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Effects of Cultivation Conditions on the Production of γ-PGA with *Bacillus subtilis* ZJU-7

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Abstract Poly-γ-glutamic acid (γ-PGA) is a kind of water-soluble and biodegradable polymer made from D- and L-glutamic acid units, which are linked by amide bonds formed between α-amino and γ-carboxylic acid groups. As a potential targeted biopolymer that can be refined from biomass directly, γ-PGA has been increasingly applied to food, cosmetic, and pharmaceutical industries. In this work, a suitable nitrogen source was screened out for the high and cost-effective production of γ-PGA in *Bacillus subtilis* ZJU-7. The effects of inoculation time and initial glucose concentration on the γ-PGA production were investigated systematically in both shake flasks and a bench-top 15-l fermentor. Under the optimized culture conditions, a high γ-PGA productivity (46.4 g/l) was obtained after 48 h cultivation at 37 °C. Finally, the large-scale fermentation of γ-PGA production was successfully scaled up to a 100-l fermentor, with the highest γ-PGA productivity for over 54.0 g/l.

Keywords Poly-γ-glutamic acid · L-glutamic acid · *Bacillus subtilis* · Cultivation conditions · Optimization · Scale up

Introduction

Since the oil price has been kept increasing and maintained over a very high level, the biorefinery has been focused on developing an alternative strategy to produce biofuel and some bulk chemicals, which are traditionally oil-based. Especially, the refining process from biomass to biopolymer is very attractive because of the great marketing demand of biopolymer and the large available quantity of biomass. Poly-γ-glutamic acid (γ-PGA) is a kind of unusual

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polypeptide made of D- and L-glutamic acid units via amide linkages between α-amino and γ-carboxylic acid groups. It can be polymerized effectively from a cheap amino acid (L-glumatic acid) in a variety of microorganisms. Besides, γ-PGA possesses many attractive properties, such as being water soluble, anionic, and edible. Therefore, various applications of γ-PGA have been developed in the fields of medicine, foods, plastics, and even oil or heavy metal ion recovery [1–3].

γ-PGA was first discovered by Ivanovics and Bruckner [4] as the capsule of *Bacillus antharacis*. Then, it has also been reported that many subspecies of *Bacillus subtilis* can produce γ-PGA significantly [5–7]. For example, Bovarnick had found that γ-PGA could be biosynthesized by *B. subtilis* 41259 and freely secreted to the culture medium. Regulation of many factors, such as carbon sources, nitrogen sources, metal ions, pH, and temperature, on γ-PGA production has been studied intensively, while the obtained results showed the difference depending on the species involved in γ-PGA production [8–10].

As a patented good γ-PGA producer, *B. subtilis* ZJU-7 was first isolated from fermented bean curd, a traditional Chinese food [11]. In our previous work, this strain can give a very good yield of γ-PGA (54.4 g/l) after 24 h cultivation at 37 °C, which was the highest compared with reported data. However, this high production of γ-PGA in *B. subtilis* ZJU-7 was supported by an expensive nitrogen source (tryptone), which was not cost-effective for the large-scale fermentation to produce this biopolymer.

In this study, we have screened out a cheaper nitrogen source to significantly reduce the cost of γ-PGA production. The effects of inoculation time and glucose concentration on γ-PGA production were investigated systematically in both shake flasks and a bench-top 15-l fermentor. Finally, the high γ-PGA productivity was successfully scaled up to a 100-l fermentor.

Materials and Methods

Strains and Culture Conditions

The *B. subtilis* ZJU-7 strain, capable of good γ-PGA production, was cultured in 250-ml flasks containing 30 ml seed medium (per liter: 10 g tryptone, 5 g beef extract, and 5 g NaCl) and incubated in a rotary shaker at 37 °C and 200 rpm for overnight. The seed culture was then inoculated (with the inoculation ratio of 5%) into the production medium (per liter: 80 g L-glutamic acid, 10 g NaCl, 1 g $CaCl_2$, 1 g $MgSO_4$, 60 g glucose, and 60 g tryptone) and grew in a rotary shaker at 37 °C and 200 rpm for 48 h to produce γ-PGA or grew 36 h to obtain the mature seed broth. For larger volume fermentation, 500 ml mature seed broth was fed into the 15-l fermentor (Shanghai Guoqiang Bioengineering Equipment Co., Shanghai, China) with 9 l production medium. The temperature was kept at 37 °C. The pH was maintained at 6.5 with automatic feeding of 25% (*v*/*v*) NH_4OH or 2 M HCl. The aeration rate was set at 1 vvm, with agitation rate ranging from 350 to 650 rpm to maintain enough dissolved oxygen. The fermentation time was about 48–72 h.

For the large-scale production of γ-PGA in 100-l fermentor (Shanghai Baoxing Bioengineering Equipment Co.), the vegetative broth, which was incubated in the 15-l fermentor for 5 h with the same conditions as described above except the agitation rate was set at 600 rpm, was used to inoculate 60 l production medium in the 100-l fermentor. During the fermentation, the aeration rate was set at 0.5–1 vvm, with the agitation rate adjusted from 200–450 rpm to maintain enough dissolved oxygen level (over 30%), and the fermentation was ended after 72 h cultivation.

Optimization of Culture Conditions

B. subtilis ZJU-7 culturing conditions were optimized both in flask scale and fermentor scale.

The effects of seven nitrogen sources [tryptone, peptone, ammonium sulfate, yeast extract (paste), yeast extract (powder), maize flour, and fish protein concentrate] on the γ-PGA production was compared with the addition level of 60 g/l in shake flasks. Among them, trytone and peptone were purchased from BBI, ammonium sulfate was chemical pure, and the rest four nitrogen sources were industrial raw materials.

The effect of seed inoculation time on the γ-PGA production was tested. Different age of seed broths with OD_{600}=2,4,6, and 8 were inoculated into flasks, respectively, and cultivated at 37 °C in a rotary shaker at 200 rpm for 36 h to evaluate the γ-PGA production.

The effect of glucose concentration on the γ-PGA production in 15-l fermentor was also tested by changing the initial concentration of glucose to 60, 80, 100, and 120 g/l, respectively.

Analysis Methods

Cell density was determined by measuring the optical density at 600 nm (OD_{600}). Viscosity was measured by a viscometer NDJ-79 (Shanghai Changji Instrument Co., Shanghai, China) at 25 °C. The concentrations of L-glutamic acid and glucose was measured by a biosensor with glucose oxidase electrode and L-glutamic acid oxidase electrode according to the manufacturer's manual.

Before the analysis of γ-PGA, the fermentation broth was centrifuged at 12,000 rpm and 4 °C for 20 min to get the supernatant. After that, 4 volumes of methanol was gently mixed up with the supernatant and stayed overnight. The precipitate containing crude γ-PGA was collected by centrifugation at 6,000 rpm and 4 °C for 10 min and re-dissolved in distilled water. The resulted solution was analyzed by a CE instrument (Agilent Technologies, Palo Alto, CA, USA) equipped with a diode array detector set at 195 nm (direct detection) and with fused-silica capillaries of 60.0 cm (length to the CCD system 46.0 cm; Supelco, Bellefonte, PA, USA). The electrophoresis system was operated under reversed polarity with constant voltage of 25 kV. A 30-mmol/l borate solution was used as electrolyte solution. The capillary was flushed sequentially with NaOH (1 min), water (1 min), and electrolyte solution (4 min) as a precondition. The running time of the electrophoresis is 15 min.

Results and Discussion

Effect of Different Nitrogen Sources on γ-PGA Production

The effects of different nitrogen sources on γ-PGA production in *B. subtilis* ZJU-7 was carried out by using seven kinds of nitrogen sources [tryptone, peptone, ammonium sulfate, yeast extract (paste), yeast extract (powder), maize flour, and fish protein concentrate]. As shown in Table 1, the inorganic nitrogen source (ammonium sulfate) did not support cell growth and γ-PGA production. The highest biomass and γ-PGA production (27.9 g/l) were achieved by using the most expensive tryptone for medium formulation. A comparable high yield of γ-PGA (23.8 g/l) was obtained by using a cheap yeast extract (paste) for medium formulation. The results indicated that yeast extract (paste) is a suitable industrial nitrogen source for large-scale γ-PGA production because the cost of nitrogen source per kilogram

Table 1 Comparison of γ-PGA production with different nitrogen sources.

Nitrogen sources	OD_{600}	γ-PGA (g/l)	The cost of nitrogen source ($)[a]
Tryptone (BBI)	27.6	27.9	161.6
Peptone (BBI)	11.16	15.2	56.4
Ammonium sulfate	0	0	–
Yeast extract (paste)[b]	25.7	23.8	1.8
Yeast extract (powder)[b]	13.2	19.7	7
Maize flour[b]	5.4	8.3	4.7
Fish protein concentrate[b]	19.2	15.6	2.8

[a] The cost of nitrogen source for the production of 1 kg PGA

[b] All the nitrogen sources were purchased as industrial raw material.

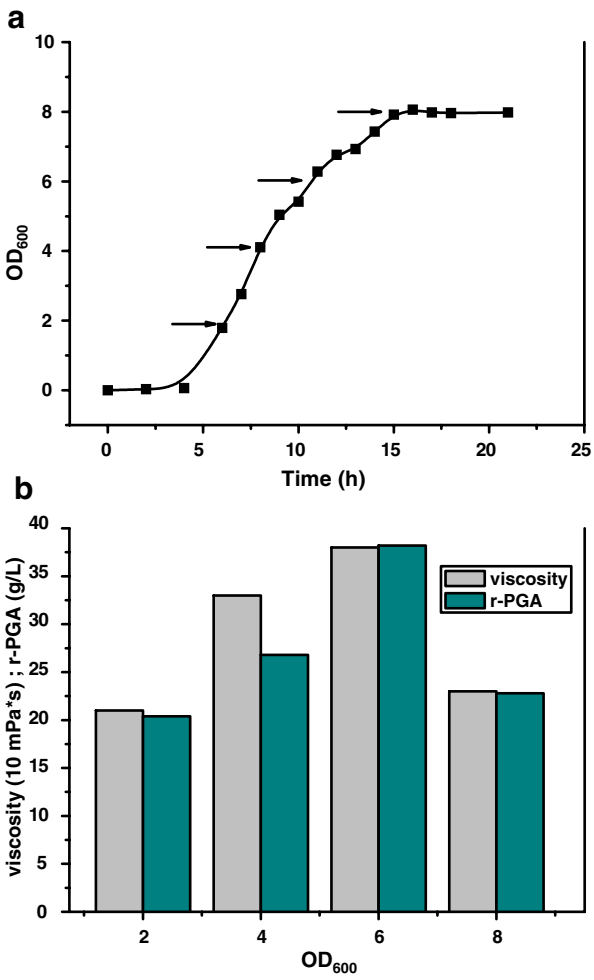


Fig. 1 Effect of inoculation time on γ-PGA production by *Bacillus subtilis* ZJU-7. **a** The growth curve of *Bacillus subtilis* ZJU-7; *arrow* the timing of taking the seed for the γ-PGA fermentation. **b** The γ-PGA productivity and broth viscosity after 48 h cultivation

γ-PGA was almost 100-fold cheaper than that in our previous experiments by using tryptone.

Effect of Inoculation Time on γ-PGA Production

The effect of inoculation time on γ-PGA production was tested by inoculating seeds from different growth stages into the production medium with the same percentage (15% *v*/*v*). As shown in Fig. 1, the comparison of γ-PGA production was carried out by inoculating the same production medium with a series of *B. subtilis* ZJU-7 seeds at different stages of log growth phase, including early (OD_{600} 2.0), middle (OD_{600} 4.0, 6.0), and late stage (OD_{600} 8.0). It was found that the high viscosity and γ-PGA production were not achieved when the seeds at early or late stage of log growth phases were used. The optimal inoculation time was set at the middle stage of the log phase (OD_{600} 6.0), which would give the highest γ-PGA production (37.0 g/l) and the viscosity (372.0 mPa.s). Based on the results from Table 1, the γ-PGA production was improved for about 50~60% with the suitable inoculation time.

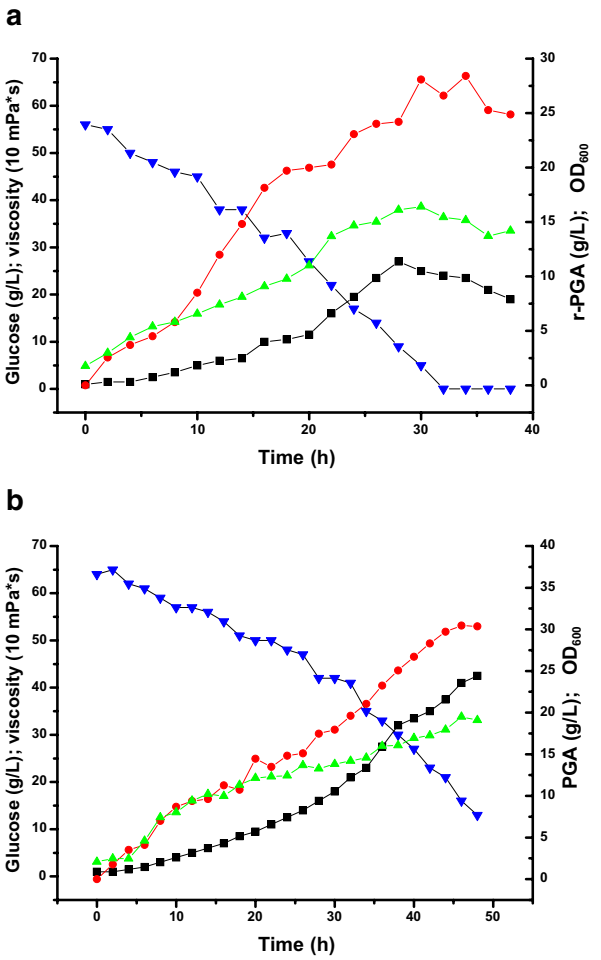
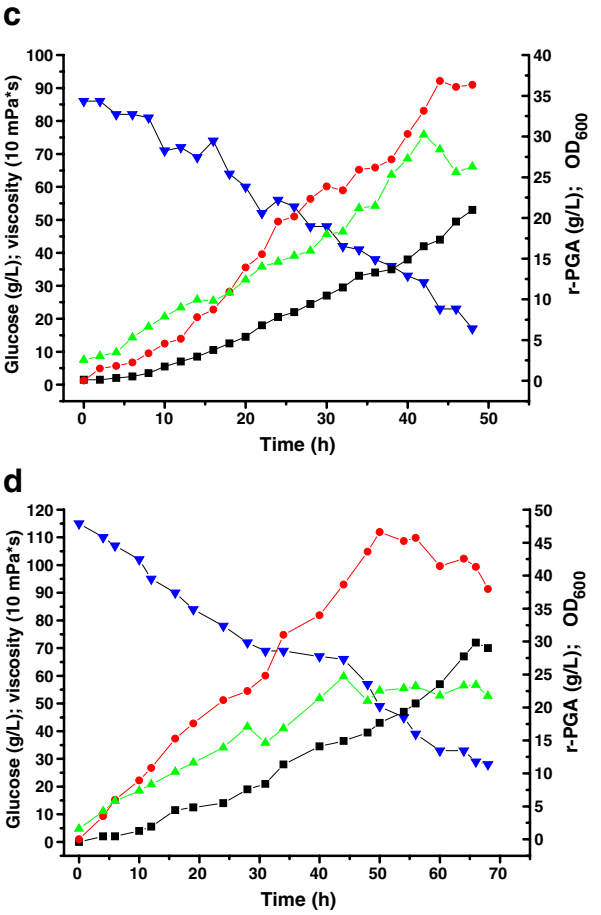


Fig. 2 Effects of initial glucose concentration on γ-PGA production by *Bacillus subtilis* ZJU-7 in the 15-l bench-top fermentor. **a**, 40 g/l glucose; **b** 80 g/l glucose; **c**, 100 g/l glucose; **d** 120 g/l glucose. *Inverted triangle* The glucoses concentration (g/l); *square*, viscosity of the broth (mPa.s); *circle* the γ-PGA concentration (g/l); *triangle* OD_{600} of the broth

Fig. 2 (continued)



Effect of Initial Glucose Concentration on γ-PGA Production

Since the in vivo biosynthesis of γ-PGA is an energy-consuming process and glucose may be utilized to form γ-PGA [8], the initial glucose concentration should be important to support cell growth and the biosynthesis of γ-PGA. Four different initial glucose concentrations (60, 80, 100, and 120 g/l) were applied to medium formulation, respectively. As shown in Fig. 2a, the biosynthesis of γ-PGA reduced very quickly when the glucose was depleted, suggesting that the γ-PGA production was affected obviously by the availability of glucose. As shown in Fig. 2b, the enhancement of the viscosity and the cell growth was extended up to 30 h cultivation when the initial glucose concentration was increased to 80 g/l. Apparently, further increase of the initial glucose concentration led to the improvement of the γ-PGA production, and the highest γ-PGA production (46.4 g/l) was achieved by applying 120 g/l glucose in the production medium. However, high residual glucose concentration was also observed to accompany with the appearance of γ-PGA, when the initial glucose concentration was more than 80 g/l. On the other hand, the secreted γ-PGA has a large molecular weight (more than 1,000,000), which makes the broth very viscous. It was found that a viscosity of 200 mPa.s in the broth seems to have no negative influence on cell growth and γ-PGA biosynthesis (Fig. 2a). Although the cell growth was enhanced by adding more glucose into the production

medium, this effect was impaired obviously when too much glucose (120 g/l) was added. Thus, it could be deduced that the high viscosity (above 400 mPa.s) due to the overaddition of glucose can exert a serious negative effect on cell growth. According to our observation (Fig. 2), it can be concluded that the more γ-PGA was produced, the higher viscosity of the broth would be, which then down-regulated the cell growth and the γ-PGA production. Considering the lower yield of γ-PGA/glucose, 120 g/l glucose was not the favorable concentration for cost-effective γ-PGA production. In order to reduce the negative effects caused by the γ-PGA-related high viscosity, some novel strategies of integrating online γ-PGA removal and cell immobilization are undergoing in our laboratory.

The Upscaled Production of γ-PGA in 100-l Fermentors

Based on the optimal results from 15-l bench-top fermentor, we scaled up the γ-PGA production from 15- to 100-l fermentor. During the cultivation, original 120 g/l glucose was used for medium formulation, and 2.0 l glucose solution (100.0 g/l) was supplemented at 44 and 64 h, respectively, to maintain the glucose concentration over 20 g/l. The γ-PGA production in the 100-l fermentor was kept increasing even the viscosity was over 400 mPa.s., and the final γ-PGA concentration has reached to 54 g/l, about 20% higher than that in 15-l fermentor. According to our observation, the availability of the agitation and aeration in 100-l fermentor was better than that in 15-l bench top fermentor, which might explain the higher γ-PGA production in the 100-l fermentor. It was also found that the viscosity rose very rapidly and reached over 2,000 mPa.s in the last 4-h cultivation; meanwhile, the γ-PGA concentration was kept almost the same. One possible reason could be the polymerization of some short-chain γ-PGA polymers, which forms a long-chain γ-PGA when the fermentation time became too long and no additional γ-PGA was synthesized during that time. This phenomenon is very interesting and important; further studies about this might provide us an approach to control the molecular weight of γ-PGA by adjusting cultivation conditions (Fig. 3).

Conclusion

In conclusion, the production medium of *B. subtilis* ZJU-7 is far much more cost-effective by replacing tryptone with yeast extract (paste). Further study showed that the γ-PGA production could rise to 46.4 g/l with the optimal inoculation time and initial glucose concentration. A 100-l fermentor, accompanied with suitable agitation, aeration, and glucose

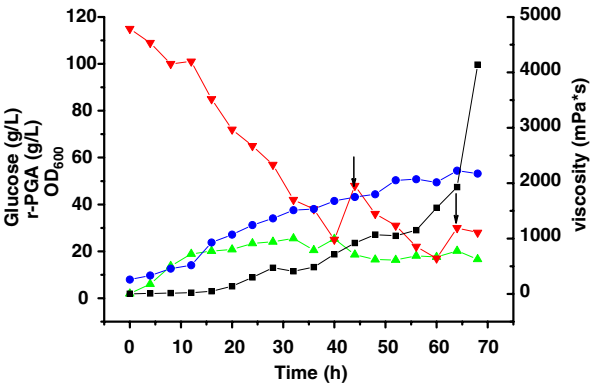


Fig. 3 Time-course studies of γ-PGA production in a 100-l fermentor. *Arrow* The timing when 2.0 l glucose solution (100.0 g/l) were supplemented, respectively; *inverted triangle* the glucoses concentration (g/l); *square* viscosity of the broth (mPa.s); *circle* the γ-PGA concentration (g/l); *triangle* OD_{600} of the broth

supply, was employed to scale up the production of γ-PGA under high viscosity, and the highest γ-PGA production of 54 g/l was achieved.

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