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# Application of response surface methodology for poly-γ-glutamic acid production by *Bacillus subtilis* ATCC 6633

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## ABSTRACT

The objective of this study was to investigate the production of poly-γ-glutamic acid (γ-PGA) by *Bacillus subtilis* ATCC 6633, using surface response methodology. Two successive factorial designs were used to determine the sucrose concentration, addition of glutamic acid and time of culture in fermentation medium for the production of γ-PGA. The results of the statistical $2^3$ full factorial design indicated that the linear terms, sucrose concentration, addition of glutamic acid and fermentation time, were significant and positive for the production of the biopolymer. Based on these results, a central composite design was utilized in order to optimize the production of γ-PGA and define the best concentrations of sucrose and glutamic acid and fermentation time. The best production of γ-PGA was 20.4 g/l obtained with 132 g/l sucrose, 27.6 g/l glutamic acid and 104 h of fermentation, and the productivity for the maximal production of γ-PGA was 0.1962 g/l.h$^{-1}$. 

## KEYWORDS

Poly-γ-glutamic;
*Bacillus subtilis*;
Optimisation;
Shake-flask;
Biopolymer;
Microbial.

## INTRODUCTION

Poly(γ-glutamic acid) (γ-PGA) is an anionic polypeptide that is biodegradable, edible and non-toxic to humans and the environment. Potential applications of -PGA and its derivative have been of interest in the past few years in a broad range of industrial fields such as foods, pharmaceuticals and medicine[1], and it has been recently demonstrated that γ-PGA can be a potential adsorbent for removing mercury (II) from water/wastewater[2].

Several bacteria produce γ-PGA as an extracellular biopolymer and a by-product produced by common soil microbes such as *Bacillus subtilis* and *Bacillus licheniformis* through fermentation[2].

In order to enhance γ-PGA production, studies have found that the need for glutamic acid added to the fermentation medium varies with the species used and that this requirement classifies the bacteria into two groups: one requiring L-glutamic acid as a carbon and nitrogen source for γ-PGA production and growth, the other not requiring L-glutamic acid for this purpose[3].

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**TABLE 1 : $2^3$ Factorial experimental design investigating the effects of the sucrose and glutamic acid concentrations and culture time on production of poly-γ-glutamic acid by *Bacillus subtilis* ATCC 6633**

| Experiment | Coded levels | | | Responses | | |
|---|---|---|---|---|---|---|
| | $X_1$ | $X_2$ | $X_3$ | Biomass g/l | γ-PGA g/l | Productivity g/lh |
| 1 | -1 | -1 | -1 | 2.01 | 0.8 | 0.0333 |
| 2 | 1 | -1 | -1 | 2.80 | 1.9 | 0.0792 |
| 3 | -1 | -1 | 1 | 4.21 | 0.5 | 0.0069 |
| 4 | 1 | -1 | 1 | 4.95 | 2.9 | 0.0403 |
| 5 | -1 | 1 | -1 | 2.36 | 2.0 | 0.0833 |
| 6 | 1 | 1 | -1 | 1.23 | 3.2 | 0.1333 |
| 7 | -1 | 1 | 1 | 3.66 | 2.0 | 0.0278 |
| 8 | 1 | 1 | 1 | 6.64 | 7.1 | 0.0986 |
| 9 | 0 | 0 | 0 | 2.97 | 2.3 | 0.0479 |
| 10 | 0 | 0 | 0 | 2.36 | 2.0 | 0.0417 |
| 11 | 0 | 0 | 0 | 2.23 | 2.6 | 0.0542 |

| Variable | Real levels | | |
|---|---|---|---|
| | -1 | 0 | 1 |
| $X_1$ Sucrose (g/l) | 30 | 75 | 120 |
| $X_2$ Glutamic acid (g/l) | 10 | 15 | 20 |
| $X_3$ Culture time (h) | 24 | 48 | 72 |

The genus *Bacillus* usually utilizes glucose, glutamic acid, citric acid and/or glycerol as a carbon source for the production of γ-PGA[1,4]. Besides a carbon source, factors such as nitrogen source, medium pH and fermentation time affect the production and quality of γ-PGA[5]. Thus, the development of a cost-effective medium for production of γ-PGA requires selection of the carbon source, nitrogen source, inorganic salts and pH[6].

In fermentation, the improvement in production of a microbial metabolite is achieved by manipulating the nutritional and physical parameters by conventional or statistical methods. The conventional method involves changing one independent variable at a time, while keeping others at a fixed level. Statistical methods offer several advantages over conventional methods. They are more rapid and reliable, shortlist significant variables, help in understanding the interactions among the variables at various levels, and reduce the total number of experiments, saving time and resources[5].

The objective of this work was to study the production of γ-PGA by *Bacillus subtilis* ATCC 6633, using a statistical methodology in two steps. Initially,

**TABLE 2 : CCRD matrix and results of biomass, production and productivity of poly-γ-glutamic acid (γ-PGA)**

| Experiment | Coded levels | | | Responses | | |
|---|---|---|---|---|---|---|
| | $X_1$ | $X_2$ | $X_3$ | Biomass g/l | γ-PGA g/l | Productivity $g/lh^{-1}$ |
| 1 | -1 | -1 | -1 | 6.4 | 7.6 | 0.1360 |
| 2 | 1 | -1 | -1 | 6.3 | 8.7 | 0.1554 |
| 3 | -1 | 1 | -1 | 6.1 | 8.3 | 0.1482 |
| 4 | 1 | 1 | -1 | 6.3 | 8.5 | 0.1518 |
| 5 | -1 | -1 | 1 | 4.9 | 8.5 | 0.0817 |
| 6 | 1 | -1 | 1 | 4.3 | 5.9 | 0.0567 |
| 7 | -1 | 1 | 1 | 8.6 | 20.4 | 0.1962 |
| 8 | 1 | 1 | 1 | 4.0 | 6.9 | 0.0863 |
| 9 | -1.68 | 0 | 0 | 3.2 | 6.6 | 0.0825 |
| 10 | 1.68 | 0 | 0 | 2.8 | 6.8 | 0.0850 |
| 11 | 0 | -1.68 | 0 | 3.0 | 6.1 | 0.0763 |
| 12 | 0 | 1.68 | 0 | 3.4 | 10.3 | 0.1288 |
| 13 | 0 | 0 | -1.68 | 3.7 | 7.9 | 0.0658 |
| 14 | 0 | 0 | 1.68 | 4.0 | 6.0 | 0.0500 |
| 15 | 0 | 0 | 0 | 5.1 | 15.9 | 0.1988 |
| 16 | 0 | 0 | 0 | 5.8 | 15.6 | 0.1950 |
| 17 | 0 | 0 | 0 | 5.9 | 15.4 | 0.1925 |

| Variable | Real levels | | | | |
|---|---|---|---|---|---|
| | -1.68 | -1 | 0 | 1 | 1.68 |
| $X_1$ Sucrose (g/l) | 120 | 132 | 150 | 168 | 180 |
| $X_2$ Glutamic acid (g/l) | 18 | 20.4 | 24 | 27.6 | 30 |
| $X_3$ Culture time (h) | 40 | 56 | 80 | 104 | 120 |

the concentrations of sucrose and glutamic acid and culture time were tested using a statistical $2^3$ full factorial design. In the second step, the factors that had significant effects were optimized using a central composite rotational design (CCRD), and response surface analysis.

## MATERIALS AND METHODS

### Bacterial strain and medium

A bacterial strain of *Bacillus subtilis* ATCC 6633 was used in the present study. The medium used for maintenance contained (g/l): peptone, 5.0; yeast extract, 3.0; and agar, 2.0. Bacterial cells on agar slants were incubated at 37 ºC for 48 h and kept at 4°C, and the cultures were renewed every 4 weeks. The inoculum medium contained (g/l): sucrose, 100; yeast extract, 2.0; $KH_2PO_4$, 1.0; $(NH_4)_2SO_4$, 3.0; $MgSO_4.7H_2O$, 0.6; $MnSO_4$, 0.15; and ammonium citrate, 0.3.

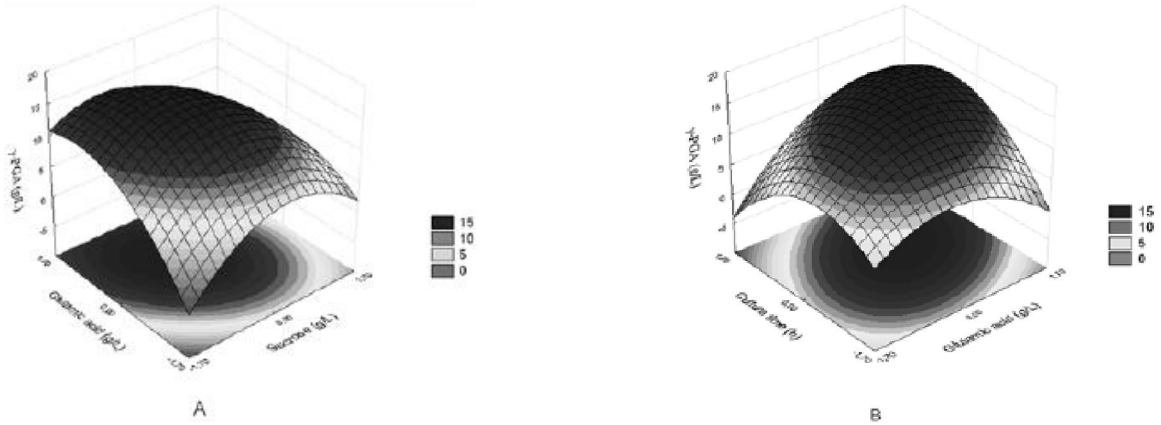


**Figure 1 : Response surface plots showing the effect of sucrose concentration and lutamic acid (A) and culture time and glutamic acid (B) on the production of δ-PGA by Bacillus subtilis ATCC 6633. In: (A) fixed culture time at level 0 (80 h) and (B) fixed glutamic acid at level 0 (24g/L)**

For the production of γ-PGA, a modified medium reported by Leonard et al.[7] was used, which contained (g/l): $NH_4Cl$, 7.0; $MgSO_4.7H_2O$, 0.5; $FeCl_3.6H_2O$, 0.04; $K_2HPO_4$, 0.5; and $CaCl_2.7H_2O$, 0.15. The carbon sources were sucrose and glutamic acid at various concentrations according to TABLES 1 and 2. The initial pH of the medium was adjusted to 7.0 using NaOH. The medium was sterilized in an autoclave for 15 min at 121°C.

## Inoculum and fermentation

A loopful of cells from a slant was transferred to 25ml of inoculum medium, in 250ml flasks and incubated at 37°C at 150 rpm for 48h. The cell concentration was determined by turbidimetry at 400nm, standardized to 0.2g/l for all fermentations.

Fermentations were carried out in 125ml Erlenmeyer flasks containing 25ml of the production medium, at 37°C. The flasks were incubated on a rotary shaker at 150 rpm and for different culture times.

## Analytical methods

After each fermentation, cultures were centrifuged at 9050 x g for 15 min, and bacterial cells suspended in 0.9% NaCl solution; cell growth was determined by turbidimetry at 400nm. The total sugars were quantified by the phenol-sulfuric acid method[8] using glucose as the standard.

The concentration of L-glutamic acid remaining in the culture was measured, after derivatization with *ortho*-phthalaldehyde[9], by high-performance liquid chromatography (HPLC, Shimadzu model LC 10AT, equipped with fluorescence detection with an excitation wavelength of 340 nm and emission wavelength of 450nm) on a reverse phase ODS-C18 column. The sample was eluted with a mobile phase consisting of sodium acetate buffer/methanol (70:30, v/v). The flow rate was set at 1 ml/min.

γ-PGA concentration was determined in the supernatant before removal of the bacterial cells by centrifugation. The supernatant containing γ-PGA was poured into four volumes of cold ethanol, and the mixture was allowed to stand for 12 h at 4°C. The precipitate was collected by centrifugation at 21,000 x g for 40 min at 4°C, and the crude γ-PGA was then lyophilized[10]. Biopolymer production was confirmed by glutamic acid analysis of cell-free broth hydrolysates with 6 M HCl at 100°C for 12 h and neutralized with 6M NaOH[11]. Glutamic acid concentration was measured by HPLC[9].

## Experimental designs and data analysis

The experiments were conducted to evaluate the effect of sucrose concentration, addition of glutamic acid and culture time on γ-PGA production by *Bacillus subtilis*, using factorial designs. In the first design, the ranges of the variables tested were concentrations of sucrose of 30-120g/l and of glutamic acid of 10-15g/l and culture time of 24-48 h. In this experimental design, the main effects and interactions of different factors, each at two different levels, were simultaneously investigated by a $2^3$ factorial design with 3 factors at 2 levels, and with 11 experimental runs. TABLE 1 shows the matrix corresponding to the three independent vari-

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**TABLE 3 : Estimate of the effects related to different factors in the experimental $2^3$ design for the production of γ-PGA**

| Factor | Effect | P |
|---|---|---|
| Mean/intercept | 15.461 | 0.000029* |
| $X_1$ | -2.120 | 0.207070 |
| $X_1^2$ | -5.174 | 0.017819* |
| $X_2$ | 2.996 | 0.090220 |
| $X_2^2$ | - 4.108 | 0.044427* |
| $X_3$ | 0.793 | 0.619240 |
| $X_3^2$ | -4.997 | 0.020678* |
| $X_1 . X_2$ | -2.950 | 0.182109 |
| $X_1 . X_3$ | -4.350 | 0.065243 |
| $X_2 . X_3$ | 3.100 | 0.163545 |

**$X_1$: sucrose (g/l); $X_2$: glutamic acid (g/l); $X_3$: culture time (h), *Significant ($p<0.05$)**

ables and their concentrations at the different coded levels and experimental data. From these results, a series of experiments was then conducted along the path of steepest ascent[10] toward the optimum region. The variables tested were sucrose concentration (120, 150 and 180g/l), added glutamic acid (20, 24.2 and 28.4g/l) and culture time (72, 81 and 89 h).

Based on the results obtained, a second design, a $2^3$ factorial CCRD (central composite rotational design) with six axial points (star points) and three replicates of center points, was used at two levels, resulting in a total of 17 experiments (TABLE 2), in order to establish the optimal conditions for the production of γ-PGA.

The responses $\hat{y}$ of interest were biomass (g/l), γ-PGA production (g/l) and γ-PGA productivity ($g/lh^{-1}$).

Statistic 7.0 software by Statsoft was used to carry out the designs and the analysis of the responses obtained.

## RESULTS AND DISCUSSION

### Assessment of the sucrose concentration, glutamic acid addition and culture time: First factorial design

Xu et al.[12] reported on the requirements for sucrose and glutamic acid precursor substrate in γ-PGA production by *Bacillus subtilis* NX-2, and Cromwick and Gross[13] confirmed that *Bacillus licheniformis* NCIM 2324 is glutamic acid dependent, and could not produce γ-PGA in absence of glutamic acid. Hence, in the present study sucrose was considered as the carbon source and glutamic acid was tested. An initial $2^3$ full factorial design was used to determine the optimal concentration of sucrose, glutamic acid addition and culture time for production of γ-PGA by *Bacillus subtilis* ATCC 6633.

**TABLE 4 : Analysis of variance for poly-γ-glutamic acid production by *Bacillus subtilis* ATCC 6633**

| Source of variation | Sum of squares | Degrees of freedom | Mean squares | Calculated F | Tabulated F |
|---|---|---|---|---|---|
| Regression | 241.37 | 9 | 26.81 | 3.15* | 1.92 |
| Residual | 59.57 | 7 | 8.51 | | |
| Total | 300.94 | 16 | | | |

***Significant ($p<0.05$)**

Based on the results presented in TABLE 1 the greatest production of γ-PGA was 7.1g/l, where: the sucrose concentration is $X_1 = +1$; glutamic acid is $X_2 = +1$; and culture time is $X_3 = +1$. A reduction in fermentation time and concentration of glutamic acid, while maintaining sucrose at 120g/l ($X_1 = +1$), resulted in a decrease of 74% in the production of the biopolymer.

Based on statistical analysis, the results were positive and significant, indicating sucrose as the most important variable ($p = 0.007414$). The interaction of sucrose with culture time also showed a significant and positive effect, and this synergistic effect between the variables resulted in increased production of γ-PGA. The curvature test indicated a lack of significance ($p = 0.34$), demonstrating that the point of maximal production of the biopolymer was not reached, making it necessary to optimize the variables by increasing the concentrations of sucrose and glutamic acid and culture time.

Jian et al.[14] optimized the parameter of the fermentation process for the production of γ-PGA by *Bacillus subtilis* in the solid state and achieved maximal production in soybean cake powder and wheat bran supplemented with glutamate and mineral salts, in 42 h at 40°C. Xu et al.[12] investigated different carbon sources and the addition of glutamic acid for the production of γ-PGA and confirmed that sucrose and glucose were the most promising sources in polymer production and that the addition of glutamic acid is essential for the production of this polymer.

With the results obtained in this first design (TABLE 1), a new set of experiments was conducted utilizing the steepest ascent design, and the best production of

γ-PGA of 16.94g/l was with 150g/l sucrose, 24.2g/l glutamic acid and a culture time of 81 h. The increase in the levels of these variables (180g/l sucrose, 28.4g/l glutamic acid and 89 h of culture time) reduced production to 13.30g/l, indicating that the steepest slope had already been reached.

**Treatment 2**

Central composite rotational design (CCRD) was used after having reached the steepest slope (TABLE 2). The point which showed the maximal production of γ-PGA (sucrose, 150g/l; glutamic acid, 24.2g/l; and culture time, 81 h) was used as the central point in this treatment of the CCRD. The results obtained are presented in TABLE 2 and the effect of the variables studied for the production of γ-PGA and the respective levels of significance in TABLE 3.

The mean production of biomass was 4.93g/l, close to the values found by other authors[15,16], and the variables did not have any significant influence on this response.

The best production of γ-PGA was 20.4g/l obtained with 132g/l sucrose, 27.6g/l glutamic acid and 104 h of fermentation (TABLE 2). The quadratic effects of the variables studied were statistically significant and negative, indicating that the increase in the concentrations of sucrose and glutamic acid as well as culture time reduced the production of γ-PGA (TABLE 3), thus confirming that the maximal production was achieved.

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Sucrose concentration was a significant and positive variable in treatment 1, where an increase to 132g/l enhanced production from 7.1 to 20.4g/l, but concentrations over 150g/l led to a reduction in production (TABLE 2).

Glutamic acid was an essential component for the synthesis of γ-PGA, where it was significant at sucrose concentrations greater than 150g/l. In the best production of γ-PGA (20.4g/l), the rate of conversion of glutamic acid to γ-PGA was 74%, where this finding is comparable to that reported by others. *Bacillus subtilis* IFO 3335 was studied by Goto and Kunioka[14] who obtained 20g/l of γ-PGA and a conversion rate of glutamic acid of 66%. Ashiuchi et al.[17] reported a production of 15.6g/l with a conversion rate of 78% with *Bacillus subtilis* (chungkookjang). Bajaj et al.[6] utilized *Bacillus licheniformi*s NCIM 2324 and increased production of γ-PGA from 5.27 to 26.12g/l and achieving a conversion rate of 130%.

The culture time influences the production of γ-PGA according to the species and culture conditions[15,17-19]. The best production was at 104 h, while a shorter or longer time (TABLE 2) resulted in a decline in biopolymer production. Other authors have explained that the decrease in production with longer times can occur due to the synthesis of polyglutamate hydrolase, the enzyme responsible for the hydrolysis of γ-PGA[15,20]. On the other hand, Kunioka[18] reported that with *Bacillus subtilis* IFO 3335, culture times longer than 40 h led to an increase in medium viscosity and cell growth, which limits oxygen availability which in turn results in reduced biopolymer production.

The influence of culture time has been described by Shih et. al.[21] utilizing *B. subtilis* C1 who demonstrated that an increase in time from 96 to 144 h increased the production of γ-PGA from 8.12 to 21.44g/l. However, some species show a good production with shorter culture times, as observed in *B. subtilis* NX-2, which can show a production of 30.2g/l in 24 h[22].

The response surfaces show the production of γ-PGA relating sucrose concentration ($X_1$) with glutamic acid concentration ($X_2$) (Figure 1A) and relating culture time ($X_3$) with glutamic acid ($X_2$) (Figure 1 B). The regions of maximal production were close to the central point indicating that this was advantageous since the values of the variables culture time and addition of glutamic acid were lower at this point.

The second degree polynomial equation obtained for the production of γ-PGA is shown in Equation (1).

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The coefficient of determination ($R^2$) was 0.80 indicating that 80% of variability of response could be explained by this model.

$$\hat{y} = 15{,}34 - 1{,}06\ X_1 - 2{,}51\ X_1^2 + 1{,}50\ X_2 - 1{,}98\ X_2^2 + 0{,}42\ X_3 - 2{,}6\ X_3^2 - 1{,}48\ X_1.X_2 - 2{,}18\ X_1.X_3 + 1{,}55\ X_2.X_3 \quad (1)$$

$\hat{y}$ : γ-PGA concentration (g/l); $X_1$: sucrose concentration (g/l); $X_2$: glutamic acid concentration (g/l); $X_3$: time (h).

The analysis of variance for the production of γ-PGA is shown in TABLE 4 and demonstrates that the regression was significant with $p<0.05$ and that the model presented has a good predictive capacity, for the purpose of biological experiments.

The productivity for the maximal production of γ-PGA was 0.1962g/lh$^{-1}$ and in the central point reached a mean of 0.1954g/lh$^{-1}$ (TABLE 2); these values are compatible with those found in the literature of 0.2 to 0.4g/lh$^{-1}$[15].

The present work made it possible to study in a new strain of *B. subtilis,* with a good potential for production of γ-PGA, the applicaton of sucrose as a carbohydrate source of low cost and the use of response surface methodology to obtain maximal production.

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## REFERENCES

[1] I.L.Shih, Y.T.Van; Bioresour.Technol., **79**, 207-225 **(2001)**.
[2] B.S.Inbaraj, C.P.Chiu, J.S.Wang, J.F.Lu, F.Y.Siao, B.H.Chen; Bioresour.Thecnol., **100**, 200-207 **(2009)**.
[3] I.L.Shih, Y.T.Van, Y.N.Chang; Enzyme Microb. Technol., **31**, 213-220 **(2002)**.
[4] Y.H.Ko, R.A.Gross; **57**, 430-437 **(1998)**.
[5] A.M.Cromwick, G.A.Birrer, G.A.Gross; **50**, 222-227 **(1996)**.
[6] I.B.Bajaj, S.S.Lele, R.S.Singhal; Bioresour.Thecnol., **100**, 826-832 **(2009)**.
[7] C.G.Leonard, R.D.Housewright, C.B.Thorne; J.Bacteriol., **76**, 499-503 **(1958)**.
[8] M.Dubois, K.A.Gilles, P.A.Roberts, F.Smith; Anal.Chem., **25**, 350-356 **(1956)**.
[9] H.W.Jarret, K.D.Cooksy, B.Ellis; Anal.Biochem., **153**, 189-198 **(1986)**.
[10] W.K.Lu, T.Y.Chiu, S.H.Hung, I.L.Shih, Y.N.Chang; Int.J.Appl.Sci.And Eng., **2**, 49-58 **(2004)**.
[11] M.Kambourova, M.Tangney, F.G.Priest; Appl.Environ.Microbiol., **67**, 1004-1007 **(2001)**.
[12] J.Xu, C.Shouwen, Y.Ziniu; Process Biochem., **40**, 3075-3081 **(2005)**.
[13] A.M.Cromwick, R.A.Gross; Int.J.Biol.Macromol. USA, **16**, 265-275 **(1995)**.
[14] X.Jian, S.Chen, Y.Ziniu; Process Biochem., **40**, 3075-3081 **(2005)**.
[15] A.Goto, M.Kunioka; Biosci.Biotechnol.Biochem., **56**, 1031- 1035 **(1992)**.
[16] H.Kubota, Y.Nambu, T.Endo; J.Poly.Sci.Part A: Polym.Chemistry, **31**, 2877-2878 **(1993)**.
[17] M.Ashiuchi, T.Kamei, D.H.Baek, S.Y.Shin, M.H.Sung, K.Soda, T.Yagi, H.Misono; Appl.Microbiol.Biotechnol., **57**, 764-769 **(2001)**.
[18] M.Kunioka; Applied Microbiol.Biotechnol., **44**, 501-506 **(1995)**.
[19] Y.Ito, T.Tanaka, T.Ohmachi, Y.Asada; Biosci.Biotechnol.Biochem., **60**, 1239-1242 **(1996)**.
[20] A.Richard, A.Margaritis; Biochem.Eng.J., **30**, 303-307 **(2006)**.
[21] I.L.Shih, P.J.Wu, C.J.Shieh; Process Biochem., **40**, 2827-2832 **(2004)**.
[22] H.Xu, M.Jiang, H.Li, D.Lu, P.Ouyang; Process Biochem., **40**, 519-523 **(2005)**.