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Production of Glutamic Acid Using *Brevibacterium roseum* with Free and Immobilized Cells

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Abstract: Experimental studies were carried out to produces L-glutamic acid with *Brevibacterium roseum* free cells, immobilized *B. roseum* and co-immobilized *B. roseum* and *Escherichia intermedia* type 1, production conditions were optimized with *B. roseum* free cells. Incubation time 96 h, pH 6.0, temperature 30°C, 150 rpm agitation and biotin 1.0 mg mL^{-1} were optimum for the maximum yield of glutamic acid. When the nutrient ingredients were optimized the glutamic acid yield increased to 40.5 mg mL^{-1}. Immobilized *B.roseum* produced 37.2 mg mL^{-1} glutamic acid. Immobilized conditions like alginate concentration, calcium ion concentration, storage period of beads and initial cell loading were optimized. Co-immobilized *B. roseum* and *Escherichia intermedia* type-1 produced 39.6 mg mL^{-1} glutamic acid.

Key words: L-Glutamic acid, *Brevibacterium roseum*, *Escherichia intermedia* type 1, immobilization, co immobilization

INTRODUCTION

The demand for amino acids as food supplements like fortification of vegetable protein and in pharmaceutical industries is fast expanding. Kinoshita *et al*. (1957) isolated L-glutamic acid producing microorganisms and subsequent research brought about the economic production of L-glutamic acid by fermentation process. This opened the way to fermentative production of various amino acids. Mono Sodium L-glutamate (MSG) is the largest product out of all amino acids and the recent survey indicates that the annual production level is of 1.5 million tons and the market is growing by about 6% per year. Fermentative production of L-glutamic acid has been employed during last few decades and the production yield has been improved considerably over the years (Tauro *et al*., 1963; Kim and Ryu, 1982). In the last few years different strategies were employed to optimize the glutamate fermentation. These include cell recycling (Izhizaki *et al*., 1993), culturing the microorganisms in the solid substrates (Nampoothiri and Pandey, 1996b), nutrient formulation (Nampoothiri and Pandey, 1995) and the use of different raw materials (Das *et al*., 1995; Nampoothiri and Pandey, 1996b). The practical importance of immobilized cells/enzymes has been successfully applied to large scale production of various fermentation products with reduced process cost. The applications of immobilized process have been highlighted from the view point of long term utilization of bio catalysts and continuous operation of stabilized systems (Fukui and Tanaka, 1982; Nampoothiri and Pandey, 1998). A number of amino acids have been produced using this methodology. Amin *et al*. (1993) made an attempt to study the formation of by-product during glucose conversion to glutamic acid using *Corynebacterium glutamicum* immobilized in polyurethane foam. Entrapment of protoplast of *Brevibacterium flavum* in matrices of agar-acetyl cellulose filtering to produce L-glutamic acid (Karube *et al*., 1985). Binding of the

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defective enzyme from an external source to free or immobilized microorganisms or immobilization of mixed culture capable of carrying out two or multi step conversions into single step conversions leads to co-immobilized cells can open up new possibilities of synergetic action and result in more yield/conversion which cannot be obtained to the same extent by separately immobilized cells (Jagannadha Rao, 1992).

The present study is aimed to determine the feasibility of glutamic acid production using free, immobilized and co immobilized cells of *Brevibacterium* and *E. intermedia* type-1 entrapped in calcium alginate beads. Glutamic acid production conditions were optimized which include fermentation, pH, temperature, gel concentration, bead size and initial biomass.

MATERIALS AND METHODS

Microorganisms

Brevibacterium roseum NCIM 2270 and *Escherichia intermedia* type-1 NCIM 2490, obtained from National Chemical Laboratory, Pune, India were used for this study.

Growth Medium and Growth Conditions

Cultures were maintained on agar slants having composition (g L^{-1}) peptone-10, Beef extract-10, Sodium chloride-5 and Agar-20. The pH of the medium adjusted to 7.0 and incubated at 37°C for 24 h. For the seed culture development, the following inoculation medium was used containing (g L^{-1}) glucose-25, yeast extract-10, urea-8, K_2HPO_4-1, $MgSO_4.7H_2O$-2.5, the pH of the medium was adjusted to 7.0 before inoculation.

Inoculum Preparation and Cell Harvest

A fully grown slant of 24 h old *Brevibacterium roseum* and *Escherichia intermedia* type 1 were scrapped off and suspended in 0.01 M citrate buffer (pH-7.0). The cell suspension was shaken thoroughly to break up the aggregates. The cell count was determined by plating 1 mL of above cell suspension, after making serial dilution. The cell count is adjusted in the range of 10^{-7} to 10^8 cells mL^{-1}. The cells were grown for 24 h at 30°C in 250 mL Erlenmeyer flasks containing 50 mL of inoculation medium on a rotary shaker at 180 rpm. Cells were separated from the inoculation medium by centrifugation and washed thoroughly with 0.01 M citrate buffer (pH 7.0).

Preparation of Calcium Alginate Beads

The cell suspension is slowly added to the ether sterilized sodium alginate (3% w/v) and mixed thoroughly with sterile glass rod. The mixture was extruded as drops into a solution of $CaCl_2$ (0.5 M). Bead size was controlled by gauge number of the hypodermic needle used during extrusion. The beads were cured in the same solution at room temperature for an hour and stored in a freshly prepared 0.1 M $CaCl_2$ solution at 4°C.

Production Medium and Conditions

Production medium contained (g L^{-1}): glucose-5.0, Urea 5, K_2HPO_4 1.2, $CaCO_3$ 16, biotin 0.05 and (mL L^{-1}) mineral solution, 10 $FeSO_4.7H_2O$; $MnSO_4.4H_2O$; $MgSO_4.7H_2O$; $ZnSO_4.6H_2O$ and NaCl each 10 mg. Glucose and mineral solution were separately autoclaved and mixed aseptically. $CaCO_3$ was sterilized by dry heat sterilization and added to the production medium. Urea and biotin were filter sterilized. The pH of the medium was adjusted to 7.0. All fermentations were carried out in 250 mL Erlenmeyer flasks containing 100 mL of production medium at 30°C on a rotary shaker.

Analytical Methods

Reducing sugars were determined by Miller (1959). Thin layer chromatography (Silica gel G, solvent moisture: n-butanol/glacial acetic acid/water 4:1:1 v/v) was used for the qualitative determination of L-glutamic acid (Brenner and Neiderwiser, 1967) and it was estimated quantitatively by a nin-hydrin color reaction (Spies, 1957).

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RESULTS AND DISCUSSION

Glutamic acid production conditions like fermentation time, pH, temperature, agitation and biotin concentration were optimized.

The influence of fermentation time on glutamic acid was shown in Table 1. The fermentation time from 36 to 108 h with 12 h increments were studied. The maximum glutamic yield is 27.6 mg mL^{-1} at 96 h of incubation and the lowest being 15.9 mg mL^{-1} at 36 h. The effect of pH on glutamic acid was determined. The pH range between 4.5 to 6.5 is used for the study. Table 2 indicates that the maximum glutamic acid (27.8 mg mL^{-1}) was recorded at pH 6.0.

The production medium was adjusted to pH 6.0 and incubated at different temperatures (26-34°C). The maximum yield of glutamic acid was observed at 30°C temperature (Table 3). The production medium with fermentation pH 6.0, 96 h and 30°C temperature were incubated at different agitation ranging from 100 to 300 rpm (Table 4). The change of agitation did not affect the glutamic acid content but a marginal increase (28.1 mg mL^{-1}) was observed with 150 rpm.

Table 1: Effect of fermentation time on glutamic acid production

Fermentation time (h)	Glutamic acid (mg mL^{-1})
36	15.9
48	16.4
60	18.5
72	21.2
84	23.0
96	27.6
108	24.6

Table 2: Effect of pH on glutamic acid production

pH	Glutamic acid (mg mL^{-1})
4.5	20.9
5.0	24.3
5.5	26.3
6.0	27.8
6.5	25.1

Table 3: Effect of temperature on glutamic acid production

Temperature (°C)	Glutamic acid (mg mL^{-1})
26.0	22.8
28.0	24.6
30.0	27.8
32.0	25.4
34.0	23.6

Table 4: Effect of agitation on glutamic acid production

Agitation (rpm)	Glutamic acid (mg mL^{-1})
100	27.5
150	28.1
200	27.8
250	25.4
300	23.9

Different biotin concentrations were employed in the production medium (0.8, 1.0, 2.0, 3.0 and 4.0 mg mL^{-1}) to determine its effect on glutamic acid. Biotin concentration of 1.0 mg mL^{-1} was found to be optimum with 32.7 mg mL^{-1} of glutamic acid. When the medium ingredients were optimized, the yield of glutamic acid increased from 37.2 to 40.5 mg mL^{-1} (data not shown).

Immobilization of *Brevibacterium roseum*

Employing the same production medium and optimum parameters, experiments were carried out with immobilization of *Brevibacterium roseum* and co-immobilization of *Brevibacterium roseum* and *Escherichia intermedia* type-1.

Immobilization of *Brevibacterium roseum*

Production of glutamic acid with immobilized *B. roseum* was carried out and optimized the parameters which include effect of alginate concentration, $CaCl_2$ concentration, storage periods of beads and initial cell loading on glutamic acid.

Effect of Alginate Concentration

Sodium alginate concentrations 2, 3 and 5% (W/V) were used for the immobilized beads. The maximum concentration of glutamic acid was obtained with 3% calcium alginate beads after 96 h of fermentation (Table 5). Sodium alginate concentration had an influence on density of the beads, higher alginate concentration showed the lower conversion efficiency, which might be due to reduced pore size of the bead. The lower alginate concentration effect the leakage of biomass from the beads, which could be due to increase in pore size of the beads. Shimmyo *et al*. (1982), Nasri *et al*. (1989), Nampoothiri and Pandey (1998) and Sunitha *et al*. (1998) also reported the similar findings.

Effect of Calcium Ions

At 0.03 M concentration, the yield of glutamic acid was more (37.0 mg mL^{-1}) than at the concentration of $CaCl_2$ higher or lower (Table 6). The concentration of $CaCl_2$ is important for the stability and pore size of bead. The calcium alginate gel is unstable in the presence of phosphates and certain cations such as Mg^{++} or K^+, which are major nutrients for living microorganisms (Lu and Chen, 1998). However, the solubilizing effect of these agents can be overcome by supplementing the growth medium with $CaCl_2$ (Nampoothiri and Pandey, 1988).

Influence of Storage Period of Beads

The calcium alginate beads were stored for 4 and 24 h. Table 7 indicates that with 4 h storage beads only 32.0 mg mL^{-1} glutamic acid was obtained after 96 h of fermentation. The prolonged storage of beads (24 h) influenced the glutamic acid yield. After 96 h fermentation the maximum yield of glutamic was 37.1 mg mL^{-1}. The cells encapsulated in properly solidified beads had better storage stability than the free cells (Lu and Chen, 1988).

Table 5: Effect of alginate concentration on glutamic acid production

Sodium alginate concentration (%)	Glutamic acid (mg mL^{-1})
2	36.5
3	37.0
4	36.0

Table 6: Effect of calcium ion on glutamic acid production

$CaCl_2$ concentration (M)	Glutamic acid (mg mL^{-1})
0.01	24.2
0.02	29.0
0.03	37.0
0.05	32.4

Table 7: Effect of storage period of beads on glutamic acid production

	Storage of beads (h)	
Fermentation time (h)	4	24
24	24.8	26.5
48	27.2	32.0
72	31.0	35.3
96	32.0	37.1

Table 8: Effect of initial biomass on glutamic acid production

Wet weight of cells/20 m/gel	Glutamic acid (mg mL^{-1})
0.25	24.2
0.50	29.0
0.75	31.1
1.00	32.4
2.00	20.3

Table 9: Production of glutamic with free/immobilized/co immobilized cells

Technique	Glutamic acid (mg mL^{-1})
Free cell-*Brevibacterium roseum*	40.5
Immobilized-*Brevibacterium roseum*	37.2
Co immobilized *Brevibacterium roseum* and *Escherichia intermedia* type-1	39.6

Effect of Initial Cell Loading

The cell concentration ranged between 0.25-2.0 g per 20 mL gel was used (Table 8). It was evident that the glutamic acid production was influenced by the initial biomass. After 96 h fermentation with 0.75 cell concentration the glutamic acid was more. The increase of cell in gels, oxygen was consumed faster then it could diffuse into beads (Gosmann and Rehm, 1986).

The mixed culture of *B. rosem* and *E. intermedia* type-1 were immobilized together in calcium alginate gel matrix were used to find out feasibility of co-immobilization for the glutamic acid production in single process. The same medium and predetermined optimum conditions which were employed for free cell and immobilization cells were used for this study. The co immobilized *B. rosem* and *E. intermedia* type1 produced 39.6 mg mL^{-1} of glutamic acid.

The results of microbial fermentation carried out using the selected production medium under similar experimental conditions revealed that the glutamic acid (40.5 mg mL^{-1}) obtained with Brevibacterium roseum is substantially greater than the yield of glutamic acid (37.2 mg mL^{-1}) obtained with immobilized *Brevibacterium roseum.* It is further observed that the co immobilized cells of *B. roseum* and *E. intermedia* type-1 produced 39.6 mg mL^{-1} glutamic acid which was higher than immobilized *Brevibacterium roseum* but slightly lower than the glutamic acid yield with *B. roseum* free cells (Table 9). The study shows that the mixed culture has greater potential for glutamic acid production instead of single organism because the conversion of glucose to α-Ketoglutarate by *B. roseum* in the first step and the subsequent conversion of α-Ketoglutarate to glutamic acid in the next step by *Escherichia intermedia* type 1.

CONCLUSIONS

Production of glutamic acid by *Brevibacterium roseum* was enhanced by the manipulation of media ingredient concentrations. Further the results obtained with co-immobilization of *B. roseum* and *E. intermedia* type 1, are quite promising. The increased production of glutamic acid by co-immobilized whole cells might be due to the presence of mixed culture with gel matrix. Hence the advantage of immobilized cells can be attributed to co-immobilized whole cells than the free cell fermentation and the immobilized cells can be recycled for the continuous production of glutamic acid.

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