Production of L-glutamic Acid by Immobilized Cell Reactor of the Bacterium Corynebacterium glutamicum Entrapped into Carrageenan Gel Beads

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Abstract: Immobilized cell reactor of the bacterium Corynebacterium glutamicum entrapped into carrageenan gel beads was constructed and used for the investigation of batch, fed-batch and continuous production of L-glutamic acid from nutritionally enriched sugarcane molasses. High final L-glutamic acid concentration (>93 g l⁻¹) was achieved, in batch fermentation, but with low productivity of 3.8 g l⁻¹ h⁻¹. Repeated batch fermentation runs utilizing the immobilized cells for L-glutamic acid production was unsatisfactory. The best results were obtained when the immobilized cell bioreactor was operated in a continuous mode and both dilution rate and penicillin supplementation were manipulated. At D of 0.4 h⁻¹ and with 20 U ml⁻¹ of the antibiotic penicillin in feeding medium, up to 73 g l⁻¹ of L-glutamic acid were recovered in reactor effluent with a yield of 75.7% and volumetric reactor productivity of 29.1 g l⁻¹.h.

Key words: L-glutamic acid • immobilized cell reactor • C. glutamicum • carrageenan gel beads • repeated batch fermentation • continuous fermentation

INTRODUCTION

L-glutamic acid is produced mainly through microbial means because chemical method produces a racemic mixture of DL-glutamic acid. This production of glutamic acid from sugar is thought to proceed predominantly through the Embden-Meyerhof-Parnas (EMP) pathway and the early steps of the tricarboxylic acid cycle, with oxygen acting as terminal electron acceptor [1]. Addition of penicillin to growing cells of Corynebacterium glutamicum triggers the excretion of high levels of glutamic acid [2].

Various fermentation techniques have been reported for the production of L-glutamic acid [3-5], but with wide variation in sugar conversion efficiency into L-glutamic acid. In all systems and among the other parameters, excretion of L-glutamic acid by bacterial cells was the rate limiting factor.

In the present study, different fermentation systems utilizing immobilized cells of the bacterium C. glutamicum were tested for L-glutamic acid production. Optimization of certain fermentation parameters during continuous operation was also considered and related to conversion efficiency.

MATERIALS AND METHODS

Microorganisms and culture media: The bacterium Corynebacterium glutamicum ATCC 13022 was obtained from the American Type Culture Collection, Rockville, Maryland USA and used in this study. It was maintained on medium (A) having the following ingredients: glucose, 40 g; K₂HPO₄, 1.0 g; MgSO₄.7 H₂O, 0.5 g; Yeast extract, 1.0 g; urea, 8 g tap water 1 l.

The production medium (medium B) contained clarified sugar cane molasses, 100 or 175 g; K₂HPO₄, 1.2 g; MgSO₄.7 H₂O, 6.2 g; K₂SO₄, 1.2 g; FeSO₄.7H₂O, 6 ppm; MnSO₄·H₂O, 6 ppm; tap water 1 l. Sugar cane molasses was replaced by 40 g l⁻¹ of glucose when the same medium was used for the propagation of actively growing cells for the purposes of cell immobilization. Sugar cane molasses was clarified according to the method described by Amin [6].

The pH of fermentation broth was maintained at 7.8 with the automatic addition of ammonia during each fermentation run. Fermentation temperature was kept at 30°C by circulation adjusted warm water through the double walled reactor. Oleic acid was used as antifoam agent. Aeration to fermentation broth was controlled at 102.0 mMO₂/l.h.
Bioreactor: Fresh active cells of the bacterium *C. glutamicum* were washed with sterile saline solution and mixed to a warm suspension of carrageenan to form cell entrapped gel beads [7]. After dryness under aseptic conditions, beads with entrapped bacterial cells were charged into double walled glass reactor. The reactor was divided into three successive compartments along the reactor height, separated by stainless steel perforated disks and keeping equal head space for each compartment. For sampling purposes, a stainless steel perforated tube was mounted properly within the reactor to allow the withdrawal of both liquid and gel beads.

Fermentation procedures: Both batch and fed-batch fermentation runs were executed in the double walled glass column with the total volume of the fermentation medium being added at the beginning of batch runs, whereas the same volume of fermentation medium was added at predetermined portions throughout the course of fed-batch fermentation runs. The total amount of sugars in feeding solutions was the same regardless the fermentation technique applied. With continuous fermentation runs different dilution rates were tested in absence and presence of the antibiotic penicillin. Oleic acid was used as antifoam agent whenever needed. The pH of fermentation broth was controlled using automatic addition of ammonia solution (16%). At regular intervals, samples from both fermentation broth and gel beads were taken and analyzed for L-glutamic acid, residual sugars, biomass and by-products.

Analytical determinations: L-glutamic acid, residual sugar were determined as previously described [8]. To measure concentrations of α-Ketoglutarate, aspartate, succinic acid, lactic acid and gluconic acid, methods described earlier [3] were used. Cell concentration in gel beads was given as dry cell weight using the method adopted by Amin and Verachtert [7].

RESULTS AND DISCUSSION

Start-up of immobilized cell reactor of the bacterium *C. glutamicum*: In order to buildup enough cell biomass entrapped into carrageenan gel beads after being charged into the reactor, production medium containing 40 g l⁻¹ of glucose, in stead of sugarcane molasses, was supplemented with 0.4 g l⁻¹ of urea and fed continuously into the reactor. A relatively low feeding rate (D = 0.02 h⁻¹) was employed. Samples were taken at regular intervals and analyzed for cell growth, L-glutamic acid and other by-products. The results are shown in Fig. 1.

Time course for cell growth and L-glutamic acid production in batch culture by immobilized cell reactor of the bacterium *C. glutamicum*: The present experiment was designed and conducted as batch culture in order to follow up pattern of both cell growth and L-glutamic acid production. Medium B containing sugarcane molasses with 100 g l⁻¹ of total sugars was fed to the reactor at
Time course of L-glutamic acid production and by-product formation by IMC of the bacterium C. glutamicum in batch culture. Aspartate and \( \alpha \)-Ketoglutarate were the major by-products. Their concentrations in reactor effluent reached 7.42 and 4.22 g l\(^{-1}\), respectively. Immobilized cell concentration reached its maximum (28.3 g l\(^{-1}\)) on day 3.1

As the fermentation proceeded, sugar consumption and L-glutamic acid production increased and concentrations of both succinic acid and lactic acid decreased markedly to 2.65 and 1.05 g l\(^{-1}\) respectively on day 6. This could be explained by a possible uptake and metabolism of these by-products by bacterial cells as demonstrated by Aida et al. \[9\]. Concentration of L-glutamic acid reached its maximum value (57.8 g l\(^{-1}\)) on day 5 and was maintained at steady state over day 6. Aspartate and \( \alpha \)-Ketoglutarate were the major by-products formed during this period. Their concentrations increased from 2.75 and 8.45 g l\(^{-1}\) on day 3 to 7.32 and 17.30 g l\(^{-1}\) on day 6 respectively. Synthesis of those two by-products is presumably due to a possible competition between certain key enzymes for common intermediates. Such competition is determined by a sequential feedback control \[10\]. Similarly, Aida et al. \[9\] found that the increase in intracellular L-glutamic acid caused feedback inhibition for both glutamate dehydrogenase and citrate synthase and resulted in accumulation of \( \alpha \)-ketoglutarate and aspartate. This must be considered to explain the simultaneous active synthesis of both aspartate and \( \alpha \)-ketoglutarate during the late accumulation period of L-glutamic acid. During this period, intracellular concentration of L-glutamic acid might have reached a critical level sufficient for a feedback control to take place and consequent cessation for the production of L-glutamic acid on day 6 (Fig. 2). Crueger and Crueger \[11\] stated that an intracellular concentration of L-glutamic acid between 25-35 µg mg\(^{-1}\) cell dry weight is capable of causing a complete cessation for L-glutamic acid synthesis.

Batch fermentation for production of L-glutamic acid from sugarcane molasses by ICR of C. glutamicum:

In order to test the capability of the ICR of C. glutamicum in producing as high final L-glutamic acid concentration as possible, the reactor was fed with production medium containing much higher concentration of clarified sugar cane molasses (175 g l\(^{-1}\) total sugars) in batch culture.
As shown in Fig. 3, the immobilized biomass supported a rapid production of L-glutamic acid and >93 g l⁻¹ of L-glutamic acid were produced in 16 hrs as no increase in L-glutamic acid concentration was recorded over the last 4 hrs. Such improved performance is most probably due to initial concentration of immobilized biomass in the reactor. Further increase in immobilized biomass took place immediately over the 1st day but slowly and reached 38.3 g l⁻¹ at the end of cultivation time. It was interesting to notice that the specific production rate (SPRG, gₚ/ g₀cell.h) recorded its maximum value over the first 4 hrs, then decreased sharply as fermentation progressed. Again, a feedback inhibition resulted from direct exposure of bacterial cells to high concentration of L-glutamic acid could be used to explain these lower values of SPRG.

Despite the relatively high final concentration of L-glutamic acid (93.0 g l⁻¹), the ICR of C. glutamicum exhibited lower yield (54.8%) and lower volumetric productivity (3.83 g l⁻¹.h) compared with ICR of similar bacterial strain entrapped into polyurethane foams having 6.2 [3], but with much higher than those reported for fermentation carried out with free suspended cell reactor of the bacterium Bacillus megaterium [12]. It is well known that high final L-glutamic acid concentration facilitates its recovery from the fermentation broth and results in a reduction in the over all production costs. The same goes perfectly well with higher yields and volumetric productivities. Therefore, decision to be taken in this regards depends mainly on local conditions which may vary from country to another.

**Repeated batch fermentations for production of L-glutamic acid by ICR of C. glutamicum from sugarcane molasses:** In view of the high final concentration of L-glutamic acid (93.0 g l⁻¹) and the unsatisfactory volumetric productivity and efficiency of sugar conversion to L-glutamic acid achieved in the above experiment, it was attempted to further investigate other fermentation techniques hoping for more improvement in ICR of C. glutamicum performances. Firstly, a repeated batch fermentation runs were investigated. The immobilized biomass of C. glutamicum was washed thoroughly by circulating a sterile saline solution into the reactor and production medium was then pumped to start batch fermentation. Five successive batch fermentation runs were performed.

It has been stated for quite long time that immobilized microbial cell systems sustain more aggressive environmental conditions compared with free suspended cell systems and support a stable production rate over long term operations [7, 13]. The obtained results (Fig. 4) did not agree with this assumption; immobilized entrapped cells of C. glutamicum supported high concentration of L-glutamic acid only during the first two fermentation runs. Thereafter, L-glutamic acid production decreased markedly to reach its lowest value of 54.35 g l⁻¹ in run 5. Although immobilized cell concentration increased within the reactor over the five successive fermentation runs, there was a sharp decrease in the specific production rate of L-glutamic acid (SPRG) from >0.5 in run 1 to 0.22 g/g.h in run 5, which could explain the dramatic decrease in L-glutamic acid production (Fig. 4). Intracellular L-glutamic acid might have increased during the successive fermentation runs and reached the critical level stated by Crueger and Crueger [11] and resulted in a feedback inhibition and the severe reduction in the specific SPRG.

Another explanation lies in the fact that cell of C. glutamicum when exposed to biotin-rich medium, such as sugar cane molasses (Imrie (1969), synthesis of short carbon chains and unsaturated fatty acid, such as oleic acid increases and results in cell membrane with low fluidity and low excretion of L-glutamic acid [15]. Thus, it was realized that there must be a certain concentration of
Table 1: Different phases of continuous production of L-glutamic acid immobilized cell reactor of the bacterium C. glutamicum

<table>
<thead>
<tr>
<th>Fermentation parameters</th>
<th>Fermentation phases</th>
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<tbody>
<tr>
<td></td>
<td>Phase 1</td>
</tr>
<tr>
<td>L-glutamic (g l⁻¹)</td>
<td>58.40</td>
</tr>
<tr>
<td>Immobilized biomass (g l⁻¹)</td>
<td>37.50</td>
</tr>
<tr>
<td>Conversion efficiency (%)</td>
<td>61.40</td>
</tr>
<tr>
<td>Productivity (g l⁻¹ h⁻¹)</td>
<td>2.92</td>
</tr>
<tr>
<td>Specific production rate (g/ g·h)</td>
<td>0.08</td>
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</tbody>
</table>

Fig. 5: Continuous production of L-glutamic acid by immobilized cell reactor of the bacterium C. glutamicum. A, B, C, D and E are steady state conditions supplementation. 1 and 2 are penicillin following changes in dilution rate and/or pencillin suplementation. 1 and 2 are *SGB is the changes in pencillin concentration and dilution rate resp. consumed sugars in growth and by-product formation

L-glutamic acid in fermentation broth that should be maintain in order to avoid feedback control phenomenon and the consequent drop in L-glutamic acid production.

Continuous production of L-glutamic acid by immobilized cell reactor of the bacterium C. glutamicum: In another experiment, it was attempted to test and identify the appropriate conditions that could sustain a stable long term operation with satisfactory final concentration of L-glutamic acid and higher yield and volumetric productivity than those recorded with batch and repeated batch runs.

A fresh immobilized cell reactor with the bacterium C. glutamicum was prepared and fed with production medium containing 100 g l⁻¹ total sugars and fermentation started under the above mentioned environmental parameters. Both penicillin supplementation and dilution rate were changed (Fig. 5). After reaching a steady state condition, one of the two parameters was changed. Samples were taken from each steady state and analyzed.

The results presented in Fig. 5 show that continuous production of L-glutamic acid was maintained for almost two week (phases A and B, Table 1) and two successive steady state periods were reached using dilution rates of 0.05 and 0.1 h⁻¹ with a final L-glutamic acid concentration of 58.4 and 63.2 g l⁻¹ respectively. However, after 50 hrs of cultivation the ICR of C. glutamicum started to perform badly. Concentration of L-glutamic acid in the effluent decreased gradually down to 50.2 g l⁻¹, with the highest levels of residual sugars and SGB; 22.6 and 16.0 g l⁻¹ respectively. This could be explained by a possible accumulation of biotin, present in sugarcane molasses [16], in microenvironment surrounding the immobilized biomass to an inhibitory level eliminating secretion of L-glutamic acid.

By increasing penicillin concentration in feeding medium from 10 to 20 U ml⁻¹, the activity of the reactor was recovered (72 h) most probably due to improved fluidity of cell membrane of bacterial cells [15].

Dilution rate was further increased and resulted in a new steady state of 72.8 g l⁻¹ with the highest conversion efficiency and volumetric reactor productivity of 75.75 and 29.1 g l⁻¹. h (Table 1).

The obtained results suggested that for a stable continuous production of L-glutamic acid with appreciable final concentration, a continuous growth of immobilized biomass within the reactor should be
Table 2: Comparison between various fermentation systems for L-glutamic acid production

<table>
<thead>
<tr>
<th>Parameters of comparison</th>
<th>Authors</th>
<th>Glut. A. (g l⁻¹)</th>
<th>Yield (%)</th>
<th>P (g l⁻¹.h)</th>
<th>Production techniques</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amin et al. [3]</td>
<td>58.50</td>
<td>74.6</td>
<td>6.2</td>
<td>Continuous culture using immobilized C. glutamicum cell into olyurethane foam cubes.</td>
</tr>
<tr>
<td></td>
<td>Aoki et al. [16]</td>
<td>53.70</td>
<td>53.7</td>
<td>-</td>
<td>Batch culture using free suspended cells of C. glutamicum.</td>
</tr>
<tr>
<td></td>
<td>Yoshioka et al. [4]</td>
<td>96.25</td>
<td>55.0</td>
<td>8.3</td>
<td>Continuous culture using free cells of Brevibacterium sp.</td>
</tr>
<tr>
<td></td>
<td>Sun-Uk et al. [5]</td>
<td>41.42</td>
<td>53.0</td>
<td>1.2</td>
<td>Batch culture using free suspended cells of C. glutamicum.</td>
</tr>
<tr>
<td></td>
<td>Al-Talhy, (2006)</td>
<td>93.00</td>
<td>54.9</td>
<td>4.7</td>
<td>Batch culture.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72.80</td>
<td>75.7</td>
<td>29.1</td>
<td>Continuous culture.</td>
</tr>
</tbody>
</table>

*P is the volumetric productivity (g glut /l/h) of the bioreactors

maintained at a relatively slow rate. Such growth is required in order to supply necessary metabolites required for the active production of L-glutamic acid and at the same time minimize the amount of consumed sugar converted into cell biomass. This was perfectly achieved through a simultaneous fine tuning of both penicillin concentration in fed medium and the dilution rate. A complete cessation to bacterial cells was proposed [4] for L-glutamic acid production utilizing the same bacterial strain, which contradict the obtained results.

The continuous system utilizing immobilized cell reactor with the bacterium C. glutamicum optimized in this study, compare very favorably with those reported in literature (Table 2). Although, Yoshioka et al. [4] reported higher final concentration of L-glutamic acid in fermentation broth, both yield and volumetric productivity of their system was markedly low being 55% and 8.3 g l⁻¹.h, respectively. In the present study, up to 75.7% and 29.1 g l⁻¹.h were achieved for yield and volumetric reactor productivity. Certainly, those values have a significant positive impact when diction for system implement on industrial scale is to be taken.

REFERENCES