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Optimized Production of Poly(γ-Glutamic acid) By *Bacillus* sp. FBL-2 through Response Surface Methodology Using Central Composite Design

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In the present study, the optimization of poly(γ-glutamic acid) (γ-PGA) production by *Bacillus* sp. FBL-2 was studied using a statistical approach. One-factor-at-a-time method was used to investigate the effect of carbon sources and nitrogen sources on γ-PGA production and was utilized to select the most significant nutrients affecting the yield of γ-PGA. After identifying effective nutrients, response surface methodology with central composite design (CCD) was used to obtain a mathematical model to identify the optimum concentrations of the key nutrients (sucrose, L-glutamic acid, yeast extract, and citric acid) for improvement of γ-PGA production. The optimum amount of significant medium components appeared to be sucrose 51.73 g/l, L-glutamic acid 105.30 g/l, yeast extract 13.25 g/l, and citric acid 10.04 g/l. The optimized medium was validated experimentally, and γ-PGA production increased significantly from 3.59 g/l (0.33 g/l/h) to 44.04 g/l (3.67 g/l/h) when strain FBL-2 was cultivated under the optimal medium developed by the statistical approach, as compared to non-optimized medium.

Keywords: Poly(γ-glutamic acid), L-glutamic acid, *Bacillus* sp. FBL-2, optimization, response surface methodology

Introduction

Poly(γ-glutamic acid) (γ-PGA) is an interesting biodegradable homopolyamide that is composed of D- and L-glutamic acid residues [1]. Although there are two types of PGA (α and γ forms), it is easier to produce γ-PGA than a-PGA by bacterial fermentation using *Bacillus* sp. [2]. γ-PGA is resistant to proteases as glutamate is usually polymerized into γ-PGA via the γ-amide linkages inside the bacterial cell [3]. The γ-PGA properties like water solubility, non-toxicity, edibility, thickening capacity, metal binding, good absorbability, and biodegradability provide for its usage as a drug carrier [4−6], an antifreeze agent, a food thickener [7−9], a flocculating agent for environmental

protection, and as a humectant in cosmetics [10, 11]. All the above-mentioned properties and applications of γ-PGA are attracting present-day investigators to study high-yield production strategies.

γ-PGA can be produced through four different chemical and biological methods: chemical synthesis, peptide synthesis, biotransformation, and microbial fermentation [12]. However, γ-PGA production through microbial fermentation is considered economical and it has some advantages such as inexpensive raw materials, minimal environmental pollution, high natural product purity, and mild reaction conditions when compared with other methods. Ivanovics and Bruckner first discovered γ-PGA from a capsule of *Bacillus anthracis* [13]. Afterwards it was

identified in archaea, bacteria, and eukaryotes. Apart from all other organisms and genera, *Bacillus* is very popular for use in γ-PGA production through fermentation. *Bacillus* strains can be classified into two major groups based on the requirement of L-glutamic acid as a carbon and nitrogen source for γ-PGA production and cell growth. L-Glutamate-dependent strains (such as *B. licheniformis* ATCC 9945a, *B. subtilis* IFO3335, *B. subtilis* F-2-01, *Bacillus* sp. RKY3) are known to produce high levels of γ-PGA by the addition of L-glutamate into the medium [14–16]. On the other hand, some other strains such as *B. subtilis* 5E, *B. subtilis* TAM-4, *B. subtilis* C10, *B. licheiformis* A35, and *Bacillus* sp. SAB-26 could produce γ-PGA through a *de novo* γ-PGA synthesis pathway [13]. γ-PGA is synthesized in a ribosome-independent manner, and then protein synthesis inhibitors like chloramphenicol have no inhibition effect on its production. Depending on the strains, γ-PGA is produced in different molecular weights ranging from 100 to 10,000 kDa [1].

Although the microbial production of γ-PGA is well established, the production cost still remains high because of the cost of both raw materials and the recovery process. Generally, in the fermentation process, the substrate cost is very high, *i.e.* nearly 60% of the total production cost. Therefore, it is essential to focus on the development of a cost-effective alternative medium. It requires selection of carbon source, nitrogen source, and inorganic salts. Recently, many research groups have investigated the optimization of growth conditions to increase yield, manipulation of enantiomeric composition, and alteration of the molecular mass of the γ-PGA [17–20]. Medium components and fermentation conditions can be manipulated by conventional or statistical methods. The conventional method is a very tedious and time-consuming process where we can only study one variable at a time while keeping others at a fixed level. However, advanced statistical methods provide possibility of studying many variables at one time and of studying the interactions among the medium and fermentation factors, and it is also fast and reliable. This in turn reduces the total number of experiments and saves the cost of the nutrients [20, 21]. Therefore, the aim of the present research is enhancement of γ-PGA production through the optimization of medium components by central composite design (CCD) for response surface methodology (RSM) using a newly isolated *Bacillus* sp. FBL-2.

Materials and Methods

Bacterial Strain and Culture Medium

Bacillus sp. FBL-2 KCTC 12962BP isolated from cheonggukjang, a fermented soybean paste, was utilized for γ-PGA production. The 16S rRNA gene sequence of strain FBL-2 was submitted to the GenBank database (https://www.ncbi.nlm.nih.gov/genbank), under accession number LY513821. To preserve the culture, 50% (v/v) glycerol as a cryoprotectant was added to the culture and then kept at −70°C in a deep freezer until usage. The stock culture was activated by inoculation into the culture medium, followed by cultivation at 37°C on a shaking incubator (Vision Scientific Co., Korea) at 200 rpm.

The medium for growth and maintenance consisted of glucose 10.0 g/l, yeast extract 3.0 g/l, L-glutamic acid 20.0 g/l, KH_2PO_4 1.0 g/l, and $MgSO_4 \cdot 7H_2O$ 1.0 g/l (pH 7.0). The production medium was composed of glucose 10.0 g/l, yeast extract 4.0 g/l, L-glutamic acid 30.0 g/l, KH_2PO_4 1.0 g/l, and $MgSO_4 \cdot 7H_2O$ 1.0 g/l (pH 7.0). The composition and amount of production medium used were different depending on the experimental conditions. A detailed experimental condition of the production medium was given later.

Fermentation

The bacterial strain was cultured at 37°C for 24 h in the shaking incubator (Vision Scientific Co.) at 200 rpm by inoculating 2 ml of the stock culture into 100 ml growth medium in a 250-ml Erlenmeyer flask. The fermentation for γ-PGA production was carried out at 37°C for 12 h in the shaking incubator at 200 rpm by inoculating 3.0% (v/v) growth culture into 100 ml production medium in a 250-ml Erlenmeyer flask.

To investigate the effect of carbon sources on γ-PGA production by *Bacillus* sp. FBL-2, the medium components except glucose in the production medium were kept the same as above. The carbon source, including glucose, lactose, fructose, sucrose, galactose, maltose, glycerol, and xylose was added to a 250 ml-Erlenmeyer flask at a concentration of 10 g/l, and the cells were inoculated and aerobically cultivated at 37°C for 12 h in the rotary shaker at 200 rpm. To investigate the effect of nitrogen sources on γ-PGA production by *Bacillus* sp. FBL-2, the medium components except yeast extract in the production medium were kept the same as above. The nitrogen source, including yeast extract, beef extract, malt extract, tryptone, peptone, urea, ammonium sulfate, ammonium chloride, and corn steep liquor was added to a 250 ml-Erlenmeyer flask at a concentration of 4 g/l, and the cells were inoculated and aerobically cultured at 37°C for 12 h in the rotary shaker at 200 rpm.

Statistical Experimental Design for Response Surface Methodology

Reaction surface methodology (RSM) was carried out through the central composite design (CCD) to investigate which combination of key interactions and independent variables of γ-PGA production could lead to the maximum response value. RSM is a combination of statistical methods for selecting the optimum experimental conditions that require the minimum number of experiments.

$$x_i = \frac{X_i - X_0}{\Delta X_i}$$

The experimental variables were coded by using the above equation, where X_i is the actual value of the independent variable, X_0 is the independent variable value at the center point, ΔX_i is the step change value, and x_i is the coded value of each independent variable.

In this study, the concentration of KH_2PO_4 and $MgSO_4 \cdot 7H_2O$ in the production medium was kept at 1.0 g/l, and four important components such as sucrose, L-glutamic acid, yeast extract, and citric acid were used as the independent variables for the designed sets of experiment. As shown in Table 1, to investigate the nature of the response surface in the optimum region, a 2^4 factorial CCD with eight axial points and six replicates of center points was used at five levels, resulting in the total number of 30 experiments.

To optimize the production of γ-PGA, the following second-order polynomial equation was used for statistical analysis.

$$y = b_0 + \sum b_i x_i + \sum b_{ij} x_i x_j + \sum b_{ii} x_i^2$$

where y is a predicted value, b_0 is a constant, b_i and b_{ii}, and b_{ij} are first-order coefficients, second-order coefficients, and interaction coefficients, respectively. x_i is the independent variable of i, $x_i x_j$ is the interaction between independent variables, and x_i^2 is the second order coefficient. The quality of fit of the model equation was described by the coefficient of determination, R^2, and the significance of statistics was determined by an F-test. The significance of the regression coefficients was investigated by a t-test. The computer software used was Design-Expert, version 9.0.0 by Stat-Ease, Inc. (USA).

Analytical Methods

The cell growth was monitored by measurement of optical density using a UV-1600 spectrophotometer (Shimadzu Co., Tokyo, Japan) at 660 nm, which was then converted to dry cell weight (DCW, g/l) based on the liner relation of DCW and optical density. The viscosity of the culture broth containing γ-PGA was measured by DV2T digital rheometer equipped with a spindle CP-42 (Brookfield, Middleboro, MA, USA) at 25°C and 10 rpm for 30 sec. The measured viscosity was corrected by silicon oil standards (44.8 cP and 496 cP at 25°C).

γ-PGA was determined by alcohol precipitation according to the modified method reported by Kunioka and Goto [15]. The fermentation broth was diluted and centrifuged at 32,000 ×*g* for 30 min. The resulting supernatant was poured into 4 volumes of cold ethanol. The precipitate was collected and washed with ethanol, then dissolved and dialyzed against deionized water overnight. The dialyzed solution was centrifuged and the supernatant was lyophilized to prepare pure γ-PGA.

Results and Discussion

The eight different carbon sources and nine nitrogen sources were selected for screening and evaluating their potentiality in producing high quantity γ-PGA using the shake flask culture method. Among the various carbon sources evaluated, sucrose showed the highest levels of production of γ-PGA and DCW followed by glucose (Table 2). These results are in accordance with Shi *et al.* [22] where they examined 7 different carbon sources and found sucrose as the best with similar γ-PGA yield and DCW.

Table 1. Central composite design matrix of four independent variables.

Run number	Levels of independent variables			
	Sucrose (x_1, g/l)	L-Glutamic acid (x_2, g/l)	Yeast extract (x_3, g/l)	Citric acid (x_4, g/l)
1	30	75	10	15
2	70	75	10	15
3	30	125	10	15
4	70	125	10	15
5	30	75	20	15
6	70	75	20	15
7	30	125	20	15
8	70	125	20	15
9	30	75	10	25
10	70	75	10	25
11	30	125	10	25
12	70	125	10	25
13	30	75	20	25
14	70	75	20	25
15	30	125	20	25
16	70	125	20	25
17	90	100	15	20
18	10	100	15	20
19	50	50	15	20
20	50	150	15	20
21	50	100	5	20
22	50	100	25	20
23	50	100	15	10
24	50	100	15	30
25	50	100	15	20
26	50	100	15	20
27	50	100	15	20
28	50	100	15	20
29	50	100	15	20
30	50	100	15	20

Table 2. Effect of carbon sources on γ-PGA fermentation by *Bacillus* sp. FBL-2.

Carbon sources (10.0 g/l)	γ-PGA (g/l)	Dry cell weight (g/l)	Viscosity (cP)	Productivity (g/l/h)
None	0.46	0.99	1.70	0.038
Lactose	0.63	1.04	2.33	0.053
Fructose	3.63	1.49	17.13	0.303
Sucrose	4.60	1.81	20.94	0.383
Galactose	1.25	1.09	4.45	0.104
Maltose	2.86	1.17	12.79	0.238
Glucose	3.96	1.74	19.11	0.330
Glycerol	0.86	0.63	3.91	0.072
Xylose	0.63	0.86	2.14	0.053

However, some of the previous reports concluded that citric acid and glycerol were better carbon sources for the production of γ-PGA than glucose or sucrose [20, 23, 24]. In our present work, the results showed that sucrose was the best carbon source for γ-PGA accumulation and cell growth, which suggests that the metabolic pathways in *Bacillus* sp. FBL-2 might be different from other glutamic acid-dependent strains for γ-PGA production. In general, glucose is the preferable carbon source for the production of γ-PGA which mostly utilizes the TCA cycle. It can be also concluded that *Bacillus* sp. FBL-2 utilizes the general TCA cycle for γ-PGA production after hydrolyzing the sucrose to glucose and fructose. Anju *et al.* [25] studied the utilization of lignocellulosic renewable resources for the production of γ-PGA and reported that the highest γ-PGA yield was from rice straw hydrolyzates, where glucose was the main carbon source.

In nitrogen sources investigated, yeast extract showed the highest production of γ-PGA and peptone resulted in

the highest cell growth (Table 3). The results indicated that inorganic nitrogen sources such as ammonium sulfate and ammonium chloride could not support γ-PGA synthesis and cell growth. Shi *et al.* [22] have screened 7 different nitrogen sources and reported that incorporation of organic nitrogen sources increased the yields of γ-PGA and no inorganic nitrogen sources had a significant effect. At the same time, many of the researchers used inorganic nitrogen sources for γ-PGA production [23, 24]. Goto and Kunioka [24] have observed the enhancement of γ-PGA by the addition of ammonium sulfate (5 g/l) and suggested that the free amino group was necessary for γ-PGA production and was readily available from inorganic nitrogen salts such as ammonium sulfate. According to the above reports, it can be concluded that the selection of nitrogen source is purely dependent on the microbial strain and there is no universal/single suitable source.

Based on the primary screening process, sucrose (x_1), L-glutamic acid (x_2), yeast extract (x_3), and citric acid (x_4) were selected as the independent variables for further optimization by RSM. A central composite factorial design of 30 experiments was conducted to examine the combined effect of these medium components on γ-PGA production. The *P*-values were used as the tool to check the significance of each variable, which, in turn, are necessary to understand the pattern of the mutual interactions between the selected variables. The ANOVA (analysis of variance) result of the optimization study indicated that the model terms, x_1, x_2, x_4, $x_2 x_3$, $x_2 x_4$, $x_3 x_4$, x_1^2, and x_2^2, were significant ($p < 0.05$) (Table 4). The lower the *P*-value is, the more significant the corresponding variable. The variables and the corresponding *P*-values suggest that among the variables tested in the present study, sucrose (x_1), L-glutamic acid (x_2), and citric acid (x_4) independently show their significance. The interaction effects between L-

Table 3. Effect of nitrogen sources on γ-PGA fermentation by *Bacillus* sp. FBL-2.

Nitrogen sources (4.0 g/l)	γ-PGA (g/l)	Dry cell weight (g/l)	Viscosity (cP)	Productivity (g/l/h)
None	1.63	0.76	1.83	0.136
Beef extract	3.98	1.72	17.17	0.332
Yeast extract	4.50	1.85	19.64	0.375
Malt extract	1.74	0.83	2.23	0.145
Tryptone	4.39	1.51	19.33	0.366
Peptone	3.83	1.33	23.38	0.319
Urea	0.00	0.22	1.23	0.000
Ammonium sulfate	2.63	0.77	5.93	0.219
Ammonium chloride	2.29	0.80	5.67	0.191
Corn steep liquor	1.67	0.82	3.29	0.139

Table 4. Analysis of variance (ANOVA) for the response surface quadratic model.

| Source	Sum of squares	Degree of freedom	Mean square	F-value	p-value Prob. > F
Model	2,233.08	14	159.51	122.44	< 0.0001
x_1	285.94	1	285.94	219.49	< 0.0001
x_2	69.56	1	69.56	53.4	< 0.0001
x_3	8.32	1	8.32	6.39	0.0232
x_4	779.53	1	779.53	598.38	< 0.0001
x_1x_2	0.61	1	0.61	0.47	0.5034
x_1x_3	1.45	1	1.45	1.11	0.3088
x_1x_4	0.3	1	0.3	0.23	0.6399
x_2x_3	17.98	1	17.98	13.8	0.0021
x_2x_4	330.42	1	330.42	253.64	< 0.0001
x_3x_4	58.26	1	58.26	44.72	< 0.0001
x_1^2	382.78	1	382.78	293.83	< 0.0001
x_2^2	326.49	1	326.49	250.62	< 0.0001
x_3^2	8.97	1	8.97	6.89	0.0191
x_4^2	8.05	1	8.05	6.18	0.0252
Residual	19.54	15	1.3		
Lack of fit	17.87	10	1.79	5.36	0.0389
Pure error	1.67	5	0.33		
Corrected total	2,252.62	29			

$R^2 = 0.9913$; adjusted $R^2 = 0.9832$; adequately precise = 43.877; CV = 4.94%.

glutamic acid (x_2) and yeast extract (x_3), L-glutamic acid (x_2) and citric acid (x_4), and yeast extract (x_3) and citric acid (x_4) also showed good significance. Other interactions were found to be insignificant. The model F-value was 122.44, and the F-value for lack of fit was 5.52. The high F-value and non-significant lack of fit indicate that the model is a good fit. The p-values for the model (<0.0001) and for lack of fit (0.0389) suggested that the obtained experimental data was a good fit with the model. The regression equation coefficients were calculated and the data were fitted to a second-order polynomial equation. The response, γ-PGA production (y) by *Bacillus* sp. FBL-2, can be expressed in terms of the following regression equation:

$$y = 28.87 + 3.45x_1 + 1.7x_2 - 0.59x_3 - 5.70x_4 - 0.20x_1x_2 - 0.30x_1x_3 - 0.14x_1x_4 + 1.06x_2x_3 - 4.54x_2x_4 - 1.91x_3x_4 - 3.74x_1^2 - 3.45x_2^2 - 0.57x_3^2 + 0.54x_4^2$$

The regression equation obtained from the ANOVA showed that the R^2 (multiple correlation coefficient) was 0.9913, where $R^2 > 0.75$ usually indicates fitness of the model. This is an estimate of the fraction of overall variation in the data accounted by the model, and thus the model is capable of explaining 99.13% of the variation in

response. The 'adjusted R^2' is 0.9832, and this result indicates that the model should be acceptable. For a good statistical model, the R^2 value should be in the range of 0–1.0, and the nearer to 1.0 the value is, the more fit the model is deemed to be. The 'adequate precision value' of the present model was 29.50, and this suggests that the model can be used to navigate the design space. The 'adequate
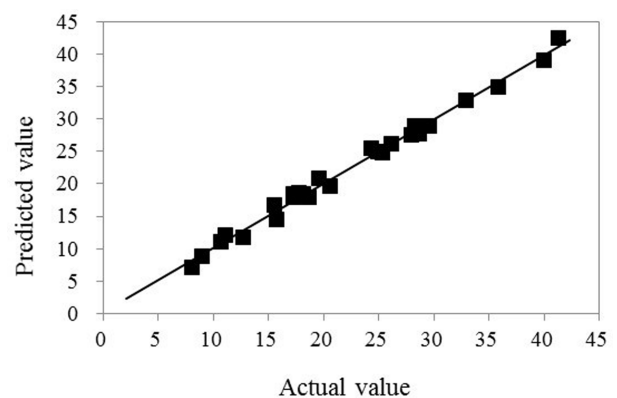

Fig. 1. Correlation of predicted values versus experimental values of the response surface methodological model developed by central composite design.

precision value' is an index of the signal-to-noise ratio, and values of higher than 4 are essential prerequisites for a model to be a good fit. As shown in Fig. 1, the correlation between predicted and observed values showed very good linearity.

In order to determine the optimal levels of each variable for maximum γ-PGA production, 3-dimensional response surface plots were constructed by plotting the response on the z-axis against any two independent variables, while maintaining other variables at their optimal levels. As shown in Figs. 2A–2F, γ-PGA production could not increase further with increasing sucrose or L-glutamic acid
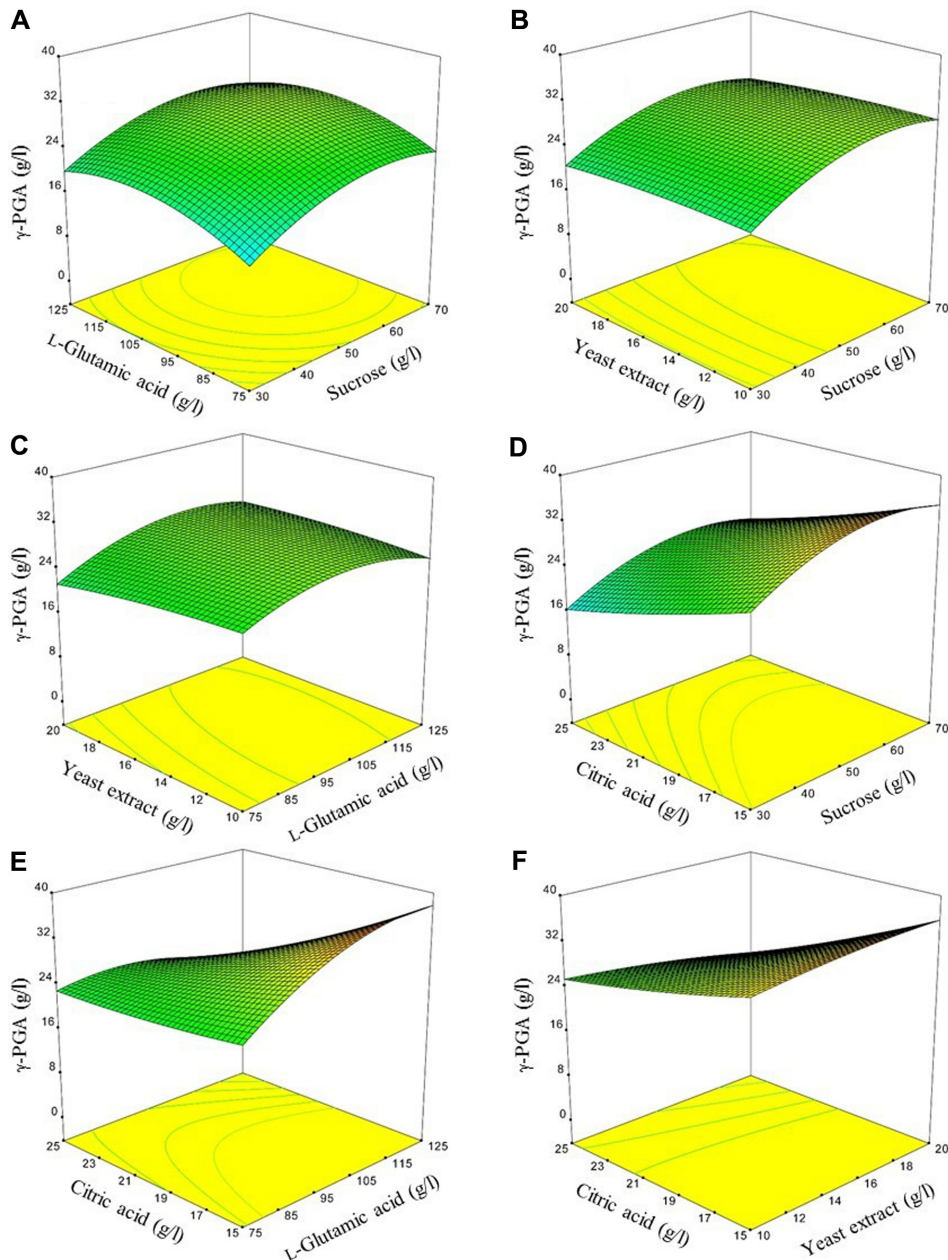
**Fig. 2.** Response surface and contour plots of poly(γ-glutamic acid) production by *Bacillus* sp. FBL-2 showing the mutual interactions of independent parameters.

Other variables except for two variables in each figure were maintained at center point level. (**A**), sucrose and L-glutamic acid; (**B**), sucrose and yeast extract; (**C**), sucrose and citric acid; (**D**), L-glutamic acid and yeast extract; (**E**), L-glutamic acid and citric acid; (**F**), yeast extract and citric acid.

and showed very good center point (optimum levels). From the central point of the contour plot, the optimal process parameters were identified. A linear increase in γ-PGA secretion was observed when the citric acid and yeast extract concentrations were increased, and no concomitant decline in γ-PGA production was observed. A similar profile was observed in Fig. 2E with citric acid and L-glutamic acid concentration. The experimental data were fitted into the aforementioned equation, and the optimum levels of each variable were determined to be as follows: sucrose 51.73 g/l, L-glutamic acid 105.30 g/l, yeast extract 13.25 g/l, and citric acid 10.04 g/l.

Fig. 3 shows the profiles of cell growth, γ-PGA production, viscosity, and productivity when *Bacillus* sp. FBL-2 was cultured under the optimized medium factors, and it was observed the cells were very quickly grown within 12 h of incubation period with maximum DCW of 1.28 g/l. There was no decline in the growth after 12 h, which suggests the optimized medium with sufficient amounts of L-glutamic acid and sucrose. The experiment was conducted for only 12 h and maximum production of 44.04 g/l γ-PGA was obtained with 19.59 cP viscosity. This amount of γ-PGA production under the optimized condition was almost similar to the predicted γ-PGA production (43.52 g/l). γ-PGA production was not considerably increased after 12 h probably because this fermentation was performed under the flask cultivation (data not shown). On the other hand, the maximum γ-PGA production using the non-optimized medium was only 3.96 g/l with 19.11 cP viscosity. From the above results, it can be concluded that a significant improvement (11.12-fold) in the production of γ-PGA was achieved using sucrose and yeast extract as carbon and nitrogen sources within a lesser incubation period (Table 5). The close relationship between the predicted and experimental response values from the investigation demonstrated the validity and acceptability of the statistical model for the optimization of the medium nutrients, allowing for maximum yields.

The present investigation shows that the amount of sucrose and L-glutamic acid in the culture medium are important for high γ-PGA productivity in *Bacillus* sp. FBL-2. γ-PGA synthesis in *B. subtilis* is an ATP-consuming
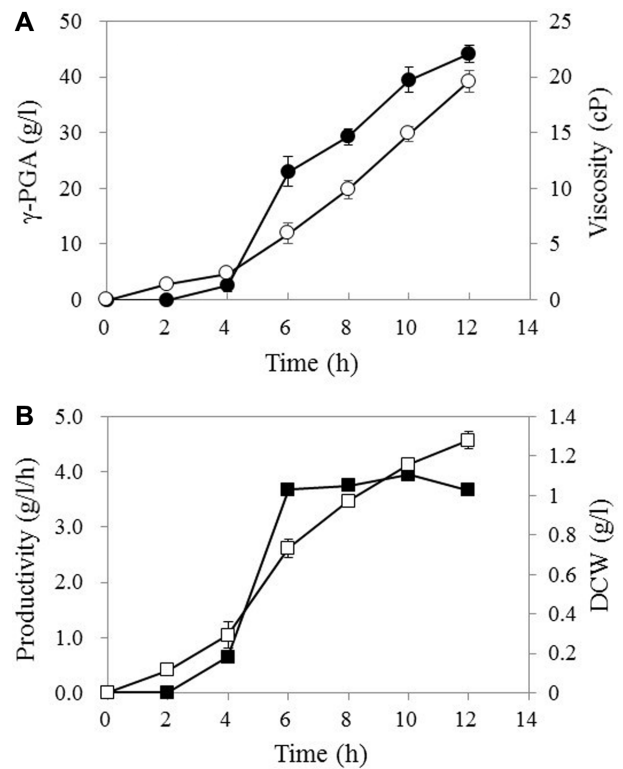
Fig. 3. Profiles of poly(γ-glutamic acid) production, viscosity, cell growth, and volumetric productivity in shake flask experiments using the optimized media.
Symbols: -●-, γ-PGA; -○-, viscosity; -■-, DCW (dry cell weight); -□-, productivity.

bioprocess. The rate of conversion from L-glutamic acid to γ-PGA should be considered very important for reducing the cost of large-scale fermentation-based production of γ-PGA [26]. It is very important to note that the nitrogen source, yeast extract concentration (10 g/l) used in the present study is low when compared to previous studies [22, 27, 28]. It is very essential to use less nitrogen source because it is very expensive and it will be 20−30% of the total medium cost. γ-PGA has recently been produced on a large scale using several bacterial strains. Table 6 shows the comparison of γ-PGA fermentation by *Bacillus* sp. FBL-2 with other literature reported previously. The highest γ-

Table 5. Poly(γ-glutamic acid) fermentation results of *Bacillus* sp. FBL-2 cultured by using non-optimized and optimized medium components.

	γ-PGA (g/l)	DCW (g/l)	Viscosity (cP)	Productivity (g·l⁻¹·h⁻¹)	Fold production
Non-optimized	3.96 ± 0.67	1.74 ± 0.06	19.11 ± 1.14	0.33	1.0
Optimized	44.04 ± 1.54	1.28 ± 0.04	19.59 ± 0.95	3.67	11.12

Table 6. Poly(γ-glutamic acid) fermentation results of *Bacillus* sp. FBL-2 and other microorganisms reported previously.

Strains	Fermentation medium components	Culture conditions	γ-PGA (g/l)	Productivity (g·l⁻¹·h⁻¹)	Ref.
B. subtilis ZJU-7	Sucrose 60 g/l, tryptone 60 g/l, L-glutamic acid 80 g/l, NaCl 10 g/l	500-ml flask, 200 rpm, 37°C, initial pH 7.0	54.4	2.267	22
B. methylotrophicus SK19.001	Glycerol 30 g/l, peptone 50 g/l, sodium citrate 15 g/l	250-ml flask, 200 rpm, 30°C, initial pH 7.2	33.84	0.940	27
B. licheniformis NCIM 2324	Glycerol 62.4 g/l, L-glutamic acid 20 g/l, citric acid 15.2 g/l, $(NH_4)_2SO_4$ 8 g/l, K_2HPO_4 1 g/l, $MgSO_4 \cdot 7H_2O$ 0.5 g/l, $MnSO_4 \cdot 7H_2O$ 0.05 g/l, $CaCl_2 \cdot 2H_2O$ 0.2 g/l	250-ml flask, 200 rpm, 37°C, initial pH 6.5	35.75	0.331	29
B. licheniformis SAB-26	Glucose 20 g/l, casein hydrolysate 8 g/l, $(NH_4)_2SO_4$ 2 g/l, K_2HPO_4 5 g/l, KH_2PO_4 5 g/l	250-ml flask, 200 rpm, 37°C, initial pH 7.0	33.5	2.792	30
B. licheniformis ATCC 9945a	Glycerol 80 g/l, citric acid 12 g/l, L-glutamate acid 20 g/l, NH_4Cl 7 g/l, K_2HPO_4 0.5 g/l, $MgSO_4 \cdot 7H_2O$ 0.5 g/l, $MnSO_4 \cdot H_2O$ 0.104 g/l, $CaCl_2 \cdot 2H_2O$ 0.15 g/l, $FeCl_3 \cdot 6H_2O$ 0.04 g/l	300-ml flask, 110 rpm, 37°C, initial pH 6.5	34.93	0.364	32
B. licheniformis WX-02	Glucose 80 g/l, L-glutamic acid 30 g/l, sodium citrate, sodium nitrate 10 g/l, NH_4Cl 8 g/l, $CaCl_2$ 1 g/l, $K_2HPO_4 \cdot 6H_2O$ 1 g/l, $MgSO_4 \cdot 7H_2O$ 1 g/l, $ZnSO_4 \cdot 7H_2O$ 1 g/l, $MnSO_4 \cdot 7H_2O$ 0.15 g/l	250-ml flask, 180 rpm, 37°C, initial pH 7.2	39.96	1.11	33
Bacillus sp. FBL-2	Sucrose 51.73 g/l, L-glutamic acid 105.23 g/l, yeast extract 13.25 g/l, citric acid 10.04 g/l, K_2HPO_4 1.0 g/l, $MgSO_4 \cdot 7H_2O$ 1.0 g/l	250-ml flask, 200 rpm, 37°C, initial pH 7.0	44.04	3.67	This study

PGA productivity reported by Shi *et al.* [22] was 2.267 g/l/h (54.4 g/l γ-PGA) using the *B. subtilis* ZJU-7 cultivated at 37°C for 24 h. Bajaj *et al.* [29] reported a maximum γ-PGA production of 35.75 g/l using *B. licheniformis* NCIM2324. Soliman *et al.* [30] reported a maximum γ-PGA production of 33.5 g/l using *Bacillus* sp. SAB-26, and Cao *et al.* [31] reported a much lower amount of γ-PGA (4.36 g/l). *B. licheniformis* ATCC 9945a produced 34.93 g/l of γ-PGA but the volumetric productivity was only 0.64 g/l/h [32]. Cai *et al.* [33] reported 1.11 g/l/h of γ-PGA productivity by flask fermentation of *B. licheniformis* WX-02. As shown in Table 6, *Bacillus* sp. FBL-2 could produce a high amount of γ-PGA with the highest volumetric productivity even though the compositions of production medium were simple compared with the other investigations. Therefore, compared with the strains in Table 6, *Bacillus* sp. FBL-2 has some advantages such as high productivity (3.67 g·l⁻¹·h⁻¹), short incubation period (12 h), and utilization of less nitrogen source (10 g/l). To the best of our knowledge, the γ-PGA productivity reported in the present study is highest in the literature. The findings of this study indicate that media optimization and the use of *Bacillus* sp. FBL-2 organisms have significant scope for use in the industrial production of γ-PGA. In addition, the results presented here could be efficiently used in the area of γ-PGA production from inexpensive bioresources such as agricultural residues or food wastes [34].

This study was conducted in an attempt to optimize medium composition for maximum γ-PGA production. The eight carbon sources and seven nitrogen sources were screened, and sucrose and yeast extract were selected as carbon and nitrogen sources, respectively, based on the preliminary experiments. In order to optimize low-cost fermentation medium with precursors, such as L-glutamic acid and citric acid for γ-PGA production, RSM study was carried out using the screened variables. The most significant medium components appear to be sucrose 51.73 g/l, L-glutamic acid 105.30 g/l, yeast extract 13.25 g/l, and citric acid 10.04 g/l. The maximum γ-PGA concentration (44.04 g/l) was obtained from this study with a much shorter incubation period (12 h) at 37°C. As a result, the γ-PGA productivity could reach to 3.67 g·l⁻¹·h⁻¹. This represents an 11.12-fold enhancement over the productivity observed with non-optimized medium. Thus, the statistical methods (RSM) may be effective for optimizing bioprocessing conditions for developing low-cost, large-scale methods of producing this important γ-PGA biomaterial in the future.

Acknowledgments

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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