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Research Note

An Efficient Strategy to Overproduce Glutamic Acid in *Corynebacterium Glutamicum* Fermentation

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In this paper, effect of penicillin addition in enhancing the secretion of glutamic acid by *Corynebacterium glutamicum* was studied. The proper time of adding penicillin to maximize glutamic acid production was found to drift at repeated trials. In contrast, maximum acid productivity was obtained when penicillin was injected at a proper biomass concentration of about 7.7 gram dry weight per lit (gdw/l). Moreover, rate of consumption of sugar and ammonia through the course of glutamic acid fermentation was monitored. In production phase, these two rates were correlated properly with a ratio of 3.2:1 which is comparable to the theoretical stoichiometric value of 5:1. Therefore, through feeding a mixture of molasses and ammonia at the above mentioned ratio, an online double control of both sugar and pH in a fed-batch process based on single pH measurements becomes feasible.

INTRODUCTION

A major concern in glutamic acid production by *Corynebacterium glutamicum* is inclusion of the acid within the cell due to the influence of molasses biotin on the cell wall [1]. A common practice to handle this problem is to benefit from the counter-effect of penicillin or an acylated surfactant containing saturated fatty acyl residues on the cell wall permeability [2,3]. This induces an increase in the saturated fatty acid content of the lipids and a large decrease in the phospholipid content of the cells [4]. The time and amount of penicillin addition are important parameters in this respect and are to be determined empirically.

Another problem in glutamic acid fermentation is the effect known as carbon metabolite repression [5]. Because of this effect, running the fermentation in a fed-batch mode of operation, in which concentration of the carbon source is maintained at a reasonably low level, is crucial in obtaining a high yield and productivity in this process. To achieve this goal, it is necessary to have means to make online measurements of sugar concentration in the fermentation media. Although glucose biosensors have been introduced recently for online monitoring of sugar concentration in the broth [6], growth of biomass onto the active site of the probe could easily deteriorate its performance [7]. Consequently, an effective strategy to monitor and/or control the carbon source concentration in fermentation media must be investigated.

In this research, these two problems were studied. Therefore, growth and glutamic acid production of a strain of *Corynebacterium glutamicum* was monitored and data was collected to analyze the nature of the problems. Based on the findings of these experiments, a strategy is recommended to increase the product yield in this bioprocess.

MATERIALS AND METHODS

The strain used in this study was *Corynebacterium glutamicum* ATCC 1532, obtained from the Persian Type Culture Collection (PTCC) of the Iranian Research Organization for Science and Technology (IROST). Seed and main culture media were optimized as introduced in the literature [8]. Table 1 illustrates the composition of these media.

The preculture media were made in 0.1 M Tris buffer at pH of 7. 60 ml of the media was fed into 500 ml shake flasks and after steam sterilization at 121°C for 20 minutes, was innoculated by *corynebacterium glutamicum* and cultivated at 32°C and 200 rpm for

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Table 1. Initial composition of seed and main cultures.

	Seed Culture	Main Culture
Molasses (total sugar)	50-60 g/l	50-60 g/l
Yeast extract	5.0 g/l	0.5 g/l
$(NH_4)_2SO_4$	—	4.0 g/l
KH_2PO_4, K_2HPO_4(1:1)	1.5 g/l	1.5 g/l
$MgSO_4.7H_2O$	0.4 g/l	0.4 g/l
$MnSO_4.4H_2O$	2.0 ppm	2.0 ppm
$FeSO_4.7H_2O$	2.0 ppm	2.0 ppm

16-20 h. Fermentation media were also made in 0.1 M Tris buffer but at a pH of 7.8. Cell cultivations were made both in 500 ml shake flasks and in a 5 l laboratory fermenter (Chemap AG, Switzerland). 10% seed culture was used for innoculation and impeller speed was set at 500 rpm. pH was maintained at 7.8 by injecting a 12% ammonia solution through the course of fermentation. Sugar was kept at the desired level by regular measurements of its concentration and feeding the proper amount of a 50% diluted sterile molasses solution. Biomass concentration was measured via turbidimetry at 610 nm. Total sugar concentration was assayed by the phenol-sulphuric acid method [9]. Thin layer chromatography was used for qualitative and quantitative analysis of glutamic acid in the broth [10].

RESULTS AND DISCUSSION

Investigations on using penicillin to direct the produced glutamic acid out of the cell were conducted in 500 ml shake flasks. Eight of these flasks were similarly innoculated by the seed culture and cultivated at 32°C and 200 rpm for 56 h. Then at two hour intervals, biomass concentration of one of the flasks was measured and a dosage of 6 units of penicillin per ml of the broth was injected into the flask. This operation was conducted for all of the flasks along the initial fermentation time of 16 h. At the end, glutamic acid concentration was assayed in all of the flasks and plotted against the addition time of penicillin in the corresponding flasks. To obtain meaningful results, this practice was repeated 3 times, for which the records are illustrated in Figure 1.

The best time of penicillin addition in these 3 runs was not repeatable and drifted between 4, 6 and 8 h. Therefore, no general conclusion could be drawn based on this analysis. However, when the final maximum acid concentration in these 3 runs were plotted against the biomass concentrations upon the addition of penicillin, a much better situation was encountered. Figure 2 shows the graph obtained in this manner. Interestingly, all of the collected data in the three runs follow the same trend and coincide in the plot. Based on this finding, the best time for addition of penicillin is when the biomass concentration in the broth rises to about 7.7 g/l.

Performance of the cell culture in the 5 l fermenter at an aeration rate of 1.2 vvm is illustrated in Figure 3. This run was operated in a batch mode and only ammonia solution was fed intermittently to control the

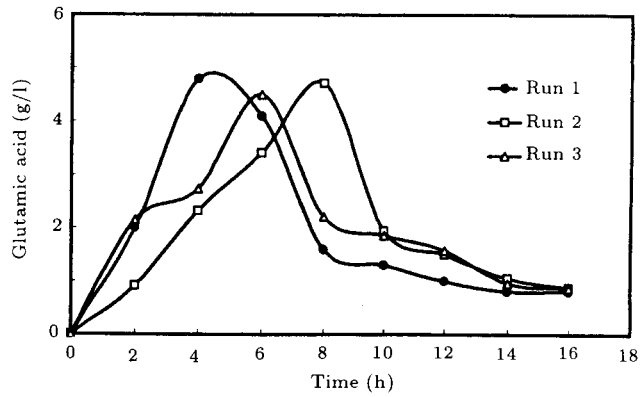

Figure 1. Effect of addition time of penicillin on the extracellular concentration of glutamic acid.

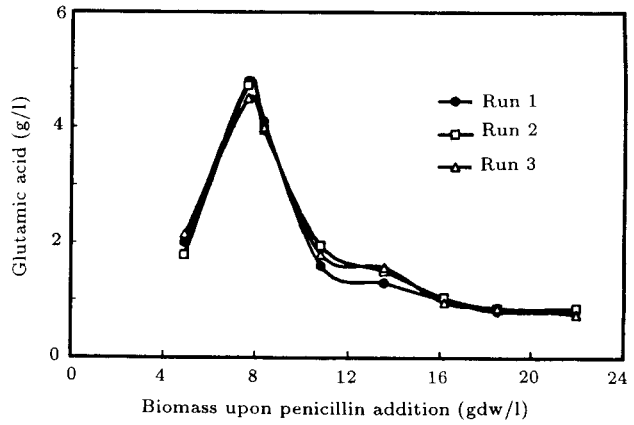

Figure 2. Correlation between the maximum production of extracellular glutamic acid and the biomass concentration upon the addition of penicillin.

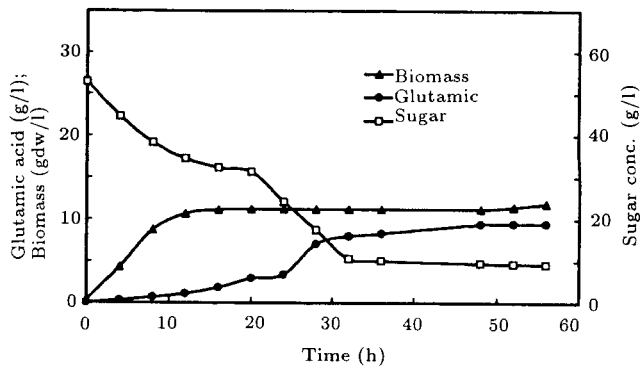

Figure 3. Trends of the changes in biomass, sugar and glutamic acid concentrations in a batch process at the aeration rate of 1.2 vvm.

pH at 7.8. Within 15 h from innoculation, biomass concentration rose to a maximum of about 11 g/l while sugar content dropped from 50 g/l to the initial plateau of about 30 g/l. In this period, glutamic acid was not secreted significantly and the final concentration was about 2 g/l.

No extra growth of biomass was observed after 15 h while after a lag of 5-10 h, sugar consumption resumed again and a sharp rise of glutamic acid to as high as 10 g/l was noticed. These observations may be a symptom of a non-growth associated nature of glutamic acid production. Such an assumption has also been implicitly used by other researchers [11]. In this case, aside from the fraction which is stripped out of the liquid and carried away via the exit airflow, part of the ammonia acts to balance secretion of glutamic acid and maintain the desired pH while the rest is digested along with carbon source in the cell metabolism. In pseudo steady state condition, which is attained in the production phase of fermentation, this could lead to a linear proportionality between sugar and ammonia rates of consumption. If this hypothesis is justified experimentally, a simple control strategy based on pH measurements could be designed and implemented to overcome carbon repression and enhance productivity.

Figure 4 presents results collected from a fed-batch fermentation at an aeration rate of 1.2 vvm in which sugar concentration was kept between lower and upper limits of 20 and 35 g/l. A preliminary. rise of glutamic acid accompanied the biomass growth up to about 10 h from the beginning of the culture. Again, the major increase in the acid content happened after 10 h when biomass concentration stayed at a constant value of about 9 g/l. The final acid concentration of 30 g/l in this experiment as compared to the maximum level of 10 g/l in batch operation clearly demonstrates the effectiveness of fed-batch operation to remedy the carbon catabolite repression governing the batch operation.

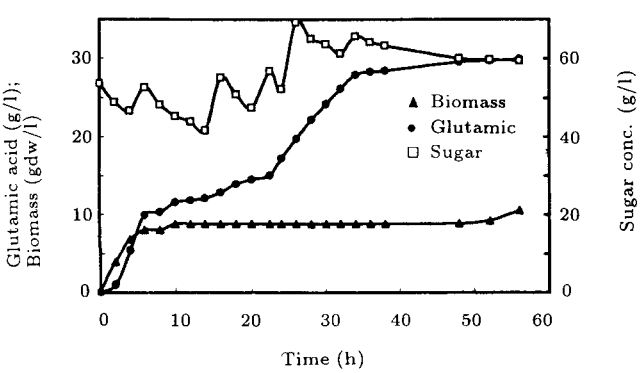

Figure 4. Trends of the changes in biomass, sugar and glutamic acid concentrations in a fed-batch process at the aeration rate of 1.2 vvm.

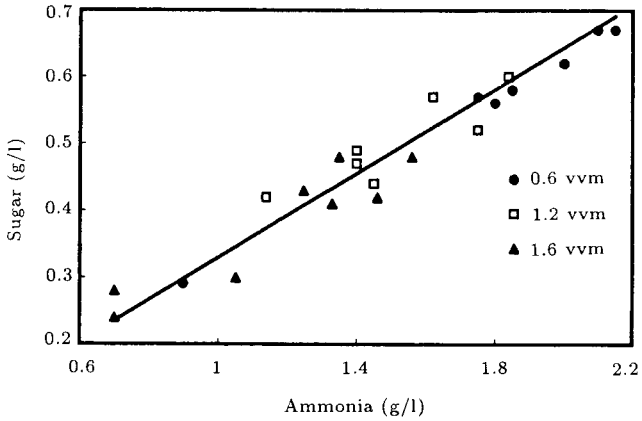

Figure 5. Correlation between sugar and ammonia concentrations in fed-batch processes at 3 different aeration rates of 0.6, 1.2 and 1.6 vvm.

Two more fed-batch runs at aeration rates of 0.6 and 1.6 vvm were conducted to collect data on the correlation of sugar and ammonia consumptions. In these experiments, the amount of sugar and ammonia fed to the fermenter to control pH and carbon at predetermined values was recorded through the course of fermentation. The correlations in the growth and death phases were not linear and the overall ratio of sugar to ammonia consumption amounted to 4.5 and 1.25, respectively. Figure 5 shows the data collected in the production phase of the three fed-batch experiments operated at 3 different aeration levels. The standard deviation of the regression between the whole collected data in the production phase of different fermentations is 95%. Slope of the line, namely the ratio of consumption of sugar to ammonia, is 3.2. The theoretical value of this ratio could be determined assuming no cell growth and only glutamic acid production. The overall biochemical stoichiometry of the production of glutamic acid may be written as:

$$C_{12}H_{22}O_{11} + 4NH_3 + 1.5O_2 \rightarrow$$

$$2C_5H_{12}O_4N_2 + 2CO_2 + 6H_2O \; . \qquad (1)$$

Based on this balance, the theoretical consumption of sugar to ammonia ratio is 342/68 # 5.0. The 1.8 unit deviation between the theory and practice may well be ascribed to evaporation and/or stripping of ammonia by the aeration stream. In any case, the observed linear correlation is reasonably good to be the basis of a new feeding strategy in this process. By adding the carbon source to the ammonium solution with a 3.2:1 ratio, pH could act effectively as the controlled variable for both pH and sugar concentration control. In this case, the need to online monitoring of sugar is eliminated and the control objective of the bioprocess becomes easily feasible.

CONCLUSIONS

Maximum secretion of glutamic acid happens when penicillin is injected at a specific biomass concentration presumably depending on the strain and other bioprocess characteristics. In this research, concentration was considered and a value of about 7.7 g/l was obtained for the strain used in this study.

Moreover, experimental measurements in this study demonstrate a constant ratio of 3.2:1 for sugar to ammonia consumption in the production phase of glutamic acid fermentation by *Corynebactrium glutamicum*. Therefore, a control algorithm in which pH measurement is used to feed a proper amount of sugar-ammonia solution at the above ratio could provide easy and feasible means to control the fed-batch operation of this process.

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