International Journal of Innovation and Applied Studies
ISSN 2028-9324 Vol. 13 No. 3 Nov. 2015, pp. 656-662

http://www.ijias.issr-journals.org/

Study on Batch Fermentation Kinetics of L-Lysine by *C. glutamicum* immobilized on complex carriers of Alginate and Bacterial Cellulose and investigate its reused ability for further fermentative cycle

Tran Thi Minh Tam, Nguyen Thi Huong Giang, and Nguyen Thuy Huong

Department of Biotechnology,
Ho Chi Minh City University of Technology,
Ho Chi Minh, Vietnam

ABSTRACT: L-Lysine fermentation by *Corynebacterium glutamicum* obtained productivity improvements from D-glucose. Immobile *Corynebacterium glutamicum* on carrier brings many significant advantages for lysine production such as high reused ability, saving seed preparation stage and high adaptation ability of immobile cells to the fermentation environment. Simultaneously, immobile cells have a long phase for lysine excretion. L-Lysine fermentation kinetics by immobile cells is studied in the basic way to solve problems related to cell physiology. Thereby, optimization techniques are carried out more easily to determine the most suitable nutrient medium as well as fermentation conditions and result in obtaining highest yield L-Lysine. L-lysine concentration was improved to the level of 28.767±0.231 gram per liter and lysine production yield was 92.6% obtained under operating conditions of fermentation time 40 hours by immobile *Corynebacterium glutamicum* cells. In the fermentation process of lysine production by immobilized *Corynebacterium glutamicum* on complex carrier of A-BC, the reused ability of immobilized product was 11 fermentation cycles and total fermentation time is 440 hours and total L-Lysine production yield is 0.711 ± 0.009 gram per liter per hour.

KEYWORDS: Batch kinitics, L-Lysine, *C. glutamicum*, alginate, bacterial cellulose, reusing finished immobile cells.

1 INTRODUCTION

L-Lysine is one of the most important amino acids necessary for constructing the many types of protein in the human body and animal. L-Lysine is indispensable, however it cannot be synthesized endogenously for satisfy the metabolic need. Therefore, it is required for nutrition of animals as well as humans and the only way to have enough L-Lysine for protein synthesis is to obtain it in the diet or take supplements. L-Lysine is present used in the pharmaceutical, food, feed milling and cosmetic industry. The intensive research on the L-Lysine biosynthetic pathways and their regulation and the search for microorganisms capable of over-producing this amino acid were carried out to apply for industrial production. The steadily increasing L-Lysine market demand also stimulated the improvement of established fermentation process.

In practice, L-Lysine was produced by batch fermentation because of its convenience and easy performance for producing amino acid. Fermentation is generally modeled by kinetic equations giving the time evolutions for biomass, substrate, and product concentrations [1]. Information on fermentation process kinetics is potentially valuable for the improvement of batch process performance; it is essential for fed-batch or continuous process designs [2]. In fermentation processes, conversion yield from the carbon source and productivity are the most important factors. The metabolism depends on the characteristics of the strain used in the fermentation. On the other hand, the growth rate of a strain, the rate of sugar utilization or the culture conditions are also significant factors that affect productivity strongly. Kinetics study shows a set of results for the cell growth, glucose consumption, and product formation for L-Lysine fermentation process using *Corynebacterium glutamicum*. Kinetic parameters were determined by Rubina Nelofer showed that the L-Lysine synthesis by free cells *C. glutamicum* depends on both the growth rate and biomass concentration [3].

Corresponding Author: Tran Thi Minh Tam

It is very promising to produce L-Lysine by using immobilized *C. glutamicum* cells. The advantages of this production process is that time, effort and expense are minimized during breed preparation period and thus results in improving efficiency of the L-Lysine fermentation. Many methods namely adsorption, covalent bonding, cross linking, entrapment and encapsulation are widely used for immobilization. Presently, the simple, common and efficient immobilization technique is entrapment on alginate and adsorption – incubation onto bacterial cellulose. To improve the quality and performance of immobilized cell biocatalysts, various combinations of natural gel with other carrier have been studied. A complex of alginate and bacterial cellulose (A-BC) was used as a combined carrier to immobilize *C. glutamicum* for L-Lysine fermentation. The A-BC carrier helps immobilized products to withstand the conditions of agitation during fermentation and keeps the locked cells in gel structure without diffusing into the fermentation medium. In addition, A-BC carrier also helps immobilized cell maintaining physiological state at which they are ready to excrete L-Lysine into fermentation medium [4].

Fermentation process by immobilized *C. glutamicum* has many meaningful advantages that help the L-lysine productivity is kept stable after several reused cycle. Using alginate as a carrier, immobilized product of *C. glutamicum* had ability to reuse 4 times and L-lysine production yield reaches 0.56 ± 0.10 gram per liter per hour. The immobilized *C. glutamicum* into BC carrier had 8 reused cycles and L-lysine production yield reached 0.62 ± 0.10 gram per liter per hour. Therefore, L-lysine production using immobilized *C. glutamicum* cells on complex carrier of alginate and bacterial cellulose in fermentation process seems to be very promising. This immobilization technique is able to overcome the mechanical and chemical drawback of alginate carriers and enhance the advantages of bacterial cellulose carriers [4, 5].

This study is carried in order to clarify kinetics of L-Lysine production by batch culture using *C. glutamicum* immobilized on A-BC which can be used as a promising carrier in modern fermentation industry. Kinetic parameters such as biomass, substrate concentration, L-Lysine, specific growth rate were estimated in order to give a fuller and more efficient exploitation of L-Lysine fermentation process.

2 MATERIAL AND METHODS

2.1 THE MATERIALS AND CULTURAL MEDIUM

The micro-organism species*: C. glutamicum* is provided by the Vietnam Type Culture Collection

The carrier: The utilized alginate is provided by the producer Sigma – Ahdrich. Alginate is powdery, light brown and its moisture content is below 15%. The 1% alginate solution is prepared and kept at 25^0C. Its viscosity is 5-40 Cps and pH is from 5 to 8. Bacterial Cellulose is obtained by the fermentation process of *Acetobacter xylinum*. Bacterial Cellulose is shaped square blocks with 1 x 1 x 1 cm^3, white and its moisture content is approximately 6%.

The cultural medium: *C. glutamicum* grows in the minimal medium with glucose (20g/L), peptone (10g/L), yeast extract (5g/L), NaCl (5g/L), agar (15g/L), pH ~ 7,2, the temperature at 30^0C, the agitation rate of 150 rpm.

The fermentation medium and conditions: They are optimized for Lysine fermentation by *C. glutamicum*, in particular, glucose (50 g/L), $(NH_4)_2SO_4$ (46,8 g/L), KH_2PO_4 (1,8 g/L), biotin (20 µg/L), thiamin (150 µg/L), tween 20 (5 mL/L), corn solution liquid (100 mL/L), the initial culture rate is reached 13.2 million colony-forming units, at 30 ± 2^0C, the initial pH in fermentation medium is 7 ± 0.2, the agitation rate is 150 ± 5 rpm.

2.2 EXPERIMENT DESIGNS

2.2.1 THE IMMOBILE PROCESS ON COMPLEX CARRIERS OF ALGINATE AND BACTERIAL CELLULOSE

The *C. glutamicum* immobilization process is a combination between entrapment method of alginate and "adsorption – incubation" method of BC. The immobilized procedure is carried out by 3 stages. First, bacteria were attached onto the BC surface and the adsorbed inside BC structure due to its porous properties. Second, alginate was used to enclose the immobilized product of BC and *C. glutamicum*. Finally, the complex carrier was incubated to increase the density of cells inside BC's space [4, 5]. *C. glutamicum* was immobilized onto complex carrier of A-BC according to optimized process which its immobilized productivity reached 90% and cell density a gram finished product is 49.9 ± 0 1 million cells.

2.2.2 BATCH FERMENTATION KINETICS OF L-LYSINE BY *C. GLUTAMICUM* ON COMPLEX CARRIERS OF ALGINATE AND BACTERIAL CELLULOSE

Immobilized *C. glutamicum* cells were used to study the L-Lysine fermentation process. The working volume of the reactor is 1000 mL. The samples were collected during fermentation time in order to determine targets such as the cell

density outside and inside carriers, residual sugar and L-Lysine concentration in the fermentative solution. And then kinetics parameters such as growth rate, substrate metabolize ability and L-Lysine productivity were analyzed.

Expected results: The average growth rate, the maximum specific growth rate and substrate utilization rate were determined after finishing batch fermentation of L-Lysine production by *C. glutamicum* on complex carriers of alginate and bacterial cellulose.

2.2.3 INVESTIGATE REUSED ABILITY OF *C. GLUTAMICUM* IMMOBILIZED ON COMPLEX CARRIERS OF ALGINATE AND BACTERIAL CELLULOSE FOR FURTHER FERMENTATION CYCLE

The finished products were used for L-Lysine production through many fermentation cycles. Samples were collected to determine parameters such as density of remaining cells, L-Lysine concentration of each cycle. And then, escaped cell rate, total fermentation time, total L-Lysine productivity and average yield of finished products were analyzed.

Expected results: reuse frequency of finished products and average L-Lysine production yield were determined through fermentative cycles.

2.3 ANALYZED METHODS

Analyzing the cell density of immobilized finished products: the finished products were mechanically broken and totally released micro-organisms. After diluting the cell suspension, inoculums were spread over surface of culture medium, and then the colonies were counted after 24 hours brewing.

Total cell density: sum of cell density in fermentative fluid and cell density in finished products.

Density of free cell: the cell density in fermentative fluid.

Residual sugar was determined as glucose in the fermentative fluid by the colorimetric DNS (3, 5-dinitrosalicylic acid) method of Miller [6].

Quantitative estimation of L-Lysine in the fermentative fluid was made by Acidic ninhydrin method of Chinard and by HPLC method [7].

Glucose utilization concentration constant K_s (g.L^{-1}.h^{-1}): $K_S = \frac{S_i - S_r}{\tau}$. Where, S_i (g/L) is the original biomass concentration, S_r (g/L) is the biomass concentration after the time interval τ, τ (hours) is the fermentation time

L-Lysine production yield H (mol.mol^{-}): $\mathrm{Y}\left(\frac{\mathrm{P}}{\mathrm{S}}\right) = \frac{180}{146} * \frac{P_i - P_o}{S_u} * 100\%$. Where, P_i (g/L): L-Lysine concentration in fermentative fluid after the time interval τ and S_u (g/L): Concentration of utilized glucose after the time interval τ.

The proportion of washed cells outside the carriers (R, %): $R_i = \frac{X_o - X_i}{X_o} * 100\%$. Where, X_o: the number of colony-forming units in carriers which are used adding as initial inculum in bioreactor; X_i: the number of colony-forming units in carriers which are checked after fermentation cycle ending.

3 RESULTS AND DISCUSSION

3.1 BATCH FERMENTATION KINETICS OF L-LYSINE BY *C. GLUTAMICUM* ON COMPLEX CARRIERS OF ALGINATE AND BACTERIAL CELLULOSE

Batch culture is a close system in which all necessary medium components and the inoculum are added at the beginning and not during period of fermentation. In batch fermentation, there are 4 following phases: lag phase, log phase exponential phase, stationary phase and death phase [8]. Kinetic parameters such as residual substrate concentration, consumed substrate concentration, specific growth rate, L-Lysine productivity and L-Lysine yield were estimated to study the physiology of *C. glutamicum* immobilized on complex carriers of alginate and bacterial cellulose during batch fermentation of L-Lysine production.

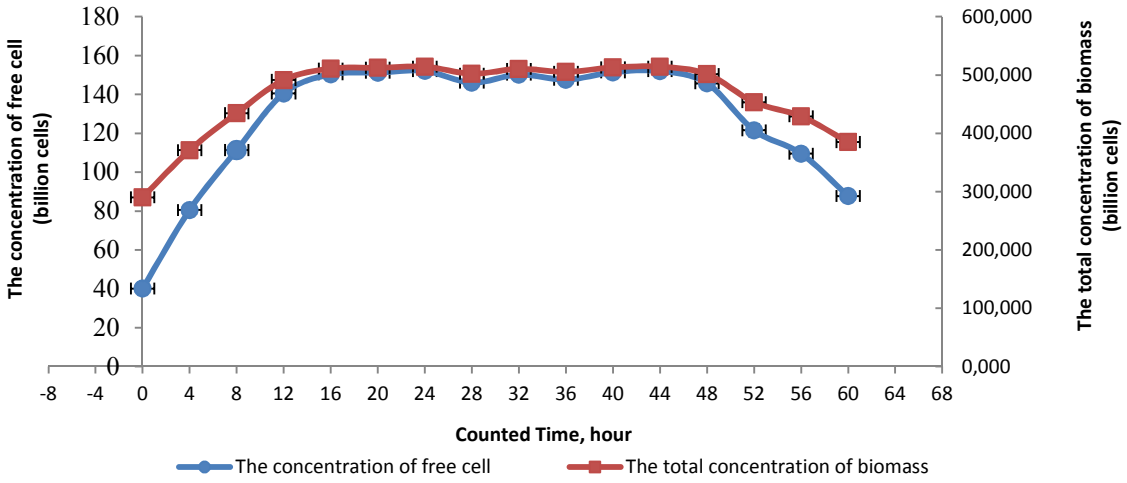

Fig. 1. The change about the concentration of cells during fermentation time

Figure 1 shows the change of biomass concentration during fermentation time. The total cell density was determined by summing of the cell density of free cells in fermentation fluid and the immobilized cells in the carrier. Initial concentration of free cells was 40 billion cells per litter, but it quickly grew up and almost increased 3.75 fold in log phase. Whereas, the biomass concentration of immobilized cells just increases about 1.6 fold. This suggests that the proliferation of free cell was stronger than that of immobilized cells because it has ability to contact directly with the nutrient medium. In addition, a part of the immobilized cells were washed out of the carrier and that was also a reason for the increase of free cells concentration. On the surface of carrier, biomass is increased and degraded more rapidly than that inside carrier. Because the optimal nutrient medium for the cell growth process has ability to contact directly with the microorganism located in the surface of carriers. Contrary, there is a contacting limitation between substrate as well as other components in the fermentation medium and microorganism which were clocked inside the structure of carrier [9]. Immobilized *C. glutamicum* cells onto the A-BC carriers have no lag phase or very short lag phase, results in increasing the biomass quickly in the first 8 hours and maintaining cell density stably during the residual time of fermentation process, prolong 36 hours.

Immobilized *C. glutamicum* cells are able to absorb and use substrate equivalently to free cells. Figure 2 shows the relationship between L-Lysine concentration and consumed substrate during the fermentation process. Fermentation of L-Lysine by the immobilized cells was carried out for 60 hours. Maximum L-Lysine concentration was produced in 8 to 40 hours and L-Lysine production constant (K_p) reached 0.98. L-Lysine fermentation was completed in two different time phases; first, physiologic state of growth and second, L-Lysine production phase. Because the lag phase was very short, most of the carbon source was consumed in the L-Lysine producing phase. Therefore, the substrate utilization rate was high in 8 to 32 hours and the substrate utilization constant (K_s) is 1.38. After first 40 hours, residual sugar in culture medium was 12 gram per liter. L-Lysine production yield is high at 92.6% and L-Lysine concentration in fermentative fluid reached 28.8 gram per liter. Comparing with the study of Nelofer, maximum L-Lysine concentration produced by free cells is lower than that of immobilized cells by 6.71 gram per liter. Further time of fermentation, L-Lysine production yield was decreased and L-Lysine productivity was not significantly increased ($p > 0.05$ compared with the time at 40^{th} hour). In terms of substrate utilization rate, the highest rate was reached at 1.1 gram per liter per hour after 32 hours and L-Lysine production yield is 91%. At this time, *C. glutamicum* used substrate for cell survival, cell growth and L-Lysine biosynthesis. Substrate utilization rate and production yield of free cells are 0.38 gram per liter per hour and 54.5%, respectively [3]. Obviously, substrate utilization rate and lysine production yield had greater values for the immobilized cells than the free cells, which means that batch culture by immobilized cells used more substrate as compared to the batch culture by free cells.

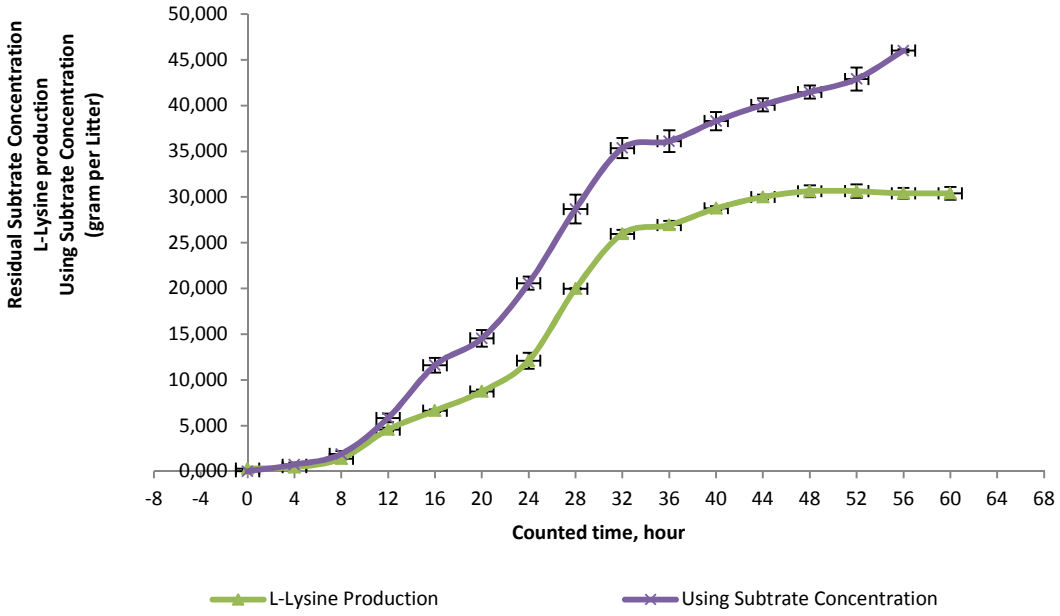

Fig. 2. Relationship between substrate concentration and L-Lysine

We are interested in the physiology of immobilized *C. glutamicum*. In the first 12 hours, the free cells grew strongly and its specific growth rate is 0.102. Meanwhile, the immobilized cells are limited by the narrow space of the carrier, contact of substrate and oxygen. Therefore, the average growth rate is just 0.04. Comparing with the kinetics analysis of batch fermentation of L-Lysine production by Rubina Nelofer (2007), we conclude that immobilized *C. glutamicum* has change in cell physiology [10]. Fermentation time of immobilized *C. glutamicum* cells (40 hours) is shorter than that of free cells (60 hours). *C. glutamicum* cells immobilized into the A-BC carrier have shorter fermentation time (40 hours) compared with *C. glutamicum* cells immobilized alginate-gluteraldehyde (72 hours) [5].

Based on the kinetics analysis of batch fermentation of L-Lysine production by immobilized *C. glutamicum* into A-BC carrier, we conclude that immobilized *C. glutamicum* has change in cell physiology. In addition, lag phase, fermentative cycle, fermentation time of immobilized cells are shorter than that of free cells. However, the L-Lysine productivity is not significant different between immobilized cells and free cells. These are meaningful advantages of the immobilized cells in fermentation process.

In conclusion, fermentation time is 40 hours, L-Lysine production yield is 92.6% and L-Lysine concentration in culture medium is 28.767±0.231 gram per liter.

3.2 INVESTIGATE RESUSED ABILITY OF *C. GLUTAMICUM* IMMOBILIZED ON COMPLEX CARRIERS OF ALGINATE AND BACTERIAL CELLULOSE FOR FURTHER FERMENTATION CYCLE

The immobilized products were used for L-Lysine fermentation process through many cycles. At the time of begin and finish of each fermentation cycle, sample was collected to analyze following targets: density of residual cells inside carrier, L-Lysine concentration.

Thereby, the escaped cell rate, fermentation time, total L-Lysine productivity and average yield of immobilized product were determined at the end of each fermentation cycle.

Table 1. The proportion of escaped cells after each fermentation cycle

The number of fermentation cycle	The proportion *of escaped* cells	The number of fermentation cycle	The proportion *of escaped* cells	The number of fermentation cycle	The proportion *of escaped* cells
1	11.60 ± 1.44[a]	**5**	33.20 ± 0.61[d]	**9**	41.73 ± 1.39[f]
2	18.27 ± 4.09[b]	**6**	34.53 ± 0.93[d]	**10**	45.20 ± 1.89[g]
3	25.87 ± 2.20[c]	**7**	36.13 ± 1.16[e]	**11**	49.20 ± 1.67[h]
4	31.87 ± 0.35[d]	**8**	38.00 ± 1.15[e]	**12**	51.33 ± 1.09[h]
The different letters in the table were significant in meanings (p < 0,05)					

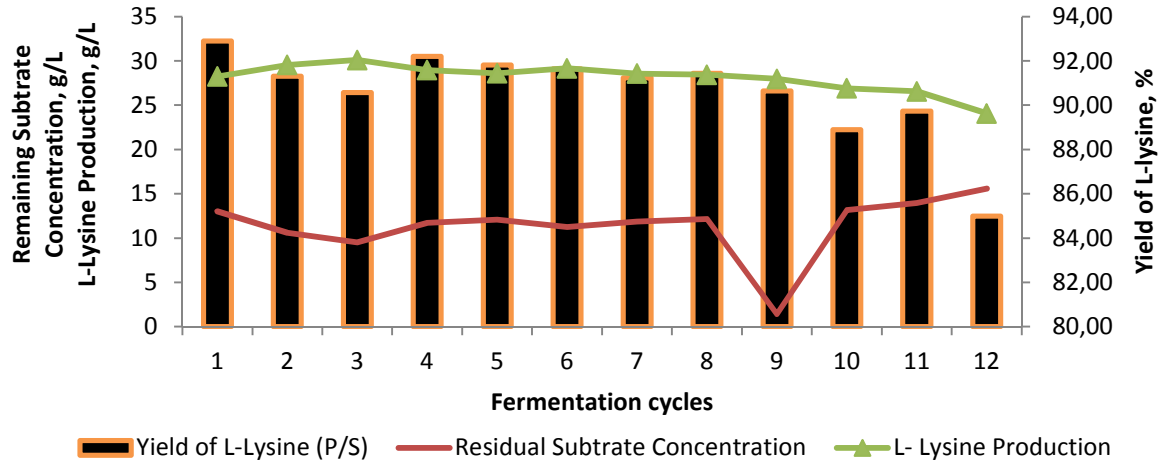

Fig. 3. Relationship between substrate concentration and L-Lysine after each fermentation cycle

The escaped cell rate after 4 fermentation cycles reduced steadily by 6 -8% compared with the previous cycle. From 5th cycle to 11th cycle, the escaped cell rate is lightly reduced by 1 -3 % compared with the previous cycle. From the 12th cycle, the escaped cell rate is decreased by 7% compared with the previous cycle and the structure of some immobilized products was broken. Obviously, in the first cycles, the escaped cell rate is high because cells attaching on the outer surface of carrier were washed out and then a part of cells inside carrier also were released into culture medium. When the cell proliferation occurs, there are the competition free hole inside the carrier structure and results in releasing cells into culture medium [11]. Although the cell density is reduced through the reused cycles, substrate utilization rate is kept constant (the residual sugar fluctuated from 12 ± 1 gram per liter after each cycle). This is explained as follows: a part of cells cannot contact directly with substrate or just contact with low amount of nutrient medium that is just enough for cells survive without synthesizing L-Lysine, so that utilized sugar concentration is not high. After each cycle, a part of cells were washed away, result in increasing free space inside the carrier and thus creating opportunity for contacting between cells and substrate. Therefore, cells can survive growth and synthesize L-Lysine. In sum, the residual sugar concentration is not significantly changed after each cycle. From the 12th cycle, the residual sugar concentration in the fermentation fluid is increased. Because of reducing density of *C. glutamicum* inside carrier, the sugar is not used totally by cells in bioreactor. In the first 11 cycles, L-Lysine concentration is in range of 27-30 gram per liter, equivalent to fermentation by free cells. From 12th cycle, L-Lysine concentration is reduced. L-Lysine production yield in the first 11 cycles is about 90%, and then it decreased in next cycle.

By comparion with the same cells that were immobilized onto simple carrier, such as, alginate, bacterial cellulose. The reused ability of these immobilized cells onto alginate, bacterial cellulose were 4, 8 fermentation cycles, respectively. Total fermenting time of immobilized product on alginate was lowest and just was 288 hours. Whereas, the immobilized product onto BC and A-BC showed higher total fermenting time and were 384 and 440 hours, respectively. Lysine production yield of immobilized cells onto Alginate, BC and A-BC were 0.56, 0.62 and 0.71 gram per liter per hour, respectively. Obviously, the application of immobilized *C. glutamicum* onto A-BC for lysine fermentation showed highest result because this finished products are able to overcome some drawback of alginate and have all the advantage of BC carrier [4, 5].

In the fermentation process of L-Lysine production by immobilized *C. glutamicum* into complex carrier of A-BC, reused ability of immobilized product is 11 fermentation cycles and total fermentation time is 440 hours and L-Lysine production yield is 0.711 ± 0.009 gram per liter per hour.

4 CONCLUSION

L-lysine concentration was improved to the level of 28.767±0.231 gram per liter and lysine production yield was 92.6% obtained under operating conditions of fermentation time 40 hours by immobile *C. glutamicum* cells on complex carriers of alginate and bacterial cellulose. And 40^{th} hour of fermentation process is the most suitable time to end running of each cycle in the survey period. In the fermentation process of lysine production by immobilized *C. glutamicum* on complex carrier of A-BC, the reused ability of immobilized product was 11 fermentation cycles and total fermentation time is 440 hours and total L-Lysine production yield is 0.711 ± 0.009 gram per liter per hour. The results obtained here indicate that L-lysine can be produced more efficiently by immobilized *C. glutamicum* by comparison with the previous results obtained with free cells. Specific growth rate was 0.102 and log phase is extremely short. These kinetic parameters show the change of cell physiology of immobilized *C. glutamicum* compared with free cells. L-lysine will be produced by immobile *C. glutamicum* cells promises to bring many benefits in industrial scale.

ACKNOWLEDGEMENTS

This research is the part of the post-graduated thesis of Tran Thi Minh Tam and Nguyen Thi Huong Giang. We are students of Department of Biotechnology of University of Ho Chi Minh City in Vietnam. We thank the school's administrators and the lectures have been helped us to study.

REFERENCES

[1] Bouville, M., Fermentation kinetics including product and substrate inhibitions plus biomass death: a mathematical analysis. Biotechnology letters, 2007. 29(5): p. 737-741.
[2] Gaden, E.L., Fermentation process kinetics. Biotechnology and bioengineering, 2000. 67(6): p. 629-635.
[3] Nelofer, R., et al., L-lysine production by the homoserine auxotrophic mutant of Corynebacterium glutamicum in stirrer fermenter. PAKISTAN JOURNAL OF ZOOLOGY, 2007. 39(3): p. 159.
[4] Trần Thị Minh Tâm, N.T.H., Optimization of Corynebacterium glutamicum immobilization process on bacterial cellulose carrier and its application for lysine fermentation. IOSR Journal of Engineering 2014. 04 (07): p. 33 -38.
[5] Tam, T.T.M. and N.T.H. , Optimization of Corynebacterium glutamicum Immobilization on Alginate and Investigation into its Storage Conditions. International Journal Of Modern Engineering Research (IJMER, 2014. 4(7): p. 66-71.
[6] Myara, I., C. Charpentier, and A. Lemonnier, Optimal conditions for prolidase assay by proline colorimetric determination: application to iminodipeptiduria. Clinica Chimica Acta, 1982. 125(2): p. 193-205.
[7] Gimba, C.E., et al., Journal of Chemical and Pharmaceutical Research, 2014, 6 (8): 39-43. Journal of Chemical and Pharmaceutical Research, 2014. 6(8): p. 39-43.
[8] Todaro, C.M. and H.C. Vogel, Fermentation and biochemical engineering handbook. 2014: William Andrew.
[9] Chibata, I. and L.B. Wingard, Immobilized microbial cells: applied biochemistry and bioengineering. Vol. 4. 2014: Elsevier.
[10] Nelofer, R., Q.B. Syed, Shahjahan , and M. Nadeem, L-lysine production by the homoserine auxotrophic mutant of Corynebacterium glutamicum in stirrer fermenter. Pakistan Journal of Zoology, 2007. 39(3): p. 159.
[11] Guisan, J.M., Immobilization of enzymes and cells. Vol. 22. 2006: Springer.