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VIABILITY OF CALCIUM ALGINATE ENTRAPPED *LACTOBACILLUS CASEI* DURING CONTINUOUS LACTIC ACID PRODUCTION

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Abstract: *Lactobacillus casei* cells harvested in log phase (20h) were entrapped in calcium alginate (10.0g l^{-1}). When the beads were packed in a thermostated (42°C) column (30.0 x 2.0 cm), the optimum flow rate for maximum lactic acid yield (74.0%) was 18.0 ml h^{-1}. At this flow rate, the productivity obtained was 21.2g l^{-1} h^{-1}. With increase in the flow rate, viable cells in the beads were decreased. However the cell number was more than those present initially. When different concentrations of glucose in the range of 50.0 to 250.0g l^{-1} was passed at 18.0ml h^{-1}, maximum lactic acid yield (81.6%) was obtained at the concentration of 125.0g l^{-1}. With an increase in glucose concentration from 50.0 to 100.0g l^{-1}, the viable cells in the beads were increased and further increase in glucose concentration in the feed decreased the viable cell number. When the nutrient medium containing 125.0g l^{-1} glucose was passed continuously, steady state was observed after 3 days and continued for 16 days. The viable cell number in the beads was constant for 16 days.

Key Word: Flow rate, immobilization, lactic acid, *Lactobacillus casei*, viable cell count.

INTRODUCTION

Lactic acid production from bacteria is more attractive than chemical methods. One of the major hindrances with the fermentation technique is product inhibition with decrease in pH[1-3]. In conventional methods, lactic acid was removed from the fermentation broth as calcium lactate[4]. Later studies revealed that use of immobilized cells[5-9] or electrodialysis[10-11] or extractive bioconversions[12-13] reduce product inhibition with increase in productivity. However viability of the entrapped cells in different polymers is another hindrance for their use[6,14-15]. In this paper we present some studies of the viability of *Lactobacillus casei* entrapped in calcium alginate during continuous operations under different conditions.

METHODS AND MATERIALS

Organism
Lactobacillus casei DSM 20021

Analytical methods

Glucose and lactic acid were measured by HPLC (Shimadzu, Auto injector). Sugar Pak™, (Waters, Millipore, USA) stainless steel column (300 x 6.5, microparticulated

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silica gel) was used and the mobile phase was deionized water. Reference time for glucose was 8.6min. Samples collected at different time intervals were prepared for lactic acid estimation by filtering through a 0.45mμ Milipore filter. Viable cell count of the immobilized *L.casei* was determined by dissolving 2.0g (wet weight) of entrapped cells in 30.0 ml of 0.1M phosphate buffer (pH 6.5) and growing the cells in MRS agar plate (MRS broth, 50.0g l^{-1} and agar, 20.0g l^{-1}).

Cultivation and harvesting of L. casei for immobilization

The inoculum was prepared in MRS broth (52.0g l^{-1}, 50.0ml) at pH 6.5 by inoculating with 100μl *L. casei* from frozen sample and incubating at 42°C for 14 h (300 rpm). Nutrient medium (2.5l) in a fermenter (working volume 5.0l) was inoculated with 2.5ml of inoculum and incubated at 42°C while mixing. The pH was maintained by titrating with sterile 8N NaOH. Nutrient medium at pH 6.5 contained the following in gl^{-1}, glucose 150.0, yeast extract, 10.0; K_2HPO_4, 0.5; KH_2PO_4, 0.5 and sodium citrate, 1.0 and salt solution, 1.0 ml. The salt solution contained in gl^{-1} $MgSO_4.7H_2O$, 50.0; $MnSO_4.H_2O$, 3.1; Fe $SO_4.7H_2O$, 2.0 and ascorbic acid, 5.0. *L. casei* cells were harvested at 20h by centrifuging (12000 rpm) in a refrigerated centrifuge at 10°C for 15min. Cells washed three times with saline were used for immobilization. *L. casei* harvested at 20h (in log phase for immobilization had the optical density of 12.8 (620nm), dry weight of 6.9g l^{-1} and the viable cell count of 9.0×10^{10}.

Immobilization of L. casei

L. casei cells (10.0g wet weight) suspended in 30.0ml of sterile sodium alginate solution (10.0g l^{-1}) were extruded by syringe through a needle (0.7 mm diameter) into 10.0% (w/w) glucose – 0.05M $CaCl_2$ (1.0 l) solution and the beads were left in the solution for 2.0 h. Beads were washed with 0.05M maleate – 0.05M $CaCl_2$ buffer (pH 6.5). Immobilized cells in the beads were incubated with nutrient medium for 72h and washed with sterile 0.05M $CaCl_2$ solution. Beads were packed in a jacketed column (30.0 x 2.0 cm) for further studies. Temperature of the column was maintained by circulating hot water from a water bath at 42°C.

Optimization of conditions for continuous lactic acid production

Effect of flow rate: Nutrient medium was pumped at flow rates ranging from 6.0 to 36.0ml h^{-1} into the column. For each flow rate the column was repacked with freshly entrapped cells. Residual glucose and lactic acid in the spent medium were estimated. Viable cells in the beads were determined.

Effect of glucose concentration: Nutrient medium containing varying concentration of glucose (from 50.0 to 250.0g l^{-1}) was pumped at optimum flow rate into the column.[2] The experiment was continued as above.

Continuous fermentation of glucose to lactic acid: Nutrient medium containing optimized amount of glucose was pumped into the column at the optimum flow rate. Fractions were collected at different time intervals and analysed for lactic acid.

RESULTS

Optimum conditions for continuous lactic acid production

Glucose was completely fermented when the flow rate was increased up to 12.0ml h^{-1}. But lactic acid yield has decreased with an increase in flow rate (Figures 1 and 2). Lactic acid productivity was increased from 8.8 to 21g$l^{-1}h^{-1}$ when the flow rate was increased from 6.0 to 24.0ml h^{-1}. Viable cells entrapped in the beads before passing the medium was 23.0 x 10^7cells g^{-1} wet bead. With an increase in flow rate from 6.0 to 36.0ml h^{-1} the viable cell count in the bead was higher than what was present initially (Figure 1). Even though the productivity (21.5g l^{-1} h^{-1}) was highest at the flow rate of 24.0ml h^{-1}, 18.0ml h^{-1} was selected (where the productivity was 21.2g l^{-1} h^{-1}), because at this flow rate lactic acid formed was about 18.0% more than that at 20.0ml h^{-1}.

Optimum concentration of glucose for maximum lactic acid production was 125g l^{-1} at the optimum flow rate (18.0ml h^{-1}, Figure 3). When the glucose concentration was increased from 50.0 to 250.0g l^{-1}, maximum productivity of 22.3g $l^{-1}h^{-1}$ was obtained at the glucose concentration of 150.0g l^{-1} (Figure 4). Above 125g l^{-1} glucose in the medium, the residual glucose also increased (Figure 3). The viable cell count in the freshly entrapped beads was 23.0 x 10^7 cells g^{-1} wet bead. With increase in glucose concentration, proliferation of the cells has taken place and the cell number has increased up to 91.8 x 10^{13} while the glucose concentration was 100.0g l^{-1}. But with further increase of glucose concentration, the cell multiplication has decreased (Figure.3).

Continuous fermentation of glucose by immobilized *L. casei*

The steady state of lactic acid production was observed after 3 days and was continued for 16 days without problem. But after the 17th day, lactic acid production started to decline. The cell number in the beads showed increase in number initially but after the third day the increase was not significant with an increase in the cell leakage into the medium. However by the 10th day it was observed that the cell leakage was pronounced and the disintergration of the beads had started. By the 26th day leakage of the cells was obvious due to breaking of the beads. The process was terminated by the 32nd day (Figure 5).

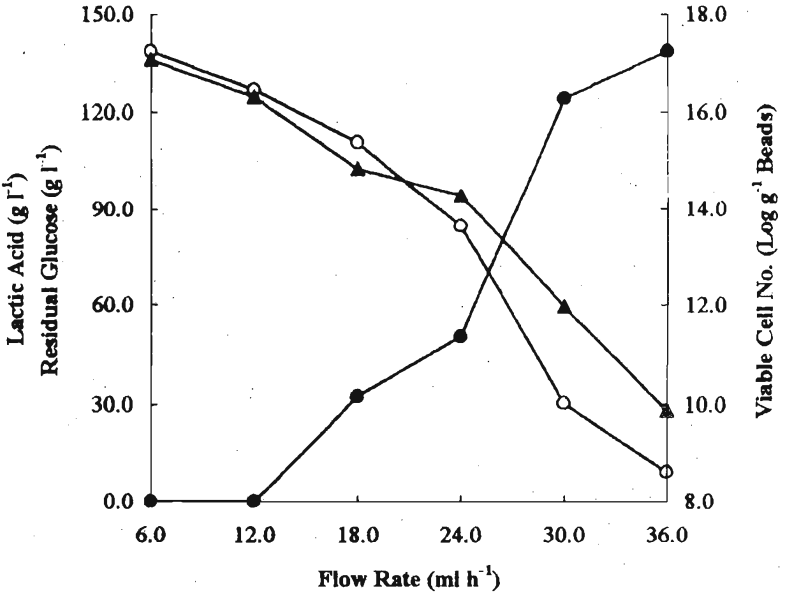

Figure 1 : Effect of flow rate on (○) lactic acid production and (●) residual glucose in the medium and (▲) viable cells in the beads when nutrient medium was passed at pH 6.5 and 42°C into a column packed with calciumn alginate entrapped *Lactobacillus casei.*

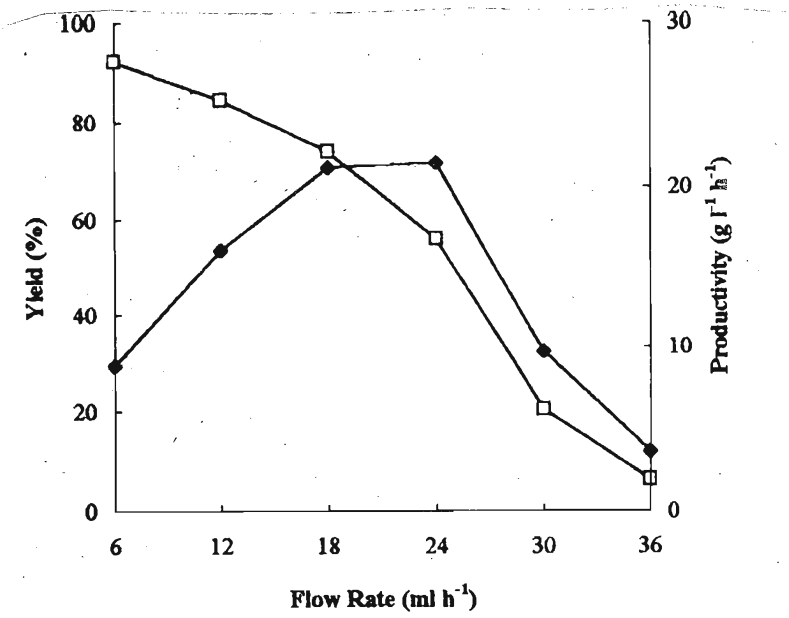

Figure 2: Effect of flow rate on lactic acid (□) yield and (■) productivity when nutrient medium was passed at pH 6.5 and 42°C into a column packed with calcium alginate entrapped *Lactobacillus casei.*

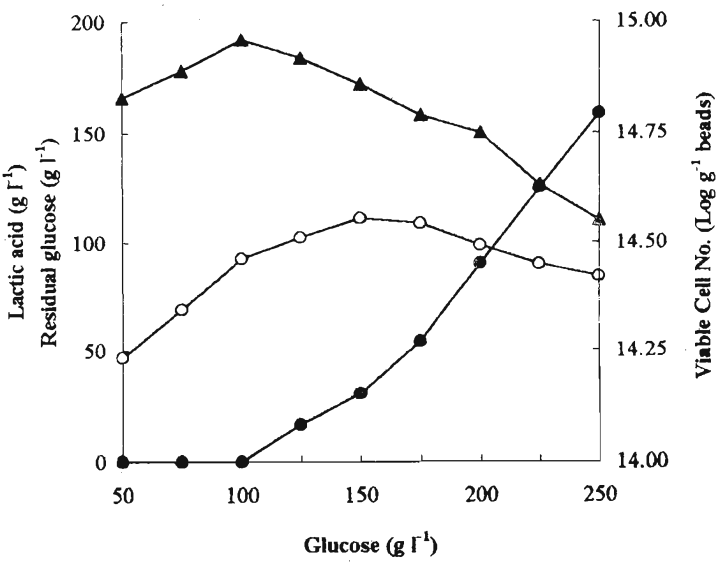

Figure 3: Effect of glucose concentration in nutrient medium on (○) lactic acid production,(●) residual glucose in the medium and (▲) viable cells in the beads when nutrient medium was passed at optimum flow rate (18.0 ml h^{-1}), pH 6.5 and 42°C into a column packed with calcium alginate entrapped *Lactobacillus casei.*

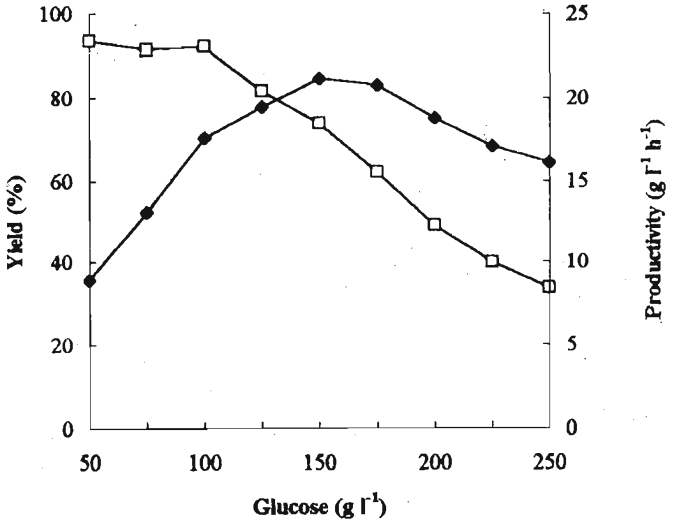

Figure 4: Effect of glucose concentration present in nutrient medium on lactic acid (□) yield and (■) productivity when nutrient medium was fed at 18.0 ml h^{-1}. pH 6.5 and 42°C into a column packed with calcium alginate entrapped *Lactobacillus casei.*

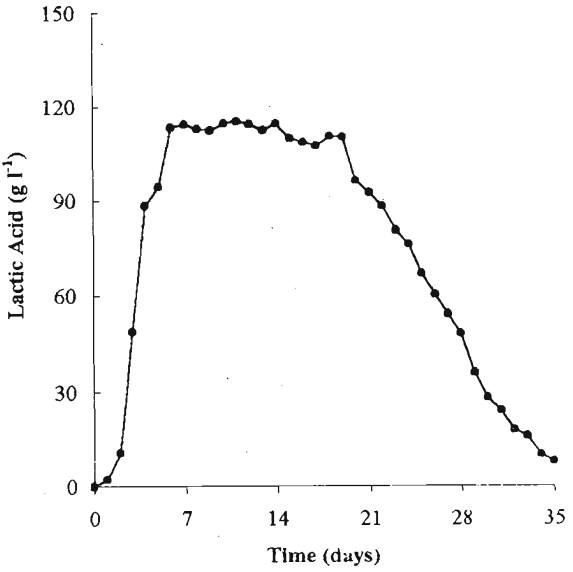

Figure 5: Continuous lactic acid production by calcium alginate entrapped *Lactobacillus casei* when nutrient medium containing 125 g l^{-1} glucose was fed at 18.0 ml h^{-1} in to the column, pH 6.5 and 42°C.

DISCUSSION

The increase in flow rate decreased glucose consumption. Reduction in lactic acid yield was due to the reduction in contact time between the cells and the substrate and leakage of the cells from the beads. Boyaval and Goulet[16] have reported that the decalcification of the calcium alginate by lactic acid was pronounced at low dilution rates and the level of the leakage of the cells was also high in such cases. Thus, it is better to change to a flow rate, which gives higher productivity and product yield to avoid decalcification of the calcium alginate beads. *Lactobacillus helveticus* entrapped in *k*-carrageenan-locust bean gum gel at a dilution rate of 1.2 l h^{-1} gave a productivity of 28.5g $l^{-1}h^{-1}$ [17], which is comparable to our results. The viable cell count in the immobilized preparation was increased even when the flow rate was increased. Thus the cell multiplication more than compensated for the cell leakage. This also indicated that the sodium alginate did not show toxicity to *L. casei*. However the inhibitory effect of free chitosan was demonstrated for lactic acid bacteria growth.[14-15]

Increase in glucose concentration beyond 100.0g l^{-1} has decreased the cell multiplication. This is due to the osmotic effect of high sugar concentration and lactic acid inhibition on cell metabolism.[1] Diffusional limitations for the substrate and product in the microenvironment would have been prominent with increased cell number in the beads. All these factors would have contributed to the decreased cell viability with increased glucose concentration.

The main defect in our experiment was that in the feed medium no calcium was included.[18-19] Calcium ions which stabilize calcium alginate would have been taken away by lactate ions produced during fermentation.[20] Wada *et al.*[21] have reported the weakening of the gel network during growth of the cells. *L. helveticus* entrapped in calcium alginate has shown a productivity of 8.0g l^{-1} h^{-1} with 50% conversion of lactose (5.0g l^{-1}) where the flow rate of the substrate was 400ml h^{-1}. After a week's operation, clogging in the packed bed was observed.[17] Stenroos *et al.*[22] reported that calcium alginate entrapped *L. delbrueckii* has yielded 97.0% of lactic acid and the biocatalyst with a half life of 50.0 days. In our experiment the lactic acid yield was 92.0%. In the experiment of Stenroos *et al.*[22] the steady state was reached after one week and in our case it was faster (3 days). With *L. bulgaricus* immobilized in calcium alginate, 50-60% lactic acid was obtained and the process continued for 25 days while reaching the steady state after 10 days.[24] In *k*-carrageenan- locust bean gum gel beads four different strains of mesophilic lactic acid bacteria were entrapped and the productivities were high for eight weeks.[25] However microscopic observation showed a progressive destruction of the beads and release of viable cells from peripheral cavities of the gels.[25]

L. casei cells entrapped in calcium alginate yielded maximum lactic acid at 18.0ml h^{-1} flow rate with a productivity of 21.2g l^{-1} h^{-1}. With increase in the flow rate, viable cells in the beads were decreased. However the cell number was more than those present initially. Maximum lactic acid yield (81.6%) was obtained at the glucose concentration of 125.0g l^{-1}. The viable cells in the beads increased with increase in glucose concentration. When the nutrient medium containing 125g l^{-1} glucose was passed continuously, steady state was reached after 3 days and continued for 16 days and the viable cell number in the beads was constant for 16 days.

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