Optimization of fermentation conditions for protease production from Bacillus subtilis

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Abstract: In the paper, the fermentation conditions and fermentation medium of bacillus subtilis protease production were optimized to improve the outcome of the protease produced from bacillus subtilis to provide premium raw materials for the subsequent preparation of chitin from crab shell. The optimal fermentation conditions are temperature at 39°C, pH value of 7.5, fermentation time of 60h, inoculum size of 5%, filling volume of 80/250mL, and rotational speed at 180r/min; the optimal medium component ratio is 1.25% for starch mass concentration, 1% for yeast paste mass concentration, and 0.3% for sodium dihydrogen phosphate mass concentration. The activity of protease produced from bacillus subtilis GC021 is 174.87U/mL under the optimal fermentation conditions and at the optimal medium component ratio.

1. Introduction

Chitin is a kind of natural biological polysaccharide high polymer material and widely used in food, chemical, pharmaceutical, agricultural environmental protection and other fields. Crab shell is an ideal material for chitin preparation for its highly 17%-18% chitin content [1-3], easy availability, and low cost. When preparing chitin from crab shell, the traditional preparation process adopts a large amount of strong acid for decalcification and deproteinization [4-5], impacting the quality of chitin and the recycle of other by-products in the crab shell, corroding production equipment corrosion, and causing serious environmental pollution[6]. Microbial fermentation to produce acids and enzymes and decalcify and deproteinize crab shell can reduce environmental pollution and recycle many valuable crab shell by-products, such as astaxanthin and fatty acid, thus improving resource utilization [7-8].

Bacillus subtilis is a species of bacillu and, an aerobe commonly used in industrial production, with strong protease activity[9]. In the paper, the fermentation conditions and fermentation medium for protease production from bacillus subtilis were optimized to improve the outcome of protease to provide premium raw materials for the subsequent preparation of chitin from crab shell.

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2. Testing Material

2.1 Strain

Bacillus subtilis GC021: Preserved in the lab.

2.2 Materials

Agar-slant medium: 1g peptone,,0.5g beef paste, 0.5g sodium chloride, 2g agar, 100mL distilled water, 7.2 in pH value, and sterilization at 121°C for 20 min.

Seed medium: 1g peptone, 0.5g beef paste, 0.5g sodium chloride, 100mL distilled water, 7.2 in pH value, and sterilization at 121°C for 20 min.

Initial fermentation medium: 1g peptone, 0.5g beef paste, 1g sucrose, 0.2g disodium phosphate, 100mL distilled water, 7.0 in pH value, and sterilization at 121°C for 20 min.

2.3 Reagents

Sucrose, maltose, soluble starch, glucose, lactose, ammonium nitrate, ammonium sulfate, peptone, urea, beef paste, yeast powder, yeast paste, dipotassium phosphate, dipotassium phosphate, disodium phosphate, potassium dihydrogen phosphate, sodium dihydrogen phosphate, Folin-phenol reagent, casein, trichloroacetic acid, anhydrous sodium carbonate, etc., all of which are analytically pure.

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3. Testing Method

3.1 Analysis method

Folin-phenol method was adopted to determine the protease activity: 1.0mL crude enzyme and 2mL 0.5% casein solution were mixed in two testing tubes which were preheated in the water bath at 30°C for 5min and then kept warm in the water bath for 10 min. 3.0mL 10% trichloroacetic acid solution was added into the mixture after the latter was taken out and then centrifuged at 3,500r/min for 10min. 1.0mL Folin-phenol reagent and 5.0mL 0.55mol/L Na2CO3 solution were added into 1.0mL centrifugal supernatant, with the mixture kept warm in the water bath for 15 min before the determination of the light absorption value at A680nm. The tube with added inactivated enzyme solution was taken as the control sample.

Enzyme activity[10]: It was specified that under the reaction conditions, each 1μg tyrosine produced from hydrolyzed casein per minute was counted as one unit of enzyme activity (U).

3.2 Preparation of Crude Enzyme

The activated agar-slant strain (cultivated at 35°C for 20h) was transferred to the seed medium by using transferring loop and oscillated on the shaker at a speed of 160r/min at 36°C for 24h. Then the strain with the inoculum size of 5% was transferred in 50mL fermentation medium and oscillated on the shaker at a speed of 160r/min at 36°C for 60h. The fermentation broth was centrifuged in the refrigerated centrifuge at 3,500r/min for 10min to obtain the supernatant that was crude enzyme.

3.3 Optimization Test of Fermentation Conditions

Enzyme activity was determined according to the method in 3.1 where protease activity level was taken as the evaluation index to examine the effects of different fermentation conditions on activity of protease produced from bacillus subtilis GC021. Initial fermentation conditions: Fermentation temperature at 36°C, medium initial pH of 7.0, fermentation cycle of 48h, inoculum size of 5%, filling volume of 60mL, and rotational speed at 160 r/min.

3.4 Optimization Test of Fermentation Medium

Enzyme activity was determined according to the method in 3.1 where protease activity level was taken as the evaluation index to examine the effects of different carbon sources, nitrogen sources, phosphates and their concentrations on activity of protease produced from bacterial strain.

4. Results and Discussion

4.1 Optimization Test of Fermentation Conditions

As shown in Figure 1, when the fermentation temperature is 30℃-39℃, enzyme activity is gradually increasing, while enzyme activity is rapidly decreasing in the range of 39℃-45℃. Two main reasons are involved, on one hand, the enzyme is denatured and inactivated due to its poor heat resistance; on the other hand, most of the bacteria have died at high temperature. Therefore, the fermentation temperature adopted in this experiment is 39℃.

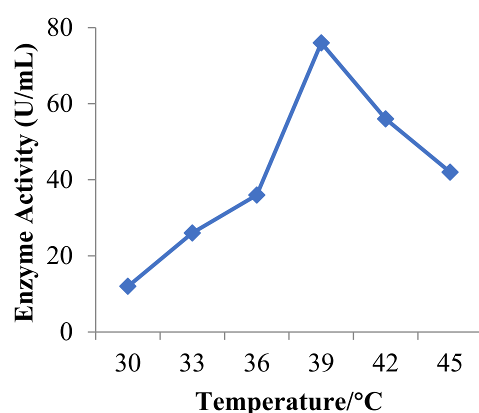

Figure 1. Effect of Temperature on Activity of Fermentation Produced Enzyme

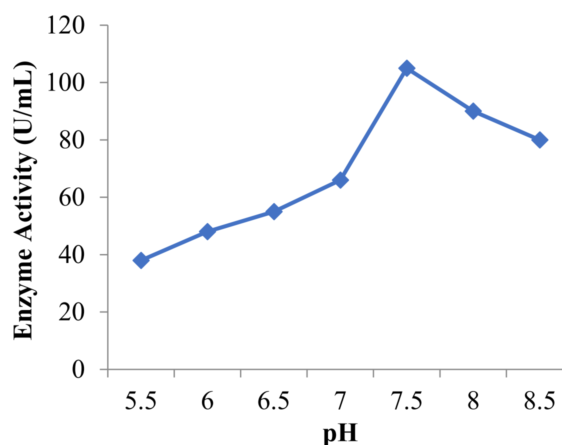

Figure 2. Effect of Initial pH Value of Medium on Activity of Fermentation Produced Enzyme

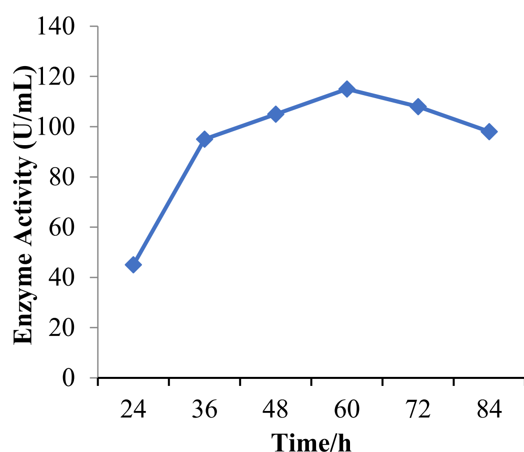

Figure 3. Effect of Fermentation Cycle on Activity of Fermentation Produced Enzyme

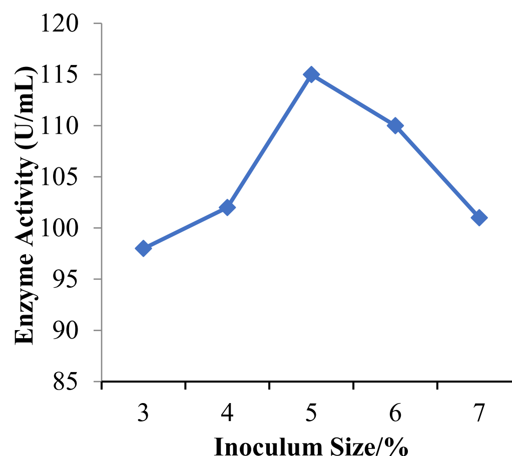

Figure 4. Effect of Inoculum Size on Activity of Fermentation Produced Enzyme

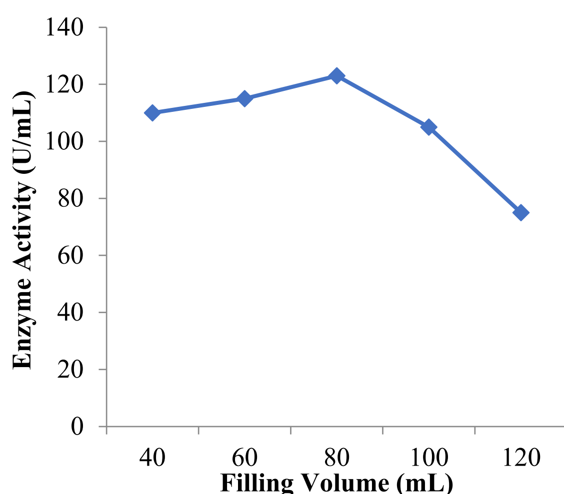

Figure 5. Effect of Filling Volume on Activity of Fermentation Produced Enzyme

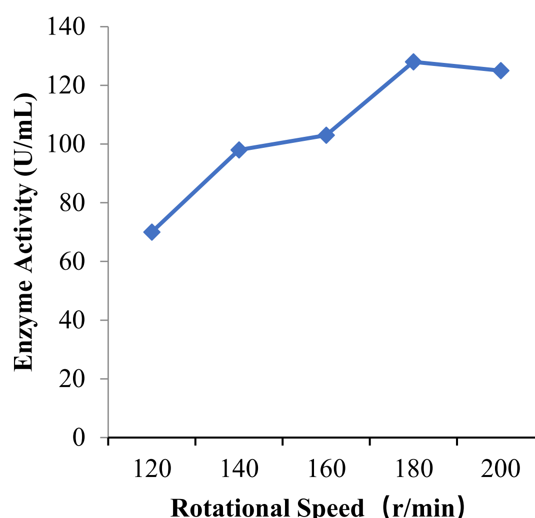

Figure 6. Effect of Rotational Speed on Activity of Fermentation Produced Enzyme

Figure 2 shows that when the pH is between 5.5 and 7.0, enzyme activity increases slowly. Enzyme activity increases significantly between 7 and 7.5, and it decreases gradually when the pH increases. Therefore, the pH of fermentation is determined to be 7.5, indicating that the enzyme produced from this strain is weak alkaline .

In Figure 3, protease activity increases with the extension of culture time at the early stage, reaching the maximum value at 60h for strain fermentation. Then protease activity, however, deceases, possibly because the accumulation of other metabolites of the strain inhibited protease activity. Therefore, 60h is adopted as the optimum fermentation cycle.

It can be seen from Figure 4 that when the inoculation amount is 5%, enzyme activity is the highest. It shows that, when the inoculation amount is 3%-4%, the amount of enzyme will also decrease due to the small amount of cell; but when the inoculation amount is 6% - 7%, the cell amount is too large, the nutrients have been consumed during the growth period of the cell, and the concentration of the nutrients has decreased during the enzyme production period, which is not conducive to the enzyme production, so the protease production has decreased.

As shown in Figure 5, protease activity reaches the maximum value at the filling volume of 80/250mL. Therefore, this filling volume is selected as the optimal shake flask filling volume for protease production.

Bacillus subtilis is an aerobic organism, which can adjust the ventilation rate by changing rotational speed of the shaking table. The test results are shown in Figure 6.With the increase of rotational speed, the protease secretion ability of the strain is constantly strengthened in the range of 120-180 r/min, indicating that the increased rotational speed facilitates oxygen dissolution and enzyme production. In order to ensure that the strain can obtain oxygen to the maximum extent, rotational speed 180 r/min is selected for fermentation and cultivation.

4.2 Optimization Test of Fermentation Medium

The results are shown in Figure 7. Different carbon sources have a great impact on the protease production of the strain. Starch is an ideal carbon source. According to relevant data, low initial medium sugar concentration is not conducive to cell growth, while high initial medium sugar concentration will produce feedback inhibition on cell enzyme production. Starch may gradually form monosaccharide during the hydrolysis process, so it not only meets the requirements of cell growth but also does not inhibit the enzyme production. On this basis, the effect of different concentrations of starch on the enzyme activity is studied, as shown in Figure 8, the optimal mass concentration of starch in the medium was determined to be 1.25% .

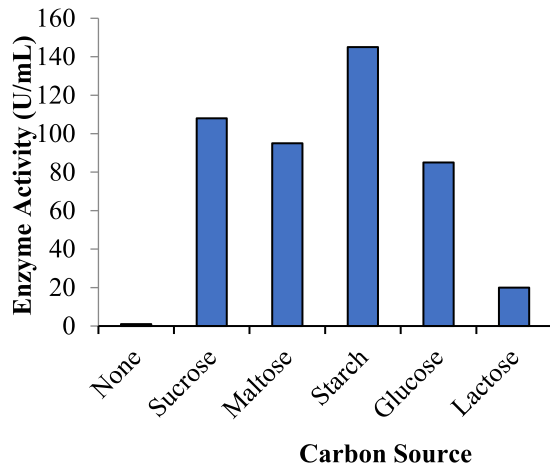

Figure 7. Effect of Different Carbon Sources on Activity of Fermentation Produced Enzyme

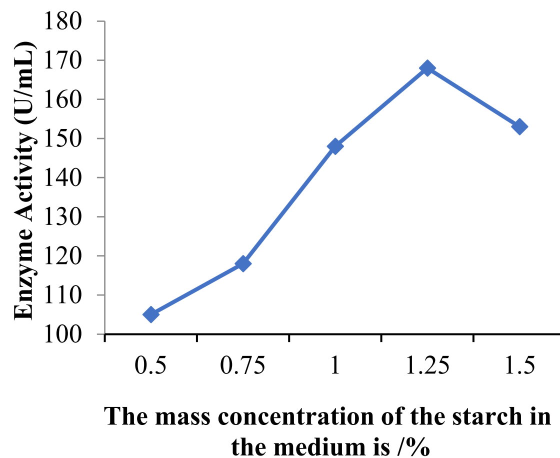

Figure 8. Effect of Starch Concentration on Activity of Fermentation Produced Enzyme

Nitrogen source is the main source of bacterial protein synthesis and genetic material, and also one of the sources of nitrogen in metabolites. It can be seen from Figure 9 that different nitrogen sources also have a great impact on the protease production of the bacteria. The protease activity is the highest when the yeast paste is used as the nitrogen source, so the yeast paste is selected as the best nitrogen source. On this basis, the effect of different mass concentrations of yeast paste on the enzyme activity is studied, as shown in Figure 10, when the mass concentration of yeast paste is 1%, the protease activity is the highest.

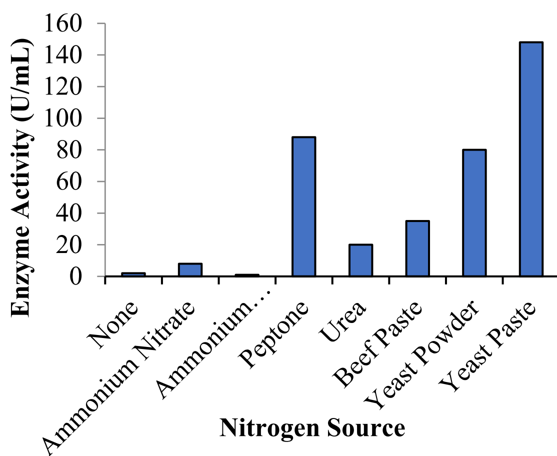

Figure 9. Effect of Different Nitrogen Sources on Activity of Fermentation Produced Enzyme

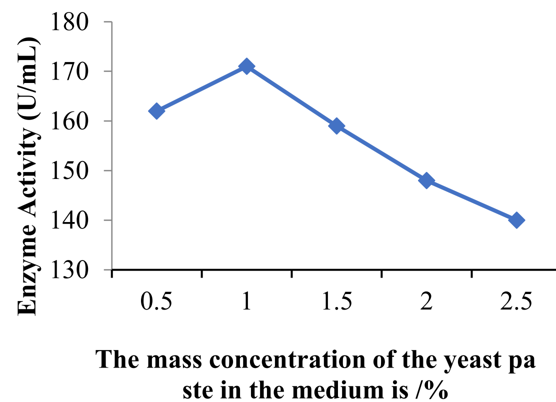

Figure 10. Effect of Yeast Paste Concentration on Activity of Fermentation Produced Enzyme

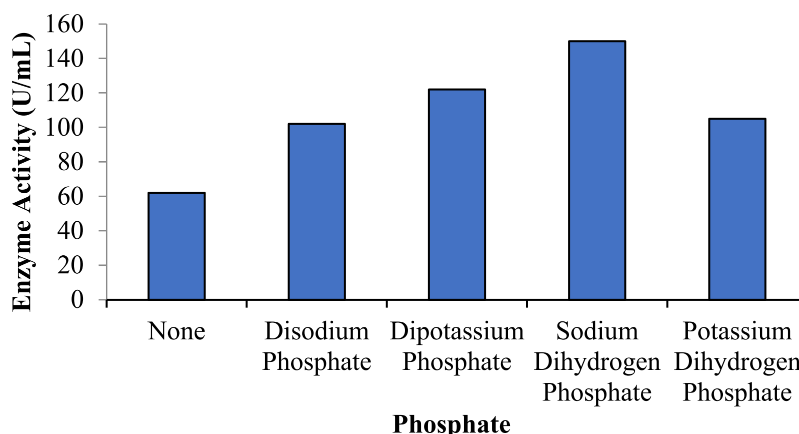

Figure 11. Effect of Different Phosphates on Activity of Fermentation Produced Enzyme

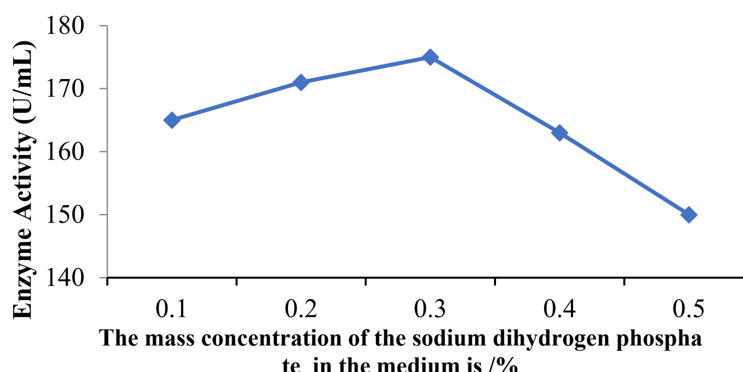

Figure 12. Effect of Sodium Dihydrogen Phosphate on Activity of Fermentation Produced Enzyme

As shown in Figure 11, phosphate has a significant effect on enzyme production, among which sodium dihydrogen phosphate has the greatest effect. Therefore, it is selected for further test. On this basis, as shown in Figure 12, the optimal mass concentration of sodium dihydrogen phosphate in the medium is determined to be 0.3%, with enzyme activity being 175.33U/mL under this condition.

4.3 Verification Test

According to the method in 3.1, 3 groups of parallel fermentation tests were carried out under the optimal fermentation conditions and in the optimal medium, with the tested enzyme activities of 173.68U/mL, 176.36U/mL, and 174.56U/mL, and the average enzyme activity of 174.87U/mL, consistent with the test results.

5. Conclusion

When producing protease from bacillus subtilis GC021, the optimal fermentation conditions (temperature at 39°C, pH 7.5, fermentation time of 60h, inoculum size of 5%, filling volume of 80/250mL, and rotational speed at 180r/min) and the optimal medium component ratio (starch mass concentration of 1.25%, yeast paste mass concentration of 1%, sodium dihydrogen phosphate mass concentration of 0.3%) were determined through test. The activity of protease produced from bacillus subtilis GC021 is 174.87U/mL under the optimal fermentation conditions and at the optimal medium component ratio.

Acknowledgments

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