Optimization of γ-Polyglutamic Acid Production by *Bacillus subtilis* ZJU-7 Using a Surface-response Methodology

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Abstract The components of the media used to elicit the biosynthesis of poly-γ-glutamic acid (γ-PGA) by *Bacillus subtilis* ZJU-7 were investigated, particularly the carbon and nitrogen sources. Of the 7 carbon sources investigated, sucrose induced the highest rate of γ-PGA productivity; among the nitrogen sources, tryptone had the best effect for γ-PGA production. A 2^{6-2} fractional factorial design was used to screen factors that influence γ-PGA production significantly, and a central composite design was finally adopted to formulate the optimal medium. γ-PGA productivity improved approximately 2-fold when the optimal medium was used compared with the original nonoptimized medium, and volumetric productivity reached a maximum of 58.2 g/L after a 24-h cultivation period.

Keywords: poly-γ-glutamic acid, L-glutamic acid, *Bacillus subtilis*, response surface methodology

INTRODUCTION

Poly-γ-glutamic acid (γ-PGA) is a water-soluble and biodegradable polymer made of D- and L-glutamic acid units linked by an amide bond between α-amino and γ-carboxylic acid groups. Various applications for γ-PGA have been developed in the food, cosmetic, and pharmaceutical industries [1-3]. Highly water-absorbent and biodegradable γ-PGA derivatives have the potential to serve as substitutes for petroleum-based hydrogels and thermoplastic polymers [4]. γ-PGA may also be able to serve as an adaptation agent in various environmental applications, such as heavy-metal ion absorption [5]. Growth in the number of applications of γ-PGA has been paralleled by growth in the demand for methods of greatly reducing the cost of producing and marketing its applications.

γ-PGA acid was first discovered by Ivanovics *et al.* as a component of the *Bacillus anthracis* capsule [6]. Thereafter, it was identified in other *Bacillus* species, including *B. licheniformis*, *B. megaterium*, *B. subtilis*, and *B. amyloliquefaciens* [7-10]. For example, the mucilage produced by *B. natto* is a mixture of γ-PGA and fructan, both of which are produced by this organism. A relatively high yield of γ-PGA (50 g/L) has been reported under a variety of culture conditions, but further effort is still needed to improve the current γ-PGA production process through strain discovery and bioprocess optimization. A new *B. subtilis* strain (*B. subtilis* ZJU-7) was recently isolated from fermented bean curd (a traditional Chinese food) and has demonstrated the potential to produce large amounts of PGA [11].

Response surface methodology (RSM) is a common technique used in biotechnology to optimize bacterial growth conditions in culture that may be useful in promoting γ-PGA production [12,13]. Specifically, RSM is used to determine the optimal values for such media parameters as pH, temperature, degree of aeration [14] and feeding rates [15]. This optimization process involves 3 major steps: performing statistically designed experiments, estimating the coefficients in a mathematical model, and predicting the response and checking the adequacy of the model. The maximum response rate can then be calculated based on the mathematical model [16].

In this study, the effects of various sources of carbon and nitrogen on γ-PGA biosynthesis were evaluated. RSM was applied to identify the key controllable factors that most significantly affect the γ-PGA yield in the culture medium. A central composite design (CCD) was used to optimize the levels of these controllable factors in culture in order to formulate a medium that provides optimal support for the production of γ-PGA by *B. subtilis* ZJU-7.

MATERIALS AND METHODS

Materials

B. subtilis ZJU-7 was isolated from fermented bean curd, maintained in the China General Microbiogical Culture Collection Center (CGMCC; accession number CGMCC 1250) at 4°C, and sub-cultured every 4 weeks.

Standard γ-PGA was obtained from the Sigma Chemi-

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cal Company (St. Louis, MO, USA); the other chemicals used in this study were certified as reagent grade. The media used for the slant, seed, and basal cultures were as follows. Slant medium: glucose 10 g/L, tryptone 10 g/L, L-glutamic acid 10 g/L, and NaCl 5 g/L; seed medium: the same as the slant medium, plus 0.1 g/L $MgSO_4$ and 0.1 g/L $CaCl_2$; basal culture medium: the same as the seed medium, plus 10 g/L glucose, 10 g/L tryptone, and 20 g/L L-glutamic acid. RSM was used to assess the effects of these media ingredients on γ-PGA production by varying the composition of the media. The initial pH was adjusted to 7.0 by adding HCl or NaOH. In all cases, the media were sterilized by autoclaving for 20 min at 121°C.

Cultivation

Aliquots (30 mL) of each medium were dispensed into 500-mL flasks and incubated at 37°C while being shaken at a speed of 200 rpm. Samples of each medium were taken after a 24-h fermentation period, then centrifuged and used for a concentration assay. All of these experiments were carried out in duplicate.

Analysis

γ-PGA was purified using a methanol precipitation method proposed by Goto and Kunioka [17]. Cells were separated from the broth by centrifugation for 20 min at 12,000 rpm and 4°C. Four volumes of methanol were then poured into the supernatant, which was left undisturbed overnight, except for gentle stirring. The precipitate was collected by applying centrifugation to the supernatant for 10 min at 6,000 rpm and 4°C, then dissolving the residue in distilled water to a concentration of 10 g/L and removing it by filtration. The aqueous solution was desalted by dialysis (cutoff MW 10,000) against 11 volumes of distilled water for 12 h with 3 water exchanges. The absence of free L-glutamic acid and polysaccharides in the solution was confirmed by L-glutamate dehydrogenase-coupled assay [18] and the phenol-sulfuric acid method [19], respectively. The final solution was lyophilized, and the dry matter was determined to be γ-PGA.

The cell density was determined by measuring the optical density at 660 nm. The dry cell weight (g/L) was measured by centrifuging 4 to 8 mL of the cell suspension in preweighed tubes, washing it with distilled water, and drying it at 95°C to a constant weight.

Cultivation Medium Optimization and Experimental Design

Selection of Carbon and Nitrogen Sources

The composition of the medium that was used for γ-PGA production with *B. subtilis* ZJU-7 was optimized on a flask scale. *B. subtilis* ZJU-7 colonies were transferred from the agar slant medium to 100-mL flasks each containing 10 mL of the slant medium without agar and incubated in a rotary shaker at 37°C at 200 rpm for 12 h. Thereafter, 1 mL of this culture medium was transferred to 50 mL of the seed medium in a 500-mL flask and cultured aerobically at 37°C for 24 h while being shaken at a speed of 200 rpm. A 1-mL portion of the seed culture was transferred to a 250-mL flask containing 30 mL of the basal culture medium, which then underwent the same conditions as the seed culture. Seven carbon sources (glucose, sucrose, maltose, lactose, starch, citrate acid, and glycerol) were used to examine the effect of each carbon source on γ-PGA production. Seven nitrogen sources (peptone, tryptone, yeast extract, ammonium sulfate, soy bean, maize flour, and fish protein concentrate) were evaluated at a concentration of 20 g/L, to which 20 g/L of the optimized carbon source and 40 g/L of L-glutamic acid added.

RSM Design

The RSM strategy was described in detail by Strobel and Sullivan [20]. A 2^{6-2} FFD was used to screen the most important factors influencing γ-PGA production and eliminate the insignificant ones to yield a smaller, more manageable set of factors. In FFD, low and high factor settings were coded −1 and +1, respectively, and the midpoint setting was coded as 0; the coded values and natural values are shown in Table 1. The coded value X_i is defined as follows:

$$X_i = (x_i - x_{i0} / \delta_i) \quad (1)$$

where x_i is the corresponding natural value, x_{i0} is the natural value in the center of the domain, and δ_i is the increment of x_i corresponding to one unit of X_i. The experimental design and results are shown in Table 4. The linear model obtained is expressed as following:

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i \quad (2)$$

If the mean of the center points exceeds the mean of the factorial points, the optimum is close to or within the experimental design space. If the mean of the center points is less than the mean of the factorial points, the optimum is outside the experimental design space and the method of steepest ascent should be applied. The direction of steepest ascent is parallel to the contour of the normal response curve generated by Eq. (2) and passes through the center point of FFD. Increment is the direct proportion to regression coefficients β_i. Experiments should be performed along the steepest ascent path until the response does not increase further; that point should be close to the optimal point and can be used as the center point.

Once critical factors were identified and a significant gross curvature was detected in the design space, the CCD was carried out to obtain a quadratic model consisting of trials plus a star configuration to estimate quadratic effects and central points to estimate pure process variability and to reassess the gross curvature with maximization of γ-PGA production being the expected response. When several factors are involved, the model can be expressed as follows:

$$y = \beta_0 + \beta_i \sum x_i + \beta_{ij} \sum x_i x_j + \beta_{ii} \sum x_{ii}^2 \quad (3)$$

Table 1. Factors and level in FFD

Factors	Factor level		
	−1	0	+1
Sucrose	20	40	60
Tryptone	20	40	60
L-glutamic acid	60	90	120
NaCl	10	20	30
$MgSO_4$	0.1	0.2	0.3
$CaCl_2$	0.1	0.2	0.3

where y is the measured response; β_0, β_i, β_{ij}, and β_{ii} are the intercept term, linear coefficient, interactive coefficient, and quadratic coefficient, respectively; and x_1, x_2, and x_3 are coded independent variables. Low and high factor settings are coded −1 and +1, and the midpoint is coded 0. The factor settings of trails that ran along axes drawn from the middle of the cube through the center of each face of the tube are coded +1.681 or −1.681. An SAS package (SAS Institute, Cary, NC, USA) was used to analyze the results.

RESULTS AND DISCUSSION

Effects of Carbon Sources on γ-PGA Production

The effects of various carbon sources on *B. subtilis* ZJU-7 growth and γ-PGA production were studied by fixing 20 g/L tryptone and 40 g/L L-glutamic acid, respectively, in the medium. Under this condition, glucose supported the maximum biomass, whereas sucrose induced the highest γ-PGA productivity rate (20.3 g/L) (Fig. 1). In fact, sucrose was the best carbon source for γ-PGA production and was used as sole carbon source in subsequent experiments.

Influence of Nitrogen Source on γ-PGA Production

The effects of various nitrogen sources on γ-PGA production were then studied by fixing sucrose 20 g/L as the sole carbon source in the media studied. None of the inorganic nitrogen sources added to these media increased cell growth or γ-PGA productivity (Fig. 2). Tryptone proved to be the most suitable source; cell density and γ-PGA productivity (19.5 g/L) were the highest when it was added compared with no more than 15 g/L γ-PGA produced when other sources of nitrogen were used. For this reason, tryptone was the only nitrogen source added to the media used in this study.

Culture Medium Optimization Using the RSM Strategy

Fractional Factorial Design

FFD is widely used to optimize culture media for bacterial growth [21]. In earlier studies, sucrose (X_1) and tryptone (X_2) were selected to serve as the main carbon and nitrogen source, respectively. Other ingredients included L-glutamic acid (X_3) as the precursor for γ-PGA synthesis and inorganic salts (NaCl [X_4], $MgSO_4 \cdot 7H_2O$ [X_5], and $CaCl_2$ [X_6]). A 2^{6-2} fractional factorial design was adopted in this study; corresponding experimental and regression results are shown in Tables 2 and 3, respectively. The variance value of $P<0.05$ was used as a cutoff for significance. The factorial analysis of variance indicated that when γ-PGA production by *B. subtilis* ZJU-7 is taken into consideration, the concentrations of sucrose (X_1), tryptone (X_2), and L-glutamic acid (X_3) were considered significant and the concentrations of NaCl, $MgSO_4 \cdot 7H_2O$, and $CaCl_2$ were considered nonsignificant. Interactions among these variables were not significant. A linear regression equation could be obtained by applying the following fractional factorial equation:

$$Y = 31.01 + 3.207X_1 + 1.916X_2 - 2.452X_3 \quad (4)$$

The regression coefficients and determination coefficient (R^2) for the linear regression model of γ-PGA production are presented in Table 3. The model was highly significant ($P<0.05$) and $R^2 = 0.651$.

Steepest Ascent Path

The direction of the steepest ascent path can be deter-

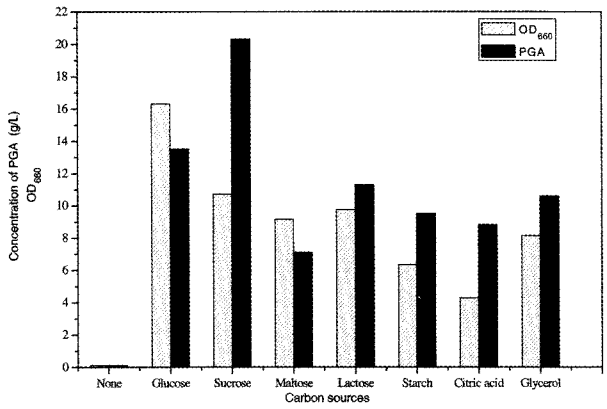


Fig. 1. The effects of various carbon sources on γ-PGA production by *B. subtilis* ZJU-7.

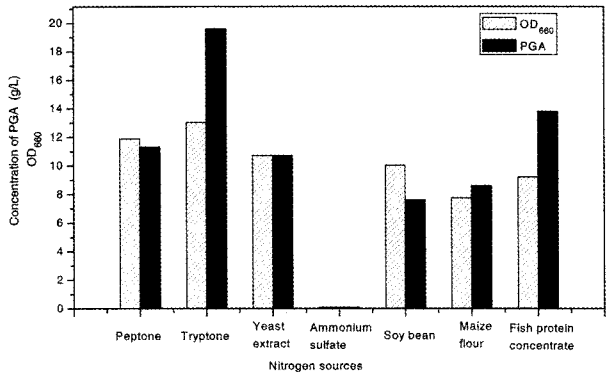


Fig. 2. The effects of various nitrogen sources on γ-PGA PGA production in *B. subtilis* ZJU-7.

Table 2. Experimental design and results of FFD

OBS	X_1	X_2	X_3	X_4	X_5	X_6	γ-PGA (g/L)
1	−1	+1	+1	+1	+1	−1	27.07
2	+1	+1	+1	+1	+1	+1	35.88
3	−1	+1	−1	+1	−1	+1	34.61
4	−1	−1	+1	+1	−1	−1	28.43
5	−1	−1	−1	+1	+1	+1	25.86
6	+1	−1	−1	−1	−1	+1	39.29
7	−1	+1	+1	−1	−1	+1	27.18
8	−1	+1	−1	−1	+1	−1	36.72
9	−1	−1	−1	−1	−1	−1	26.87
10	+1	+1	−1	−1	+1	+1	37.91
11	+1	−1	+1	−1	+1	−1	38.54
12	+1	−1	+1	+1	−1	+1	23.33
13	−1	−1	+1	−1	+1	+1	22.06
14	+1	−1	−1	+1	+1	−1	34.75
15	+1	+1	−1	+1	−1	−1	38.06
16	+1	+1	+1	−1	−1	−1	32.35
17	0	0	0	0	0	0	28.87
18	0	0	0	0	0	0	27.68
19	0	0	0	0	0	0	26.09
20	0	0	0	0	0	0	28.67

mined using the regression results obtained with Eq. (3). Because the concentrations of NaCl, $MgSO_4$, and $CaCl_2$ were insignificant, they were kept low. Sucrose (X_1), tryptone (X_2), and L-glutamic acid (X_3) were significant factors, the coefficients of X_1 and X_2 were positive, and the coefficient of X_3 was negative. This means that increasing the concentrations of X_1 and X_2 while decreasing the concentration of X_3 may have a positive effect on γ-PGA productivity. This concentration of sucrose was chosen as a standard because its coefficient is the highest. One basal increment is defined as a 0.15% (w/v) decrease in the L-glutamic acid concentration. The design and results of this experiment are shown in Table 4. The highest γ-PGA productivity in OBS 8 was 53.16 g/L, and γ-PGA production decreased after the L-glutamic acid concentration was increased. These results indicate that the concentrations of sucrose, tryptone, and L-glutamtic acid in OBS 8 were nearly optimal; therefore, OBS 8 was chosen as the center point to optimize the medium composition. The negative effect of increasing the L-glutamic acid concentration on γ-PGA productivity is probably caused by the inhibitory effect of L-glutamic acid on the enzyme system required for γ-PGA synthesis; some new fermentation modes, such as the fed-batch strategy, may be applied to eliminate this inhibitory effect.

Central Composite Design

The concentrations of sucrose (X_1 = 56.0 g/L), tryptone (X_2 = 49.6 g/L), and L-glutamic acid (X_3 = 78 g/L) in OBS 8 were chosen to optimize the composition of the media used for the CCD experiment. The specific experimental design and results are shown in Table 5. An equation for γ-PGA production was developed based on a regression analysis of the experimental data:

$$Y = 52.416 + 8.875X_1 + 9.130X_2 + 3.002X_3 - 7.009X_1^2 - 5.797X_2^2 - 4.026X_3^2 \quad (5)$$

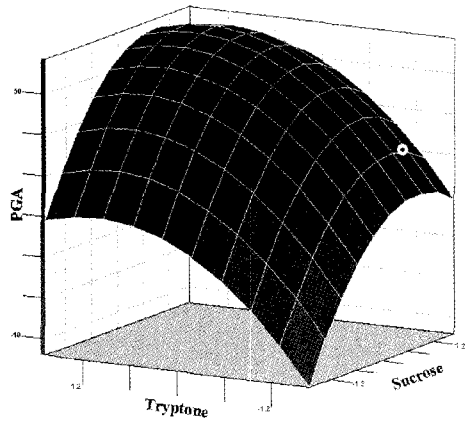
Using this equation, we obtained results that were highly significant ($P<0.01$), and the value of the determination coefficient (R^2 = 0.901) was satisfactory. The effects of sucrose, tryptone, and L-glutamic acid on γ-PGA production are shown in Figs. 3~5. Maximum γ-PGA productivity was obtained when the initial concentrations of sucrose, tryptone, and L-glutamic acid were 59.80, 53.54, and 81.05 g/L, respectively. A maximum PGA productivity of 60.95 g/L was predicted using this RSM. The mean PGA productivity for 3 tests (58.31 ± 1.27) for experiments using the predicted optimal medium coincided with the predicted value. Compared with the result after the 2^{6-2} FFD was applied, improvement in γ-PGA productivity of approximately 10% was attained by adopting the CCD design. The corresponding optimal medium for efficient γ-PGA production was finalized using the following concentrations: sucrose, 59.80 g/L;

Table 3. Regression results of FFD

Parameter	Unstandardized coefficients		Standardized coefficients	t	*Sig*	95% Confidence interval for B	
	B	Std error	Beta			Lower bound	Upper bound
(Constant)	31.011	0.921		33.665	0.001	29.021	33.001
Sucrose	3.207	1.030	0.536	3.114	0.008	0.982	5.432
Tryptone	1.916	1.030	0.320	1.860	0.086	−0.309	4.141
L-glutamic acid	−2.452	1.030	−0.410	−2.381	0.033	−4.677	−0.227
NaCl	−0.808	1.030	−0.135	−0.785	0.447	−3.033	1.417
$MgSO_4$	0.542	1.030	0.091	0.526	0.608	−1.683	2.767
$CaCl_2$	−1.042	1.030	−0.174	−1.012	0.330	−3.267	1.183

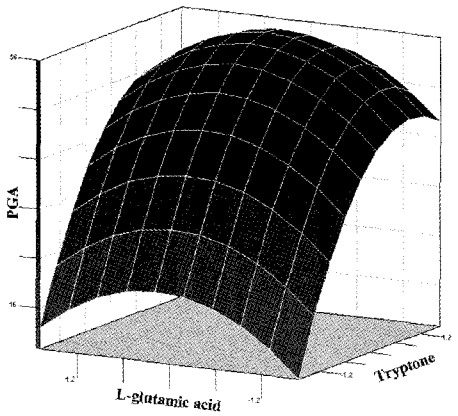
Table 4. Results of the steepest ascent path experiments

OBS	X_1 (%, w/v)	X_2 (%, w/v)	X_3 (%, w/v)	γ-PGA (g/L)
Origin	4.00	4.00	9.0	26.07
1	4.20	4.12	8.85	27.34
2	4.40	4.24	8.70	30.28
3	4.60	4.36	8.55	32.54
4	4.80	4.48	8.40	35.71
5	5.00	4.60	8.25	38.09
6	5.20	4.72	8.10	43.61
7	5.40	4.84	7.95	48.73
8	5.60	4.96	7.80	53.16
9	5.80	5.08	7.65	50.43
10	6.00	5.20	7.50	47.69
11	6.20	5.32	7.35	41.89



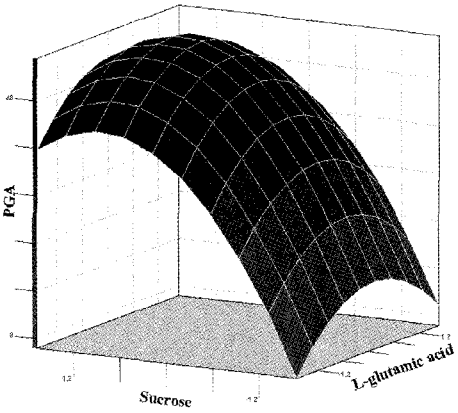
Fixed levels: L-glutamic acid = 1.5E-6

Fig. 3. Response surface plot for the effect of sucrose (X_1) and tryptone (X_2) on γ-PGA production.



Fixed levels: Sucrose = 1.5E-6

Fig. 4. Response surface plot for the effect of tryptone (X_2) and L-glutamic acid (X_3) on γ-PGA production.



Fixed levels: Tryptone = 1.5E-6

Fig. 5. Response surface plot for the effect of sucrose (X_1) and L-glutamic acid (X_3) on the γ-PGA production.

tryptone, 53.54 g/L; L-glutamic acid, 81.05 g/L; NaCl, 10 g/L; $MgSO_4 \cdot 7H_2O$, 1.0 g/L; and $CaCl_2$, 1.0 g/L.

These experiments show that the amount of sucrose and nitrogen in the culture medium are important for achieving high γ-PGA productivity in *B. subtilis* ZJU-7. γ-PGA synthesis in *B. subtilis* is an ATP-consuming bioprocess [22]. It was also found that the rate of conversion from L-glutamic acid to γ-PGA improved from 48.3 to 68.2% after medium optimization. This effect is very important for reducing the cost of large-scale fermentation-based production of γ-PGA.

γ-PGA has recently been produced on a large scale using several bacterial strains. Ogawa *et al.* [23] reported a maximum productivity of 35 g/L using a *B. licheniformis* ATCC9945a strain cultivated at 40°C for 35 h. The highest γ-PGA productivity reported by Kubota [24] was 50 g/L using the *B. subtilis* F201 strain cultivated at 37°C for 6 days. Compared with these strains, *B. subtilis* ZJU-7 has some advantages, such as high productivity (2.43 g L^{-1} h^{-1}) under optimized culture conditions, which is more than twice the highest rate (1.0 g L^{-1} h^{-1}) reported elsewhere. Volumetric productivity of γ-PGA was also the highest reported in the literature. The findings of this study indicate that media optimization and the use of *B. subtilis* ZJU-7 organisms for bioprocessing have great potential for use in the commercial production of γ-PGA.

CONCLUSION

The findings of this study suggest that the best carbon and nitrogen sources for γ-PGA production in *B. subtilis* ZJU-7 are sucrose and tryptone, respectively. The most important media components appear to be glucose, tryptone, and L-glutamic acid, with an optimal composition (based on the RSM strategy) of sucrose 59.80 g/L, tryptone 53.54 g/L, L-glutamic acid 81.05 g/L, NaCl 10 g/L, $MgSO_4 \cdot 7H_2O$ 1.0 g/L, and $CaCl_2$ 1.0 g/L. The maximum γ-PGA productivity observed in this study was 58.2

Table 5. The design and results of CCD

OBS	X_1	X_2	X_3	Y (g/L)
1	−1	−1	−1	21.65
2	−1	−1	+1	24.39
3	−1	+1	−1	32.84
4	−1	+1	+1	29.68
5	+1	−1	−1	33.42
6	+1	−1	+1	31.35
7	+1	+1	−1	57.49
8	+1	+1	+1	51.46
9	−1.681	0	0	16.38
10	+1.681	0	0	49.69
11	0	−1.681	0	17.43
12	0	+1.681	0	55.49
13	0	0	−1.681	26.74
14	0	0	+1.681	56.19
15	0	0	0	51.26
16	0	0	0	53.04
17	0	0	0	50.24
18	0	0	0	52.98
19	0	0	0	54.17
20	0	0	0	52.66

g/L, which was achieved after 24-h cultivation at 37°C; this represents an approximately 2-fold improvement over the results seen with a nonoptimized medium. Thus, RSM may also be efficient for optimizing bioprocessing conditions and as a basis for developing low-cost, large-scale methods of producing this important poly-amino acid biomaterial in the future.

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