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# Determination of Immobilization Process Parameters of *Corynebacterium glutamicum* on Kappa carrageenan, Its Application in L-lysine Fermentation and The Investigation Into Its Storage Conditions

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**ABSTRACT:**
The parameters of the immobilized process of Corynebacterium glutamicum on kappa carrageenan 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 kappa carrageenan carrier reached at 78% ± 2%. Optimal parameters were 3 grams kappa carrageenan per 100 militters sterile water and 58.58 million cfu/mL, forming gels at $10^0$C for 25 minutes and the speed when soaking particles of 150 rpm for 120 minutes in 0.58 M potassium chlorua solvent. The immobile finished products are applied in L-lysine production, their reusing ability is 3 times and the total yield of L-lysine was accumulated 93 g/L in medium during 96 fermented hours. The L-lysine productivity of the batch fermentation was 0.969 $g.L^{-1}.h^{-1}$. And the set-up storage conditions are the mixed solvent of $CaCl_2$ 0.5% (w/v) and KCl 0.5% (w/v); pH is 7.0 in $4^0$C. After 60 storage days, the survival cell rate was remained 51%.

***Keywords:*** *Corynebacterium glutamicum*, kappa carrageenan, entrapment, micro-entrapment, Plackett-Burman, Response Surface Methods, Central Composite Designs, L-lysine production, batch fermentation

## I. INTRODUCTION

L-Lysine is an essential amino acid which cannot be synthesized by human being and animals. The L-Lysine is received from *Corynebacterium glutamicum* which can be applied in a few factories all over the world. *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 entrapment techniques [4]. One of solutions to upgrade the productivity of the L-Lysine is using immobilized *Corynebacterium glutamicum* cells on some carriers.

Many techniques have been used previously for enzyme immobilization, including entrapment, cross-linking, adsorption, or a combination of these methods. *Corynebacterium glutamicum* is a bacterium which has been immobilized onto a wide variety of solid supports such as alginate, bacterial cellulose, k-carrageenan, chitosan, gelan, agarose, polyvinyl alcohol polymers or complex matrix. K-carageenan has been used for the immobilization of cells and enzymes by entrapment techniques. Carageenan matrix suffers weak mechanical and thermal stability.

Kappa carrageenan is linear sulfated polysaccharide extracted from the red algae (*Rodophyceae*). The viscosity of kappa carrageenan depends on derivation of the algae, melting temperature, the presence of appropriate cations (example potassium or calcium) and pH of the liquor [1]. In the presence of calcium, kappa carrageenan forms a stiff and brittle gel. However, it forms very firm and elastic gels in the presence of potassium salts. pH tolerance of kappa carrageenan ranges between 4.0 and 10.0, it is not stable in poor acid liquor. Kappa carrageenan thickens from 0.02% to 1.5% and gels above 1.5% concentration. The carrageenan solution need to be heated over 79$^o$C to be completely hydrated and as it cools down below 45$^o$C, it starts to gel. Increasing the amount of potassium or calcium avaible will make the gel stronger and allow gels at higher setting temperatures and lower concentrations.

*Corynebacterium glutamicum* is immobilized on kappa carrageenan 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 kappa carrageenan 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. *Corynebacterium glutamicum*, provided by Vietnam Type Culture Collection, has overproducing pathway, so it is chosen in study on lysine production. In this study, this bacterium was used as an object in all experiments. 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* is provided by the Vietnam Type Culture Collection

**The carrier**: the utilized kappa carrageenan is provided by the producer Vietnam. Kappa carrageenan is powdery, light brown and its moisture content is approximately 15%. The 1% kappa carrageenan solution is prepared and kept at $25^0$C. Its viscosity is 5-40 Cps and pH is from 4.0 to 10.0.

**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 $30^0$C, the agitation rate of 150 rpm [5]. After 24 fermentating hours, the cell rate in broth was 200 billion clone form units.

**The fermentation medium:**

Glucose (50g/L), $(NH_4)_2SO_4$ (32g/L, $KH_2PO_4$ (1 g/L), $MgSO_4.7\ H_2O$ (0.1 g/L), $FeSO_4$ (10 mg/L), $MnSO_4$ (10 mg/L), $ZnSO_4$ (10 mg/L), $CuSO_4$ (1 mg/L), Biotin (20µg/L), $CaCO_3$ (10 g/L), Thiamine (150 µg/L), Tween 20(5mL/L), Corn Solution Liqid (120 mL/L).

**The fermentation condition:** temperature $30^0$C, initial pH in medium 7.0 ± 0.3, adjusted by $NH_3$ 25% solution.

### 2.2. Optimizing the *Corynebacterium glutamicum* immobilization process on Kappa carrageenan

The inoculum was introduced in seed culture and was incubated in a rotary shaker at 150 rpm, $30^0$C. 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 kappa carrageenan. Seven factors were examined in the Plackett-Burman matrix with different 12-run.We determined the immobilization productivity for each validation formula and analyzed 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 (RSM-CCD) and determined function of the polynomial regression accurately to describe relations between the immobilization yield and factors having great impacts on the entrapment techniques.

### 2.3. Lysine fermentation in the immobilized cell on kappa carrageenan carrier

Bioreactors 1000 milliliters of fermentation medium and 10% (w/v) immobilized cell on kappa carrageenan. Response: lysine field, residual sugar and the rate of escape cell after the cycle of Lysine fermentation.

### 2.4. The survey of the conditions to maintain the immobile *Corynebacterium glutamicum* final products on kappa carrageenan

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 solvent to soak the finished product: deionzed water, NaCl (0.50 – 0.85 – 1.00 % w/v), KCl (0.50 – 1.00 % w/v), $CaCl_2$ (0.50 – 1.00 % w/v), $CaCl_2$ : KCl (0.50 : 0.50 % w/v), $CaCl_2$ : KCl (1.00 – 1.00 % w/v), $CaCl_2$ : KCl (0.50 – 1.00 % w/v), $CaCl_2$ : KCl (1.00 – 0.50 % w/v) | - pH of solvent: 7.0<br>- The maintaining temperature: $4^0$C<br>- The storage time: 3 days |
| 2 | pH of the solvent: 5 – 6 – 7 – 8 | - The maintaining temperature: $4^0$C<br>- The storage time: 3 days<br>- The solvent to soak the finished product: chosen in the experiment 1 |
| 3 | The maintaining temperature ($^0$C): 0 – 4 – room temperature (between 30 and 32$^0$C) | - The storage time: 3 days<br>- The solvent to soak the finished product: chosen in the experiment 1<br>- The pH of the liquor: chosen in the experiment 2 |
| 4 | The storage time (days): 7 – 10 – 14 – 30 – 60 | Others conditions are chose in all experiments above. |

### 2.5. The analyzing method

#### 2.5.1. Analyzing L-lysine in fermentation broth

**Qualitative assay:** Ascending Thin Layer Chromatography was employed for the detection of L-lysine in the culture broth. The solvent systems used included n-butanol: acetic acid: water (1:2:4, v/v). The spots were visualized by spraying with a solution of 0.5% ninhydrin in butanol.

**Quantitative estimation:** The concentration of L-lysine was assayed by acidic ninhydrin method of Chinard (1952) which is modified by Chung – Lung Hsieh (1995).

#### 2.5.2. Analyzing residual sugar in fermentation broth

Residual sugar was measured as glucose in the supernatant fluid by colorimetric DNS method of Miller (1959).

#### 2.5.3. Analyzing the immobilized finished products

Immobilized product is mechanically soaked 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: $H = \frac{the\ immobilized\ cells\ in\ kappa\ carrageenan}{the\ number\ of\ cells\ added}.100\ (\%)$

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

The L-lysine production yield: $Y = \frac{Lysine\ \frac{g}{L}}{Using\ Sugar\ \frac{g}{L}}.100\,(\%)$

## III. RESULTS

### 3.1. Optimizing the parameters to immobilize *Corynebacterium glutamicum* on the kappa carrageenan carrier:

The main effect of all factors which were established in Plackett – Burman matrix was given in 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 kappa carrageenan carrier by the entrapment techniques. Those are the initial inoculum ($X_2$) and the concentration of KCl ($X_5$). The polynomial regression is determined according to the simple function as given below:

$$Response, y\ (\%) = 84.4 + 3.96\,x_2 - 9.03\,x_5 \quad (3.1)$$

The two factors, $X_2$ and $X_5$, suit the model of (3.1) R-sq = 96.4%. This is acceptable. We went on conducting 9 initial 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 initial experiments were established by center points and corner points of two chosen factors above. The p-value of Lack-of-fit test was 0.005 and R-sq was 98.85 %. This meant that the immobilization efficiency and two main effective factors were not in the linear relationship.

The highest cell immobilization efficiency was run-5 ($x_2$ = -1, $x_3$ = -1) of all experiments, which was reached 71.6 ±2.95 %. In this case, we determined the relationship between the main effective factors and response (the cell immobilization efficiency). The poly-nominal regression equation was determined below:

$$Response, y\ (\%) = 135.1 - 0.613x_2 - 22.0x_5 - 0.0009x_2^2 + 2.97x_5^2 + 0.0238x_2x_5$$

(3.1.)

In the above equation, y (%) symbols for cell immobilization efficiency, $x_2$ ($10^6$ cells/mL) symbols for initial inoculum and $x_5$ (mol/L) symbols for concentration of KCl.

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 concentration of kappa carrageenan (%) | $X_1$ | 2 | 3 | 4 | +0.792 | 0.106 |
| The initial inoculum (million cells/mL) | $X_2$ | 100 | 200 | 300 | -0.334 | 0.001 |
| The temperature to form gels ($^0$C) | $X_3$ | 5 | 10 | 15 | +3.338 | 0.155 |
| The forming gels duration (mins) | $X_4$ | 20 | 25 | 30 | -2.140 | 0.605 |
| The concentration of KCl (M) | $X_5$ | 1 | 2 | 3 | -18.060 | 0.009 |
| The speed when soaking particles (rpm) | $X_6$ | 100 | 150 | 200 | +0.310 | 0.940 |
| The soaking duration (mins) | $X_7$ | 60 | 120 | 180 | -0.008 | 0.908 |
| R-sq = 96.4%; p < 0.05 was considered significant | | | | | | |

The regression equation (3.1) showed that two factors (added initial inoculum factor and concentration of KCl factor) were positively affected on the formation of the cell immobilization efficiency. In fact, the initial inoculum factor had the strongest influence level in all factors. This factor was decreased, the response would be increased. The concentration of KCl factor was also decreased to improve the cell immobilization efficiency. And, the response was been high, the 2-way interaction ($X_2$ * $X_5$) was increased. However, they are non-linear, they change in certain limitation. We identified the function of the poly-nominal regression accurately, the experiments for response surface methodology having the central composite designs (RSM-CCD) with the main effective factors (-α, -1, 0, +1, +α) were conducted. The cell immobilization efficiency and the average immobilized cell on kappa carrageenan were got responses.

*Table 3*. *Corynebacterium glutamicum* cell immobilization efficiency and average immobilized cell on Kappa carrageenan carrier by RSM-CCD experiments.

| Runs | Code units | | Uncoded values | | Response, Y (%) |
|---|---|---|---|---|---|
| | $X_2$ | $X_3$ | $X_2$ ($10^6$ cells/mL) | $X_5$ (mol/L) | |
| 1 | 0.0 | 0.0 | 200.00 | 2.00 | 58.55±1.82 |
| 2 | 0.0 | 0.0 | 200.00 | 2.00 | 57.11±1.96 |
| 3 | -1.4 | 0.0 | 58.58 | 2.00 | 87.57±2.32 |
| 4 | 0.0 | 0.0 | 200.00 | 2.00 | 56.14±2.21 |
| 5 | -1.0 | -1.0 | 100.00 | 1.00 | 91.60±2.95 |
| 6 | +1.4 | 0.0 | 341.42 | 2.00 | 49.50±0.75 |
| 7 | -1.0 | +1.0 | 100.00 | 3.00 | 77.60±4.12 |
| 8 | +1.0 | +1.0 | 300.00 | 3.00 | 56.83±2.05 |
| 9 | 0.0 | 0.0 | 200.00 | 2.00 | 56.92±2.10 |
| 10 | 0.0 | -1.4 | 200.00 | 0.59 | 64.50±3.12 |
| 11 | 0.0 | +1.4 | 200.00 | 3.41 | 47.01±1.25 |
| 12 | +1.0 | -1.0 | 300.00 | 1.00 | 61.30±2.81 |
| 13 | 0.0 | 0.0 | 200.00 | 2.00 | 57.11±1.81 |

The analysis of variance indicated that the above poly-nominal regression function got R-sq = 91.41%. We used response optimizer tool in Minitab 17 to indentify the combination of predictor values that jointly optimize one. The highest predict response value (y, %) was 91.6% with established optimal parameters as below: 3 grams kappa carrageenan per 100 militters sterile water and 58.58 million cfu/mL, forming gels at $10^0$C for 25 minutes and the speed when soaking particles of 150 rpm for 120 minutes in 0.58 M potassium chlorua solvent. However, in experiments with optimal factors, the cell immobilization efficiency was achived 78% ± 2% and the cell density in carrier was 50 ± 0.07 million cfu/g of finished product.

In fact, the gel form ability of kappa carrageenan depends on many factors including the concentration of kappa carrageenan in mixed solution and in the presence of cation as well as temperature to make gels. In the study, optimum parameters for the cell *Corynebacterium glutamicum* immobilization on kappa carrageenan carrier were identified by screening experiments.

The results of screening experiments were determinated two in seven factors that should be changed to achive the optimal immobilization efficiency. Those were the initial inoculum ($X_2$) and the concentration of potassium salts. In this immobilization process, $X_2$ and $X_5$ factors were set up higher than those in optimal parameters for the immobilization process while the remaining factors in the immobilization process were fixed. In fact, the cell density in carrier depends on space (both the number of space and square of space) in carrier and the ability of making linkages. The initial inoculum rate is high to compete the space in carrier and to increase their leakages, therefore, the the cell immobilization efficiency is low. Moreover, in the presence

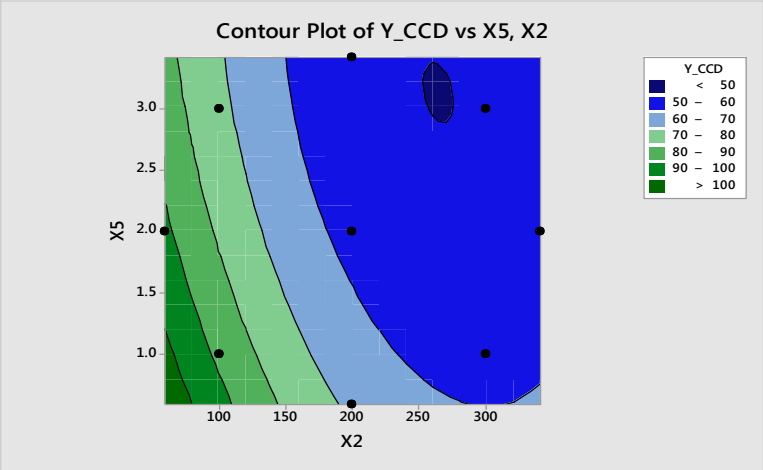


Figure 1. The equation contours (3.2) show the relationship between H (%) and $X_2$, $X_5$

of potassium salts, kappa carrageenan forms very firm and elastic gels. Increasing the amount of potassium will make the gel stronger and the cell will go difficultily through carrier to immobile in carrier. In comparison to the cell immobilization on alginate, the cell immobilization efficiency is lower and the cell density in kappa carrageenan carrier is higher.

### 3.2. Lysine fermentation on *Corynebacterium glutamicum* on Kappa carrageenan

The immobile finished products of *Corynebacterium glutamicum* were applied in L-lysine production. The batch fermentation was set up with optimum parameters such as fermentation medium, conditions, counting fermentation time. Batch fermentation was analyzed by determinating some responses including remaining sugar, using sugar, L-lysine, the washout cell rate and yield of L-lysine production.

*Table 4*. Analyzing the batch fermentation parameters in L-lysine production by the immobile finished products on kappa carrageenan

| Cycling fermentation | Lysine, g/L | Sugar remaining, g/L | Sugar using, g/L | The washout cell rate, % | Yield of L-lysine production, % |
|---|---|---|---|---|---|
| 1 | $30.94 \pm 0.22^a$ | $10.24 \pm 0.03^a$ | $39.76 \pm 0.03^a$ | $13.15 \pm 2.01^a$ | $77.83 \pm 0.53^a$ |
| 2 | $32.74 \pm 0.18^a$ | $14.24 \pm 0.72^b$ | $35.76 \pm 0.72^b$ | $29.39 \pm 1.05^b$ | $91.59 \pm 2.32^b$ |
| 3 | $29.58 \pm 0.44^a$ | $14.70 \pm 0.06^b$ | $35.3 \pm 0.06^b$ | $37.72 \pm 2.43^c$ | $83.79 \pm 1.39^c$ |
| 4 | $23.39 \pm 0.36^b$ | $14.74 \pm 0.09^b$ | $35.3 \pm 0.09^b$ | $53.88 \pm 1.90^d$ | $66.34 \pm 1.04^d$ |

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 L-lysine fermentation, Lysine production was stable during three-reuse. The highest production was 32.74 ± 0.18 g/L in the second cycling, it was larger than that in the first cycling, flowed by in the third and the fourth, 29.59, 23.39 g/L, respectively. On other hand, the finished products were consumed approximately amount of sugar during four reusing times. In the first, the remaining sugar was the least. Additionally, the washout cell rate after reusing was determinate by density of survival cell in the carrier, they decreased gradually from 13.15 % to 53.88%, after the first and

the fourth, respectively. Other response was considered that was yield of L-lysine production, the maximum yield was in the second reuse, 91.59%. To sum up, the immobile finished products on kappa carrageenan were used in L-lysine fermentation that they had three reusing times and the total yield of L-lysine was reached 93 g/L during 192 fermented hours. The productivity of the batch fermentation was 0.485 $g.L^{-1}.h^{-1}$, was higher than that using the immobile finished products on alginate.

In stable fermentation condition, the immobile finished products on kappa carrageenan were used in L-lysine production, were different from amount of L-lysine, amount of sugar using and the washout cell rate. In the first cycling, the amount of L-lysine was low but, *Corynebacterium glutamicum*, it used large amount of sugar. Because bacteria need the amount of substrate to sharp biomass then it excretes L-lysine in medium. In the next reusing, the bacteria are adopted and have stable density of cell in medium, they use sugar to produce L-lysine in medium, so that, the yield of L-lysine production is high. However, the yield decreases gradually because the washout cell rate decreases after every reusing, the amount of biomass in medium decreases.Using immobilized *Corynebacterium glutamicum* on kappa carrageenan carrier for Lysine fermentation had many advantages. The Lysine yield was increased due to short fermenting time per batch and immobilized cell reusing. Immobilized cell were used for fermentation had not lag stage as the result of saving time because cells had already been adapted to the environment. Moreover, we had not to prepare innoculum, it means, we did not spend time and money preparing medium [3].

In L-lysine fermentation, Lysine production was stable during three-reusing times. The highest production was 32.74 ± 0.18 g/L in the second fermentation cycle and larger than that in the first once, followed by the third and the fourth cycles, 29.59, 23.39 g/L, respectively. On other hand, the finished products were consumed approximately amount of remaining sugar during four reusing times. In the first cycle, the remaining sugar was the least. Additionally, the washout cell rate after reusing was determined by density of survival cell in the carrier and it incereased gradually from 13.15 % to 53.88% after fourth reusing time. Other response was considered that was yield of L-lysine production, the maximum yield was in the second reuse, 91.59%. To sum up, the immobile finished products on kappa carrageenan were used in L-lysine fermentation that they had three reusing times and the total yield of L-lysine was reached 93 g/L during 192 fermented hours. The productivity of the batch fermentation was 0.485 $g.L^{-1}.h^{-1}$ and higher than that using the immobile finished products on alginate.

In stable fermentation condition, the immobile finished products on kappa carrageenan were used in L-lysine production, there are differences between amount of L-lysine, amount of sugar using and the washout cell rate in each reusing cycle. In the first fermentation, *Corynebacterium glutamicum* used large amount of sugar, but the amount of synthesized L-lysine was not high. Because bacteria need a large amount of substrate to increase biomass sharply then it excretes L-lysine in medium. In the next reusing cycle, the bacteria have stable density of cell in medium, they use sugar to produce L-lysine in medium, so that, the yield of L-lysine production is high. However, the yield decreases gradually because the washout cell rate increases after each reusing time, result in reducing the amount of biomass in medium.Using immobilized *Corynebacterium glutamicum* on kappa carrageenan carrier for Lysine fermentation had many advantages. The Lysine yield was increased due to short fermenting time per batch and ability to reuse immobilized cells. Immobilized cells were used for fermentation had not lag stage as the result of saving time because cells had already been adapted to the environment. Moreover, we had not to prepare innoculum, it means, we did not spend time and money preparing medium [3].

**3.3. Examination the storage conditions**

Approximately 13.244 ± 3.652 g of finished products were put in every 50 mL of storage solvent (mean ± SD).

**3.3.1 The influence of the maintaining solvents**

We analyzed the finished products of 12 validation formulas to examine the five solvents: sterile water, KCl liquor (0.5 – 1.0%), NaCl liquor (0.50 – 0.85 – 1.00 % w/v), $CaCl_2$ liquor (0.5 – 1.0%), and mixed ratio between $CaCl_2$ and KCl. We determined the cell survival percentage after 72 hours maintaining under the $4^0$C condition.

The percentage of survival cells preserved in mixed solvent group was higher than that in others. In particular, the group stored in mixed solvents ($CaCl_2$ and KCl) had over 60% of survival cells and others had less than 50% of survival cells in finished products after 3rd storage day. The mixed solvent of $CaCl_2$ 0.5% and KCl 0.5% with ratio 1 : 1 had the highest survival cell percentage (98%) over the runs and followed by the solvent of $CaCl_2$ 1% and KCl 1% with ratio 1 : 1 had 86% of survival cells. The percentage of survival cells in remaining mixed solvents was approximately 80%. Storing finished products in sterile water had the least percentage of survival cells for 72 storage hours. To study other influenced factors in finished product prervation, the mixed solvent of $CaCl_2$ 0.5% and KCl 0.5% was chosen.

Table 5. The survival percentage of cells at different solvents

| Solvents | The survival cell rate, % | Solvents | The survival cell rate, % |
|---|---|---|---|
| Sterile Water | 23.04 ±0.17[a] | $CaCl_2$ 0.50% | 45.97±3.83[c] |
| NaCl 0.50% | 37.63±2.99[b] | $CaCl_2$ 1.00% | 48.92+4.77[d] |
| NaCl 0.85% | 39.24±2.19[b] | $CaCl_2$:KCl (0.50:0.50%) | 98.72+2.18[e] |
| NaCl 1.00% | 44.67±7.54[c] | $CaCl_2$:KCl (0.50:1.00%) | 77.32+3.99[f] |
| KCl 0.50% | 49.92±3.51[d] | $CaCl_2$:KCl (1.00:1.00%) | 86.08+4.65[g] |
| KCl 1.00% | 38.23±4.19[b] | $CaCl_2$:KCl (1.00:0.50%) | 80.79+3.47[h] |
| 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 | | | |

**3.3.2 The influence of pH**

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

Table 6. The survival percentage of immobile cells at the different pH

| The survival cell percentage after 72-hour incubation (%) | 5 | 6 | 7 | 8 |
|---|---|---|---|---|
| | 54.24 ± 2.27[a] | 78.81 ± 2.38[b] | 98.78 ± 0.56[c] | 83.12 ± 1.2[b] |
| | Data presented as mean ± standard deviation<br>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 | | | |

All runs designed the different pH of storage solvent, pH of storage solvent was 7.0 had the highest proportion of survival cells (98.7%), followed by at pH = 8 and pH = 6 had 83% and 78%, respectively. The remaining runs had less than 55%. To sum up, pH = 7 was used to examine the influence of other factors that used to maintain the finished products.

**3.3.3 The influence of storage temperature**

Examine the change of the temperatures that was used to store the final product from 0 to $40^0$C. Determine the immobilized cell survival percent after 72 hours soaking in the 0.5 % $CaCl_2$ and 0.5 % KCl solvent with ratio 1:1, pH = 7.

Table 7. The survival cell percent at different maintaining temperatures

| The survival cell percentage after 72-hour incubation (%) | $0^o$C | $4^o$C | Room temperature ($30^0$C – $32^0$C) |
|---|---|---|---|
| | 54.37 ± 1.53[a] | 98.66 ± 1.06[b] | 79.47 ± 1.80[c] |
| | Data presented as mean ± standard deviation<br>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 | | |

At different maintaining temperatures, the highest survival cell percent of immobile cells was at $4^0$C (98,6%). The second highest was at room temperature ($30^o$C – $32^o$C) (79.47%). And the temperature stored immobile cells was chosen at $4^0$C.

**3.3.4 The influence of storage duration**

The survival cell rate was identified after different storage duration (7 – 10 – 14 – 30 – 60, days) in optimal conditions which were studied above.

*Table 8*. The survival cell percent during storage duration

| The survival cell percentage after storage duration (%) | 7 days | 10 days | 14 days | 30 days | 60 days |
|---|---|---|---|---|---|
| | 95 ± 1.00[a] | 93.33 ± 0.58[b] | 83.00 ± 1.00[c] | 72 ± 1.00[d] | 51.13 ± 1.53[e] |
| | Data presented as mean ± standard deviation<br>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 finished products were preserved in the best storage condition which the study was interested in above, but the number of *Corynebacterium glutamicum* clone form units was decreased over storage time. In seventh storage day, the survival cell rate was gone down by 5 % and it was significantly droped when the storage duration was prolonged. In fact, 7%, 17%, 28% were the percentages of the dead cells after storage duration for 10 days, 14 days, 30 days, respectively. After $60^{th}$ storage day, the survival cell rate was remained 51 %.

The *Corynebacterium glutamicum* immobilized products on kappa carrageenan or alginate or other carriers 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 kappa carrageenan gel. All conditions such as inoculum conditions of *Corynebacterium glutamicum* and stable conditions of finished products are set up to store the immobile products. 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 and in the presence of potassium salt, kappa carrageenan forms very firm and elastic gels. Thus, the mixed solution including $CaCl_2$ 0.5% and KCl 0.5% with ratio 1:1 was chosen to store the finished products. Nevertheless, too high concentration of salt in the solvent affects the damage of the membrane of *Corynebacterium glutamicum* [9]. pH solvent and temperature were set up to store finished products that immobilized on kappa carrageenan were the same those on alginate.

## IV. CONCLUSION

L-lysine production by the immobile finished products has many benefits. The productivity is higher than that from the free cell. The immobilized process of *Corynebacterium glutamicum* on kappa carrageenan is simple, economical and easy to do. The storage conditions are susceptible.

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