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Optimization of Fermentation Conditions for γ-PGA Production by *Bacillus subtilis* QM3

Bingqi Dong, Qingping Hu*

School of Life Science, Shanxi Normal University, Taiyuan, China
Email: *hqp72@163.com

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Abstract

γ-Polyglutamic acid (PGA) is a high molecular polymer polymerized by glutamic acid (Glu). In the γ-PGA production pathway, the production of γ-PGA by microbial fermentation in the laboratory has become the main way of γ-PGA synthesis because of its convenient operation, low cost, and considerable effect. In order to find the high yield of γ-PGA strain and increase the yield of γ-PGA produced by microorganisms, *B. subtilis* QM3 was used as the experimental material to optimize the nutrition conditions and culture conditions of γ-PGA production by QM3 fermentation. The results showed that the optimum medium composition and fermentation conditions for the production of γ-PGA by *B. subtilis* QM3 fermentation were as follows: glucose 4%, yeast extract 1%, sodium glutamate 1%, $MgSO_4 \cdot 7H_2O$ 0.025%, K_2HPO_4 0.2% department NH_4Cl 0.3%, initial pH 6, 250 ml flask, liquid volume 30 ml, 121˚C sterilization 20 min, inoculum 2%, shaker speed 220 rpm 42˚C shaker culture for 72 hours, at this time, the yield of γ-PGA was the highest, reaching 124.58 g·L.

Subject Areas

Biochemistry

Keywords

B. subtilis QM3, γ-PGA, Condition Optimization

1. Introduction

γ-PGA is a kind of amino acid polymer synthesized by microorganisms, which is formed by the condensation of glutamic acid monomer with γ-carboxyl group and amino group [1]. Many advantages of γ-PGA make it applied in many fields:

in the field of environmental protection, γ-PGA can be used as a mixture of heavy metal ions and reflective substances; in the field of food, it can be used as a thickener and astringent agent for food; in the field of agriculture, the use of γ-PGA can significantly improve the emergence rate of seeds, and can also be used as a slow-release agent for pesticides and fertilizers. At present, γ-PGA is mainly produced by microbial fermentation [2]. The material metabolism of microorganisms will be affected by external culture conditions, and the appropriate external environment is conducive to the growth and reproduction of microorganisms themselves. In the process of producing γ-PGA by *B. subtilis* QM3, the composition of the culture medium and fermentation conditions affect cell growth, intracellular γ-PGA synthetase system and γ-PGA secretion [3]. In this chapter, a single-factor experiment was designed to optimize the medium composition and fermentation conditions, and then the fermentation conditions were optimized by orthogonal design to determine the best composition and fermentation conditions of laboratory shake flask culture medium, in order to improve the fermentation yield of γ-PGA. This not only further develops the fermentation background of *B. subtilis* QM3, but also provides good fermentation conditions and a theoretical basis for laboratory fermentation to produce γ-PGA, and provides excellent strains for industrial large-scale production of γ-PGA, so as to reduce the production cost of γ-PGA and increase the yield.

2. Experimental Procedure

All the reagents below are analytically pure.

B. subtilis QM3 preserved strain was selected and cultured in sterilized beef extract peptone Agar medium at 37˚C for 24 hours as an activated strain. The single colony of activated bacteria was inoculated into a seed medium at 37˚C, 220 r/min, and incubated in a shaker for 24 hours as seed liquid. The seed liquid was inoculated in the fermentation medium at 37˚C, 220 r/min and shaker culture for 48 h according to 4% inoculation amount, and the fermentation broth containing γ-polyglutamic acid was obtained [4]. 2 g of sodium hydroxide was prepared into 2% sodium hydroxide solution and 2% sodium hydroxide solution was used as solvent, 0.5 g CTAB was taken, and 2% sodium hydroxide solution was fixed to 100 ml to prepare CTAB solution of 5 g/L. Accurately weigh the γ-PGA standard, and prepare the γ-PGA standard solution of 8 g/L, 16 g/L, 24 g/L, 32 g/L, 40 g/L and 48 g/L, Take the above standard solution of 2 ml into the test tube, add the 2 ml CTAB solution, time from the time of adding, fully oscillate, avoid bubbles during the period, determine the absorbance value under the wavelength 250 nm during 3 min [5], and draw the standard curve of γ-PGA, as shown in **Figure 1**. The fermentation broth was 5000 r/min, centrifuged 30 min, the pH value of the supernatant was adjusted to 2, and then 4 times the volume of precooled ethanol was added. The mixture was placed overnight at 4˚C for 10 h [6]. The mixture 5000 r/min, centrifugal 10 min [7]. The supernatant was discarded and the retained product was precipitated until the residual ethanol was

completely volatilized. A proper amount of distilled water was added to dissolve the precipitation and fully oscillated. The content of γ-PGA in the fermentation broth was determined by CTAB-NAOH turbidimetry. Each group of data was repeated three times, and the results were expressed by average ± standard error.

The culture medium is commonly used to cultivate microorganisms in the laboratory, and the composition of the medium varies according to the types of bacteria, but most media contain carbon sources, nitrogen sources, trace metal elements, and some unique substances needed for bacterial growth. In the medium for fermentation culture of γ-PGA, the initial medium components are as follows: glucose 4%, yeast extract 0.8%, sodium glutamate 2%, $MgSO_4 \cdot 7H_2O$ 0.025%, K_2HPO_4 0.2%, NH4Cl 0.2%. Different bacterial culture conditions will also affect the strain growth and product accumulation. Under laboratory conditions, the main factors affecting the number of strains and product content are temperature, pH, inoculation amount, inoculation time and liquid volume. Therefore, in order to optimize the process of producing γ-PGA by *B. subtilis* fermentation QM3 and maximize its yield, 10 groups of single-factor experiments were carried out to investigate the effects of nutrients: carbon source, nitrogen source, sodium glutamate, dipotassium hydrogen phosphate, ammonium chloride and fermentation conditions: temperature, pH, liquid volume, inoculation time and inoculation amount on the yield of γ-PGA. When all the experiments were completed, three factors that were significantly related to the yield of γ-PGA in the fermentation conditions were selected and analyzed by orthogonal experiment, and the experimental conditions with the highest yield of γ-PGA were obtained.

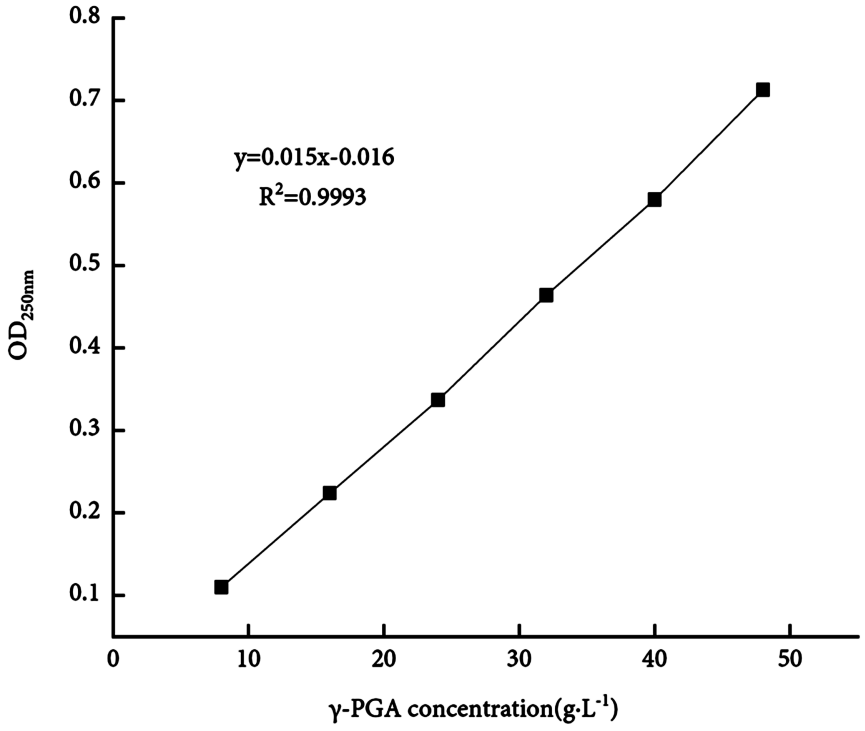


Figure 1. γ-PGA standard curve.

3. Results

3.1. Effect of Carbon Source Type and Concentration on γ-PGA Yield

The types of carbon sources in microbial culture medium have important effects on the growth of microorganisms and the accumulation of target products [8]. It is a carbon nutrition used by microorganisms to construct the carbon skeleton of bacteria and metabolites and to provide energy for life. Different microorganisms have different abilities to utilize carbon, and different types of carbon sources have different effects on bacterial growth and metabolic activity [9]. As shown in **Figure 2**, when glucose was used as a carbon source, the yield of γ-PGA was the highest, and when citric acid was used as a carbon source, the yield of γ-PGA was the lowest. As shown in **Figure 3**, after determining the optimal carbon source as glucose, the yield of γ-PGA increased at first and then decreased with the increase of glucose concentration in the fermentation broth. When the glucose concentration was 4%, the yield of γ-PGA was the highest. Therefore, 4% glucose is the optimal carbon source and concentration in the fermentation medium.

3.2. Effects of Types and Concentrations of Nitrogen Sources on the Yield of γ-PGA

Nitrogen sources also play an important role in the growth and metabolism of microorganisms, which can provide substances for the synthesis of cellular proteins, nucleic acids, etc [10]. Microbial growth and synthetic products are closely related to the type and concentration of nitrogen sources, which can greatly

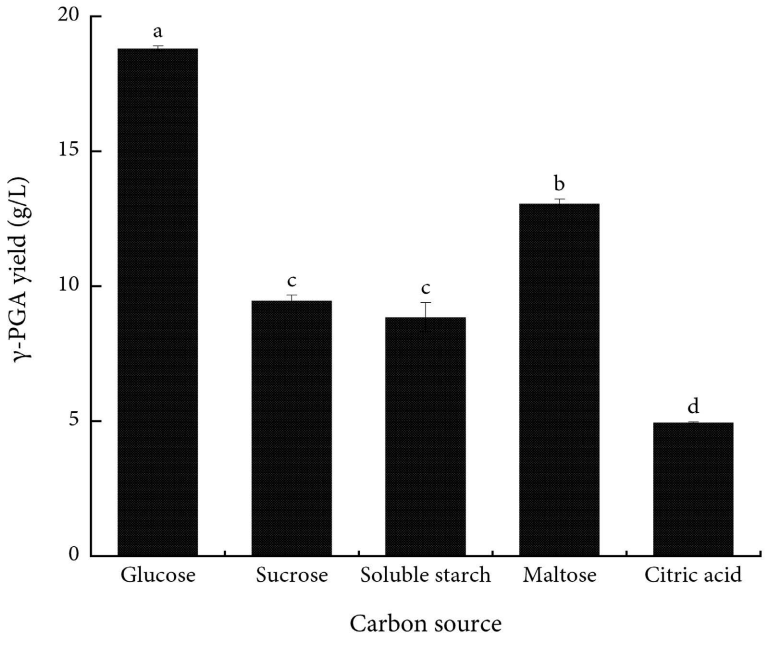
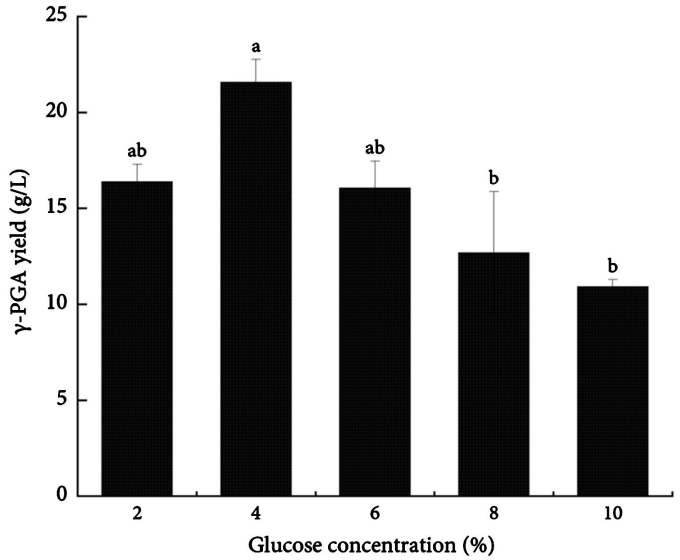


Figure 2. Effect of carbon sources on γ-PGA yield.

affect the production of secondary metabolites [11]. As shown in Figure 4 and Figure 5, the yield of γ-PGA was the highest when yeast extract was used as a nitrogen source, and the accumulation of polyglutamic acid was the lowest when peptone was used as a nitrogen source for strain QM3. After determining the optimal inorganic nitrogen source as yeast extract, the yield of γ-PGA first decreased and then increased with the increase of the concentration of yeast extract in the fermentation broth. When the concentration was 1%, the yield of polyglutamic acid was the highest. Therefore, 1% yeast extract is the optimal nitrogen source and concentration of the fermentation medium.



Significance Level: 0.05

Figure 3. Effect of glucose concentration on the yield of γ-PGA.

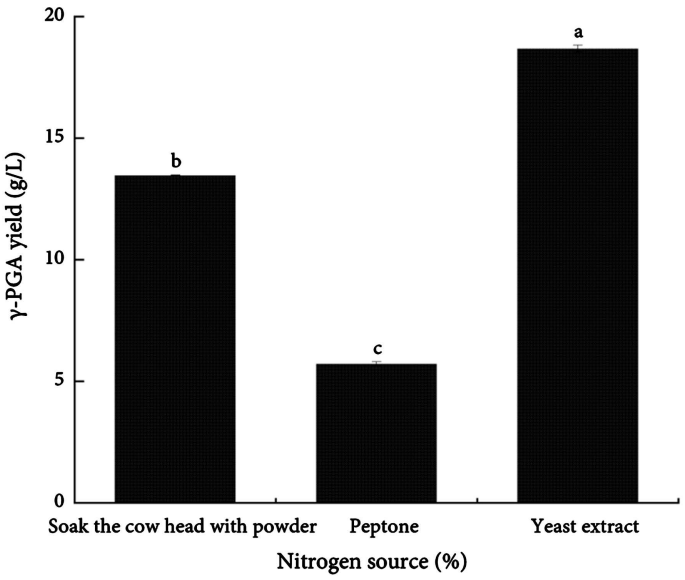


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Figure 4. Effect of nitrogen sources on γ-PGA yield.

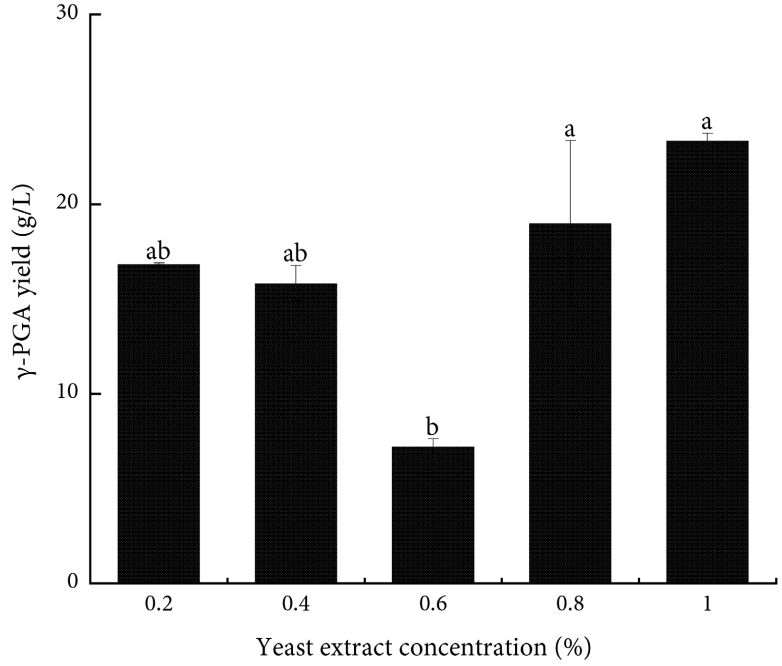

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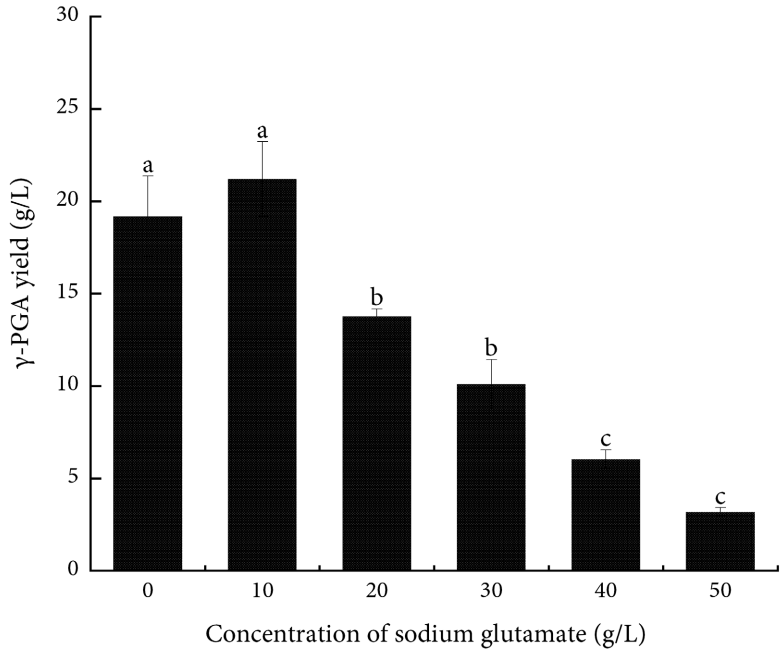
Figure 5. Effect of yeast extract concentration on the yield of γ-PGA.

3.3. Effect of Concentration of Sodium Glutamate on the Yield of γ-PGA

γ-PGA is synthesized by fermentation by microorganisms, and its research is mainly focused on *B. anthracis*, *B. licheniformis*, *B. subtilis* and other strains of Bacillus [12]. According to the nutritional requirements of cell growth, γ-PGA synthesizing bacteria can be divided into two categories according to whether L-glutamate is needed or not [13]: One is glutamate-dependent, that is, L-glutamate is needed to accumulate γ-PGA, which mainly includes *B. anthracis*, *B. subtilis* MR-141, *B. licheniformis* ATCC-9945, *B. subtilis* IFO3335 and *B. subtilis* Fly2-01, etc., and the other is glutamate-independent, that is, it can accumulate γ-PGA without L-glutamate, such as *B. subtilis* 5E, *B. licheniformis* A35, *B. subtilis* TAM-4, etc [14]. As shown in **Figure 6**, with the increase in the concentration of sodium glutamate in the fermentation broth, the yield of γ-PGA increased at first and then decreased. When the concentration of sodium glutamate was 10 g/L, the yield of γ-PGA was the highest. Combined with the results of whole genome sequencing of *B. subtilis* QM3, QM3 is a glutamate-independent strain, and γ-PGA can be synthesized without adding exogenous glutamate. When the concentration of sodium glutamate in the fermentation broth is 0, that is, without adding exogenous glutamate, strain QM3 can still produce γ-PGA, and the yield is not low. Proper addition of exogenous glutamate will increase the yield of γ-PGA, which is consistent with the results of whole genome sequencing. Therefore, sodium glutamate of 10 g/L is the most suitable concentration of sodium glutamate in the fermentation medium.



Significance Level: 0.05

Figure 6. Effect of concentration of sodium glutamate on the yield of γ-PGA.

3.4. Effect of Dipotassium Hydrogen Phosphate Concentration on the Yield of γ-PGA

In the process of microbial fermentation to produce γ-PGA, the main metal ions that affect the yield of γ-PGA are K^+, Mg^{2+}, Na^+, Mn^{2+} and so on. Wan-Taek Ju *et al.* found that adding the appropriate amount of K_2HPO_4 and $MnSO_4$ to the production of γ-PGA in Bacillus RKY3 fermentation had a certain effect on the yield of γ-PGA [15]. K_2HPO_4 can promote the growth of bacterial cells. As shown in **Figure 7**, when the concentration of dipotassium hydrogen phosphate is 2%, the production of γ-PGA is the highest, and then the production of polyglutamic acid decreases with the increase of the concentration of dipotassium hydrogen phosphate. Therefore, dipotassium hydrogen phosphate of 2 g/L is the optimum concentration of dipotassium hydrogen phosphate in the fermentation medium.

3.5. Effect of Ammonium Chloride Concentration on the Yield of γ-PGA

Nitrogen sources are divided into inorganic nitrogen sources and organic nitrogen sources, common organic nitrogen sources such as urea, NH_4Cl, NH_4NO_3, $(NH_4)_2SO_4$ and so on. As shown in **Figure 8**, with the increase of the concentration of ammonium chloride in the fermentation broth, the yield of γ-PGA first increased and then decreased, and when the concentration was 3 g/L, the yield of polyglutamic acid was the highest; when ammonium chloride was not added in the fermentation broth, QM3 could also accumulate γ-PGA, but the relative yield was not high. Therefore, the γ-PGA of 3 g/L is the optimum concentration of ammonium chloride in the fermentation medium.

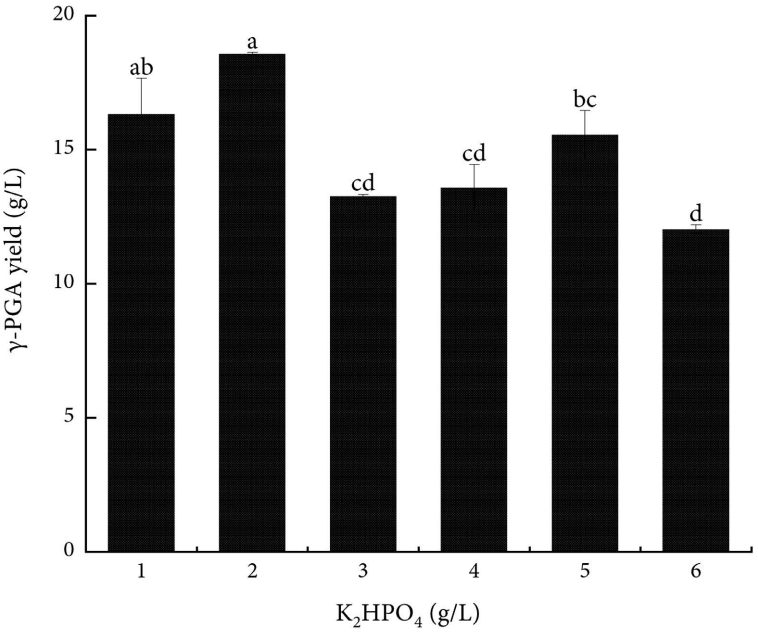


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Figure 7. Effect of concentration of dipotassium hydrogen phosphate on the yield of γ-PGA.

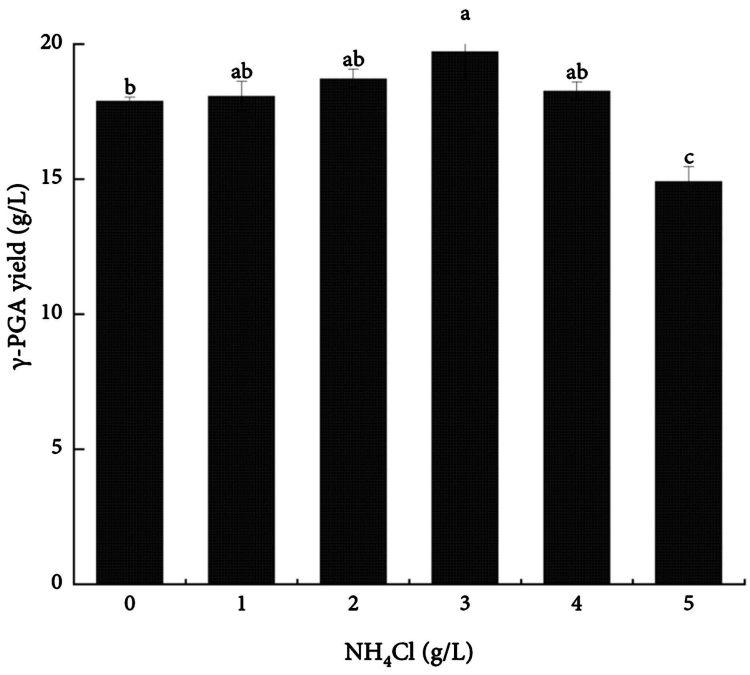

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Figure 8. Effect of ammonium chloride concentration on the yield of γ-PGA.

3.6. Effect of Temperature on the Yield of γ-PGA

Temperature has an important effect on the growth of microorganisms and the accumulation of target products [16]. Each kind of microorganism has an optimal growth temperature, that is, the temperature when the microbial growth

rate reaches the fastest. Therefore, in order to increase the yield of γ-PGA produced by microbial fermentation, the optimum temperature of each γ-PGA producing strain should be found [17]. As shown in **Figure 9**, the yield of γ-PGA in fermentation broth increased at first and then decreased with the increase of temperature, and the yield of γ-PGA was the highest when the temperature was 37˚C. Therefore, 37˚C is the optimum temperature for γ-PGA fermentation of strain QM3.

3.7. Effect of pH on the Yield of γ-PGA

PH is an important environmental factor, which has an important impact on the growth, reproduction and metabolism of microorganisms. As shown in **Figure 10**, with the gradual increase of pH, the yield of γ-PGA decreased continuously, and the yield of γ-PGA was the highest when pH was 6. It is possible that the weak acidity of the culture medium contributed to the accumulation of γ-PGA. Therefore, pH = 6 is the most suitable pH for strain QM3 to ferment and culture γ-PGA.

3.8. Effect of Liquid Loading on the Yield of γ-PGA

In the process of fermentation, the aeration condition will be directly affected by the amount of liquid, and the aeration condition will have a great influence on the metabolism of polyglutamic acid [18]. As shown in **Figure 11**, with the increase of liquid volume, the yield of γ-PGA increased at first and then decreased. When the liquid volume was 40 ml, the yield of polyglutamic acid in fermentation broth was the highest. With the increase of liquid volume, the amount of

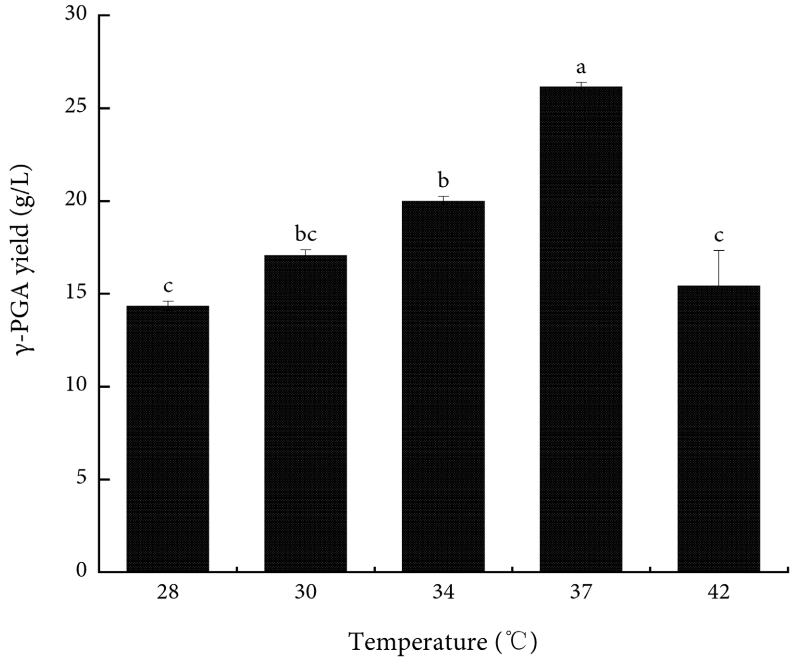

Figure 9. Effect of ammonium chloride concentration on the yield of γ-PGA.

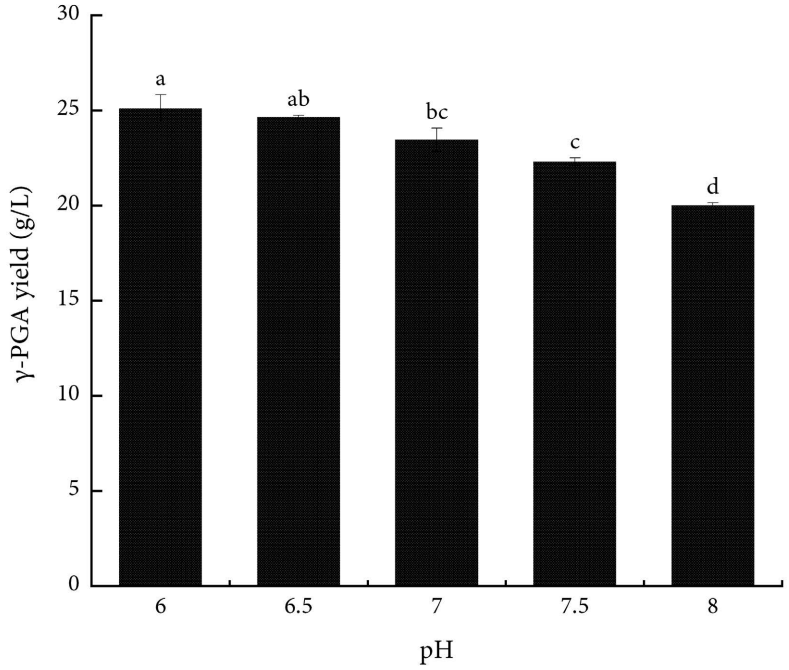

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Figure 10. Effect of pH on γ-PGA production.

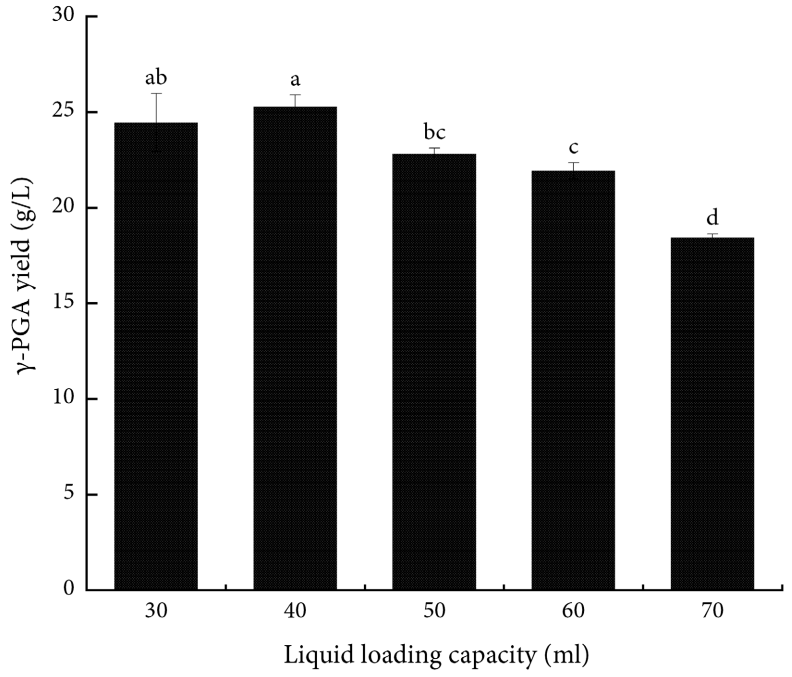

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Figure 11. Effect of liquid loading on the output of γ-PGA.

dissolved oxygen of bacteria decreased slowly, which was not conducive to cell growth, thus the accumulation of γ-PGA decreased. Therefore, the optimum amount of liquid in the fermentation medium was 40 ml.

3.9. Effect of Inoculation Time on γ-PGA Yield

In the process of fermentation, the growth and metabolism of bacteria have a certain regularity. As shown in Figure 12, the yield of γ-PGA increased at first and then decreased with the extension of fermentation time, and the yield of γ-PGA in the fermentation broth was the highest at 72 h. Therefore, the optimum inoculation time of fermentation medium was 72 hours.

3.10. Effect of Inoculation Amount on the Yield of γ-PGA

Different inoculum size means different initial cell concentrations, which affects the end time of fermentation and the yield of γ-PGA, and its size affects the length of cell growth lag period and the rate of cell multiplication [19]. As shown in Figure 13, when the inoculation amount is 2%, the yield of γ-PGA in the fermentation broth is the highest. Therefore, the optimum amount of inoculation in the fermentation broth was set at 2%.

3.11. Orthogonal Experiment

The optimum fermentation conditions of each factor were determined by the above single-factor experiment. In order to further study the effect of the interaction of various factors on the yield of γ-PGA, according to the results of the single-factor experiment, the three factors that had the greatest influence on γ-PGA, namely, temperature, fermentation time and liquid volume, were selected to carry out L9 (3^3) three-factor and three-level orthogonal design, and the yield of γ-PGA was optimized. The orthogonal experimental design and results are shown in Table 1.

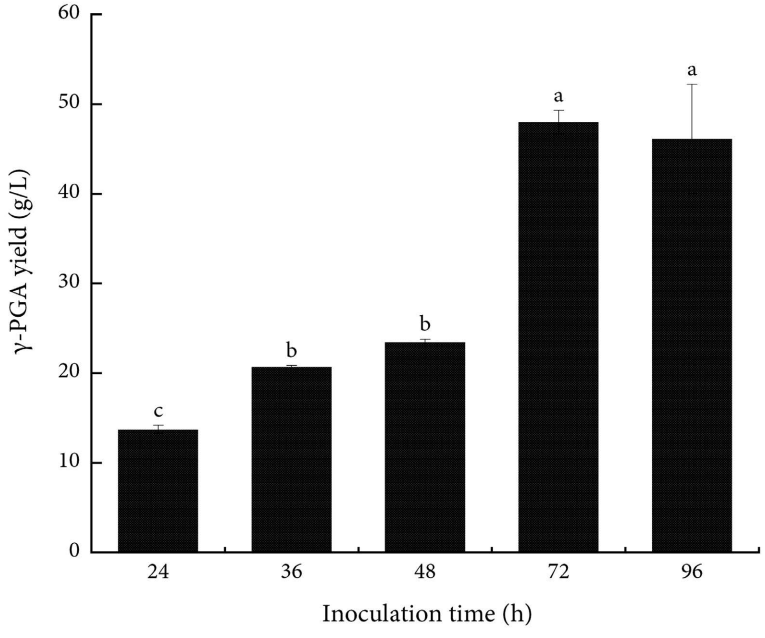
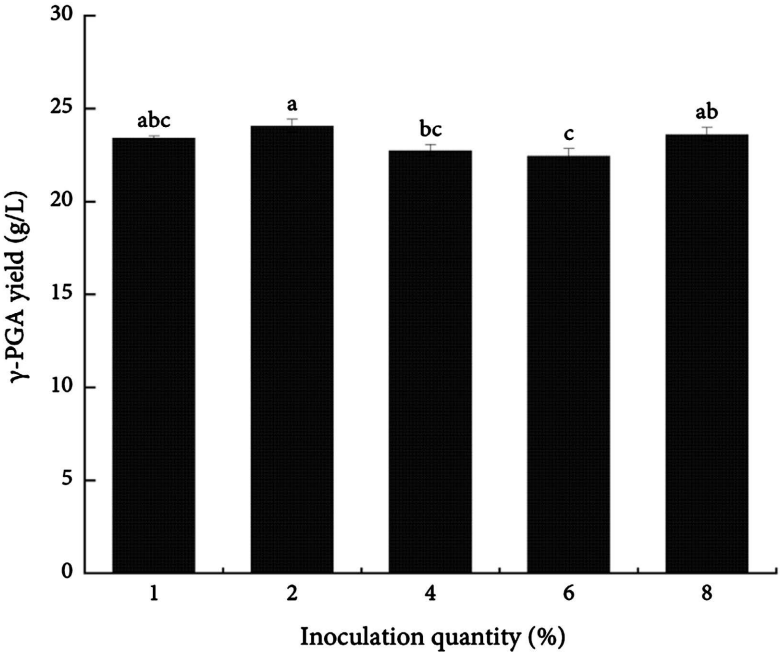


Figure 12. Effect of inoculation time on the yield of γ-PGA.



Significance Level: 0.05

Figure 13. Effect of inoculation size on the yield of *γ*-PGA.

Table 1. Orthogonal experimental design and results.

Experiment No	A Temperature (˚C)	B inoculation time (h)	C liquid volume (ml)	γ-PGA yield (g/L)
1	1	1	1	46.51
2	2	1	2	39.45
3	3	1	3	40.73
4	1	2	2	34.35
5	2	2	3	38.85
6	3	2	1	124.58
7	1	3	3	26.82
8	2	3	1	82.21
9	3	3	2	77.81
k1	107.68	126.69	253.3	
k2	160.51	197.78	151.61	
k3	243.12	186.84	106.4	
$\bar{k}1$	35.89	42.23	84.43	
$\bar{k}2$	53.50	65.93	50.54	
$\bar{k}3$	81.04	62.28	35.47	
R range	45.15	23.7	48.96	

The larger the R range is, the greater the influence of factors on the experimental results. As shown in Table 1, the order of the factors affecting the yield of polyglutamic acid from high to low is as follows: liquid volume (C) > temperature (A) > fermentation time (B). The amount of liquid was the main influencing factor, followed by temperature, and the fermentation time had the least effect on the yield, R2 to 0.973. Based on the above analysis, the optimum technological conditions were determined as follows: the amount of liquid was 30 ml, the temperature was 42˚C, the inoculation time was 72 h, and the yield of polyglutamic acid was 124.58 g/L.

4. Discussion

In order to explore the effects of different carbon sources on the production of γ-PGA by *B. subtilis* QM3, compared with citric acid, sucrose, maltose and soluble starch, strain QM3 has a stronger ability to use glucose than other sugars, which is consistent with the results of QM3 genome sequencing. Strain QM3 can use glucose as the endogenous substrate to synthesize γ-PGA, so as to accumulate γ-PGA and increase yield. When exploring the effect of sodium glutamate concentration on the production of γ-PGA, the content of γ-PGA could still be detected in the fermentation broth without exogenous glutamate, which indicated that QM3 was a glutamate-independent strain. In the culture environment without exogenous glutamate, γ-PGA could still be accumulated by other substances through its own metabolic system, which was consistent with the results of whole genome sequencing. The production cost was reduced, and an appropriate amount of sodium glutamate could increase the production of γ-PGA. With the increase in the concentration of sodium glutamate, the accumulation of γ-PGA decreased, which may reflect the negative regulation relationship between the content of sodium glutamate and the accumulation of γ-PGA. This regulation mechanism remains to be confirmed.

5. Conclusion

In this experiment, *B. subtilis* QM3 was used as the experimental strain, the nutritional conditions and fermentation conditions were optimized at first, and then the fermentation conditions were optimized by orthogonal design. The optimum medium composition and fermentation conditions were determined as follows: glucose 4%, yeast extract 1%, sodium glutamate 1%, $MgSO_4 \cdot 7H_2O$ 0.025%, K_2HPO_4 0.2% NH_4Cl 0.3%, initial pH 6, 250 ml triangle flask, liquid volume 30 ml, 12˚C sterilization 20 min, inoculum 2%, shaking speed 220 r/min. The yield of γ-PGA was 124.58 g/L after shaker culture at 42˚C for 72 h. In the study, it was found that the fermentation of this culture condition strain produced a high yield of γ-PGA and reduced the production cost, which could provide excellent fermentation strains for industrial large-scale production of γ-PGA, thus further promoting the industrialization process of microbial production of γ-PGA.

Acknowledgements

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Conflicts of Interest

The authors declare no conflicts of interest.

References

[1] Sung, M.H. (2005) Natural and Edible Biopolymer Ploy-Gamma-Glutamic Acid: Synthesis, Production, and Applications. *The Chemical Record*, **5**, 352-356. https://doi.org/10.1002/tcr.20061

[2] Perez-Camero, G. (1999) Biosynthesis and Ultrasonic Degradation of Bacterial Ploy (Gamma-Glutamic Acid). *Biotechnology and Bioengineering*, **63**, 110-115. https://doi.org/10.1002/(SICI)1097-0290(19990405)63:1<110::AID-BIT11>3.3.CO;2-K

[3] Liu, J. (2015) Fermentation Preparation of *Bacillus subtilis* Study on γ-Polyglutamic Acid. Ph.D. Thesis, Ocean University of China, Shandong.

[4] Li, H.J., Fang, J, Jiang, C.X., *et al.* (2015) γ-Study on Polyglutamic Acid Fermentation Process. *Food Research and Development*, **36**, 79-82.

[5] Zhang, Q.Q., Jin, X.Q., Chen, J.X, *et al.* (2012) In Fermentation Broth γ-Study on Rapid Determination Method of Polyglutamic Acid Content. *Food Industry Science and Technology*, **33**, 294-296, 300.

[6] Zhang, B.S., Chen, Y.H., Zhang, L., *et al.* (2022) One Strain γ-Screening and Identification of Polyglutamic Acid Producing Strains and Optimization of Fermentation Medium. *Journal of Changshu Institute of Technology*, **36**, 91-98.

[7] Ju, L., Ma, X. and Zhang, J. (2011) γ-Fermentation and Water Holding Properties of Polyglutamic Acid. *China Brewing*, **7**, 57-60.

[8] Ko, Y. and Gross, R. (1998) Effects of Glucose and Glycerol on Gamma-Ploy (Glutamic Acid) Formation by Bacillus Licheniformis ATCC9945a. *Biotechnology and Bioengineering*, **57**, 430-437. https://doi.org/10.1002/(SICI)1097-0290(19980220)57:4<430::AID-BIT6>3.0.CO;2-N

[9] Lu, M.Y., li, Z.X., Du, M., *et al.* (2021) Effect of Culture Medium on Microbial Corrosion. *Chinese Journal of Corrosion and Protection*, **41**, 757-764.

[10] Han, Y.X. (2014) Screening, identification and application of a microbial flocculant producing strain. Ph.D. Thesis, Guangxi University, Nanning.

[11] Wang, F.Y. and Ran, G.Y. (2022) Microbial Fermentation Synthesis γ-Optimization of PGA Fermentation Conditions. *Journal of Sichuan University of Light and Chemical Technology*, **35**, 17-25.

[12] Negus, D., Burton, J., Sweed, A., *et al.* (2013) Poly-γ-D-Glutamic Acid Capsule Interferes with Lytic Infection of Bacillus Anthracis by *B. anthracis*-Specific Bacteriophages. *Applied and Environmental Microbiology*, **79**, 714-717. https://doi.org/10.1128/AEM.02682-12

[13] Wang, H., Yang, L.P., Qiao, J., *et al.* (2011) Research Progress of γ-Polyglutamic Acid. *Shandong Food Fermentation*, **4**, 30-34.

[14] Hidetoshi, K., Toshio, M., Kazumichi, U., *et al.* (1993) Production of Poly (γ-Glutamic Acid) by *Bacillus subtilis* F-2-01. *Bioscience*, *Biotechnology*, *and Bio-*

chemistry, **57**, 1212-1213. https://doi.org/10.1271/bbb.57.1212

[15] Ju, W.-T., Song, Y.-S., Jung, W.-J., *et al.* (2014) Enhanced Production of Poly-γ-Glutamic Acid by a Newly-Isolated *Bacillus subtilis*. *Biotechnology Letters*, **36**, 2319-2324. https://doi.org/10.1007/s10529-014-1613-3

[16] Kunioka, M. (1995) Biosythesis of Ploy (Mamma Glutamic Acid) from L-Glutamin Ditric Acid and Ammonium Sulfate in *Bacillus subtilis* IFO3335. *Applied Microbiology and Biotechnology*, **44**, 501-506. https://doi.org/10.1007/BF00169951

[17] Fang, J.N., Lei, J., Xu, L.S., *et al.* (2018) Microbial Fermentation Production γ-Research Progress of Polyglutamic Acid. *Journal of Applied and Environmental Biology*, **24**, 1041-1049.

[18] Qian, Y. (2022) Yield γ-Isolation Identification and Application of Polyglutamic Acid Producing Strains. Ph.D. Thesis, Hubei University of Technology, Wuhan.

[19] Sang, L. (2004) Production by Microbial Fermentation γ-Polyglutamic Acid. Ph.D. Thesis, Nanjing University of Technology, Nanjing.