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Contribution of Glycerol on Production of Poly(γ-Glutamic Acid) in *Bacillus subtilis* NX-2

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Abstract Glycerol would stimulate the production of poly(γ-glutamic acid) (γ-PGA) and decrease its molecular weight in *Bacillus subtilis* NX-2. When 20 g/l glycerol was added in the medium, the yield of γ-PGA increased from 26.7±1.0 to 31.7±1.3 g/l, and molecular weight of γ-PGA decreased from $2.43\pm0.07\times10^6$ to $1.86\pm0.06\times10^6$ Da. In addition, it was found that the decrease of γ-PGA chain length by glycerol would lead to the decrease of broth viscosity during the fermentation and enhanced the uptake of substrates, which could not only improve cell growth but also stimulate γ-PGA production. Moreover, it was also found that glycerol could effectively regulate molecular weight between $2.43\pm0.07\times10^6$ and $1.42\pm0.05\times10^6$ Da with the concentration ranging from 0 to 60 g/l. This was the first time to discover such contribution of glycerol on γ-PGA production in *Bacillus* genus. And the effects of glycerol on molecular weight of γ-PGA would be developed to be an approach for the regulation of microbial γ-PGA chain length, which is of practical importance for future commercial development of this polymer.

Keywords *Bacillus subtilis* NX-2 · Fermentation · Glycerol · Molecular weight · Poly(γ-glutamic acid)

Introduction

Poly(γ-glutamic acid) (γ-PGA) is a homopolymer of D- and L-glutamic acid units produced by microbes. It has broad applications in fields of medicine, foods, plastics, and many others [1]. Production of γ-PGA was most extensively studied, and work has been carried out on the nutritional requirements to increase the yield of γ-PGA [2–5]. Glycerol has been reported to be an important nutriment in γ-PGA production in *Bacillus licheniformis* ATCC 9945a, a famous γ-PGA producer, as it could obviously increase the yield of γ-PGA [5]. However, glycerol was scarcely employed in γ-PGA production in *Bacillus subtilis*, as the

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function of glycerol on γ-PGA production in *B. subtilis* was not remarkably effective in previous reports [1].

On the other hand, molecular weight is an important feature of microbial γ-PGA for the effect that it has on polymer properties [6]. However, γ-PGA produced by *Bacillus* genus generally has relatively high molecular weight, and such a high molecular size polymer is too viscous, rheologically unmanageable, and difficult to be modified by chemical reagents [1]. Therefore, it is useful to decrease γ-PGA molecular weight during fermentation course to obtain the polymer with relatively low molecular weight.

In this work, we found that the glycerol present in the medium would not only increase the yield of γ-PGA but also decrease the molecular weight of γ-PGA in *B. subtilis* NX-2. This was the first time to discover such contribution of glycerol on γ-PGA production in *Bacillus* genus, and it would provide a brand new understanding of the regulation of γ-PGA biosynthesis.

Materials and Methods

Microorganism

B. subtilis NX-2 was isolated from soil sample [7]. It was deposited in China General Microbiological Culture Collection Center with the accession number of CGMCC No. 0833.

Production of γ-PGA by Flask Culture

B. subtilis NX-2 was first inoculated into 50 ml of seed medium containing glucose 20 g/l, L-glutamate 10 g/l, peptone 5 g/l, $K_2HPO_4{\cdot}3H_2O$ 2 g/l, $MgSO_4$ 0.3 g/l in 500-ml flask and aerobically incubated at 32.5 °C for 16 h with shaking at 220 rpm. Seed culture (0.8 ml) was then transferred to 500-ml flask containing 80 ml of basal medium comprising glucose 40 g/l, glutamate 40 g/l, $(NH_4)_2SO_4$ 10 g/l, $K_2HPO_4{\cdot}3H_2O$ 2 g/l, $MgSO_4$ 0.3 g/l, and $MnSO_4$ 0.03 g/l, the final pH was adjusted to 7.5, and glycerol was added as described in "Results and Discussion." The flask culture was incubated at 32.5°C in a rotary shaker at 220 rpm for 48 h.

Purification of γ-PGA

Upon completion of the fermentation at 48 h, the culture was centrifuged for 30 min (10,000 rpm, 4 °C). The cells were discarded, and the supernatant was dialyzed extensively through a membrane with 1×10^4 Da cutoff in distilled water for 3 days; then, the resultant sample was added to seven volumes of ethanol to precipitate γ-PGA. The obtained precipitate was dissolved in distilled water and lyophilized to give pure γ-PGA.

Analytical Methods

Dry cell weight was determined from 10-ml cell suspensions that were harvested by centrifugation, washed with distilled water, and dried at 80 °C for 24 h to a constant weight. The concentrations of glucose and glutamate were measured enzymatically using a bioanalyzer (SBA-40C, Shandong Academy of Sciences). The concentration of glycerol was determined enzymatically using analysis kit purchased from Sigma. Samples removed

from shake-flask cultures were passed through 0.22-μm filters and then treated according to detailed procedures described in the product information. The volumetric yield and molecular weight of γ-PGA was measured by gel permeation chromatography system following the method reported previously [8]. Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy was performed with a Varain Unity Inova 600 spectrometer, and samples for NMR were dissolved in D_2O solution.

Statistical Evaluation

All measurements were made at least in triplicates. For each parameter, mean and standard deviation (SD) were calculated followed by one-way analysis of variance (ANOVA) with post hoc mean comparison by Tukey true significant difference test, and a significance level of $p<0.05$ was chosen. All statistical analyses were performed using Statistica 6.0 software package.

Results and Discussion

Improvement of γ-PGA Production with the Addition of Glycerol

As far as we know, there were few reports about employment of glycerol in γ-PGA fermentation in *B. subtilis* due to the unremarkable effect of glycerol on γ-PGA production in this species. However, it was interesting to note that glycerol could effectively influence γ-PGA fermentation process in *B. subtilis* NX-2. As shown in Fig. 1a, glycerol had a positive effect on γ-PGA production in this strain. When 20 g/l glycerol was added at the beginning of fermentation, the yield of γ-PGA increased from 26.7±1.0 to 31.7±1.3 g/l, an increase by 18.7%, and the biomass was also comparatively higher than that without glycerol. On the other hand, molecular weight of γ-PGA decreased with glycerol during the fermentation, and it decreased from $2.43\pm0.07\times10^6$ to $1.86\pm0.06\times10^6$ Da (a decrease by 23.5%) at the end of fermentation (Fig. 1b). It seemed that glycerol began to take effect at the very beginning of γ-PGA formation. This was a unique discovery in γ-PGA production, as to our knowledge, there was no report about the decrease of γ-PGA molecular weight by the addition of glycerol in medium. Moreover, glycerol could effectively decrease the fermentation viscosity (Fig. 1c). When glycerol was added in the medium, both the extracellular glucose and glutamate were consumed faster than those without glycerol (Fig. 1d), indicating that the decrease of fermentation viscosity would relieve the mass transfer limitation and stimulate the uptake of extracellular substrates, which would not only improve cell growth but also stimulate γ-PGA production in *B. subtilis* NX-2.

Effect of Initial Concentration of Glycerol on γ-PGA Production

The effect of initial concentration of glycerol on γ-PGA production was shown in Fig. 2. Glycerol had a positive effect on both cell growth and γ-PGA biosynthesis in *B. subtilis* NX-2, with the initial concentration ranging from 10 to 40 g/l ($p<0.05$), and the optimum concentration for γ-PGA production was 20 g/l. While in the case of *B. licheniformis* ATCC 9945a, high level of glycerol was used (80 g/l) [3]; this was also the advantage of our strain, as the low concentration of glycerol required would efficiently lower the industrial cost. On the other hand, the results of one-way ANOVA indicated significant

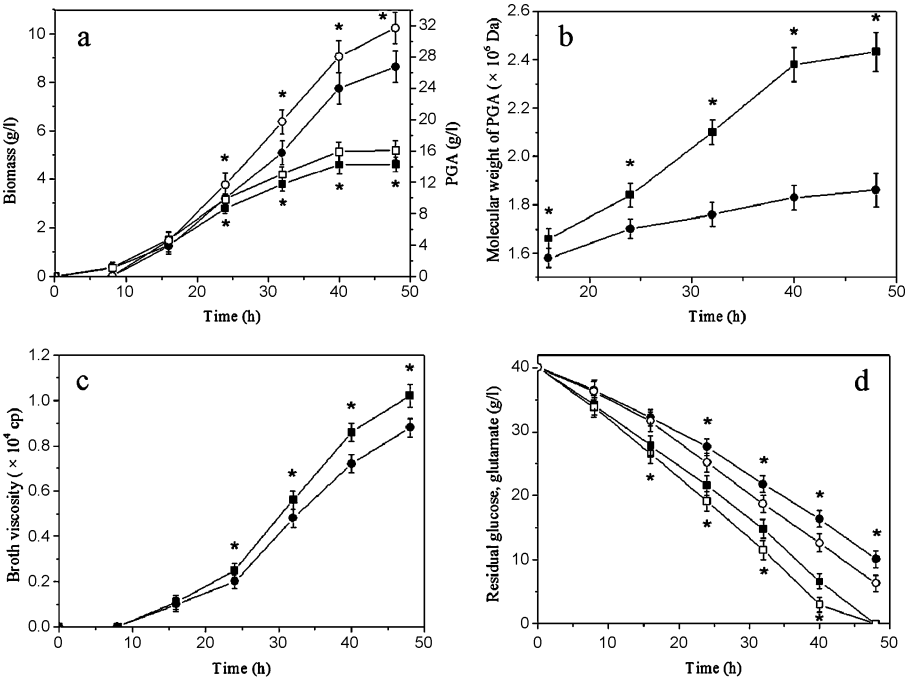

Fig. 1 Time profile of poly(γ-glutamic acid) (γ-PGA) fermentation. **a** Biomass without glycerol (*filled square*), biomass with 20 g/l glycerol (*empty square*), yield of γ-PGA without glycerol (*filled circle*), yield of γ-PGA with 20 g/l glycerol (*empty circle*); **b** molecular weight of γ-PGA without glycerol (*filled square*), molecular weight of γ-PGA with 20 g/l glycerol (*filled circle*); **c** broth viscosity without glycerol (*filled square*), broth viscosity with 20 g/l glycerol (*filled circle*); **d** residual glucose without glycerol (*filled square*), residual glucose with 20 g/l glycerol (*empty square*), residual glutamate without glycerol (*filled circle*), residual glutamate with 20 g/l glycerol (*empty circle*), both the initial concentrations of glucose and glutamate were 40 g/l. *$p<0.05$, significant differences between control and samples with glycerol

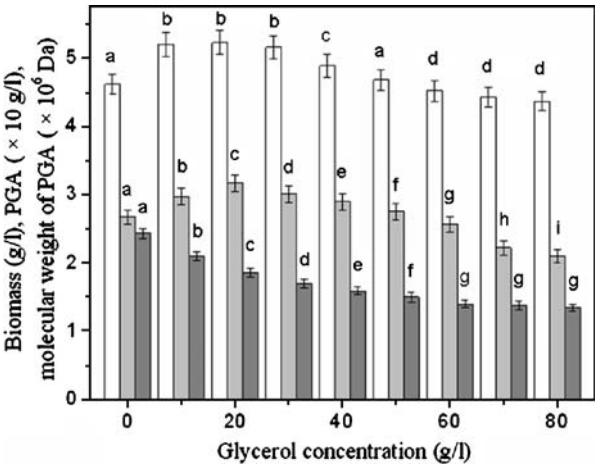

Fig. 2 Effect of glycerol concentration on poly(γ-glutamic acid) (γ-PGA) fermentation, biomass (*white bar*), yield of γ-PGA (*light gray bar*), molecular weight of γ-PGA (*dark gray bar*). The *superscript letters* indicated the statistical significance of differences at $p<0.05$; the values followed by the same letter with the same parameter types were not significantly different

differences among various groups with respect to molecular weight ($p<0.05$). The post hoc test showed that the control without glycerol had significantly higher values of γ-PGA molecular weight than another eight groups, and there was a significant difference ($p<0.05$) between six groups with glycerol concentration ranging from 10 to 60 g/l, except for the concentration of 60, 70, and 80 g/l ($p>0.1$). It indicated that γ-PGA molecular weight decreased remarkably from $2.43\pm0.07\times10^6$ to $1.42\pm0.05\times10^6$ Da with glycerol concentration increasing from 0 to 60 g/l, and it kept nearly constant despite further increase of glycerol concentration to 80 g/l. Therefore, it was convenient for us to effectively modulate γ-PGA molecular weight by altering glycerol concentration between 0 and 60 g/l to meet different requirements in different fields, which could leave out the downstream hydrolysis process and reduce the cost in γ-PGA production.

Effect of Glycerol Addition Time on γ-PGA Production

The effect of glycerol addition time on γ-PGA production in *B. subtilis* NX-2 was also studied. Twenty grams per liter glycerol was added in the medium at interval of 8 h; after incubation for 48 h, the production of γ-PGA was determined, and the results are shown in Table 1. Glycerol showed nearly the same effect ($p>0.05$) on yield (around 31.5 ± 1.5 g/l) and molecular weight of γ-PGA (around $1.87\pm0.08\times10^6$ Da) as long as it was added before 16 h, and the effect gradually weakened ($p<0.05$) when it was added thereafter. It seemed that the effect of glycerol might be related to the process of γ-PGA biosynthesis. On the other hand, since glycerol with high concentration would bring inhibition to cell growth (Fig. 2), it was suggested that glycerol could be added in the medium around 16 h; this would lessen the initial inhibition on cell growth.

Utilization of Glycerol during Fermentation

When 20 and 80 g/l glycerol were added in the medium, respectively, no glycerol was consumed by *B. subtilis* NX-2 during the whole fermentation process (data not shown). While in *B. licheniformis* ATCC 9945a, glycerol was utilized from the very beginning, and a total amount of 30 to 40 g/l glycerol had been consumed until the end of fermentation when 80 g/l glycerol was added in the medium [2, 9]. The difference of glycerol consumption between the two strains might be due to the differences of species and medium composition.

In fact, it has been reported that glycerol added in the medium could also decrease molecular weight of some other biopolymers, such as poly(hydroxyalkanoate) and ε-poly-L-lysine. And

Table 1 Effect of glycerol addition time on poly(γ-glutamic acid) (γ-PGA) molecular weight.

Glycerol addition time (h)	γ-PGA yield (g/l)	Molecular weight ($\times10^6$ Da)
Without glycerol	26.7 ± 1.0^a	2.43 ± 0.07^a
0	31.7 ± 1.3^b	1.86 ± 0.06^b
8	31.6 ± 1.3^b	1.86 ± 0.06^b
16	31.2 ± 1.2^b	1.89 ± 0.06^b
24	30.2 ± 1.2^c	1.96 ± 0.06^c
32	29.1 ± 1.2^d	2.13 ± 0.06^d
40	27.8 ± 1.2^e	2.38 ± 0.07^a

Superscript letters indicated the statistical significance of differences at $p<0.05$; the values followed by the same letter within rows were not significantly different.

the mechanism was that the esterification of glycerol and these biopolymers occurred, which led to the inhibition of monomers incorporation into the carboxyl terminus [10, 11]. As no glycerol was consumed by *B. subtilis* NX-2, glycerol would not be covalently bonded to γ-PGA, and it was also characterized by ^{1}H NMR in this work (data not shown). As a result, glycerol might have another particular effect mechanism in γ-PGA production in this strain, and more work should be investigated further to elucidate the variation of γ-PGA biosynthesis with the presence of glycerol.

Conclusion

Glycerol could decrease the molecular weight of γ-PGA in *B. subtilis* NX-2 and then decrease the broth viscosity and enhance the uptake of substrates, which was beneficial for cell growth and γ-PGA production in this strain. As a result, the yield of γ-PGA in this strain was also increased with the presence of glycerol. This was the first time to discover such contribution of glycerol on γ-PGA production by *Bacillus* genus, and the improved yield of γ-PGA reached 31.7±1.3 g/l and the conversion rate of glutamate was 79±3%, which was at a relative high level compared with values reported in the literature [12, 13].

In addition, we also learned some information of the effect of glycerol in this strain. It might be related to the process of γ-PGA biosynthesis but was not due to the inhibition of glutamate monomers incorporation into the carboxyl terminus of γ-PGA. It was deduced that glycerol might influence γ-PGA polymerase to alter γ-PGA biosynthesis. In fact, it was such a complex function of glycerol and was still unclear as to why the addition of glycerol resulted in such a variation in γ-PGA molecular weight, and more work should be further investigated to elucidate the biosynthesis mechanism of γ-PGA with the presence of glycerol.

Moreover, it was expected that glycerol would modulate γ-PGA molecular weight more effectively through further investigation for the mechanism features, which would not only deepen the knowledge of γ-PGA biosynthesis process but also establish a general foundation for the regulation of microbial γ-PGA molecular weight.

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