Cell entrapment of *Lactobacillus casei* subsp*. casei* ATCC 39392 for lactic acid production

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Abstract
In this study, lactic acid production by repeated batch fermentation using cell entrapped methods was compared. Barium alginate beads, agar gel and polyurethane foam cubes were employed as carriers to immobilize *Lactobacillus casei* subsp*. casei* for the purpose of L (+)-lactic acid production. Increasing concentrations of lactic acid during fermentation were better tolerated by barium alginate entrapped cells. Alginate beads had a considerable effect on lactic acid production and reduced the fermentation time by half. The volumetric productivity with barium alginate and agar immobilized cells were 0.625 and 0.425 (g/lh) respectively, whereas it was 0.375 (g/lh) for conventional free-cell fermentation. Beside biocompatibility, barium alginate immobilized cells exhibited good mechanical strength during repetitive fermentations and could be used in repetitive batch cultures for more than 40 days. The novelty of this study is lactic acid production by repeated batch fermentation with immobilized *L. casei* using polyurethane foam (PUF) in an economical culture medium composed of whey and corn steep liquor supplemented by glucose.
Keywords: Cell immobilization, Entrapment, Lactic acid production, *Lactobacillus casei* subsp*. casei.*

INTRODUCTION

Lactic acid, the first biotechnically produced chemical, has a wide range of applications in the food, pharmaceutical and cosmetic industries (Panesar, 2007) primarily as an acidulant, preservative agent and as a precursor for the production of emulsifiers such as stearoil-2 lactylates, in the baking industries (Mattey *et al*., 1992). Lactic acid is classified as GRAS (generally recognized as safe) by the Food and Drug Administration (FDA) in the US and other regulatory agencies. New applications such as its use as a monomer for the production of biodegradable plastics (Shen and Xia, 2006) and as an environmental-friendly chemical and solvent will increase future lactic acid demands (Hongo *et al*., 1986).

Conventional batch processes for lactic acid production suffer from reduced cell growth and productivity due to severe product inhibition (Datta *et al*., 1995). The efficiency of this process can be improved within limits by varying culture conditions and medium composition. Immobilization of bacteria has been one of the means for retention of high cell densities in the bioreactor (Goncalves *et al*., 1992). Immobilized cell technology has been successfully employed for various types of fermentation processes using lactic acid bacteria. Traditional fermented dairy products (yogurt, cheese, cream) as well as starter culture and metabolites have been produced with a higher productivity using immobilized cells (Norton and Vuillematd, 1994). In fact *L. casei* cells have been entrapped in certain support materials for lactic acid production. Lactic acid production from whey by immobilized *L. casei* in agar has been found to be more effective than polyacrylamide (Tuli *et al*., 1985). Calcium pectate gel (Panesar *et al*., 2007) and chemically modified chi-

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tosan beads have also been used as supports for *L. casei* immobilization (Goksungur *et al*., 2005). Alginate has so far been a popular matrix for immobilization of lactic acid bacteria. It is composed of mannuronic and glucoronic acid and can be connected by Ca^{2+} through binding of consecutive blocks of glucoronic acid (Shapiro and Cohen, 1997; Fett *et al*., 1986). Other supports used for immobilization include ceramic beads or porous glass (Goncalves *et al*., 1992), poraver recycled glass beads (Senthuran *et al*., 1999), and gluten pellets (Chronopoulos *et al*., 2002).

The objective of this study was comparison of a number of immobilization methods and selection of an appropriate carrier for lactic acid production using the *L. casei* strain at the laboratory scale. The performed investigations also included attempts to increase productivity, and choosing appropriate conditions needed for the batch process using a selected carrier. This knowledge can eventually be used for setting up a system allowing complete conversion of high concentrations of substrate during a shorter period of time but with a longer operational life time.

MATERIALS AND METHODS

Bacterial strains and culture conditions: *L. casei* sub sp. *casei* ATCC 39392 was obtained from the Persian type culture collection (PTCC, Tehran, Iran). Cells were cultured on slants of de Man, Rogosa and Sharpe (MRS) agar. An inoculum of 10^8 cells per ml was obtained by growing the anaerobic culture in a 250 ml Erlenmeyer flask containing 50 ml of MRS broth medium (Panesar *et al*., 2007). The cultures were incubated at 30°C (in order to obtain a better growth rate) with shaking at 150 rpm for 36 h on a rotary shaker incubator (Clim-o-shake, System, Kuhner, Switserland) (Mirdamadi *et al*., 2002). The medium used for lactic acid production contained (g/l): glucose 80, whey 50, corn steep liquor (CSL) 20, sodium citrate 0.1, $MgSO_4.7H_2O$ 0.2, $MnSO_4.H_2O$ 0.03, $FeSO_4.H_2O$ 0.03. It was sterilized by autoclaving at 121ºC for 15 min. This production medium was optimized by the one factor at a time method (Unpublished data). Whey (from Pegah dairy industries, Iran) and CSL (from Glocosan, Iran) were used as industrially and economically available substrates. The other media and salts were obtained from Sigma and Merck (USA, Germany).

Free cells of *L. casei* were cultured in a 250 ml Erlenmeyer flask containing 50 ml of production medium after inoculation with 5 ml of preculture containing 10^8 cells/ml (size of inoculum was 10% (v/v) of production medium). Fermentation was carried out at 42ºC (in order to obtain a better lactic acid production rate) with shaking at 180 rpm on a rotary shaker incubator until the glucose was consumed entirely. The pH value of the medium was maintained at approximately 4.8-5 by adding 1% (w/v) calcium carbonate (sterilized separately) during the process of fermentation. (Mirdamadi *et al*., 2002) The fermentation with immobilized cells was performed under the same conditions as the free cells. The fermentation with free cells was used as a control. Two ml of the culture samples were taken every 24 h for the measurement of pH, glucose consumption and lactic acid production. Each experiment was performed in triplicate.

Immobilization of cells

Entrapment in alginate: Cells (1×10^8 cfu) were harvested from MRS broth during early stationary phase routinely by centrifugation at $5500\times g$ (refrigerated centrifuge, Model J2-21, Beckman, USA) for 10 min. The supernatant was removed and the pellet was mixed with 40 ml of 2% (w/v) sodium alginate (Merck, Germany) that was prepared previously by dissolving 0.8 g of sodium alginate in 40 ml of distilled water. The bead-forming solution was dropped into 2% (w/v) $BaCl_2$ solution with mild stirring maintained for 30 min at room temperature in order to cure. The resulting beads which had 2 mm diameters were washed twice with normal saline and used as the biocatalyst (Shen and Xia, 2007).

Entrapment in polyurethane foam: Polyurethane foam (PUF) cubes (1×1×1 cm) were weighed and placed into 250 ml Erlenmeyer flasks containing 50 ml of MRS broth and autoclaved at 121ºC for 15 min (Dong *et al*., 2000). Five ml of 24 h grown MRS culture broth was then inoculated into each flask and incubated with the same conditions as the preculture. After 24 h the culture broth was decanted completely and fresh production medium was transferred into each flask (PUF-1). in second procedures (PUF-2) the MRS broth pellet was mixed with two subunits of PUF (Eli-Chem Resins System UK Ltd). After hardening, the foam was cut into blocks (1 ×1 ×1 cm) and used as the biocatalyst.

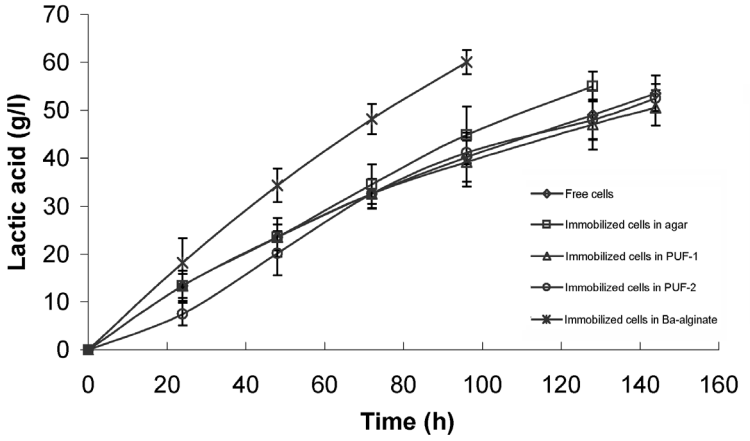

Figure 1. Production of lactic acid by free and different carrier immobilized cells of *L. casei*.

Entrapment in agar: Cells were harvested from MRS broth by centrifugation at 5500 ×*g* for 10 min. The supernatant was removed and the pellet mixed with 10 ml of previously sterilized 2% (w/v) agar. The hot agar solution was cooled to 45ºC and *L. casei* cells were added with slow stirring and the agar-cell suspension was poured onto a sterile plastic plate. The mixture was allowed to solidify. The solid layer was cut into 0.5 cm blocks and used as the biocatalyst. All the immobilization steps were carried out under aseptic conditions.

Analytical methods: Biomass concentration was determined by measuring optical density at 610 nm (OD_{600}) (Unicam 8620 uv/vis spectrometer, England). Glucose was determined using an enzymatic kit (Boehringer Mannheim Biochemica), Total lactate was determined calorimetrically using p-phenyl phenol, according to the method by Kimnberely and Taylor (1996).

RESULTS

The effect of Immobilization on productivity: Comparative data on lactic acid production and the yield and productivity of fermentations by free cells and different carrier immobilized cells are shown in Figures 1 and 2, respectively. As shown in Figure 2, there were not any remarkable differences between free and immobilized cells with respect to fermentation yields. However, there were noticeable differences in volumetric productivity of fermentations. The volumetric productivity of barium alginate and agar immobilized cells were 0.625 and 0.425 (g/lh) respectively, whereas it was 0.375 (g/lh) for conventional free-cell fermentation. Results also indicated no further increase by polyurethane immobilized cells. The productivity of fermentation by barium alginate was enhanced by 68% whereas in the case of agar immobilized cells, it was enhanced by 13% in comparison to conventional free-cell fermentation.

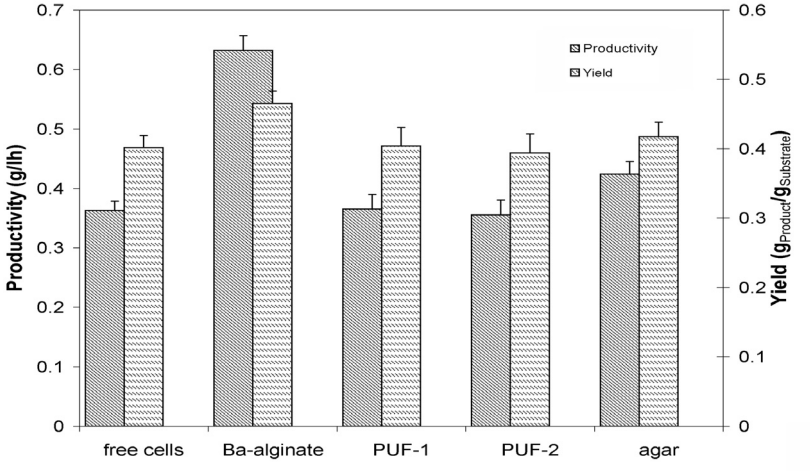

Figure 2. Comparison between the yield and productivity of the fermentations between free and immobilized *L. casei* in different carriers.

Table 1. Repetitive batch cultures by alginate beads.

Batch Number	Fermentation time (h)	Lactic acid (g/l)	Yield ($g_{product}/g_{substrate}$)	Productivity (g/lh)
1	96	61.52 ± 0.03	0.473	0.641
2	96	60.13 ± 0.191	0.463	0.626
3	102	57.93 ± 0.053	0.446	0.568
4	120	54.56 ± 0.110	0.425	0.455
5	144	50.17 ± 0.225	0.386	0.348
6	192	43.85 ± 0.596	0.337	

Table 2. Repeated batch cultures by PUF cubes.

Batch Number	Fermentation time (h)	Lactic acid (g/l)	Yield ($g_{product}/g_{substrate}$)	Productivity (g/lh)
1	144	54.52 ± 0.349	0.425	0.378
2	150	50.13 ± 0.191	0.386	0.334
3	168	47.93 ± 0.091	0.375	0.285
4	192	41.56 ± 0.265	0.319	0.216

Repeated batches using immobilized cells: The repeated batch fermentations were conducted using barium alginate and PUF immobilized cells. The fermentation was performed until total glucose was consumed. Then supernatant was decanted and beads and cubes were washed with normal saline and then transferred into the fresh medium for a second round of fermentation. The process of fermentation was carried out for 40 days with beads and 30 days with cubes. Increase in lactic acid production with barium alginate immobilized cells was observed up to the 4th cycle followed by a gradual decrease in lactic acid production (Table 1). However, there was no such increase with PUF immobilized cells (Table 2). Moreover, in the case of barium alginate immobilized cells, fermentation time reached 192 h after 6 batches, but for PUF immobilized cells this time period was achieved after 4 batches, which further confirms that barium alginate is a more effective carrier for lactic acid production. It should also be noted that the presence of higher concentrations of lactic acid caused a decrease in the stability of barium alginate structure.

DISCUSSION

Several methods for cell immobilization exist. The choice depends on the specific task and is determined by factors of which most can be assessed only from practical experience. However, certain general aspects should be taken into account. If the immobilized cells are expected to catalyze a particular chemical reaction, the method of immobilization will depend on the nature of this reaction. The catalyst must be stable under conditions of its immobilization. Mechanical properties of the support, specially the gel strength, its physical shape, and chemical resistance, are also important. These properties determine how easily the cells to be immobilized are washed out from the gel during immobilization. Among various cell immobilization methods entrapment in calcium alginate beads has commonly been used for cell immobilization of lactic acid bacteria (Wang *et al*., 1995). However calcium alginate gels are chemically unstable on contact with various cation-chelating agents such as phosphate, citrate and lactate which can cause bead disruption and dissolution. It has been reported that barium alginate beads are chemically and physically more stable in electrolyte solutions than conventional calcium alginate beads (Lee *et al*., 1993).

As shown in Figure 1, a maximum concentration of lactic acid (60 g/l) was obtained by *L. casei* entrapped in barium alginate after 96 h of fermentation. Namura *et al*. (1987) reported that the maximum concentration of lactic acid (80 g/l) was obtained by *Lactobacillus delbrueckii* entrapped in calcium alginate beads after 120 h of fermentation, while Roukas and Kotzekidou (1990) reported 41.3 (g/l) of lactic acid with co-immobilized *L. casei* and *Lactobacillus lactis* cells after 48 h of fermentation. Yoo *et al*. (1996) reported that the highest concentration of lactic acid (98.1 g/l) was obtained by *L. casei* entrapped in barium alginate after fermentation time (32 h) in Glucose

yeast extract medium (GY medium) containing yeast extract 30 (g/l) as a supplement. However, medium composition plays a vital role in the improvement of the efficiency and economics of the ultimate lactic acid production. Taking this factor into account, in this research yeast extract which is a costly nitrogen source was replaced with a less expensive source, corn steep liquor, though the consequent decrease in the yield and productivity of fermentation was inevitable.

Although the entrapment methods using soft gels such as barium alginate are mostly employed, there are some disadvantages using alginate gels. Sun and Furusaki (1990) reported that using the gel-entrapment methods, the limitation of oxygen supply by the diffusional resistance of the gel matrix might decrease the fermentation rate and it may affect L (+)-lactic acid transformation. PUF is a popular matrix for cell immobilization in bioremediation applications such as hydrocarbon degradation in oil pollutions and other toxic waste treatments (Oh *et al*., 2000). PUF has pores that are large relative to cellular dimensions, making it possible for high levels of cellular adhesion within the pores. Moreover, its highly porous structure facilitates total surface exposure. Dong *et al*. (1996) have used *Rhizopus oryzae* immobilized on PUF for the purpose of lactic acid production. According to their results, there is no difference between the yield of fermentation by free and immobilized cells, however, they were able to reduce fermentation time from 30 to 10 h. In this study the yield of fermentation by immobilized *L. casei* on PUF cubes was compatible with the results obtained by Dong *et al*. (1996). As it is shown in Figure 2, there is no significant difference in the productivity of the fermentation between free and PUF immobilized cells. Agar seems to be a better carrier than PUF, but it is not mechanically strong and since the reaction is performed at high shaking speed, agar can not be a good choice for fermentations lasting for long periods. Roukat and Kotzekidou (1990) have found that calcium alginate is a better support for lactic acid production than κ-carrageenan, agar and polyacrilamide gels. Our results are in agreement with Roukat and Kotzekidou and besides alginate's favorable characteristics for use as a matrix, such as its biocompatibility, low toxicity and easy bead formation by ionotrophic gelation, it is also more desirable for use in lactic acid production processes on the laboratory scale. On the other hand the highly porous structure of PUF facilitates total surface exposure, but our data show that alginate acts as a better carrier for lactic acid production.

According to the experiments of this study, immobilization of *L. casei* subsp. *casei* on barium alginate is the best approach for lactic acid production on a laboratory scale. It also has the capability of being used during repeated batch cultures for lactic acid production. Further investigations are underway to increase the strength of the barium alginate beads.

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