Effect of corona electric field on the production of gamma-poly glutamic acid based on bacillus natto

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Abstract. Bacillus Natto is an important strain for gamma-poly glutamic acid (γ-PGA) production. The mutagenesis of Bacillus Natto 20646 under corona electric field and the screening of high γ-PGA producing mutant were investigated. A new mutant bacillus natto Ndlz01 was isolated from Bacillus Natto 20646 after mutation in corona electric field at 9kV for 2min. The Ndlz01 exhibited genetic stability of high γ-PGA producing ability even after five generation cultures. When the bacterium was mutated in streamer discharge state at 9kV for 2min, its death rate was more than 90%. Compared with the yield of γ-PGA based on the original Bacillus Natto 20646, the γ-PGA yield of mutant bacillus natto Ndlz01 increased from 2.6 to 5.94 g/L, with an increase rate of 129.78%.

1. Introduction
The gamma-poly glutamic acid (γ-PGA) is a kind of new environment friendly biopolymer material, which consists of D-Glutamic and L-Glutamic connected by amino bond [1]. With the advantages of biodegradability, biocompatibility, edibility, especially the characteristic of non-toxic both to human and environment, the γ-PGA is being widely applied in several fields, such as medicine, food, cosmetics, sewage treatments and so on [2]. Among several common ways to synthesize γ-PGA, the biosynthesize is chosen in present paper, because this method processes gentle culture conditions, short production period and higher extraction efficiency. In addition, the producing process and the molecular weight are easily controlled and the way is environment friendly.

At present, UV, DES, NTG and microwave are the common methods to mutate the bacterium domesticy. Most of γ-PGA is produced by the mutant strains and its yield increased by in the range of 30% to 135.3%. In the present paper, the Bacillus natto is mutated in corona electric field with the aim to improve the yield of γ-PGA from Bacillus natto. Up to now, there is lack of information on the mutagenesis of Bacillus natto by corona electric field.

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2. Materials and methods

2.1 Materials, reagents and apparatus

Bacillus natto20646 was chosen as the original bacterium, which was provided by Tianjin Institute of Industrial Microbiology (1.01034).

Chemical reagents of beef extract, peptone, agar, cane sugar, yeast extract, MgSO₄, CaCl₂, NaCl, MnSO₄·H₂O, K₃HPO₄, KH₂PO₄ and sodium glutamate are all analytical purity grade. KBr is spectroscopical purity grade.

The ultraviolet and visible spectrophotometer (HITACHI U-2900) was used to monitor the growth curve of bacteria. The Infrared spectrometer (Nicolet Awatar 330) was used to identify the γ-PGA. The PCR apparatus (ABI Veriti) was used to identify the mutant Bacillus natto.

2.2 Medium

The fluid nutriment medium with pH of 7.0 was prepared as follows: bean sprouts soup100 mL, cane sugar 5.0 g /L. The seed medium with pH of 7.5 was prepared as follows: beef extract 5 g /L, peptone 10 g /L, cane sugar 30 g /L, yeast extract 5 g /L, NaCl 5 g /L, MnSO₄·H₂O 0.4 g /L, K₂HPO₄ 2 g /L, KH₂PO₄ 4 g /L, MgSO₄ 0.5 g /L and CaCl₂ 0.25 g /L. Fermentation medium with pH of 7.5 was prepared as follows: peptone 10 g /L, cane sugar 30 g /L, yeast extract 5 g /L, sodium glutamate 30 g /L, NaCl 5 g /L, MnSO₄·H₂O 0.4 g /L, K₂HPO₄ 2 g /L, KH₂PO₄ 4 g /L, MgSO₄ 0.5 g /L and CaCl₂ 0.25 g /L. The solid-state medium with pH of 7.5 was prepared as follows: beef extract 10 g /L, peptone 10 g /L, cane sugar 30 g /L and agar 10 g /L NaCl 10 g /L.

2.3 Method

2.3.1 Cultivating condition. Dilution-plate method was used to cultivate the bacteria and each culture dish contains 25 mL solid-state medium. The culture dish was put up side down and cultivated at 37 ℃ in a constant-temperature incubator for 48h. Cultivating condition of seed was as follows: bacteria was inoculated into a 250 mL conical flask which contains 50 mL seed medium and inoculation quantity was 2 %, the bacteria was cultivated on a rotary shaker (200 rpm) at 37 ℃ for 24 h. Cultivating condition of fermentation was as follows: the bacteria was inoculated into a 250 mL conical flask which contains 50 mL fermentation medium and inoculation quantity was 2 %, the bacteria was cultivated on a rotary shaker (260 rpm) at 37 ℃ for 24 h.

2.3.2 Plate separation. The bacterial suspension was obtained by the bacillus natto seeds cultivated in seed medium for 24 h. After a diluting process, 20 μL of diluted suspension was taken and coated uniformly on the solid-state medium. After that, the solid-state medium was put into a constant-temperature incubator and cultured at 37 ℃ for 48 h. Then, the single colony was picked up and dissolved into the sterile water for further use.

2.3.3 Gram stain. Gram stain was carried out as the direction and examined by microscope.

2.3.4 Infrared Spectrum. The γ-PGA was composed of D-Glutamic and L-Glutamic by amino bond. The amino bond had a series of characteristic absorption peak, which was easy to be identified by infrared spectroscopy [3]. The sample was prepared by squashing method and characterized by infrared spectrometer in 4000–400 cm⁻¹ at room temperature.

2.3.5 Growth curve. The cultivating time determines the density and vitality of the bacteria. Cultivated the bacillus natto seed in seed medium for 34 h, and then take 3 mL to measure its ABS value at OD₆₆₀ every two hours, using the blank seed medium as the reference.

2.3.6 High voltage corona electric field. Pin-plate equipment was used to generate corona electric field, which was shown in Figure 1.
The bacillus natto 20646 was mutated in two corona electric fields, respectively. Both the critical state and the streamer discharge state were chosen. The critical voltage was 3.5 kV and the treatment time included 15 min, 20 min and 25 min. Because of the wide streamer discharge state, three different voltages (5, 7 and 9 kV) were chosen to mutate the original bacteria. The treatment time included 1, 2 and 3 min, respectively.

The bacillus natto seed was cultivated in seed medium for 12 h. When growing to exponential growth phrase, it was made into bacterial suspension. Firstly, 7 mL of bacterial suspension was put into culture dish with a diameter of 7 cm and shaken slightly to make it distribute uniformly in the culture dish. Secondarily, it was mutated in corona electric field according to the conditions mentioned above; finally, the mutated bacterial suspension was collected and diluted by ten-fold dilution method, and inoculated on the solid-state medium.

2.3.7 Primary screening. The mutant strains were coated on the solid medium immediately after mutation. If the yield of one strain was large enough, obvious pucker on intermediate protrusions of the colony would appear and obvious threadlike strains could be observed when it was drawn by inoculation needle. A high γ-PGA yield of the strain corresponded to obvious puckers and longer threadlike strains.

2.3.8 Freeze-drying way to secondary screening. After fermenting for 24 h, the fermented liquid was centrifuged and the supernatant was taken out. Then the supernatant was mixed with four volumes of absolute ethanol and held for 19 h to precipitate the γ-PGA at 4°C under static condition. Finally, the sediment was obtained by centrifuging and eluting method and was frozen below -20 °C. After freeze-drying, the sediment was weighed by electronic balance.

2.3.9 PCR identification. Because the 16SrDNA sequence (GenBank) of bacillus subtilis and bacillus natto were homologous, the primers were designed by choosing the conserved fragment in both ends of the 16SrDNA sequence, and PCR was amplified by using colony directly in 20 μL system.

2.3.10 Genetic stability of the high yield bacterium. Both the original bacterium and the mutated bacterium were inoculated in fermentation medium to cultivate for 24 h and were used to extract the γ-PGA. The yield of γ-PGA extracted from the original and that of the mutated bacterium was compared. In order to investigate the genetic stability of the bacterium, the bacterium was fermented further until the fifth generation.
3. Results and discussion

3.1 Gram stain
The microscopic examination revealed that the bacterium was purple and it implied that the Bacillus natto was gram-positive bacterium, which was consistent with the report in the literature.

3.2 Infrared spectrum
According to the characteristic absorption peak of amino bond reported in Ref. [4], the Fourier Transform Infrared Spectroscopy shown in Figure 2 and Figure 3 suggested that the polymer obtained in the present experiment was γ-PGA.

![Figure 2. Fourier Transform Infrared Spectroscopy of original bacterium.](image1)

![Figure 3. Fourier Transform Infrared Spectroscopy of Ndlz01 bacterium.](image2)

3.3 Measuring the growth curve
The growth curves of both the original bacterium and the mutated Ndlz01 bacterium were shown in Figure 4. Fig.4 indicated that the lag phrase began at 4 h. The bacillus natto was in the exponential growth phrase between 4 to 10 h and it reached the stationary phrase after 10 h. During the first 18h, the Ndlz01 bacterium grew faster than the original bacterium.

![Figure 4. The growth curve of Bacillus natto.](image3)
3.4 Mutagenesis of bacteria in corona electric field

After mutation, the death rate of the bacteria was calculated and the results were shown in Table 1. The data shows the death rate was 94.59% under the condition of 3.5kV for 25min, which was higher than 50%, but the mutagenesis time was too long. The death rate under the condition of 9kV for 2min reached 97.3%, which was higher than 50% and the time was acceptable.

| Time | Voltage | Time | Voltage |
|------|---------|------|---------|
| 15 min | no | 1 min | 5 kV | 5 kV | 7 kV | 9 kV |
| 20 min | 13.5 % | 2 min | 59.46 % | no | no | 70.27 % |
| 25 min | 94.59 % | 3 min | nearly100 % | nearly100 % | nearly100 % |

3.5 Primary screening

Compared the colony shape of the mutant bacterium with that of original bacterium, a lot of puckers were found on the edge of one culture dish. Drawn with an inoculation needle, obvious thread-like strains were observed. The corresponding mutation condition was under 9kV for 2min. This bacterium was named as Ndlz01 and reserved for secondary screening.

3.6 Freeze-drying way to secondary screening

After primary screening, the optimal bacterium named as Ndlz01 was isolated, fermented to synthesize γ-PGA. The yield of γ-PGA from Ndlz01 was compared with that from the original bacterium. The results were shown in table 2 (the γ-PGA was extracted from 50 mL fermented liquid). The table clearly showed that the yield of γ-PGA from Ndlz01 was much higher than that of original bacterium.

Table 2 The comparisons of γ-PGA yield from the original bacterium and the Ndlz01

| bacterium | G1 | G2 | G3 | G4 | G5 | Average |
|-----------|----|----|----|----|----|---------|
| Original bacterium | 117.5 mg | 121.7 mg | 144.4 mg | 144.5 mg | 122.5 mg | 130.12 mg |
| ndlz01 | 260.2 mg | 326.8 mg | 332.5 mg | 271.2 mg | 295.2 mg | 297.18 mg |
| Increment | 21.45 % | 168.53 % | 130.26 % | 87.68 % | 140.98 % | 129.78 % |

3.7 The result of PCR identification

![Electrophoretogram of the PCR production.](image-url)
The PCR reaction used colony directly. The gel electrophoresis result of the PCR production was about 1450 bp (shown in Figure 5 for 1, 2, 3 lane), it was consistent with the expected result.

3.8 The genetic stability of the high yield bacterium
Table 2 gives the measuring results of the γ-PGA yield of original bacterium and that of Ndlz01 in each generation, and the calculated results of yield increment was given too. The result showed that the genetic of the Ndlz01 was stable.

4. Conclusion
The death rate of the bacterium increased with increasing voltage and treatment time. Under the optimal condition of 9 kV for 2 min, the death rate reached 97.3%. After mutation, the optimal bacterium was isolated and named as Ndlz01. The yield of γ-PGA from Ndlz01 increased from 2.6 g/L (130.12 mg per 50 mL fermented liquid) to 5.94 g/L (297.18 mg per 50 mL fermented liquid), the increase rate reached 129.78%. And what’s more, Ndlz01 exhibited good genetic stability. These results indicated that the bacillus natto was sensitive to the corona electric field, and a positive effect corona electric field on mutation of bacillus natto existed.

The identification of mutant bacterium according to analysis of PCR revealed that the bacterium used was really the bacillus natto. The growth curves after 18 hours indicated that the growth rate of Ndlz01 was slower than that of the original bacterium, which may be attributed to the fact that the nutrients in the medium of Ndlz01 is less than that in the medium of original bacterium. So it’s valuable to make further research to find out a way to keep the bacteria growing fast all the time, which contributes to improving the production.

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