation of lactose by *Propionibacterium acidipropionici:* Effects of pH. Biotechnology and Bioengineering 38: 571-578.

Youseef, C.B., V. Guillou, and A.O. Dichara. 2000. Modelling and adaptive control strategy in lactic acid fermentation process. Control of Engineering Practice 8: 1297-1307.

Yun, J.S., Y.J. Wee, and H.W. Ryu. 2003. Production of optically pure L(+)lactic acid from various carbohydrates by batch fermentation of Enterococcus faecalis RKY1. Enzyme and Microbial Technology 33: 416-423.