International Journal of Applied Science and Engineering
2004. 2, 1: 49-58

Use of Response Surface Methodology to Optimize Culture Medium for Production of Poly-γ-glutamic Acid by *Bacillus licheniformis*

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Abstract: Response surface methodology (RSM) was employed to study the effects of four medium components (L-glutamic acid, citric acid, glycerol and NH_4Cl) on the production of poly-γ-glutamic acid (PGA) in flask cultures by *Bacillus licheniformis* CCRC 12826 (ATCC 9945) with initial medium pH 6.5. The optimal PGA yield (35.34 g/L, average of four repeats) was appeared at the region where the respective concentrations (in g/L) of glutamic acid, citric acid, glycerol and NH_4Cl were around 34.0, 26.0, 146.0 and 11.0, respectively. The optimized composition derived from RSM regression was (in g/L) glutamic acid 34.70, citric acid 27.00, glycerol, 145.45 and NH_4Cl 10.49. With this composition, the PGA production was 33.20 g/L (average of eight repeats) after 96 h of cultivation, while the predicted maximum production was 35.52 g/L. The optimized medium resulted in significant increase of the PGA yield by *B. licheniformis* CCRC 12826 in shake-flask cultivation without any feeding process. In comparison with glutamic acid, citric acid and glycerol, NH_4Cl represents a crucial role in PGA biosynthesis.

Keywords: Bacillus licheniformis; response surface methodology; poly-γ-glutamic acid.

1. Introduction

Poly-γ-glutamic acid (γ-PGA or PGA) is an unusual anionic, naturally occurring, water-soluble polyamide. It is biodegradable, edible and nontoxic toward humans and the environment. The potential applications of PGA and its derivatives have been of interest in the past few years in the broad range of industrial fields such as food, cosmetics, medicine and water-treatment [1]. Therefore, the application of PGA is versatile, safe and environmental friendly. The development of this biomaterial is also both economically and environmental valuable.

Several bacteria produce PGA as an extracellular viscous material [2-5], all of which belong to the *Bacillus* genus. In order to enhance the PGA productivity, researchers have investigated the nutrient requirements for PGA production and found that the nutrient requirements varied according to the strain used. In these studies, researchers tried to clarify the metabolic pathway for PGA syn-

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Accepted for Publication: Feb. 20, 2004

thesis, researchers found that there are different mechanistic systems for PGA production in different bacteria, indicating that PGA production in diverse in microorganisms [6]. According to the nutrient requirements, *B. licheniformis* is generally one of the glutamic acid dependent bacteria. The glutamic acid independent bacteria such as *B. licheniformis* A35 usually used glucose as major carbon sources for PGA production [4]. Although *B. licheniformis* ATCC 9945a used citric acid and glycerol as carbon sources [7-8], it is glutamic acid dependent. The role of glutamic acid in this example is presumably an activator for the PGA enzyme system but not as a carbon source [7]. Besides carbon sources, factors such as nitrogen source, ionic strength, aeration, and medium pH affected the productivity and quality of PGA [8]. A recent report showed that pH 6.5 was optimal for attaining increased PGA yields and maintaining high specific productivity for *B. licheniformis* ATCC 9945a cultures [8]. Besides, citrate utilization occurred more rapidly to greatly extent at pH 6.5. This result indicated that the formation of PGA from citrate at pH 6.5 was of increased importance. However, we have found that *B. licheniformis* CCRC 12826 (ATCC 9945) required the presence of glutamic acid, citric acid, glycerol and ammonium chloride (NH_4Cl) in the culture medium for PGA production and PGA was later proved to be a homopolymer of glutamic acid [9]. We also attempted to enhance the production of PGA by *B. licheniformis* CCRC 12826 and applied statistical experimental methods (SEM) to determine the effects of three medium components (glutamic acid, citric acid and glycerol) and initial medium pH on PGA production [10]. The results showed that the initial medium pH exhibited insignificant effect on PGA production. The optimal PGA yield was 19.62 ± 1.07 g/L, which was good enough as compared to conventional medium E used in the literature by this microorganism [6,9]. However, the effect of NH_4Cl addition on PGA yield was also not investigated. It was reported that the amount of PGA increased with the increase in ammonium sulfate ($(NH_4)_2SO_4$) concentration for *B. subtilis* IFO3335, although the addition of 2.5 g/L or more $(NH_4)_2SO_4$ to medium depressed cell growth [11-12]. Therefore, the addition of $(NH_4)_2SO_4$ to the medium facilitates PGA and depresses cell growth. In this study, NH_4Cl is supposed to be similar to $(NH_4)_2SO_4$ and to be beneficial for the effective production of PGA.

RSM is a powerful technique for testing multiple process variables because fewer experimental trials are needed compared to the study of one variable at a time. Also, interactions between variables can be identified and quantified by such a technique. In this study, we applied response surface methodology (RSM), especially central composite design (CCD), to further attempt to enhance the PGA production. The CCD is conducted in the optimum vicinity to locate the true optimum values of the multiple variables. The RSM and CCD were increasingly used for optimization of various phases in some fermentation [13-17]. However, the application of this technique on optimizing the PGA production is scant. Therefore, we use RSM and CCD to demonstrate the effects of four medium components (glutamic acid, citric acid, glycerol and NH_4Cl) on PGA production by *B. licheniformis* CCRC 12826 with initial medium pH 6.5.

2. Materials and methods

2.1. Strain and culture conditions

B. licheniformis strain CCRC 12826 was purchased from the Culture Collection and Research Center (CCRC) in Food Industry Research and Development Institute (Hsinchu, Taiwan). This microorganism, obtained as a lyophilized powder in a glass ampoule sealed under vacuum, was first cultured on LB-agar (Miller) containing yeast extract (5 g/L), peptone (10 g/L), NaCl (10 g/L), agar (15 g/L), to

induce spore formation. After cultivation at 37ºC for 24 h, colonies (~ 100 mm^2) were transferred into 100mL of LB-broth (Miller), and incubated at 37ºC for 48 h with shaking at 150 rpm. After incubation, 0.5mL (1% v/v) of the above broth was inoculated into 50mL of the culture medium with various concentrations of L-glutamic acid, citric acid, glycerol and NH_4Cl in a 250-mL flask. Minor salts ingredients, K_2HPO_4 (0.50 g/L), $MgSO_4 \cdot 7H_2O$ (0.50 g/L), $FeCl_3 \cdot 6H_2O$ (0.04 g/L), $CaCl_2 \cdot 2H_2O$ (0.15 g/L) and $MnSO_4 \cdot H_2O$ (0.104 g/L), were fixed throughout the study. The culture was incubated at 37ºC, pH 6.5 with shaking at 150 rpm for 96 h. At the end of the cultivation, a viscous culture broth was obtained and stored at 4ºC for 48 h. The viscous broth was then centrifuged at 21,000 × g for 20 min to separate the cells. The viscous materials were further purified for PGA production by the procedures described further. The cell growth was monitored by measurement of optical density at 660 nm.

2.2. PGA purification

After centrifugation, the viscous cell-free supernatant was poured into four volumes of cold ethanol and to precipitate the PGA. The resultant precipitate was collected by centrifugation at 21,000 × g for 40 min and redissolved in distilled water. The crude PGA thus obtained was dialyzed at 4ºC for overnight in deionized water and lyophilized to give pure material.

2.3. RSM experimental design

Concentrations of four medium ingredients (glutamic acid, citric acid, glycerol and NH_4Cl) were optimized through RSM to predict the production of PGA. This was studied to conduct with CCD in the optimum vicinity to locate the true optimum concentrations of glutamic acid, citric acid, glycerol and NH_4Cl for PGA production. At the beginning of the studies, the analysis of a first-order model was constructed from the 2^4 factorial design, involving two concentrations of each factor was effective in searching for direction of the optimum domain. A series of single experiments was then conducted along with the path of steepest ascent toward to the optimum region when the fitted first-order model was adequate. Finally, to describe the nature of the response surface in the optimum region, a 2^4 factorial CCD with eight axial points (or so-called star points) and four replicates of center points was used at five levels, resulting in the total number of 28 experiments. The levels of each factor are given in Table 1. The experimental results of the CCD were fitted with a second-order polynomial equation by a multiple regression technique.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i x_j + \sum_{i<j} \sum \beta_{ij} x_i x_j \quad (1)$$

Table 1. Coding and assigned concentrations of variables of different levels of the central-composite design

Independent variable	Symbol	Code levels				
		-2	-1	0	+1	+2
Glutamic acid (g/L)	X_1	28	31	34	37	40
Citric acid (g/L)	X_2	10	18	26	34	42
Glycerol (g/L)	X_3	134	140	146	152	158
NH_4Cl (g/L)	X_4	1	6	11	16	21

Y is the predicted response; β_0, β_i, β_{ii}, β_{ij} are constant coefficients, and x_i, x_j are the coded independent variables or factors. The quality of fit of the second-order model equation was expressed by the coefficient of determination R^2, and its statistical significance was determined by an *F*-test. The significance of the regression coefficients was tested by a *t*-test. The computer software used was Statistica, version 5.0 by Statsoft, Inc. (Tulsa, OK USA).

3. Results and discussion

In order to establish the culture conditions for the optimization of production of PGA, several preliminary tests have been performed to evaluate the efficacy of the carbon and nitrogen sources in the PGA production from *B. licheniformis* CCRC 12826 [9-10]. Some investigations have been shown that glutamic acid, citric acid and glycerol were the suitable carbon sources, and NH_4Cl was the favorable nitrogen source for PGA production [9]. In this study, four compositions (glutamic acid, citric acid, glycerol and NH_4Cl) were studied to evaluate the approximate polynomial for all dependent variables, explaining their effects on the production of PGA. RSM and CCD were then used to optimize PGA production by *B. licheniformis* in shake-flask cultivation.

3.1. RSM experimental design

The levels of the variables for the CCD experiments were selected according to the results of the previous experiments such as the 2^4-factorial design and the path of steepest ascent (descent) design [18]. In addition, the steepest ascent (descent) procedures presumably lead us to the neighborhood of the true optimum point. The point representing high PGA production was used as the center point in the next phase of CCD. The center point of the corresponding composition was selected to be 34.0 g/L glutamic acid, 26.0 g/L citric acid, 146.0 g/L glycerol, and 11.0 g/L NH_4Cl. Minor salts ingredients were fixed throughout the study. Further evaluation was conducted with a CCD experiment. The CCD design and the corresponding experimental data were shown in Table 1 and 2, respectively. After applying multiple-regression analysis on the experimental values, the results of ANOVA for the 28 trials performed by the experimental design were obtained as shown in Table 3. The corresponding second-order response model for Eq. (1) that was found after Statistica^{TM} analysis for the regression was

$$Y_{(g/L)} = 35.34 - 3.82X_1^2 - 4.34X_2^2 - 3.83X_3^2 - 7.20X_4^2 \quad (2)$$

The statistical significance of the second-order model equation was checked by the *F*-test. The fit of the model was also expressed by the coefficient of determination R^2, which was found to be 0.707, indicating that 70.7% of the variability in the response could be explained by the model. This indicated that Eq. (2) is a suitable model to describe the response of the experiment pertaining to PGA production. The response taken from Table 3 revealed that the square coefficients of glutamic acid (X_1^2), citric acid (X_2^2), glycerol (X_3^2), and NH_4Cl (X_4^2), have a remarkable effect on the PGA yield. Moreover, all the linear and interaction terms of four factors presented insignificant effects on the PGA yield at 5% probability level. Since all coefficients of the above equation (Eq. (2)) are all negative, the response surface is suggested to have a maximum point (Figure 1).

Accordingly, three-dimensional graphs were generated for the pair-wise combination of the four factors, while keeping the other two at their optimum levels for PGA production (Figure 1 (a)~(f). Graphs are given here to highlight the roles played by various factors and also to emphasize the roles played by the physical constraints *vis-à-vis* the biosynthetic aspects in the final yield of the PGA.

Table 2. Experimental design and results of the central-composite design

No.	Glutamic acid (g/L)	Citric acid (g/L)	Glycerol (g/L)	NH_4Cl (g/L)	PGA (g/L)
1	31.0	18.0	140.0	6.0	22.17
2	31.0	18.0	140.0	16.0	23.16
3	31.0	18.0	152.0	6.0	23.67
4	31.0	18.0	152.0	16.0	23.03
5	31.0	34.0	140.0	6.0	23.52
6	31.0	34.0	140.0	16.0	28.97
7	31.0	34.0	152.0	6.0	27.11
8	31.0	34.0	152.0	16.0	22.78
9	37.0	18.0	140.0	6.0	36.66
10	37.0	18.0	140.0	16.0	28.67
11	37.0	18.0	152.0	6.0	18.87
12	37.0	18.0	152.0	16.0	23.16
13	37.0	34.0	140.0	6.0	28.14
14	37.0	34.0	140.0	16.0	21.09
15	37.0	34.0	152.0	6.0	27.23
16	37.0	34.0	152.0	16.0	26.77
17	28.0	26.0	146.0	11.0	27.67
18	40.0	26.0	146.0	11.0	29.41
19	34.0	10.0	146.0	11.0	23.94
20	34.0	42.0	146.0	11.0	31.07
21	34.0	26.0	134.0	11.0	24.98
22	34.0	26.0	158.0	11.0	32.07
23	34.0	26.0	146.0	1.0	22.26
24	34.0	26.0	146.0	21.0	21.32
25(C)	34.0	26.0	146.0	11.0	36.86
26(C)	34.0	26.0	146.0	11.0	34.82
27(C)	34.0	26.0	146.0	11.0	34.86
28(C)	34.0	26.0	146.0	11.0	34.80

The optimal concentrations for the four components as obtained from the maximum point of the model were calculated to be around 34.0, 26.0, 146.0, and 11.0 g/L for glutamic acid, citric acid, glycerol, and NH_4Cl, respectively. With this composition, the PGA yield was 35.34 ± 1.02 g/L (average of four repeats: No. 25(C), 26(C), 27(C), 28(C) shown in Table 2) after 96 h of cultivation.

3.2. Production of PGA

The optimized composition derived from RSM regression was (in g/L) glutamic acid 34.70, citric acid 26.97, glycerol, 145.45 and NH_4Cl 10.49 for the predicted maximum PGA yield response of 35.52 g/L (No. 4 shown in Table 4). Based on the predicted results described above, the time course of a typical flask cultivation using the predicted optimum medium (No. 4 in Table 4) was carried out at 37ºC with shaking at 150 rpm. The pH of the culture medium was not controlled.

The time course of PGA production as well as changes of the medium pH and the cell growth (OD_{660}) were monitored and shown in Figure 2. After approximately 12 h, the cell growth was dramatically increased due to biomass formation, while the production of PGA suddenly increased after 36 h of cultivation.

Table 3. Analysis of variance for the experimental results of the central-composite design

Factor[a]	Effect	Standard error	Sum of square	DF[b]	*F*-ratio	*t*-ratio	*p*[c]
Mean/Interc.[d]	35.3350	2.000236				17.66541	0.000000*
X_1	1.63833	1.633186	16.1048	1	1.00631	1.00315	0.334095†
X_1^2	-3.82333	1.633186	87.7073	1	5.48041	-2.34103	0.035820*
X_2	1.70667	1.633186	17.4763	1	1.09201	1.04499	0.315069†
X_2^2	-4.34083	1.633186	113.0570	1	7.06439	-2.65789	0.019713*
X_3	-0.46500	1.633186	1.2973	1	0.08107	-0.28472	0.780334†
X_3^2	-3.83083	1.633186	88.0517	1	5.50193	-2.34562	0.035515*
X_4	-0.96933	1.633186	5.6260	1	0.35154	-0.59291	0.563414†
X_4^2	-7.19833	1.633186	310.8960	1	19.42641	-4.40754	0.000708*
$X_1 \times X_2$	-1.81000	2.000236	13.1044	1	0.81883	-0.90489	0.381986†
$X_1 \times X_3$	-2.16250	2.000236	18.7056	1	1.16883	-1.08112	0.299291†
$X_1 \times X_4$	-1.58500	2.000236	10.0489	1	0.62791	-0.79241	0.442345†
$X_2 \times X_3$	3.01250	2.000236	36.3006	1	2.26825	1.50607	0.155956†
$X_2 \times X_4$	-0.38000	2.000236	0.5776	1	0.03609	0.18998	0.852261†
$X_3 \times X_4$	0.93250	2.000236	3.4782	1	0.21734	0.46619	0.648791†
Error			208.0491	13			
Total SS[e]			709.3746	27			

[a] X_1 – Glutamic acid, X_2– Citric acid, X_3 – Glycerol, X_4 – NH_4Cl.
[b] Degree of freedom.
[c] † – not significant, * $p < 0.05$, R^2 =0.707.
[d] Intercept.
[e] Sum of square.

Table 4. Medium composition optimized for PGA yield response

No.	Composition concentration (g/L)				PGA yield (g/L)
	Glutamic acid	Citric acid	Glycerol	NH_4Cl	
1	57.30[a]	24.50[a]	157.11[a]	7.00[a]	19.62±1.07[a]
2	34.00[b]	26.00[b]	146.00[b]	11.00[b]	35.34±1.02[b]
3	34.70[c]	26.97[c]	145.45[c]	10.49[c]	33.20±3.45[c]
4	34.70[d]	26.97[d]	145.45[d]	10.49[d]	35.52±0.00[d]

[a] the optimized values and the max. PGA yield response in the previous study [10]
[b] the center points in Table 2 and the PGA yield response in this study
[c] the optimized values derived from RSM regression and the PGA yield response in this study
[d] the predicted optimum values and the predicted max. PGA yield derived from RSM regression in this study

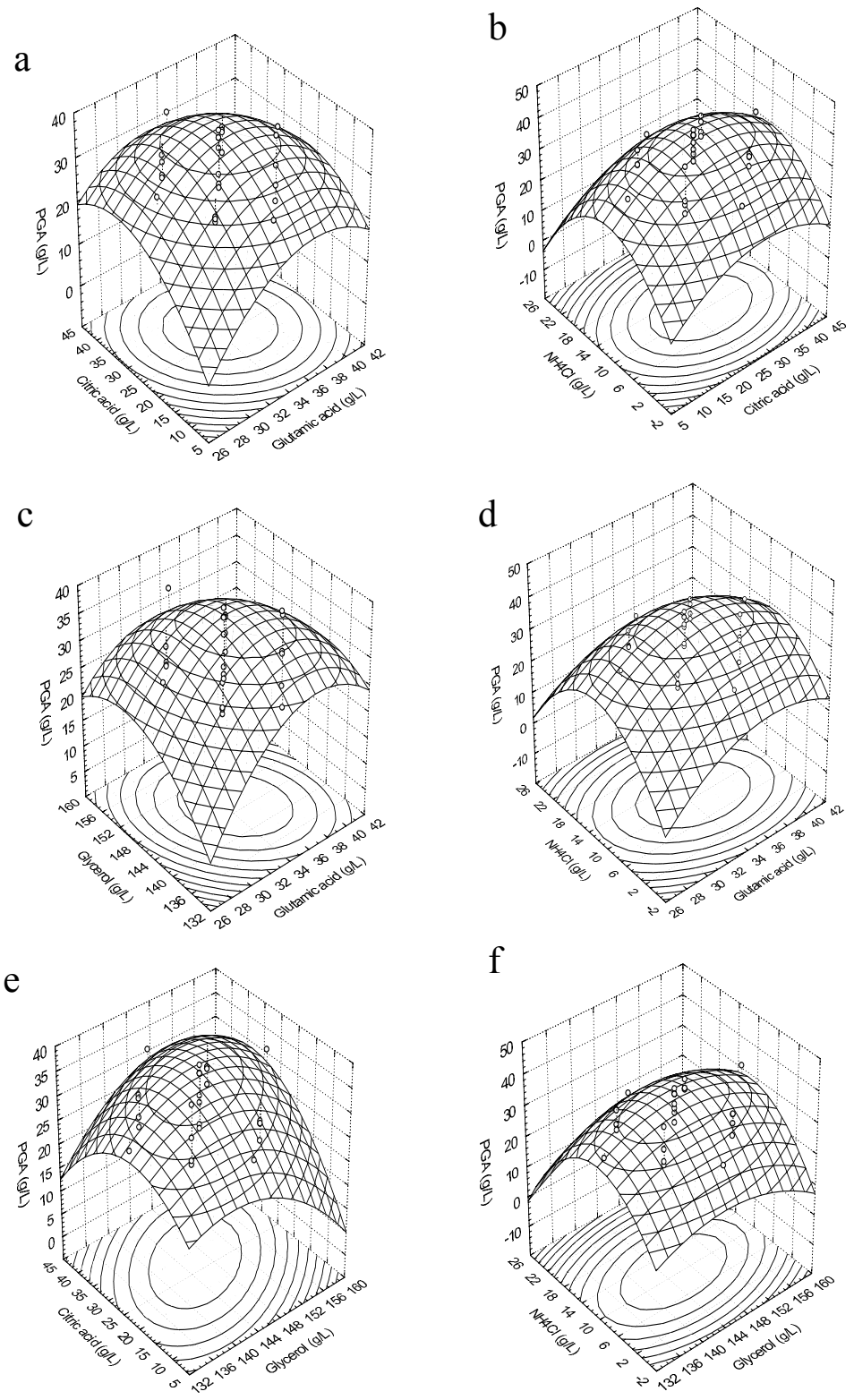

Figure 1. Response surface plots for the yield of PGA; changing components were citric acid and glutamic acid (a), NH_4Cl and citric acid (b), glycerol and glutamic acid (c), NH_4Cl and glutamic acid (d), citric acid and glycerol (e), NH_4Cl and glycerol (f)

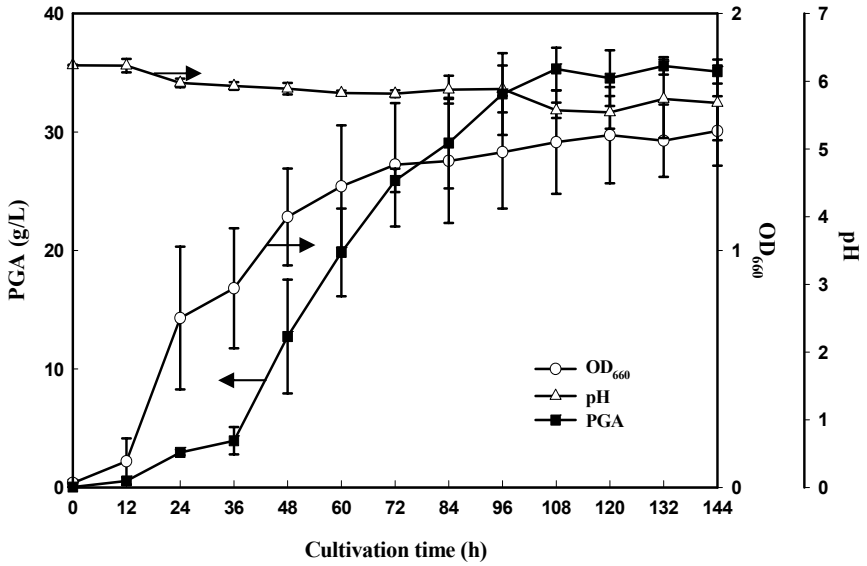

Figure 2. Time courses of PGA production, changes of pH and bacterial growth (OD_{660})

The pH profile showed that the pH of the culture medium gradually fell from 6.23 to 5.55 in about 120 h but little rose afterward. With the optimized medium as shown as No. 3 in Table 4, the overall PGA product isolated after incubation of 96 h was 33.20 ± 3.45 g/L (average of eight repeats), as shown as No. 3 in Table 4, while the predicted maximum production was 35.52 g/L. The production of PGA reached a maximum (35.32 ± 1.80 g/L) after 108 h of cultivation, due to the limitation of cell growth. This resulted in a limitation of the production of PGA. This also suggested that the production of PGA for *B. licheniformis* CCRC 12826 might be one example of a growth-associated product.

From the previous results the optimal concentrations for the four components are shown as No. 1 in Table 4, and the PGA yield response was 19.62 ± 1.07 g/L (average of three repeats). In comparison with the previous results, shown as No. 1 in Table 4 [10], the addition of NH_4Cl suggested to be increased for increasing PGA productivity while the addition of glutamic acid, citric acid, and glycerol supposed to be decreased in this study. Based on the results in this study (No. 2 & 3 in Table 4), the PGA yield dropped little. This might be caused by the little lower contents of glycerol and NH_4Cl in the optimized medium, while the contents of glutamic acid and citric acid were little higher. As is shown the effect of NH_4Cl on PGA production was much more significant than citric acid (Figure 1 (b)). This also can be seen from the surface plot for these components as shown in (Figure 1 (d) and (f)). In addition, analysis of variance indicated that the negative coefficient of the quadratic term of NH_4Cl in the second-order model was less than those of glutamic acid, citric acid and glycerol (Eq. (2)). On the other hand, the interactions between citric acid and glutamic acid, glycerol and glutamic acid as well as citric acid and glycerol were less significant, confirmed from their corresponding contour plots (Figure 1 (a), (c), (e)). This suggested that the NH_4Cl content fell in the region of maximum production and played a crucial role in PGA biosynthesis, similar to $(NH_4)_2SO_4$ [11-12].

4. Conclusions

The results of this study have clearly indi-

cated RSM is an effective method for optimization of PGA production by *B. licheniformis* CCRC 12826. The PGA production by this microorganism could be increased by about 169% from 19.62 to 33.20 g/L when the strain was cultivated in the optimized medium that developed by RSM, as compared to the optimal medium by means of SEM in the previous experiments [10]. Therefore, RSM was proved to be powerful and useful tool for enhancing PGA production by *B. licheniformis* CCRC 12826. The addition of ammonium chloride (NH_4Cl), similar to $(NH_4)_2SO_4$ [11-12], to the medium was proved to be important in term of the high productivity of PGA. Thus the results obtained in this study will be beneficial for the effective production of PGA.

Acknowledgements

This study was supported by a grant from the National Science Council, the Republic of China, with the no. NSC 89-2214-E-212-013.

References

[1] Shih, I. L. and Van, Y. T. 2001. The production of poly(γ-glutamic acid) from the microorganisms and its various applications. *Bioresource Technology*, 79: 207-225.

[2] Thorne, C. B., Gomez, C. G., Noyes, H. E., and Housewright, R. D. 1954. Production of glutamyl polypeptide by *Bacillus subtilis*. *Journal of Bacteriology*, 68: 307-315.

[3] Troy, F. A. 1973. Chemistry and biosynthesis of the poly(γ-glutamyl) capsule in *Bacillus subtilis*. 1. Properties of the membrane-mediated biosynthetic reaction. *The Journal Biological Chemistry*, 248: 305-316.

[4] Cheng, C., Asada, Y., and Aaida, T. 1989. Production of γ-polyglutamic acid by *Bacillus subtilis* A35 under denitrifying conditions. *Agricultural and Biological Chemistry*, 53: 2369-2375.

[5] Kubota, H., Matsunobu, T., Uotani, K., Takebe, H., Satoh, A., Tanaka, T., and Taniguchi, M. 1993. Production of poly(γ-glutamic acid) by *Bacillus subtilis* F-2-01. *Bioscience, Biotechnology, and Biochemistry*, 57: 1212-1213.

[6] Kunioka, M. and Furusawa, K. 1997. Poly(γ-glutamic acid) hydrogel prepared from microbial poly(γ-glutamic acid) and alkane diamine with water-soluble carbodiimide. *Journal of Applied Polymer Science*, 65: 1889-1893.

[7] Birrer, G. A., Cromwick A. M., and Gross, R. A. 1994. γ-Poly(glutamic acid) formation by *Bacillus licheniformis*: physiological and biochemical studies. *International Journal of Biological Macromolecules*, 16: 265-275.

[8] Cromwick, A. M., Birrer, G. A., and Gross, R. A. 1996. Effects of pH and aeration on γ-Poly(glutamic acid) formation by *Bacillus licheniformis* in controlled batch fermentor cultures. *Biotechnology and Bioengineering*, 50: 222-227.

[9] Shih, I. L., Van, Y. T., Yeh, L. C., Lin, H. G., and Chang, Y. N. 2001. Production of biopolymer flocculant from *Bacillus licheniformis* and its flocculation properties. *Bioresource Technology*, 78: 267-272.

[10] Shih, I. L., Van, Y. T., and Chang, Y. N. 2002. Application of statistical experimental methods to optimize production of poly(γ-glutamic acid) by *Bacillus licheniformis* CCRC 12826. *Enzyme and Microbial Technology*, 31: 213-220.

[11] Kunioka, M. and Goto, A. 1994. Biosynthesis of poly(γ-glutamic acid) from L-glutamine, citric acid and ammonium sulfate in *Bacillus subtilis* IFO3335. *Applied Microbiology and Biotechnology*, 40: 867-872.

[12] Kunioka, M. 1995. Biosynthesis of poly(γ-glutamic acid) from L-glutamine, citric acid and ammonium sulfate in *Ba-*

cillus subtilis IFO3335. *Applied Microbiology and Biotechnology*, 44: 501-506.

[13] Zertuche, L. and Zall R. R. 1985. Optimizing alcohol production from whey using computer technology. *Biotechnology and Bioengineering*, 27: 547-554.

[14] Buchanan, R. L. and Philips, J. G. 1990. Response surface model for predicting the effects of temperature, pH, sodium chloride content, sodium nitrite concentration and atmosphere on the growth of *Listeria monocutogenes*. *Journal of Food Protection*, 53: 370-376.

[15] Prapulla, S. G., Jacob, S., Chand, N., Rajalakshmi, D., and Karanth, N. G. 1992. Maximization of lipid production by *Rhodotroula gracilis* CFR-1 using response surface methodology. *Biotechnology and Bioengineering*, 40: 965-969.

[16] Haltrich, D., Press, M., and Steiner, W. 1993. Optimization of a culture medium for increased xylanase production by a wild strain of *Schizophyllum commune*. *Enzyme and Microbial Technology*, 15: 854-860.

[17] Chang, Y. N., Huang, J. C., Lee, C. C., Shih, I. L., and Tzeng, Y. M. 2002. Use of response surface methodology to optimize culture medium for production of lovastatin by *Monascus ruber*. *Enzyme and Microbial Technology*, 30: 889-894.

[18] Lu, W. K. 2003. "*Studies on Production of Poly(γ-glutamic acid) by* Bacillus licheniformis *CCRC 12826 with Response Surface Methodology*". Master Thesis of Department of Food Engineering, Da-Yeh University, Changhua, Taiwan, R.O.C. (in Chinese)