Optimization of *Corynebacterium glutamicum* Immobilization on Alginate and Investigation into its Storage Conditions

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Abstract: *The parameters of the immobilized process of Corynebacterium glutamicum VTCC – B – 0632 on alginate were identified by Plackett-Burman matrix, and the experiments were designed by response surface methodology having the central composite designs (RSM-CCD). The maximum yield of cell immobilization on alginate carrier reached at 92.6%. Optimal parameters were the cell density of 89.3 million cells/mL in the 4% sterile alginate with ratio 1:1. This mixture went through the syringe system of the 2M $CaCl_2$ solution at 20^0C with the shaking speed of 75 rpm until the gels get in shape. Then, these gels were soaked in the $CaCl_2$ liquor and shaken for 41 minutes (150 rpm). At last, the particle size of final products was 4mm and the average cell density was 14.75 million cells/gram. This immobile product is maintained under the suitable condition in the $CaCl_2$ liquor (w/v), pH=7. The cell survival percentage after 72 hours were 98% when it was stored in 4^0C, 0.5% $CaCl_2$ and pH of 7.*

Keywords: *Corynebacterium glutamicum, alginate, entrapment, micro-entrapment, Plackett-Burman, Response Surface Methods, Central Composite Designs.*

I. INTRODUCTION

Alginate is the natural polysaccharide extracted from the brown alga (*Phaeophyce*) which is discovered in the shallow water in the temperate zones. The viscosity of alginate depends on derivation of the alga, its molecular weight, temperature and pH of the liquor [1]. Under controlled conditions, alginate can create the gels in the liquor containing the II valence cations [2]. The cation Ca^{2+} is often used to immobilize cells because of its low toxicity. The capability of creating gels of alginate mainly relates to acid guluronic. The divalent cations linked with the G mass to make "an egg box" form that it is more solid than the M one [2].

Alginate is used as a carrier in the enzyme immobilization technology and it is developed into the traditional carrier in the cell immobilization technology, especially in the cell of micro-organism one. The cell immobilization in alginate carrier method which is usually used is the entrapment, in micro-encapsulation, creating cross linking [3].

Corynebacterium glutamicum is the viscous membrane bacterium, able to hold on the carrier and together. Therefore, we can use it to study the cell immobilization on some suitable carriers. The *Corynebacterium glutamicum* immobilization process is carried out by the hole trapping technology [4]. L-Lysine is an essential amino acid which cannot be synthesized by human being and animals. The too much amylaceous diets of the agricultural nations lead to the loss of this substance in the body. The L-Lysine is received from *Corynebacterium glutamicum* which can be applied in a few factories all over the world. One of solutions to upgrade the productivity of the L-Lysine is using immobilized *Corynebacterium glutamicum* cells on some carriers. *Corynebacterium glutamicum* is immobilized on alginate by entrapment methods. It is based on the inclusion of cells within a rigid network to prevent the cells from diffusing into the surrounding medium, while still allowing mass transfer of nutrients and metabolites [3]. L-lysine production using immobilized *Corynebacterium glutamicum* cells on alginate in fermentation process seems to be very promising. The advantages of this production process is that time, effort and expense are minimized during preparation period for the breed before fermenting. Consequently, efficiency of the Lysine fermentation is improved. However, application of immobilized *Corynebacterium glutamicum* in L-lysine fermentation still pointed out some disadvantages like physical carriers in fermentation medium or the ability of enzyme activity. It is one of some important reasons to study immobilization of cells [3].

II. THE MATERIALS AND METHODS

2.1 The materials and cultural medium

The micro-organism species: *Corynebacterium glutamicum* VTCC – B – 0632 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.

The cultural medium: *Corynebacterium 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 [5].

2.2. Optimizing the *Corynebacterium glutamicum* immobilization process on Alginate

The inoculums were introduced in seed culture and were incubated in a rotary shaker at 120 rpm, 30^0C. After 16 hours, the number of the cells was checked and counted and then the necessary breed density was predicted for the immobilization process with 100 mL breed liquor and alginate. Seven factors were examined in the Plackett-Burman matrix with different 12 runs. Determine the immobilization productivity for each validation formula. Analyze the factors that affect the productivity. The main factors had the value of $p < 0.05$. With the selected factors, we carried out the first experiments with the original values (+1, -1). After analyzing the initial experiments, we determined whether the factors having great impacts on the high regression equation suitably or not. Based on that, we conducted the experiments for response surface methodology having the central composite designs (RMS-CCD) and determined function of the polynomial regression accurately to describe relations between the immobilization yield and factors having great impacts on the hole trapping method.

2.3. The survey of the conditions to maintain the immobile *Corynebacterium glutamicum* final products on alginate

The aim is to determine the appropriate conditions to store the immobile *Corynebacterium glutamicum* products. We start to soak the products in some different pH liquors at different temperatures.
The major function is the survival percentage of *Corynebacterium glutamicum* after 72 hours storing according to the validation formula laid out (Table 1).

Table 1. Set up the survey experiment of the conditions

Runs	Changeable factors	Fixed factors
1	The liquor to soak (aseptic water-$CaCl_2$ (g/L): $0.5 - 1.0 - 2.0 - 3.0$)	-The weight of immobile bacterium: 10 gram -The maintaining temperature: 4^0C -pH of the liquor: 7
2	pH of the solvent: $4 - 5 - 6 - 7 - 8$	-The weight of immobile bacterium: 10 gram -The maintaining temperature: 4^0C -The solvent to soak the finished product: chosen in the experiment 1
3	The maintaining temperature (^{0}C): $0 - 4 - 10 - 15 - 20 - 30$	-The weight of immobile bacterium: 10 gram -The solvent to soak the finished product: chosen in the experiment 1 -The pH of the liquor: chosen in the experiment 2

2.4 The analyzing method

Analyzing the immobilized finished products: the immobilized *Corynebacterium glutamicum* on alginate is soaked in the sodium citrate in order to break the gels totally, and then release micro-organisms. After diluting the liquid medium, inoculums were spread over surface of culture medium, and then the colonies were counted after 24 hours brewing.

Formula:

The immobilization yield: $H = \frac{the\ immobilized\ cells\ in\ alginate}{the\ number\ of\ cells\ added} . 100$ (%)

The average cell density: $\frac{the\ number\ of\ immobile\ cells\ in\ alginate}{the\ weight\ of\ the\ finished\ product}$ (millions cells/g)

III. RESULTS

3.1. Optimizing the parameters to immobilize *Corynebacterium glutamicum* on the alginate carrier:

We analyzed the efficiency of the selecting experiments and determine the degree of these factors' impact in the fluctuation range according to different levels (Table 2). The change of the impacted levels of 2 among 7 surveyed factors has a noticeable influence on the immobilization yield of *Corynebacterium glutamicum* on the alginate carrier by the hole trapping method. Those are the density of immobilized cell suspension (X_2) and the soaking duration in $CaCl_2$ (X_7). The polynomial regression is determined according to the simple function as given below:

$$H = 61.8 - 17.69\,x_2 - 13.62\,x_7 \tag{3.1}$$

Table 2. The factors in the Plackett-Burman matrix experiments

Names of the factors	Symbols of the factors	The levels			Main effect	The p value
		Low (-1)	Medium(0)	High (+1)		
The weight of particle carriers (%)	X_1	3	4	5	+ 3.53	0.699
The density of biomass (million cells/mL)	X_2	100	200	300	-35.38	0.014
The concentration of $CaCl_2$ (M)	X_3	1	2	3	-3.56	0.696
The temperature to form gels (^{0}C)	X_4	15	20	25	+14.25	0.168
The shaking speed to form particles (rpm)	X_5	50	75	100	-11.46	0.248
The speed when soaking particles (rpm)	X_6	100	150	200	+10.05	0.301
The soaking duration (minutes)	X_7	60	120	180	-27.25	0.032
R-sq = 89.51%; p < 0.05 was considered significant						

The two factors, X_2 and X_7, suit the model of (3.1) 89.51%. This is acceptable. We went on conducting 9 experiments, 4 of them are (-1, 1) ones and 5 are the central ones. ANOVA was carried out to statistically analyze the correlation of immobilization efficiency to the two selected factors. The p-value of Lack-of-fit test was 0.849 and R-sq was 99.67 %. This means that the arrangement of the two big effect factors (Table 2) is far away from the extreme of the aimed function. To define the extreme zone, it is necessary to carry out the path of steepest ascent experiments (Table 3).

Table 3. The Path of steepest ascent extreme zone experiments

Runs	X_1	X_2	X_3	X_4	X_5	X_6	X_7	H (%)
1	4	94.3	2	20	75	150	50	91.23
2	4	88.6	2	20	75	150	40	92.6
3	4	82.9	2	20	75	150	30	89.79
4	4	77.2	2	20	75	150	20	89.29
5	4	71.5	2	20	75	150	10	89.27
6	4	65.8	2	20	75	150	0	88.64

The Path of steepest ascent extreme zone experiments was aimed to define the extreme zone of the yield of cell immobilization. The second experiment was chosen as a central degree. At this, the highest yield of all experiments was obtained. To establish the right relationship between X_2 and X_7, we analyzed the 13 RMS-CCD experiments showed by the polynomial regression equation 3.2 (R-sq = 85.35%):

$$H = 92.6 - 0.452\,x_2 + 0.325\,x_7 + 0.355\,x_2 x_7 - 1.912\,x_2^2 - 1.437\,x_7^2 \tag{3.2}$$

The maximum immobilization yield of *Corynebacterium glutamicum* on alginate was 92.65 % when the density of the added cells was 89.3 million cells/ mL, the soaking duration in $CaCl_2$ was 41 minutes, the concentration of utilized alginate is 4% (w/v), 2M $CaCl_2$, the temperature is 20^0C, the agitation rate to form gels is 75 rpm, the agitation rate when soaking is 150 rpm. The particle-shaped finished product has the diameter of $4 \pm 0,2$ mm and the cell density of $14.75 \pm 0.053\ million\ cells/g$ of finished product.
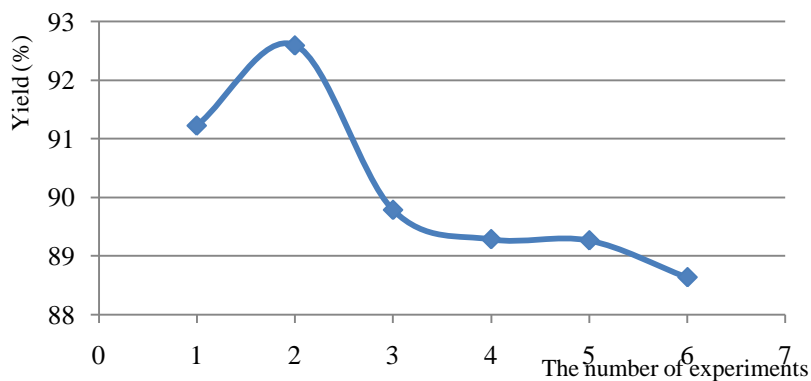
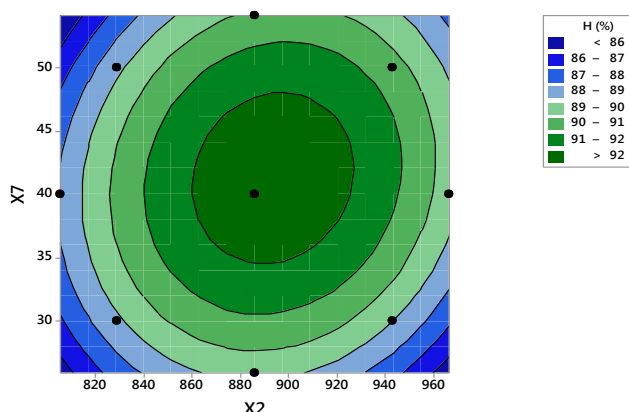Figure 1. The Path of steepest ascent extreme zone chart

Figure 2. The equation contours (3.2) show the relationship between H (%) and X_2, X_7

3.2. Examination the storage conditions

We chose the round products with no bubbles which are achieved from the optimizing process. We took them to examine. The validation formulas examined use 20 ± 0.2 g of products in 50 mL of solvent.

3.2.1 The influence of the maintaining solvents

We analyzed the finished products of 5 validation formulas to examine the two solvents: water and $CaCl_2$ liquor (0.5-1.0-2.0-3.0%). We determined the cell survival percentage after 72 hours maintaining under the 4^0C condition.

Table 4. The survival percentage of cells at different solvents

The survival cell rate after 72-hour incubation (%)	Sterile water	CaCl₂ 0.5%	CaCl₂ 1.0%	CaCl₂ 2.0%	CaCl₂ 3.0 %
	67.31 ± 7.87^a	94.8 ± 4.64^b	90.15 ± 1.05^c	83.64 ± 2.35^d	68.95 ± 0.45^a
	Data presented as mean ± standard deviation The values with the same symbols are not different from the meaning p < 0.05; the values with different symbols are different from the meaning of p < 0.05				

$CaCl_2$ was the solvent to maintain the immobile final product *Corynebacterium glutamicum* on alginate better than distilled water. The cell survival percentage after 72 hours was higher 68% and higher than in distilled water (67.3%). Especially, the 0.5% $CaCl_2$ solution (w/v) had the highest percentage (94.8%). This concentration was used for the next experiments.

3.2.2 The influence of pH

The validation formulas examined the change of pH (4-5-6-7-8) of the 0.5 % $CaCl_2$ solvent and adjusted by HCl 1N and 25% NH_3 solution, we examined the survival percentage of cells after 72 hours at 4^0C.

Table 5. The survival percentage of immobile cells at the different pH

The survival cell rate after 72-hour incubation (%)	pH=4	pH=5	pH=6	pH=7	pH=8
	65.58 ± 2.31^a	66.20 ± 2.40^a	75.49 ± 2.93^b	95.29 ± 3.66^c	83.36 ± 3.29^d
	Data presented as mean ± standard deviation The values with the same symbols are not different from the meaning p < 0.05; the values with different symbols are different from the meaning of p < 0.05				

In the limit of pH from 4 to 7, the survival cell rate increases. If we continue to raise the pH to 8, the percentage decreases less 12% than that of pH of 7. At the pH of 7, the survival rate was maximum with the value of 7. The pH of 7 is used to examine the influence of temperature that was used to maintain the finished product.

3.2.3 The influence of temperature

Examine the change of the temperatures that was used to store the final product from 0 to 40^0C. Determine the immobilized cell survival percent after 72 hours soaking in 0.5% CaCl2 solution , pH = 7.

Table 6. The survival cell percent at different maintaining temperatures

The survival cell percentage after 72-hour incubation (%)	0^oC	4^oC	10^oC	20^oC	30^oC	40^oC
	50.37 ± 0.14^a	98.31 ± 0.31^b	82.24 ± 0.09^c	87.91 ± 0.13^d	86.09 ± 0.26^d	27.35 ± 0.09^e
	Data presented as mean ± standard deviation. The values with the same symbols are not different from the meaning $p<0.05$; the values with different symbols are different from the meaning of $p<0.05$					

The cell survival percentage is the lowest one when the temperature is bad for the cells' survival, this percent gets minimum at 40^oC (27%). In the limit of 4^oC-30^oC, the survival percentage is 82% or higher. The percentage is the highest one when we maintain the finished product at 4^oC (98%).

IV. DISCUSSION

4.1 The optimizing parameters for the cell *Corynebacterium glutamicum* immobilization on alginate carrier process by entrapment method

The hole trapping technology is also called the outside forming gel. Guisan (2006) assumed that under the Ca^{2+} ion condition, the above surface of alginate immediately caused gels. After that, the Ca^{2+} ions diffused into alginate particles. This made the alginate ones inside become gels and form the network button links [3,6]. The gels were continuously soaked in the Ca^{2+} for a while to make the structures stable before it was used in the fermentation process [3]. According to Gordon F. Bickerstaff (1997), the alginate concentration did not affect the shape and the size of gels but it led solid state. When we increased the alginate concentration, the gel networks in the outside surface became dense. This increased the solid state of gels but decreased the cell metabolism [3]. Moreover, Gordon (1997), Guisan (2006) and Morch (2006) claimed that the gels got more solid when the concentration of Ca^{2+} ions was increased. However, the growth of the cells could be affected by too high concentration [3, 7]. As regards the physical space, the each gel hole had a certain size, so it contained a certain number of cells. Therefore, original suitably adjusted density of cells was able to make the immobilization efficiency maximum. Kourkoutas (2004) claimed that the large density of cells among the mesh fabrics of the linking networks blocked the polymer circuit shrinking and reduce the solid state of the whole gene mass [8].

4.2. The storage condition

The *Corynebacterium glutamicum* immobilized products on alginate has the growth that is nearly same as the *Corynebacterium glutamicum* free cells. The only difference is that disadvantages of the stored condition can be reduced by the assistance of the immobilized carrier. The examination of stored condition is based on the growth limit of *Corynebacterium glutamicum* and the condition to firm the structure of alginate gel. Morch (2006) assumed that Ca^{2+} ions had ability to firm the structure and maintain pH of solvents stably during the store process as well as the fermentation process. Thus, calcium chloride is the chosen solvent that is used to store the final products [7]. Nevertheless, too high concentration of salt in the solvent affects the damage of the membrane of *Corynebacterium glutamicum* [9]. Kourhoutas (2004) recognized that if the pH was not suitable, the solid state of gels would be decreased. However, as mentioned by Eggeling (2003), the gel structure was not affected when pH was in range 4-10, this change affected the existence of *Corynebacterium glutamicum*. He also claimed that these bacteria grew stably at the neutral pH [5]. According to Ohnishi (2003), the suitable limit of temperature for the growth of *Corynebacterium glutamicum* was from 20-40^0C [10]. The cells were able to grow within this temperature range. During cell growth, the rapidly increasing number of gels will block the formation of Ca^{2+} and the carboxyl dyads of the alginate molecules in the gel making process. As a result, the stability of gel structures goes down and the cells get out of the easily in store process [3]. Besides, during cell growth, CO_2 is released and break the gel structures [11].

V. CONCLUSION

The maximum yield of the *Corynebaterium glutamicum* immobilization on alginate process is 92.6%. The cells have density of 83.9 million cells/mL mixed in 4g of sterile alginate to get the volume of 100 ml. Let this mixture go through the syringe system of 2M CaCl$_2$ at 20^{0}C and the agitation rate of 75 rpm until the gel shape is totally created. Continue to soak these gels in CaCl$_2$ 2M in 41 minutes with the agitation rate of 150 rpm. The obtained result is that the final products have particle shape with 4 mm in diameter and the cell density is 14.75 million cells/g. Final product is soaked in 0.5% CaCl$_2$ solvent (w/v), pH = 7 and then kept at 4°C. After 72h, the percentage of cell survival is 98%. The *immobilized Corynebaterium glutamicum* final product on alginate is used as a method to upgrade the efficiency of ferment to create L-Lysine amino acid.

Acknowledgements

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