Breeding of High-yield Alkaline Protease Producing Strain by Atmospheric and Room Temperature Plasma Mutagenesis

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Abstract. To obtain alkaline protease producing strain with high-yield, the strain *Bacillus subtilis* was treated by atmospheric and room temperature plasma (ARTP) mutagenesis. The results showed that the strain had a higher positive mutation rate when the mutagenesis time was 50s. Finally, a high-yield mutant strain A59 was obtained after repeated mutagenesis by ARTP and enzyme activity increased by 23.38% from 6835U/mL to 8433U/mL. Then fermentation conditions of mutant strain were optimized by single-factor method and response surface method, and the optimum fermentation conditions were as follows: 3.6% soybean meal, 5.2% corn meal, 0.9115% Na$_2$HPO$_4$, 0.1593% Mg$^{2+}$, 0.09% Tween-80 and 5% inoculation volume. Under the conditions of the verification experiment, the enzyme activity of the A59 strain reached 14026 U/mL, which was 2.05 times that of the original strain.

1. Introduction
Alkaline proteases belong to proteases that can hydrolyze protein-peptide bonds under alkaline conditions, with a wide range of pH values between 6 and 13\cite{1}. Most of its active centers contain serine, also known as serine protease. Alkaline proteases are produced by microorganisms, particularly *Bacillus* strains \cite{2}. Alkaline proteases are widely used in detergents, food, medical, silk, leather, and other industries, especially in the detergent industry, so their demand is increasing \cite{2,3}. The hindrance for wide range application of proteases in the industry is the low yield, high production cost, and poor properties of enzymes \cite{4}. Therefore, it is still a hot research topic to improve the ability to producing enzymes and optimize culture condition.

ARTP technology is a new type of microbial mutation breeding method that has been rapidly developed in recent years. Compared with the traditional mutation methods, ARTP has the advantages of high mutation rate, good genetic stability of the mutant strain, and non-polluting nature \cite{5,6}. Compared with molecular manipulation methods, it has the advantages of simple operation, low cost, safety and non-toxicity. Therefore, ARTP was widely used in microbial mutations \cite{7}. And at least 24 bacteria and 14 fungi have been successfully applied \cite{8}.

In this paper, the aim of the study was improving alkaline protease yield of *Bacillus subtilis* by ARTP mutagenesis and obtaining a high-yield mutant strain. Then response surface tests were used to optimize fermentation conditions of the mutant strain.
2. Materials and Methods

2.1. Microorganism
The experimental strain is *Bacillus subtilis*, 2709 alkaline protease strain.

2.2. Media
Slant medium (m/v): 1% beef extract, 1% peptone, 0.5% NaCl, 2% agar, pH 7.0.
Selective medium (m/v): 1% beef extract, 1% peptone, 0.5% NaCl, 2% agar, 1% casein, pH 7.0.
Seeding medium (m/v): 1% beef extract, 1% peptone, 0.5% NaCl, pH 7.0.
Fermentation medium: 3.6% soybean meal, 5.2% corn meal; natural pH; and made up to 25 mL per bottle with 0.4% Na₂HPO₄.
The media used in this study were all sterilized at 115°C for 30 minutes.

2.3. ARTP Mutagenesis
In each ARTP mutagenesis, the cells of one loop were inoculated into 25mL seeding medium and cultured to logarithmic growth stage at 37°C on a rotary shaker (298rpm) for 6h. And its OD value was about 0.6. Then 1.5mL of the cells suspension was taken in 5mL centrifuge tube (glycerol was added, and the final concentration was 5%), and mutagenesis was carried out in ARTP mutagenesis system ARTP-M. 10μL of the cells suspension was uniformly coated on the surface of the small sterile iron block (0.5cm diameter). Under the conditions of 100W power and 10.00SLM gas volume, the suspension was treated for 10s, 20s, 30s, 40s, 50s and 60s. After each treatment, a small sterile iron block was transferred into a centrifuge tube containing 1mL sterile saline. And then the centrifuge tubes were placed on a vortex mixer for 1 minute to elute the cells attached to the small sterile iron block to none. The new cells suspension was formed and diluted in a gradient.

2.4. Calculation of Survival Rate and Mutation Rate
Survival rate: It is the ratio of colony number in different mutagenesis time to that in blank control.
Mutation rate: At different mutagenesis time, it is the ratio of mutant strains to all investigated strains under this mutagenesis time. The positive mutant strain was a mutant strain whose enzyme activity was more than 10% higher than that of the original strain, the negative mutant strain was a mutant strain whose enzyme activity was 10% lower than that of the original strain, and the non-mutant strain was between 90% and 110%. The total strain is not less than 100 strains.

2.5. Strain Selection
Initial selection: 0.1mL of the plasma-treated cell suspension was uniformly coated on the selective medium plates, and then cultured at 37°C for 48h. And the number of bacterial colonies on the medium plate was recorded. The bacterial colonies showing a bigger zone of casein hydrolysis were selected and inoculated on the slant medium. They were cultured in 37°C for 46h and maintained at 4°C.
Re-screening: The mutant strains screened by the initial selection were inoculated into 25mL fermentation medium. They were cultured at 37°C for 46h with shaking at 298rpm. The alkaline protease high-yield strains were screened out by measuring the yields of alkaline protease.

2.6. Single-factor Optimization
Based on the re-screening culture conditions of the strains, the effects of different factors in the fermentation medium on the enzyme production capacity of the strains were investigated. The factors were as follows: carbon source concentration (3.6%, 4.0%, 4.4%, 4.8%, 5.2%, 5.6%, 6.0%, 6.4%), nitrogen source concentration (2.0%, 2.4%, 2.8%, 3.2%, 3.6%, 4.0%), Na₂HPO₄ concentrations (0, 0.2%, 0.4%, 0.6%, 0.8%, 1.0%), different metal ions (Mg²⁺, Mn²⁺, Zn²⁺ and their concentrations 0, 0.05%, 0.1%, 0.15%, 0.2%, 0.3%), Tween-80 concentration (0, 0.03%, 0.06%, 0.09%, 0.12%, 0.15%), inoculation volume (1%, 2%, 3%, 4%, 5%, 6%), shaking speed (298 r/min, 270r/min, 240r/min, 210r/min, 180r/min).
2.7. Response Surface Optimization
The results of single-factor experiments showed that the main factors to improve the enzyme production were \( \text{Na}_2\text{HPO}_4 \) concentration, \( \text{Mg}^{2+} \) concentration, and Tween-80 concentration. Table 1 showed three factors and their five levels in this study. Then, a total of 20 experiments were performed by the central composite design (CCD) in the response surface methodology (RSM).

| factor          | level |
|-----------------|-------|
|                 | -α    | -1   | 0    | +1   | +α   |
| (A) Na\(_2\)HPO\(_4\) (%) | 0.6000 | 0.6811 | 0.8000 | 0.9189 | 1.0000 |
| (B) Mg\(_2\) (%) | 0.1000 | 0.1203 | 0.1500 | 0.1797 | 0.2000 |
| (C) Tween-80 (%) | 0.0900 | 0.1022 | 0.1200 | 0.1378 | 0.1500 |

3. Results and Discussion

3.1. Effect of ARTP Mutagenesis Time on Survival Rate
The strain Y9 (6835U/mL) with high enzyme activity was obtained by activating the original strain. The strain Y9 was mutated by ARTP experiments. The results showed in Figure 1. The survival rate decreased substantially as the mutagenesis time increases. The survival rate of the strain decreased slowly when the mutagenesis time were 10s to 20s. The cell damage was little during short mutagenesis time, and the survival rate was higher. The survival rate of the strain decreased sharply when the mutagenesis time were 20s to 40s. The cell damage becomes serious when the mutagenesis time surpassed 20s. The survival rate of the strain increased slightly when the mutagenesis time were 40s to 50s. The cell activated some internal repair mechanism, and the survival rate increased temporarily \[^9\]. The survival rate of the strain decreased again when the mutagenesis time were 50s to 60s.

![Figure 1. Effect of ARTP mutagenesis time on survival rate.](image-url)
3.2. Effect of ARTP Mutation Time on Mutation Rate

In Figure 2, when the mutagenesis time was 10s to 30s, the negative mutation rate was the highest, and there was no positive mutation rate. When the mutagenesis time was 40s to 60s, the negative mutation rate decreased, and the positive mutation rate first increased and then decreased. When the mutagenesis time was 50s, the positive mutation rate was the highest and higher than the negative mutation rate. It was consistent with the modern theory that when the lethal rate of mutant microorganism was between 70% and 80%, the positive mutation rate was often very high \[9\]. Considering the negative mutation rate and the positive mutation rate, 50s was chosen as the optimal mutation parameter.

By successive mutagenesis and screening, a high-yielding alkaline protease strain A59 was obtained. And the enzyme activity of strain A59 was as high as 8433 U/mL, which increased by 23.38% (the original strain Y9 was 6835 U/mL).

3.3. Single-factor Experimental Results

Based on the culture conditions of strain re-screening, the fermentation conditions were studied by single-factor experiments. The fermentation conditions were as follows: carbon source, nitrogen source, Na₂HPO₄, metal ions, Tween-80, inoculation volume, and shaking speed. The results showed in Table 2.

| factor           | Ck Ck A (U/mL) | Max | Max A (U/mL) | R (%) |
|------------------|----------------|-----|--------------|-------|
| carbon source    | 6.0% 8435      | 5.2% | 8890         | 5.39  |
| nitrogen source  | 3.6% 8891      | 4.0% | 8851         |       |
| Na₂HPO₄          | 0.40% 8891     | 0.80% | 10144       | 14.09 |
| Mg²⁺             | 0 10240        | 0.15% | 11451       | 11.83 |
| Mn²⁺             | 0 10246        | 0.05% | 10870       | 6.09  |
| Zn²⁺             | 0 10220        | 0.05% | 10326       | 1.03  |
| Tween-80        | 0 11440        | 0.12% | 12543       | 9.64  |
| inoculation volume| 2% 12564       | 5%   | 13365       | 6.37  |
| shaking speed    | 298r/min 13303 | 270r/min 13330 | 0.2 |

In Table 2, Ck was the control group, Ck A was the average enzyme activity of Ck, Max was the highest level except for Ck, Max A was the average enzyme activity of Max, R was the rate of increase. The optimum conditions were as follows: 3.6% of soybean meal, 5.2% of corn meal, 0.8% of Na₂HPO₄ concentration, 0.15% of Mg²⁺ concentration, 0.12% of Tween-80 concentration and 5% of inoculation volume. The enzyme activity was 13365 U/mL, which was 58.45% higher than the
original condition. The main factors for increasing enzyme production were Na₂HPO₄ concentration, Mg²⁺ concentration, and Tween-80 concentration. And the CCD experiment was performed accordingly.

3.4. Response Surface Experiment Results and Analysis
Three factors, Na₂HPO₄ concentration (A), Mg²⁺ concentration (B), and Tween-80 concentration (C), were selected for the CCD test. The results showed in Table 3. The variance analysis was performed on the data of Table 3 using the statistical analysis software (Design-Expert 10.0.3). The results showed in Table 4.

The quadratic regression equation between enzyme activity and three variables was obtained by response surface methodology:

\[ Y = 13484.39 - 202.07A + 105.32B - 201.87C - 79.70AB - 326.00AC - 101.41BC - 171.65A^2 - 320.21B^2 + 12.78C^2. \quad R^2 = 0.8908. \]

In the equation above, Y is the response value, that is enzyme activity of alkaline protease, and A, B, and C are the coded values of the test variables (Na₂HPO₄, Mg²⁺ and Tween-80 respectively). As shown in Table 4, the model was extremely significant (P < 0.01), and the test of lack of fit was not significant (P > 0.05), which indicated that the model had a good fitting degree. The relatively low coefficient of variation (C.V. = 1.70) indicated that the accuracy and reliability of the experiments performed were improved. The signal to noise ratio was 10.529 >4, which indicated that the model had a small error in the design interval, and the prediction was more accurate. In the model, variables A, C, interaction term AC and quadratic term B² had extremely significant effects on the model; A² had significant effects on the model; B, AB, BC, and C² had no significant effects on the model.

The strength of the factor interaction can be reflected by the contour shape. The shape of the contour line is elliptical or saddle, indicating that the two interactions are significant. The closer the shape of the contour line is to a perfect circle, the less significant the interaction between the two. The response surface 3D map showed in Figure 3.

Table 3. Design and results of the response surface experiment.

| order number | A   | B   | C   | Y    |
|--------------|-----|-----|-----|------|
| 1            | -1  | -1  | -1  | 12837.7 |
| 2            | 1   | -1  | -1  | 13272.38 |
| 3            | -1  | 1   | -1  | 13446.26 |
| 4            | 1   | 1   | -1  | 13301.26 |
| 5            | -1  | -1  | 1   | 13243.4 |
| 6            | 1   | -1  | 1   | 12113.22 |
| 7            | -1  | 1   | 1   | 13185.45 |
| 8            | 1   | 1   | 1   | 11997.31 |
| 9            | -1.681792831 | 0  | 0  | 13330.34 |
| 10           | 1.681792831 | 0  | 0  | 12895.66 |
| 11           | 0   | -1.681792831 | 0  | 12403.01 |
| 12           | 0   | 1.681792831 | 0  | 12982.59 |
| 13           | 0   | 0   | -1.681792831 | 13765.03 |
| 14           | 0   | 0   | 1.681792831 | 13504.21 |
| 15           | 0   | 0   | 0   | 13388.3 |
| 16           | 0   | 0   | 0   | 13388.3 |
| 17           | 0   | 0   | 0   | 13591.15 |
| 18           | 0   | 0   | 0   | 13620.1 |
| 19           | 0   | 0   | 0   | 13575.8 |
| 20           | 0   | 0   | 0   | 13303.5 |
Table 4. Analysis of variance table of CCD experiment.

|                          | sum of Squares | Df | Mean square | F value | P-value | Prob>F |
|--------------------------|----------------|----|-------------|---------|---------|--------|
| model                    | 4059000        | 9  | 451000      | 9.07    | 0.0009  |        |
| A- Na$_2$HPO$_4$         | 557700         | 1  | 557700      | 11.21   | 0.0074  |        |
| B- Mg$^{2+}$             | 151500         | 1  | 151500      | 3.05    | 0.1116  |        |
| C-Tween-80               | 556500         | 1  | 556500      | 11.19   | 0.0074  |        |
| AB                       | 50823.10       | 1  | 50823.10    | 1.02    | 0.3359  |        |
| AC                       | 850200         | 1  | 850200      | 17.09   | 0.0020  |        |
| BC                       | 82275.96       | 1  | 82275.96    | 1.65    | 0.2274  |        |
| A$^2$                    | 424600         | 1  | 424600      | 8.54    | 0.0153  |        |
| B$^2$                    | 1478000        | 1  | 1478000     | 29.71   | 0.0003  |        |
| C$^2$                    | 2351.98        | 1  | 2351.98     | 0.047   | 0.8322  |        |
| Residual                 | 497400         | 10 | 497400      | 4.58    | 0.0601  |        |
| Lack of fit              | 408300         | 5  | 81662.69    | 4.58    | 0.0601  |        |
| Pure error               | 89102.48       | 5  | 17820.50    | 4.58    | 0.0601  |        |
| Cor total                | 4557000        | 19 |             |         |         |        |

Figure 3a showed the interaction of Na$_2$HPO$_4$ concentration and Mg$^{2+}$ concentration on the enzyme activity of the strain when Tween-80 was at the central level and all other variables remained at zero levels. The result showed that the interaction between Na$_2$HPO$_4$ and Mg$^{2+}$ was not significant. Figure 3b showed the interaction of Na$_2$HPO$_4$ concentration and Tween-80 concentration on the enzyme activity of the strain when Tween-80 was at the central level. The result showed that Na$_2$HPO$_4$ and Tween-80 had extremely significant interactions. With the increase in the concentration of each factor, the enzyme activity of the strain increased first and then decreased. Figure 3c showed the interaction of Mg$^{2+}$ concentration and Tween-80 concentration. The result showed that the interaction between Mg$^{2+}$ and Tween-80 was not significant.

The statistical analysis software (Design-Expert 10.0.3) was used to further analyze the response surface. And the optimal levels of three main factors were: Na$_2$HPO$_4$ concentration 0.9115%, Mg$^{2+}$ concentration 0.1593% and Tween-80 concentration 0.09%. And the predicted enzyme activity of the strain reached 14065 U/mL. According to the response surface optimization parameters, three groups of parallel experiments were performed. The enzyme activities of the strain were 13812U/mL, 14091U/mL and 14175U/mL, and the average enzyme activity was 14026U/mL. It showed that F crit $=18.5128>F=0.0323$, then P$>0.05$. It shows that there is no significant difference between the experimental results and the predicted values. After optimizing fermentation conditions, the enzyme activity of the strain increased by 66.32%.

4. Conclusion
The original strain of this study is an industrial production strain. Firstly, the separation and purification experiments were carried out. The strain Y9 was selected as the original strain, and its
enzyme activity was 6835 U/mL. The strain was mutated by ARTP. The results showed that the positive mutation rate of the original strain was higher when the mutagenesis time was 50s. By several mutagenesis, a high-yield alkaline protease strain A59 was obtained, and its enzyme activity was as high as 8433 U/mL, increasing by 23.38 % than that of the original strain.

The effect of various factors on alkaline protease yield was studied by the single-factor experiment. The result showed that the optimum conditions were as follows: 3.6% of soybean meal, 5.2% of corn meal, 0.8% of Na₂HPO₄ concentration, 0.15% of Mg²⁺ concentration, 0.12% of Tween-80 concentration and 5% of inoculation volume. The enzyme activity was 13365 U/mL, increasing 58.48% than that of the original condition. Based on the single factors experiment, the concentration of Na₂HPO₄, Mg²⁺ and Tween-80 was found to be major variables in the alkaline protease fermentation production. Therefore, response surface methodology was used to study their effects on enzyme activity. The variance and significance analysis were performed, and the response model was established. Finally, the optimum conditions were as follows: 0.9115% Na₂HPO₄, 0.1593% Mg²⁺ and 0.09% Tween-80. Through response surface optimization, the predicted enzyme activity of the strain reached 14065 U/mL. The verification experiment was performed under the conditions, the average enzyme activity of the A59 strain was 14026 U/mL, and by the response surface test, the enzyme increased by 4.94%, and the condition optimization was improved by 66.32%, which was 2.05 times higher than that of the original strain.

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