Middle-East Journal of Scientific Research 7 (2): 235-240, 2011
ISSN 1990-9233

Studies on Continuous Production Kinetics of L-Lysine by Immobilized *Corynebacterium glutamicum* 13032

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Abstract: L-lysine ($C_6H_{14}N_2O_2$), one of the essential and commercially important amino acids, is found in naturally occurring proteins of all living organisms. The steadily increasing L-lysine demand necessitates indispensably its fermentative production over synthetic processes. In practice, most of the amino acids were produced by batch fermentation. Continuous fermentation processes have certain advantages over the traditional discontinuous processes. L-lysine production using immobilized cells in continuous mode seem to be very promising for future applications. The method employed for immobilization is calcium alginate cross linked with gluteraldehyde entrapment. We made an attempt to investigate the dependence of dilution rate on substrate utilization rate and cell productivity in continuous culture studies. L-lysine concentration was improved to the maximum level of 45.34 g/l and yield was 0.55 (g/g) obtained at an optimum dilution rate of 0.3 (1/h) under operating conditions of fermentation time 72 hr, pH-7.5, Temperature-30°C and glucose concentration of 90 g/L.

Key words: Continuous · *Corynebacterium* · CSTR · Immobilization · Kinetics · L-lysine

INTRODUCTION

With the advent of new uses and the growing markets of amino acids, amino acid production technology has made significant progress during the latter half of the 20th century. Amino acid industry has been expanding and this industrial growth will surely continue because of incessant efforts to improve the established production processes also can be expected to further reduce the production costs, thereby increasing the worldwide market. The development of low-cost fermentation processes for many kinds of amino acids and the recent rapid progress in biotechnology including strain improvement technology, progress in biochemical engineering and downstream processing indicate that fermentation attains the key position in the amino acid industry. Fermentation technology has played crucial roles over a period of time and currently the amino acids produced by fermentation represent chief products of biotechnology in both volume and value [1].

Out of the twenty naturally occurring amino acids, L-Lysine is one of the nine essential amino acids and commercially important amino acids. L-Lysine is added to improve the nutritional value of cereal-based diets and it is currently sold at approximately US$ 3-4/kg. Presently, 80% of lysine in the world market is made by Microbial fermentation and the remaining 20% by chemical Synthesis. L-lysine is presently being used in the pharmaceutical, food, feed milling and cosmetics industries. Thus, the outlook for this amino acid is high because of its expanding market demand [2].

In production of L-lysine by fermentation all raw materials are natural or biologically available substances. No harmful by-products have been found in L-lysine fermentation. However, useful substances remain in the spent-broth from which many byproducts could be recovered. The microorganisms, separated from the broth were found to be composed of more than 50% protein of animal feed grade quality. The spent-broth still contains various useful substances, including organic and

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inorganic nitrogen compounds, phosphorus compounds and potassium salts which could be used as fertilizer [2].

Strains like B*revibacterium flavum* [3], *Brevibactirium lactofermentum* [4] and *Corynebacterium glutamicum* [5] have been used for the last fifty years for the industrial production of L-lysine. However fermentative methods seem to be most economical and practicable means of producing L-lysine. Various such kinds of processes were investigated by Schrump *et al*. [6] Eggeling *et al.* [7] and Ekwealor *et al.* [8].

In fermentative processes, conversion, yield from the carbon source and productivity are the most important factors. The conversion depends on the characteristics of the strain used in the fermentation and most research has been concerned with the improvement of L-lysine producing strains. On the other hand, productivity is strongly affected by the growth rate of a strain, the rate of sugar utilization or the culture conditions. However, there have been very few reports on the development of fermentation process [9].

MATERIALS AND METHODS

Microorganism and Culture Conditions: The organism employed throughout this study was *Corynebacterium glutamicum ATCC13032* obtained from Institute of Microbial Technology, Chandigarh (India). It was cultured on agar slopes using actinomycetes agar medium. The growth medium, containing glucose (2 g), beef extract (1 g), bacto peptone (1 g), NaCl (0.25 g), agar (2 g), distilled water (100 ml), was maintained at pH-7.

The Production Medium Used Was as Follows: glucose (100 g), ammonium sulphate (20 g), calcium carbonate (10 g), bacto casamino acid (2 g), potassium dihydrogen phosphate (2 g), magnesium sulphate heptahydrate (0.2 g), manganese chloride tetra hydrate (3 g), thiamine hydrochloride (100 µg), d-biotin (20 µg) and distilled water (1.0 L). Freshly prepared agar slants were used to inoculate 50 ml of growth media and kept on shaker for 48 hr (150 rpm) at 30°C. 100 ml of 72 hr old culture was used to prepare immobilized beads of calcium alginate. 15% volume of beads is used throughout this study.

Immobilization Method: Cells were immobilized using calcium alginate cross linked with gluteraldehyde entrapment method [10,11]. 3% sodium alginate and 100µl gluteraldehyde were well-mixed with 0.06% cells on dry cell weight basis (DCW) (w/v), to get uniform suspension. The solution was dropped into 0.2M $CaCl_2$ solution using peristaltic pump through a cut micropipette tip (or) orifice. The beads then formed were cured for 24 hrs by incubating in 0.2M $CaCl_2$ solution and washed twice with sterile saline (0.9% NaCl solution (w/v)) and stored in saline solution at 4°C for further use. All these steps are carried out under aseptic conditions.

Fermentation Studies: Amino acids are commercially produced in batch or fed-batch processes. All of nutrients are added at the beginning of the fermentation in batch operations. In batch fermentation microorganism grows until one or more of essential nutrients is exhausted or until fermentation conditions become unfavorable (e.g. product inhibition, oxygen limitation, pH decrease in shake flasks and uncontrolled fermentations etc.). In the present study fermentation experiments with immobilized cells have been conducted in batch mode using a sterilized continuous stirred tank reactor of 1L capacity - 22 cm height and 10 cm inner diameter. Medium was loaded in to the reactor and the filtered oxygen was introduced from the top. Air filter (0.2µm) and compressed oxygen were used for the purpose. The working volume of the reactor was 600 ml.

For experiments with immobilized cells, production medium containing 60 g of immobilized beads (15%) was used throughout the experiments. The diameter of the beads was in the range 3.0-3.5 mm. The oxygen flow rate maintained for L-lysine production was 1.5 VVM (Volume of the air per volume of the reactor per minute). The ranges of variables studied can be seen from the figures. While studying, variation in a particular variable was made as per maintained values obtained from the previous experiments. The optimized values were – temperature 30°C, pH 7.0, substrate concentration 90(g/L), pressure <2 atms and agitation speed 200 rpm.

Analytical Methods: Quantitative estimation of L-lysine in the supernatant fluid was made by Acidic ninhydrin method of chinard [12]. Residual sugar was determined as glucose in the supernatant fluid by the colorimetric DNS Method of Miller [13]. Biomass in the broth was estimated in 2 ml samples after centrifugation and dried at 104°C for 3 hr.

RESULTS AND DISCUSSIONS

The present report describes about L-lysine productivity, yield of L-lysine and the factors affecting L-lysine production in continuous cultures.

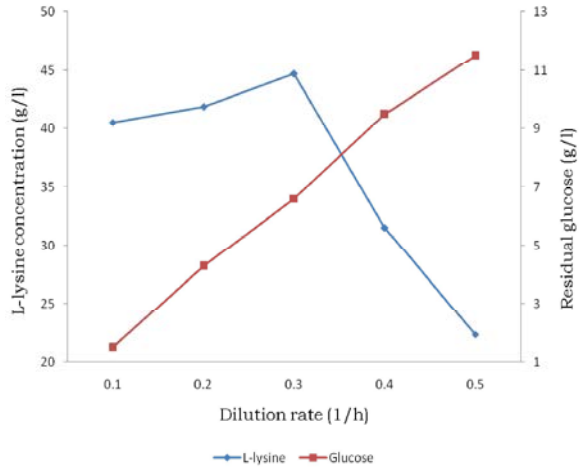

Fig. 1: Effect of dilution rate on L-lysine production by immobilized cells in continuous stirred tank bioreactor

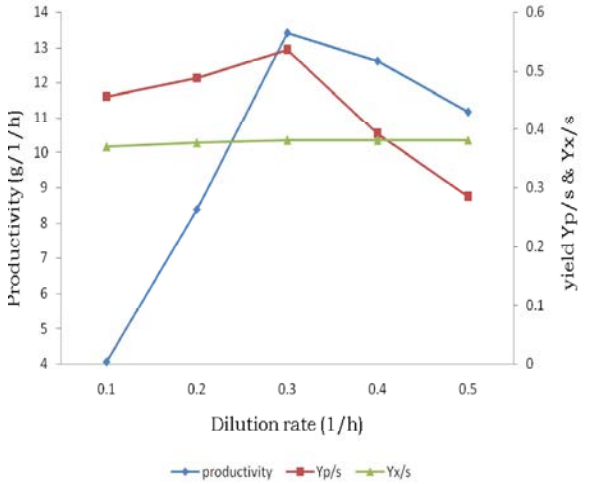

Fig. 2: Effect of dilution rate on L-lysine productivity and yield by immobilized cells in continuous stirred tank bioreactor

The continuous culture described in this study also facilitated analysis of the various factors affecting L-lysine fermentation. Earlier few scientists reported about the multistage continuous cultures and their difficulties to run continuous L-lysine production systems [3, 14]. Fermentation studies were carried out under operating conditions of fermentation time 72 hr, pH-7.5, temperature-30°C and glucose concentration of 90 g/L.

Fig. 1 shows the dilution rate versus average L-lysine and residual glucose concentration. The dilution rate is the inverse of retention time. The dilution rate indicates the retention time required for the substrate in the reactor for the L-lysine production. The optimum dilution rate indicates optimum retention time at which accumulation of L-lysine is more. In other words it indicates maximum utilization of the glucose in the less possible retention time followed by higher yields of L-lysine. The increase in dilution rate shows the decrease in residence time. In the present study, the reactor was operated at five different dilution rates viz. 0.1, 0.2, 0.3, 0.4, 0.5 (1/hr). The graph indicated maximum L-lysine production (44.7 g/l) and less glucose (6.65 g/l) at a dilution rate of 0.3 (1/hr). So the reactor exhibited optimal performance in terms of L-lysine production and sugar utilization at 0.3 (1/hr). Above this dilution rate momentous reduction in L-lysine accumulation and more residual substrate, where as below this dilution rate relatively less accumulation of lysine and more substrate consumed.

Fig. 2 shows the dilution rate versus productivity, Yield and Yield coefficient during continuous operation of CSTR. The productivity shows the efficiency of the reactor in terms of retention time and end product. The yield shows the reactor performance in terms of substrate utilization and product formation. This parameter directly relates to economics of the L-lysine production as the raw material consumption reduces if the product formation is more per unit of raw material consumption. Yield coefficient indicates good metabolic activity of the cell in terms of end product formation. In continuous operation the productivity and yield increased steadily and reached a maximum value 13.411 (g/l/h) and 0.536 (g/g) respectively at a dilution rate of 0.3 (1/h). But the yield coefficient remains almost at the same value (0.380 (g/g)). Above 0.3 dilution rate there is a momentous reduction in productivity and yield as the retention time decreased.

Figure 3 shows the biomass, residual substrate concentration and productivity of biomass at different dilution rates, where dilution rate characterizes the holding time or processing rate of the CSTR. The productivity of the biomass shows the efficiency of the fermenter to cultivate the cells in terms of retention time and biomass production. In order to get productivity the dilution rate is multiplied with biomass concentration. The productivity factor defined as above is applicable only for continuous operation of the reactor. In the present experiments the highest cell productivity of 9.5 g/l/day and minimal residual glucose concentration of 6.6 g/l were observed at the dilution rate of 0.3 (1/h). Therefore, it could be concluded that operation of the reactor at 0.3 (1/h) dilution rates is most economical for good production of cells.

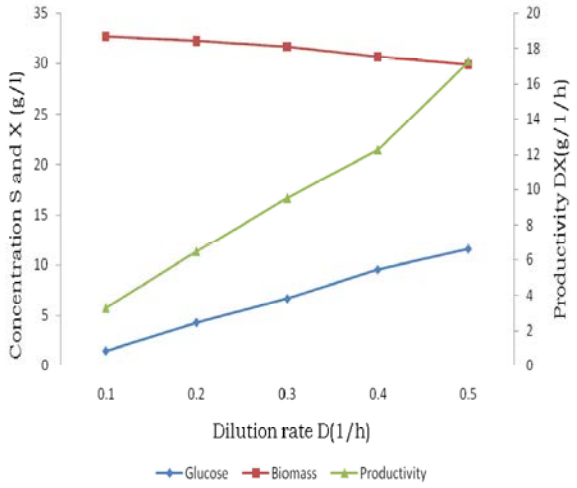

Fig. 3: Dependency of dilution rate on cell productivity for continuous culture

Table 1: Effect of dilution rate on continuous production of L-lysine by immobilized cells in stirred tank bioreactor

Dilution rate (1/hr)	Residual glucose (g/l)	L-lysine concentration (g/l)
0.1	1.492	40.527
0.2	4.285	41.855
0.3	6.615	44.705
0.4	9.480	31.520
0.5	11.512	22.332

Table 2: Effect of dilution rate on L-lysine productivity and yield

Dilution rate (1/hr)	Productivity (g/l/hr)	Yield (Yp/s)	Yield (Yx/s)
0.1	4.052	0.457	0.369
0.2	8.371	0.488	0.376
0.3	13.411	0.536	0.380
0.4	12.608	0.391	0.380
0.5	11.166	0.284	0.381

Table 3: Effect of dilution rate on biomass and glucose utilization.

D (1/hr)	S (g/l)	X (g/l)	DX (g/l/hr)
0.1	1.4925	32.68	3.268
0.2	4.285	32.2325	6.4465
0.3	6.615	31.6875	9.50625
0.4	9.48	30.6275	12.251
0.5	11.5125	29.8825	17.25125

Table 4: Evaluation of kinetic parameters for continuous production of L-lysine in stirred tank bio reactor

1/D (hr)	(S0-S)/X	1/S (lit/g)	X/D(S0-S)
10	2.708	0.703253	3.692
5	2.659	0.234798	1.880
3.333	2.631	0.154995	1.2668
2.5	2.628	0.106206	0.950
2	2.626	0.087097	0.761

Evaluation of Monod Kinetic Parameters: The Monod chemostat is an extension of the CSTR (Continuous stirred- tank reactor), which considers both the substrate consumption and the cell growth. A fresh sterile medium is fed continuously to the reactor. The reactor volume remains constant because of constant feed and constant product withdrawal.

The microbial growth kinetics by the Monod equation has led to the development of techniques to determine the constants Ks and μ_m used in the equation (1). At the same time as μ_m is in fact dependent on other parameters, such as temperature and pH, in the usual case there are specified and the design procedure requires values of Monod constants under these conditions. The yield coefficient Y will also be required in order to link calculations of microbial growth to substrate concentrations.

The measurements of these constants may be carried out using continuous fermenter experiments. In contrast to the batch fermentation methods of determining kinetic constants, the use of continuous fermenter requires more experiments to be performed, but the analysis tends to be more straightforward.

In essence, the experimental method involves setting up a continuous stirred-tank fermenter to grow the *Corynebacterium* cells on a sterile feed of the glucose media. The feed flow rate is initially adjusted to the 0.1 L/hr, of course, must produce a dilution rate of 1.0 (1/h) and the system allowed to reach steady state.

Careful measurements of the microbial mass X, the glucose concentration S and the flow rate F were made when steady state has been achieved and then operation is repeated at a series of suitable dilution rates of 0.2(1/h), 0.3(1/h), 0.4(1/h), 0.5 (1/h) respectively.

Graph between [X/D(S0-S)] Vs [1/S] is shown in Fig. 4. Similarly another graph between [(S0-S)/X] Vs [1/D] is shown in Fig. 5.

Monod Kinetic Equations:

$$[X/D(S0-S)] = [(KsY/ \mu_m)(1/S)+(Y/ \mu_m)] \quad \text{Fig. 4}$$

$$[(S0-S)/X] = [(k_d/YD) + (1/Y)] \quad \text{Fig. 5}$$

Calculations:

From Fig. 5: Linear equation was Y=0.0106X+2.605

(1/Y)= 2.605 & $k_d/ Y_{X/S}$ =0.0106

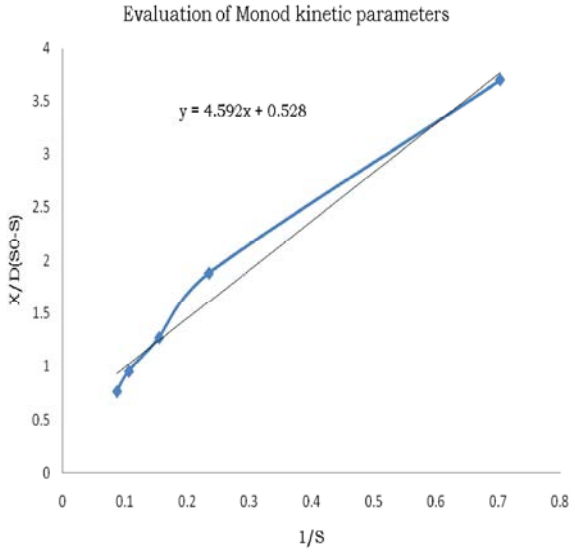

Fig. 4: Evaluation of Monod kinetic parameters for continuous culture

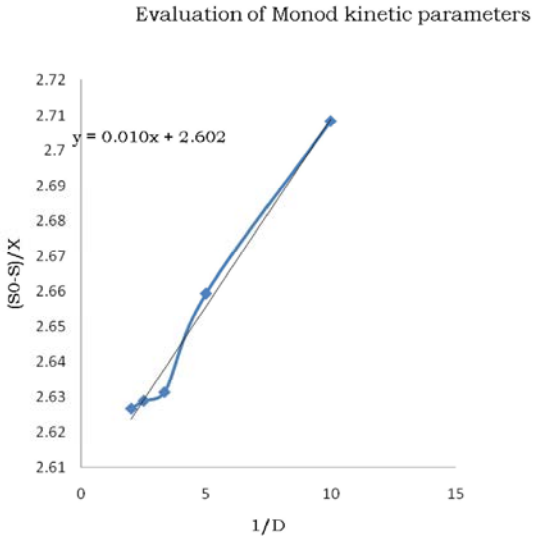

Fig. 5: Evaluation of Monod kinetic parameters for continuous culture

There fore $Y_{X/S}$ = 0.3842(g/g)

After substitution of Y value, K_d = 0.004 (1/h)

From Fig. 5.4: Linear equation was Y=4.592X+0.5288
(KsY/μ_m) = 4.5928 & (Y/μ_m) = 0.5288
After substitution of Y value,

μ_m = 0.7265 (1/h)
Ks = 8.685 (g/l)

From these equations the Monod growth parameters for L-lysine production were evaluated with chemostat. The parameters evaluated are μ_m=0.7265 (l/hr), K_s=8.685 g/l and K_d=0.004 (1/h).

CONCLUSIONS

L-lysine concentration was improved to the maximum level of 45.34 g/l and yield was 0.55 (g/g) obtained at an optimum dilution rate of 0.3 (1/h) under operating conditions of fermentation time 72 hr, pH-7.5, Temperature-30°C and glucose concentration of 90 g/L. The results obtained here indicate that L-lysine can be produced efficiently by immobilized growing *Corynebacerium* cells by comparison with the previous results obtained with free cells in stirred tank bioreactor.

List of Symbols

Yp/s	*Y*ield of product (g/g)
Yx/s	Yield of biomass (g/g)
μ	Specific growth rate of biomass (1/h)
μmax	Maximum specific growth rate (1/h)
N	Impeller speed (min-1)
Kd	Respiratory quotient (1/h))
V_L	Liquid volume (m3)
X	Biomass concentration (g/l)
S_0	Initial glucose concentration (g/l)
S	Residual glucose concentration (g/l)
P	Product concentration (g/l)
F	Feed flow rate (g/h)
D	Dilution rate (1/h)
Dmax	Maximum dilution rate (1/h)
Dopt	Optimum dilution rate (1/h)
Ks	Limiting nutrient concentration (g/l)
Qp	Specific production rate (1/h)

ACKNOWLEDGEMENTS

Authors wish to thank University Grant Commission (UGC), New Delhi for providing computer controller bioreactor at Chem. Dept., JNTU, Aanantapur under UGC project scheme and also wish to express sincere gratitude to the management of MVGR College of Engineering, Vizianagaram for their continuous encouragement for doing this work and we also wish to express our deepest sense of gratitude to the Jawaharlal Nehru Technological University (JNTU), Hyderabad for encouraging to publish this paper.

REFERENCES

1. Ikeda, M., 2003. Amino acid production processes. Adv. Biochem. Eng./Biotechnol., 79: 1-35.
2. Anastassiadis, S., 2007. L-Lysine Fermentation. Recent Patents on Biotechnology, 1: 11-24.
3. Becker, M.J., 1982. Product biosynthesis in continuous fermentation. Folia Microbiol., 27: 315-318.
4. Balch, J.F. and P.A. Balch, 1990. Prescription for nutritional healing. Avery Publishing Group Inc, New York, pp: 27-28.
5. Bercovici, D. and F. Fuller, 1995. Industrial amino acids in nonruminant animal nutrition. In: R.J. Wallace and A. Chesson, (eds) Biotechnology in animal feeds and animal feeding, VCH, Weinheim., pp: 93-113.
6. Choi, Y.J. and D.E. Tribe, 1982. Continuous production of phenyl alanine using an *Escherichia coli* regulatory mutant. Biotechnol. Lett., 4: 223-228.
7. Davis, B.D., 1952. Biosynthesis interrelations of lysine, Diamnopimelic acid and threonine in mutants of Escherichia coli., Nature, 169: 533-534.
8. Demain, A.L. and P.S. Masurekar, 1974. Lysine inhibition of in vivo homocitrate synthesis in Penicillium chrysogenum. J. Gen. Microbiol., 82: 143.
9. Sassi, A.H., N. Coello, A.M. Deschamps and J.M. Lebeault, 1990. Effect of medium composition on L-lysine production by variant strain of Corynebacterium glutamicum ATCC-21513. Bitechnol. Lett., 12(4): 294-296.
10. Jetty, A., A. Gangagni Rao, B. Sarva Rao, G. Madhavi and S.V. Ramakrishna, 2005. Comparative studies of ALR and FBR for Streptomycin production by immobilized cells of *Streptomyces sp.* Chem. Biochem. Eng. Q., 19(2): 179-184.
11. Marek, P.J., M. Kierstan and M.P. Coughlan, 1985. Immobilized cells and Enzymes. A practical approaches U.K., Ireland press limited, pp: 43-44.
12. Chinard, F.P., 1952. Photometric estimation of proline and ormithine. J. Biol.Chem., 199: 91-95.
13. Miller, G.L., 1959. Use of dinitrosalicyclic acid reagent for detection of reducing Sugar. Annal. Chem., 31: 427-431.
14. Michalski, H.J., L. Krzystek, R. Blazczyk, T. Jamroz and A. Wieczorek, 1984. The effect of mean residence time and aeration intensity on the L-lysine production in a continuous culture. In: Third Europian congress on biotechnology, Verlag Chemie, Weinheim, 2: 527-532.