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Optimization of Breeding and Cultivation Conditions for Mutant Strain with High Productivity of γ-Polyglutamic Acid

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***Bacillus* sp. LY33 was used as the original strain to breed a genetically stable mutant strain with high productivity of γ-polyglutamic acid (γ-PGA) through complex mutagenesis using diethyl sulfate (DES), nitrosoguanidine (NTG) and ultraviolet (UV) irradiation. Through fermentation by screened mutant strain, the yield of γ-PGA was up to 18.65 g/L, which exhibited an enhancement by 45.93% compared with the fermentation by original strain. The mutant strain was identified as *Bacillus subtilis* through colony and cellular morphology, and physiological and biochemical properties. The fermentation conditions in shaking flasks of the mutant strain were optimized by response surface analysis to be seed age of 10 h, inoculum size of 3%, fermentation pH of 7.0, fermentation temperature of 37°C, medium-loading volume of 50 mL in a 250 mL flask, and shaking speed of 200 r/min. The optimal medium for fermentation required 70.00 g/L glucose, 8.00 g/L yeast extract and 41.80 g/L sodium glutamate. The validation tests revealed the γ-PGA yield of 32.68 g/L under the optimal conditions, with an enhancement of 75.23% when compared with the fermentation using original strain before optimization.**

Key words: γ-Polyglutamic acid, Complex mutagenesis, Fermentation, Response surface methodology, Optimization.

γ-Polyglutamic acid (γ-PGA), as an extracellular multi-amino acid polymer synthesized by the condensation reaction between α-amino group and γ-carboxyl group in glutamic acids, is a kind of biocompatible natural polymer material without toxicity to human and environments[1,2]. Due to its thickening, moisturizing, splicing, gelation, emulsion and film-forming functions, γ-PGA has tremendous development and application prospects in the fields of medicine, food, agriculture, cosmetics, daily products and environmental protection products[3-5].

The complex chemical structure of γ-PGA results in the difficulty in its synthesis so that it is mainly produced by microbiological fermentation [6-9]. So far, microbiological fermentation methods for the synthesis of γ-PGA have made great progress, and many technological processes have been patented[10-13]. The production and sale volume of γ-PGA reveal gradual increase, but the high price of raw material up to 3000 yuan/kg in China is still the limitation of γ-PGA production due to the non-optimized fermentation conditions[14-16]. Therefore, in the present study, *Bacillus* sp. LY33 was used as the original strain to breed a genetically stable mutant strain for the high productivity of γ-PGA through shaking flask fermentation. The optimization of fermentation conditions will provide a theoretical reference for the large-scale production of γ-PGA.

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MATERIAL AND METHODS

Materials

Strains: Bacillus sp. LY33 was obtained and preserved in the culture collection, Department of Life Sciences, Longyan University, China, on slants of LB media at 4°C.

Media

Solid separation and preservation medium (LB) was composed of (in grams per liter): peptone 10, yeast extract 5, NaCl 10, agar 20, pH 7.0. Seed cultivation medium was composed of (in grams per liter): glucose 20, peptone 5, sodium glutamate 10, K_2HPO_4 2, $MgSO_4$ 0.25, pH 7.0. Fermentation basic medium was composed of (in grams per liter): glucose 30, peptone 8, sodium glutamate 30, K_2HPO_4 2, $MgSO_4$ 0.25, pH 7.5.

Reagents

L-glutamic acid (99% or more) was purchased from Sigma Chemical Co. γ-PGA was purchased from ShiNiMei Trading Company of Shanghai, China. All other chemicals used in this study were of analytical reagent grade.

Methods

Breeding of the mutant strain with high productivity of γ-PGA

Preparation of mutant bacterial suspension

The sticky colonies with large size and smooth surface of the preserved strain were selected[17-18] and cultured in an incubator with shaking speed of 200 r/min (ZHWY-2102 double-layer temperature oscillator, Zhicheng Analytical Instrument Manufacturing Co. Ltd., Shanghai, China) at 37°C until a logarithmic phase. Then, the broth was centrifuged at 10 000 r/min for 10 mins and the cells were harvested to prepare bacterial suspension with a density of 10^7 cells/mL.

Screening of mutagenesis conditions

The prepared bacterial suspension was subjected to the mutagenesis by using diethyl sulfate (DES), nitrosoguanidine (NTG) and ultraviolet (UV) irradiation, as shown in Table 1. The optimal doses of DES and NTG as well as their optimal treatment durations were explored. The mutagenesis using UV irradiation was completed at a 20 W UV lamp with an irradiation distance of 30 cm. The processed samples were diluted serially and the lethal rate of bacteria was examined by plate colony counting method. The lethal rate of selected bacteria in the range of 60-95% was defined as the positive mutation rate when compared with the original strain.

Complex mutagenesis

The prepared bacterial suspension was subjected to sequential treatments of DES, NTG and UV irradiation according to previously determined optimal conditions. The treated broth was mixed and chilled for 2 h. The sticky colonies were selected and inoculated in fermentation flasks at 37°C for shaking cultivation for 48 h. The cultivation with the highest γ-PGA production was selected as the starter strain of next step mutagenesis until the strain with high productivity of γ-PGA was screened. The strain with high productivity of γ-PGA was preserved in LB medium for future use.

Morphological, physiological and biochemical identifications of mutant strains

The morphological, physiological and biochemical identifications of mutant strains with high productivity of γ-PGA were completed for determining their basic properties.

Optimization of cultivation conditions for γ-PGA

Determination of inoculation age of the strain

The screened mutant strain with high productivity of γ-PGA was cultured at a 250 mL flask with medium-loading amount of 30 mL for 14 h. The initial fermentation pH, fermentation temperature and shaking speed were 6.5, 37 °C and 200 r/min, respectively. The OD_{660} as the evaluation indicator of bacterial growth, pH and residual glucose amount were determined at every 2 h[19-21].

Optimization of inoculum size, pH, temperature, medium-loading amount and shaking speed

The strain with appropriate inoculation age was used for the fermentation according to the designed inoculum size, pH, temperature, medium-loading amount and shaking speed to explore the effects of these factors on the production of γ-PGA, thus determining the optimal cultivation conditions of γ-PGA.

Optimization of fermentation medium for γ-PGA.

Single factor tests

On the basis of fermentation medium, the types of carbon and nitrogen sources were explored by single factor tests. Similarly, on the basis of appropriate carbon source and nitrogen source, the optimal concentrations for carbon source, nitrogen source and sodium glutamate

were also investigated by single factor tests to explore the effects of these factors on the production of γ-PGA, as shown in Table 2.

Principal component optimization by response surface methodology

On the basis of single factor test results, according to Box-Benhnken central composite experimental design principles, response surface tests with three factors and three levels were designed using Design-Expert software to explore optimal concentrations of principal components such as carbon source, nitrogen source and sodium glutamate in fermentation medium[22-24].

Determination of γ-PGA

The fermentation broth was centrifuged to remove bacteria and the supernatant was collected to determine the content of glutamic acid monomer. In addition, the supernatant was added to the tube containing 12 M hydrochloric acid at a volume ratio of 1:1 and subjected to hydrolysis at 110°C for 12 h after vacuum sealing. The total content of glutamic acid in the hydrolysates were determined by paper chromatography. The difference of both determinations was the content of γ-PGA[16,25].

RESULTS AND DICUSSION

Screening of mutant strains with high productivity of γ-PGA

Optimal induction doses and durations of DES, NTG and UV

The relationship between lethal rate and positive mutation rate after the treatments with DES, NTG and UV irradiation at various doses and treatment durations was shown in Table 3. The results showed that higher concentration and longer treatment duration could result in the increased lethal rate of bacteria. Through observing the positive mutation rate in the lethal rate of 60-95%, the optimal concentration and treatment duration of DES were 0.9% (Treatment 5) and 45 min (Treatment 4); the optimal concentration and treatment duration of NTG were 0.4% (Treatment 2) and 20 min (Treatment 3); and the optimal treatment duration of UV irradiation was 120 seconds (Treatment 5).

Complex mutagenesis

The prepared bacterial suspension was subjected to sequential treatments of DES, NTG and UV irradiation at the DES dose of 0.95 for 45

Table 1. Design of mutagenesis by single factor tests

Treatment	1	2	3	4	5	6	7
DES dose (%)	0.1	0.3	0.5	0.7	0.9	1.2	
DES treatment duration(min)	0	15	30	45	60	75	
NTG dose (%)	0.2	0.4	0.6	0.8	1.0		
NTG treatment duration (min)	0	10	20	30	40	50	
UV treatment duration (s)	0	30	60	90	150	180	210

Table 2. Design of single factor tests for optimizing principal compositions of fermentation medium

Treatment	1	2	3	4	5	6	7
Carbon source types	glucose	sucrose	starch	citric acid	glycerin		
Concentration of optimized for carbon source types (g/L)	20	30	40	50	60	70	80
Nitrogen source types	peptone	yeast extract	beef extract	corn steep liquor	ammonia sulfate		
Concentration of optimized for nitrogen source types	2	4	6	8	10	12	
Concentration of sodium glutamate (g/L)	0	10	20	30	40	50	

min, NTG dose of 0.4% for 20 min and UV irradiation for 120 s at the irradiation distance of 30 cm. After treatments, 20 colonies were selected for the fermentation in shaking flasks. The yield of γ-PGA in the fermentation broth was determined and compared with that in the fermentation broth using the original strain. As shown in Table 4, the selected 9 strains revealed higher productivity of γ-PGA than original strain. Moreover, No. 16 strain revealed the yield of γ-PGA up to 18.65 g/L with an enhancement of 45.93% when compared with the original strain. Therefore, the complex mutagenesis exhibited more obvious mutation efficiency.

No. 16 strain was subjected to the cultivation for 10 passages in LB slant medium. The shaking flask fermentation was conducted by the strain from each generation and corresponding yield of γ-PGA was determined to explore the genetic stability. As shown in Figure 1, after the passage for 10 generations, the yield of γ-PGA during the fermentation only revealed a slight decrease, suggesting that the mutant strain had better genetic stability. Therefore, No. 16 strain can be used as an excellent strain for the production of γ-PGA through fermentation method. After the passage for 10 generations, the yield of γ-PGA during the fermentation only revealed a slight decrease.

Morphological, physiological and biochemical identifications of mutant strains

Colony morphology, Gram staining, spore staining, and physiological and biochemical properties of selected No. 16 strain were examined. According to microbial taxonomy and Bergey bacterial identification Manual 9th edition, and

Table 3. Lethal rates of bacteria subjected to the treatments of DES, NTG and UV with various doses and treatment durations

Treatment		1	2	3	4	5	6	7
DES dose (%)	Lethal rate (%)	38.5	42.6	60.8	78.9	90.6	100	
	Mutation rate (%)	-	-	2.1	11.9	14.9	-	
DES treatment	Lethal rate (%)	0	60.1	80.1	90.2	100	100	
duration (min)	Mutation rate (%)	-	1.2	14.6	15.2	-	-	
NTG dose (%)	Lethal rate (%)	76.8	89.3	93.4	96.5	99.6		
Mutation rate (%)	6.4	15.3	8.2	-	-			
NTG reatment	Lethal rate (%)	0	75	85.4	90.3	95.0	99.1	
duration (min)	Mutation rate (%)	-	15.2	16.0	13.4	12.1	-	
UV treatment	Lethal rate (%)	0	50.1	73.2	88.5	92.6	94.8	96.8
duration (s)	Mutation rate (%)	-	-	0.8	4.5	7.3	3.7	-

Table 4. The yield of γ-PGA in fermentation by the mutant strain from complex mutagenesis

Strain	Yield of γ-PGA g/L	Strain	Yield of γ-PGA g/L
Original strain	12.78±0.43		
1	14.23±0.62	11	2.94±0.18
2	14.64±0.45	12	0.61±0.02
3	16.52±0.66	13	10.67±0.22
4	1.66±0.05	14	12.75±0.40
5	10.64±0.21	15	16.41±0.45
6	16.64±0.68	16	18.65±0.63
7	4.36±0.08	17	12.54±0.27
8	12.45±0.21	18	18.12±0.60
9	8.85±0.22	19	12.54±0.25
10	14.96±0.42	20	14.58±0.28

Table 5. Effect of inoculation amount on the yield of γ-PGA

Inoculation amount %	Yield of γ-PGA g/L	Inoculation amount %	Yield of γ-PGAg/L
1	18.65±0.21	4	19.45±0.25
2	18.92±0.22	5	19.32±0.22
3	19.70±0.30	6	19.26±0.14

Table 6. Effect of pH on the yield of γ-PGA

pH	Yield of γ-PGA g/L	pH	Yield of γ-PGA g/L
6.0	6.75±0.16	7.5	16.65±0.25
6.5	16.02±0.21	8.0	8.22±0.08
7.0	20.81±0.40	8.5	0.42±0.02

combined with strain colonies and morphological observation, No. 16 strain was identified as *Bacillus subtilis*[26-27].

Optimization of fermentation conditions for the production of γ-PGA

Inoculation age of strains

The growth curve of No. 16 strain was shown in Figure 2. Results indicated that bacteria entered a logarithmic growth phase after 6 h cultivation and a stable growth phase after 10 h cultivation. Meanwhile, a sharp decrease in residual sugar, bacterial autolysis and pH rebound were observed[28]. Therefore, in order to maintain its strong growth capability, the inoculation age of the strain was selected as 10 h.

On the 10th h, the bacteria reached a stable growth phase. Meanwhile, residual sugar decreased sharply, and bacterial autolysis and pH rebound were observed. Therefore, 10 h was selected as the best inoculation age of the strain.

Inoculum size, pH, temperature, medium-loading amount and shaking speed

The effects of inoculum size, pH, temperature, medium-loading amount and shaking speed on the production of γ-PGA were shown in Table 5 to 9. The results indicated that the optimal fermentation conditions were inoculation amount of 3%, fermentation pH of 7.0, fermentation temperature of 37°C, medium-loading amount of 50 mL in a 250 mL flask, and shaking speed of 200 r/min.

Optimization of principal components in fermentation medium for the production of γ-PGA

Optimal types and concentrations of carbon and nitrogen sources

The effects of different carbon and nitrogen source types and concentrations on the production of γ-PGA were shown in Table 10. Results indicated that the optimal carbon source was glucose and the highest yield of γ-PGA was observed at the glucose concentrations of 50, 60 and 70 g/L; similarly, the optimal nitrogen source was yeast extract with the highest yield of γ-PGA at the yeast extract concentration of 8, 10 and 12 g/L. Therefore, these six variables were selected as the factors and levels of the response surface design.

Sodium glutamate concentration

The effect of sodium glutamate concentration on the yield of γ-PGA was shown in Figure 3. The results indicated that the selected strain was a glutamate-dependent strain. In the absence of sodium glutamate, γ-PGA could not be synthesized and the yield of γ-PGA was increased as the concentration increase of sodium glutamate. In contrast, the conversion rate of glutamic acid revealed a decreasing trend when sodium glutamate reached up to a certain level, thus resulting in the reduction of γ-PGA yield[29]. The highest yield of γ-PGA was observed at sodium glutamate concentrations of 30, 40 and 50 g/L. Therefore, these three variables were selected as the factors and levels of response surface tests.

Optimization of principal components in fermentation medium

Taking γ-PGA yield as the evaluation index, the Box-Benhnken design and response

Table 7. Effect of fermentation temperature on the yield of γ-PGA

Fermentation temperature	Yield of γ-PGA g/L	Fermentation temperature	Yield of γ-PGA g/L
28	12.75±0.29	37	21.05±0.40
32	16.02±0.41	40	16.22±0.42

Table 8. Effect of medium-loading amount on the yield of γ-PGA

medium-loading amount mL/250mL	Yield of γ-PGA g/L	medium-loading amount mL/250mL	Yield of γ-PGA g/L
10	4.75±0.21	40	18.65±0.41
20	6.02±0.22	50	22.82±0.50
30	12.21±0.30	60	16.22±0.41

Table 9. Effect of shaking speed on the yield of γ-PGA

Shaking speed r/min	Yield of γ-PGA (g/L)	Shaking speed r/min	Yield of γ-PGA (g/L)
100	12.75±0.18	200	23.25±0.28
150	16.02±0.21	250	18.22±0.22

Table 10. Effects of carbon and nitrogen source types and concentrations on the yield of γ-PGA

Treatment	1	2	3	4	5	6	7
Carbon source types	glucose	sucrose	starch	citric acid	glycerin		
Yield of γ-PGA(g/L()	23.2±0.15	19.25±0.12	8.59±0.05	15.68±0.11	14.26±0.10		
Glucose concentrations(g/L)	20	30	40	50	60	70	80
Yield of γ-PGA(g/L)	12.56±0.10	15.26±0.12	17.25±0.14	19.12±0.13	23.89±0.17	20.32±0.15	18.54±0.18
Nitrogen source types	peptone	yeast extract	beef extract	corn steep liquor	ammonia sulfate		
Yield of γ-PGA(g/L)ÿ	15.23±0.12	24.56±0.18	16.35±0.12	7.56±0.04	7.05±0.05		
Yeast extract concentrations(g/L)	2	4	6	8	10	12	
Yield of γ-PGA(g/L)	7.05±0.05	11.23±0.10	13.65±0.11	25.69±0.19	22.36±0.18		

surface design with three factors and three levels as well as corresponding results were shown in Table 11. The regression analysis of experimental data was completed by Design-Expert7.0 software. The binary regression equation between γ-PGA yield and the variables was achieved to be

$$Y=-162.86750+2.11003A+2.36375B+5.56197C-0.077625AB+0.014750AC+5.87\times10^{-3}BC-0.014920A^2+0.10512B^2-0.079420C^2$$

Through analyzing the regression model equation, the results were shown in Table 12. The results showed that this model had a statistical significance ($P = 0.0004 < 0.05$) and no significance in non-fit variables ($P = 0.1988 > 0.05$), suggesting that the regression equation had a small error and could better describe the relationship between various factors and response values. Meanwhile, this equation can be used for the determination of optimal proportion of principal components in fermentation medium of γ-PGA. In addition, on the basis of variance analysis of each factor, the order for affecting the yield of γ-PGA from strong to weak was sodium glutamate, glucose and yeast extract. Among these factors, sodium glutamate and glucose revealed a significant impact on the yield of γ-PGA; in contrast, yeast extract did not reveal an obvious effect on the yield of γ-PGA.

Factor-response surface analysis results were shown in Figure 4. According to the prediction by resultant model, the optimal compositions of fermentation medium for the production of γ-PGA were 70.00 g/L glucose, 8.00 g/L yeast extract and 41.80 g/L sodium glutamate. Under the optimal fermentation conditions, the yield of γ-PGA reached up to 32.74 g/L. During 3 parallel experiments according to above optimal fermentation conditions, the average yield of γ-PGA was 32.68 g/L, which revealed an enhancement by 75.23% when compared with the yield of γ-PGA (18.65 g/L) before optimization. Meanwhile, this result was close to the theoretically predicted value of 32.74 g/L. Moreover, this method also revealed better reproducibility so that the parameters obtained from response surface methodology are accurate, reliable and feasible.

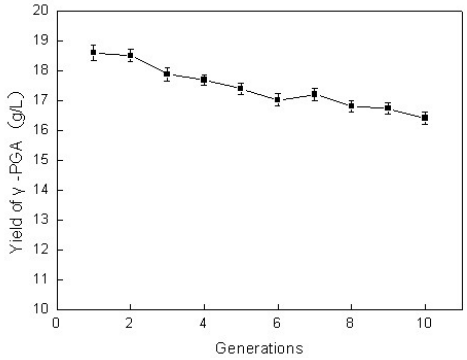
Response surface plot for the effect of cross-interaction between glucose (A) and yeast extract (B) on γ-PGA production. b. Response surface plot for the effect of cross-interaction

Table 11. Box-Benhnken design, arrangement and corresponding results

Test number	AGlucose(g/L)	BYeast extract(g/L)	CSodium glutamate(g/L)	Yield of γ-PGA (g/L)
1	1(70)	0(10)	-1(30)	19.54
2	0(60)	1	-1	20.54
3	0	0	0(40)	30.87
4	0	-1(8)	1(50)	25.69
5	-1(50)	0	-1	20.32
6	0	0	0	31.69
7	0	0	0	30.35
8	1	1(12)	0	30.81
9	-1	-1	0	25.45
10	0	0	0	29.12
11	1	0	1	25.27
12	0	0	0	31.74
13	-1	0	1	20.15
14	0	1	1	24.08
15	0	-1	-1	22.62
16	1	-1	0	31.72
17	-1	1	0	30.75

Table 12. Results of the variance analysis of regression model

Source	Sum of squares	Df	Mean square	F Value	P Value	Significance
Model	332.23	9	36.91	19.16	0.0004	* *significant*
A	14.23	1	14.23	7.39	0.0299	* *significant*
B	0.061	1	0.061	0.032	0.8635	
C	18.51	1	18.51	9.61	0.0173	* *significant*
AB	9.64	1	9.64	5.00	0.0604	
AC	8.7	1	8.7	4.52	0.0712	
BC	0.055	1	0.055	0.029	0.8704	
A^2	9.37	1	9.37	4.86	0.0632	
B^2	0.74	1	0.74	0.39	0.5539	
C^2	265.58	1	265.58	137.83	< 0.0001	* *significant*
Residual	13.49	9	1.93			
Lack of Fit	8.79	3	2.93	2.50	0.1988	*not significant*
Pure Error	4.69	4	1.17			
Cor Total	345.72	16				



After the passage for 10 generations, the yield of γ-PGA during the fermentation only revealed a slight decrease

Fig. 1. The yield of γ-PGA in fermentation by the strain with various generations

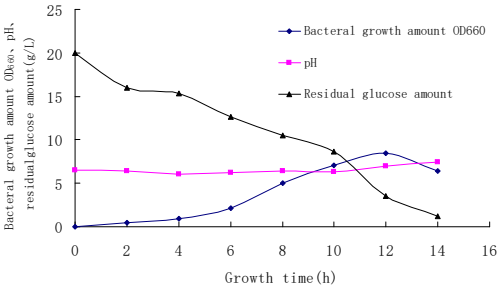


Fig. 2. Growth curve of No. 16 strain

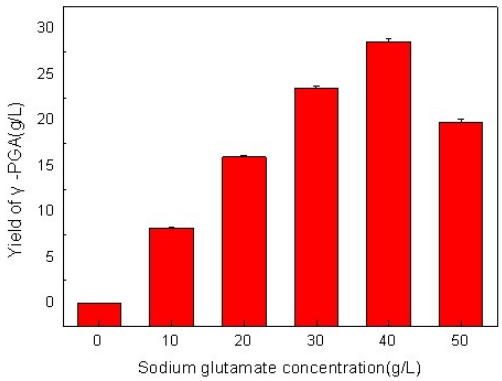
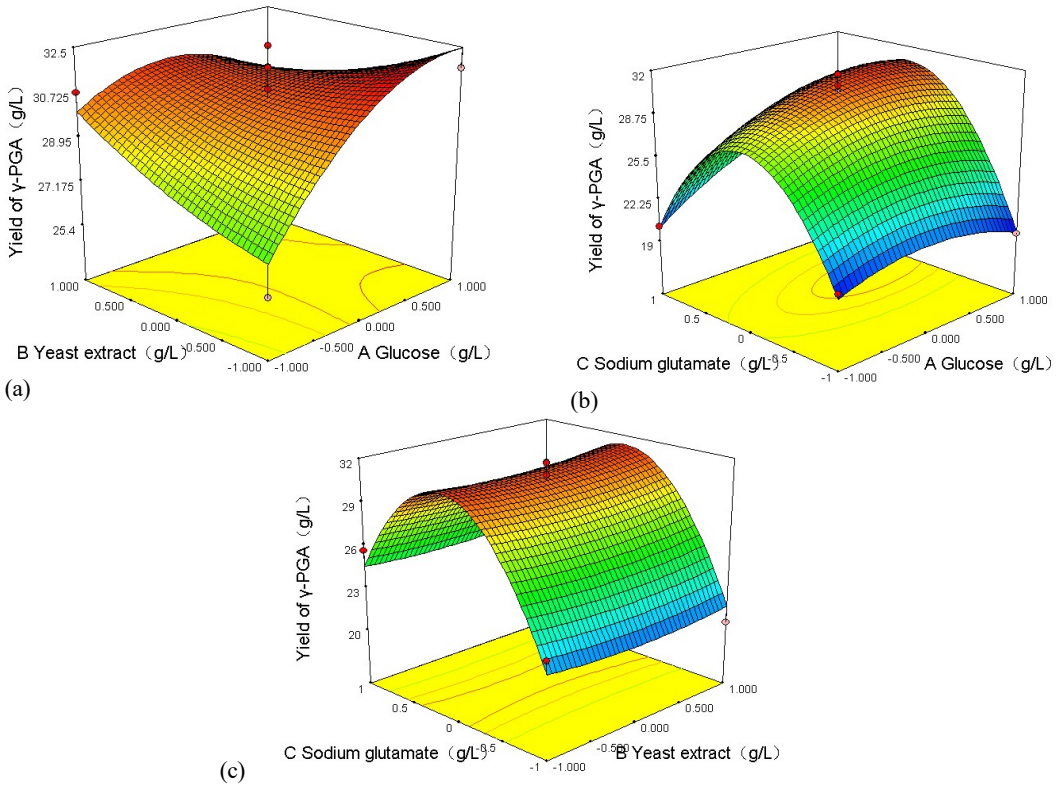


Fig. 3. Effect of sodium glutamate concentration on the yield of γ-PGA



(a) (b) (c)

Fig. 4. Response surface plots for the effects of cross-interactions among these factors on the yield of γ-PGA

between glucose (A) and sodium glutamate (C) on γ-PGA production. c. Response surface plot for the effect of cross-interaction between yeast extract (B) and sodium glutamate (C) on γ-PGA production.

CONCLUSION

Bacillus sp. LY33 as the original strain was used to breed a genetically stable strain with high productivity of γ-PGA through the

mutagenesis of DES, NTG and UV irradiation. The yield of γ-PGA through mutant strain fermentation was up to 18.65%, which revealed an enhancement of γ-PGA production by 45.93% when compared with the γ-PGA yield using original strain fermentation. The selected mutant strain was identified as *Bacillus subtilis* through colony morphological, physiological and biochemical identifications.

In addition, the fermentation conditions and fermentation medium of the mutant strain were also optimized. The optimal fermentation conditions required inoculation age of 10 h, inoculation amount of 3%, fermentation pH of 7.0, fermentation temperature of 37 °C, medium-loading volume of 50 mL in a 250 mL flask and shaking speed of 200 r/min. Similarly, the optimal compositions of fermentation medium included 70.00 g/L glucose, 8.00 g/L yeast extract and 41.80 g/L sodium glutamate. Under the optimal conditions, the yield of γ-PGA was up to 32.68%, which revealed an enhancement by 75.23% when compared with the yield of γ-PGA through the fermentation by original strain (18.65 g/L). Moreover, compared with the previous studies that the optimal γ-PGA yields were 23.32 g/L and 14.88 g/L obtained from Yanping Xu group and Pei Du group[14,16], our yield of γ-PGA was also significantly higher than previously reported results, suggesting that, in the present study, the breeding and fermentation conditions of the mutant strain for producing γ-PGA were optimized and excellent performance was achieved, which provided a theoretical reference for the large-scale production of γ-PGA in the future.

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REFERENCES

1. Ashiuchi, M., Misono, H. Biochemistry and molecular genetics of poly-gamma-glutamate synthesis. *Appl Microbiol Biotechnol*., 2002; **59**(1): 9-14.
2. Bhatt, R., de Vries, P., Tulinsky, J., Bellamy, G., Baker, B., Singer, J. W. ,Klein, P. Synthesis and in vivo antitumor activity of poly (l-glutamic acid) conjugates of 20S-camptothecin. *J Med Chem*.,2003; **46**(1): 190-3.
3. Mitsuiki, M., Mizuno, A., Tanimoto, H., Motoki, M. Relationship between the antifreeze activities and the chemical structures of oligo- and poly(glutamic acids)s. *J Agric Food Chem*., 1998; **46**(3): 891-5.
4. Shih, I. L., Van, Y. T. The production of poly-(γ-glutamic acid) from microorganisms and its various applications. *Bioresour Technol.,* 2001; **79**(3): 207-25.
5. Veronse, F. M., Ceriotli, G., Calicets, P. Slow release of narciclasine from matrixes obtained by radiation induced polymerization. *J Control Release.*, 1991; **16**(3): 291-3.
6. Bajaj, I.B., Lele, S.S., Singhal, R.S. A statistical approach to optimization of fermentative production of poly(gamma-glutamic acid) from Bacillus licheniformis *NCIM 2324. Bioresour Technol.*, 2009; **100**(2): 826-32.
7. Kunioka, M. Biosynthesis and chemical reactions of poly(amino acids) from microorganisms. *Appl Microbiol Biotechnol.*,1997; **47**(5): 469-75.
8. Shi, F., Xu, Z., Cen, P. Efficient production of poly-gamma-glutamic acid by Bacillus subtilis ZJU-7. *Appl Biochem Biotechnol.*, 2006; **133**(3): 271-82.
9. Shih, I.L., Van,Y.T., Shen, M.H. Biomedical applications of chemically and microbiologically synthesized poly(glutamic acid) and poly (lysine). *Mini Rev Med Chem.*, 2004; **4**(2): 179-88.
10. Kino, K., Ara,i T., Arimura, Y. Poly-á-glutamic acid synthesis using a novel catalytic activity of rimK from *Escherichia coli* K-12.*Appl Environ Microbiol.,*2011; **77**(6): 2019-25.
11. Konno, A., Taguchi, T., Yamaguchi, T. Bakery products and noodles containing poly(γ-glutamic acid). US/4888193 1989-12-19.
12. McLean, R.J.C., Beauchemin, D., Clapham, L., Beveridge, T.J. Metal binding characteristics of the gamma glutamyl capsule polymer of Bacillus licheniformis ATCC9945. *Appl Environ Microbiol.,*1990; **56**(12): 3671-77.
13. Sakai, K., Sonoda, C., Murase, K. Bitterness relieving agent. JP/021390 2000-04-20.
14. Du, P., Zhu, L.J., Chen, S.X. Fermentation medium optimization forγ-PGA production of *Bacillus subtilis* HD-F9 by response surface methodology. *Journal of Henan University (Natural Science) (Chinese Journal).,*2010; **40**(5): 498-503.
15. Wang,J., Shao, L.Q. Synthesis, Chemical Modification and Application of γ-Polyglutamic Acid. *Chemistry and Bioengineering (Chinese*

Journal), 2008; **25**(4): 17-20.

16. Xu, Y.P., Wang, S.Y., Li, H.Z., Chen, J. Screening of high productive mutant of γ-Polyglutamic acid and its fermentation conditions. *Journal of Wuxi University of Light Industry (Chinese Journal).,* 2004; **23**(5): 6-10.
17. Bajaj, I.B., Singhal, R.S. Enhanced production of poly (gamma-glutamic acid) from *Bacillus licheniformis* NCIM 2324 by using metabolic precursors. *Appl Biochem Biotechnol.,* 2009; **159**(1): 133-41.
18. Jeong, J.H., Kim, J.N., Wee, Y.J., Ryu, H.W. The statistically optimized production of poly(gamma-glutamic acid) by batch fermentation of a newly isolated *Bacillus subtilis* RKY3. *Bioresour Technol*., 2010; **101**(12): 4533-39.
19. Birrer, G.A., Cromwick, A.M., Gross, R.A. Gamma-poly(glutamic acid) formation by *Bacillus licheniformis* 9945a: physiological and biochemical studies. *Int J Biol Macromol.,* 1994; **16**(5): 265-75.
20. Kedia,G., Hill, D., Hill, R.,Radecka, I. Production of poly-gamma-glutamic acid by *Bacillus subtilis* and *Bacillus licheniformis* with different growth media. *J Nanosci Nanotechnol.,*2010; **10**(9): 5926-34.
21. Xing, Y., Zhang, L., Cong, W., Yue, L., Chen, C., Ma, J. Application of a pH feedback-controlled substrate feeding method in glutamic acid fermentation. *Sheng Wu Gong Cheng Xue Bao* .,2011; **27**(10): 1457-63.
22. Ko, Y. H.ÿGross, R. A. Effect of glucose and glycerol on poly(glutamic acid) formation by *Bacillus licheniformis* ATCC 9945a, *Biotechnol Bioeng*.,1998; **57**(4): 430-7.
23. Richard, A., Margaritis, A. Optimization of cell growth and poly(glutamic acid) production in batch fermentation by *Bacillus subtilis*. *Biotechnol Lett*.,2003; **25**(6): 465-8.
24. Xiong, C., Shouwen, C., Ming, S., Ziniu, Y. Medium optimization by response surface methodology for poly-gamma-glutamic acid production using dairy manure as the basis of a solid substrate. *Appl Microbiol Biotechnol*., 2005; **69**(4): 390-6.
25. Hui, M., Tian, Q. Batch fermentation analysis of poly(γ-glutamic acid) formation by bacillus *Bacillus subtilisa* B53. *Journal of Henan University of Technology (Natural Science Edition) (Chinese Journal) .,*2010; **28**(1): 13-16.
26. Ito, Y., Tanaka, T., Ohmachi, T., Asaday, Y. Glutamic acid independent production of Poly(γ-glutamic acid)by *Bacillus subtilis* TAM-4. *Biosci Biotechnol Biochem*., 1996; **60**(8): 1239-42.
27. Sanda, F., Endo, T. Chemical synthesis of poly gamma-glutamic acid by po1ycondensation of gamma-glutamic acid dimmer: Synthesis and reaction of poly-gamma-glutamic acid methyl ester. *J Polym Sci Pol Chem*.,2001; **39**(5): 732-41.
28. Kambourova, M., Tangney, M., Priest, F. G. Regulation of polyglutamic acid synthesis by glutamate in Bacillus licheniformis and *Bacillus subtilis*. *Appl Environ Microbiol.,* 2001; **67**(2): 1004-7.
29. Richard, A., Margaritis, A. Empirical modeling of batch fermentation kinetics for poly(glutamic acid) production and other microbial biopolymers. *Biotechnol Bioeng.,* 2004; **87**(4): 501-15.