환경위생공학, 제23권 1호(2008)
Journal of Environmental and Sanitary Engineering, Vol. 23, No. 1, pp.25~31 (2008)

Isolation and identification of *Bacillus megaterium* producing Alkaline α-amylase

Shiru Jia[1] · Yong-Deok Choe[2] · Hoon Cho[3*]

[1]*Biochemical Engineering Lab., Tianjin University of Science & Technology 1038 Dagunanlu, Tianjin 300222, China.*
[2]*Department of Environmental Engineering and Science, University of Seoul, Seoul 130-743, Korea.*
[3]*Department of Polymer Science & Engineering, Chosun University, Gwangju 501-759, Korea*

Abstract

A bacterial strain, *Bacillus megaterium* L-49 has been isolated and identified that produces alkaline α-amylase. The cell is ellipsoidal, about 1.0-1.2 × 3.0-3.6 μm in diameter, Gram-positive, motile, and central partial central. Growth occurs in media containing 7% of NaCl. This strain could utilize D-glucose, lactose, xylose, sucrose, mannose, and maltose, and but it does not utilize D-fructose, and glycogen. Among the various concentrations of saturated ammonium sulfate, the retractation ratio in range of 70 to 100% was about 93%. However, in the case of acetone, it was about 98.7%. EDTA has activating effect and Ca2+ has no effect on alkaline α -amylase activity. The alkaline α-amylase has low thermal stability. The optimal temperature for reaction is 50℃The alkaline α-amylase activity maintained stabilizing at pH 6-11 and the optimal pH for reaction was 9-10.

Key words : *Bacillus megaterium*, alkaline α-amylase, retractation ratio.

* Corresponding author E-mail : hcho@chosun.ac.kr

Ⅰ. INTRODUCTION

Enzymes used in wash industries have been developed in variety and quantity for several years. Alkaline α-amylases, belong to these enzymes, are used not only in wash industries but also in textile and paper industries as well as food production. In addition, hydrolyzing starch in alkaline conditions is benefit to gelatinization at low temperature. Therefore, study of alkaline α-amylase may provide a new method of hydrolyzing starch. α-amylase obtained from *Aspergillus niger* is acid-resistant. However, in the case of α-amylase obtained from *Bacillus subtilis* BF7568 is active in pH range between 5.0 and 7.0. These amylases all have been produced in industry. In late eighties, strains which produced highly thermostable alkaline α-amylase were isolated from different soil samples by India scholar.[1,2] Alkaline α-amylase has not been sold as commodity yet. Although the *Bacillus* sp. producing alkaline amylases, have pH optima between 9.2 and 10.5, all have narrow pH activity curves and most rapidly lose activity at temperatures above 40℃. Although some alkaline α-amylase hasbeen reported, the study was only concerning strain selection and properties of enzyme.[3,4]

In this article, we isolated and identified *Bacillus megaterium* which secretes alkaline α-amylase in high alkaline media. In addition, properties of the alkaline α-amylase were investigated.

Ⅱ. MATERIALS AND METHODS

2.1. Strain isolation

Strains were isolated from different soil samples using a selective medium, which contains 0.5% yeast extract, 0.5% peptone, 1% soluble starch, 0.1% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 1% Na_2CO_3. For the solid medium, agar was used at a concentration of 2.5%. Firstly, soil samples were spread on the medium in the culture plate and incubated at 30℃ for 48 h, and then several amylase producing bacterial colonies were selected from the samples after observing the diameter of concentric zone of excretive starch. Each isolate was subsequently transferred to 500 mL conical flask containing 50 mL liquid medium and allowed to grow in shake culture for 48 h at 30℃. The culture fluid was finally centrifuged and supernatant used for amylase activity assay. The strain that yielded a high level of alkaline α-amylase in alkaline media (pH 11) was selected and identified.

2.2. Alkaline α-amylase activity assay

0.2 ml of methyl ethane sulphonate was added into 20 ml of diluted culture solution, and incubated at 30℃ for 40 and 60 min. After detoxification carried out by dilution, the organism was cultured for 16 h and subsequently diluted as unicellular suspended liquid by saline. This solution was irradiated by ultraviolet radiation (15W, 30 cm), and then 0.1 ml of this solution was added on the culture plate containing 50 μg/ml ampicillin and cultured at 30℃ for 3 days for selecting the mutant4. The strain was kept at 4℃ on the agar slants containing 0.5%yeast extract, 0.5% peptone, 0.1% K2HPO4, 0.02% MgSO4•7H2O , 1% Na2CO3 and 2.5% agar.

2.3. Cultural conditions

Alkaline α-amylase production was carried

out by growing the organism in a liquid medium with the same composition as the isolation medium. Fifty milliliters medium was placed in a 500 mL conical flask and inoculated with the culture. Culture was continued at 30℃ for 72 h in a reciprocating shaker. Before assay, the cells were separated by centrifugation at 5000 g. The clear supernatant was used as crude enzyme preparation.

2.4. Analytical Procedure

Extracellular alkaline α-amylaseactivity was determined by measuring the decrease in iodine color. The reaction contained 1mL diluted enzyme (cell from supernatant), 3 mL boracic acid-borax buffer (pH 9.0) and 1mL of 11% starch solution incubated at 40℃ for 10 min. The reaction was stopped by adding 0.5 mL of this solution to 1 mL of 1N HCl. Two milliliters of 0.01% iodine solution and 6.5 mL distilled water were added to this acidified solution. The optical density of blue-colored solution was determined at 680 nm. The same procedure was repeated using 1 mL distilled water instead of the enzyme sample in order to measure the optical density without the enzyme. One unit of enzyme activity (DUN) is defined as the quantity of enzyme that causes 10% reduction of blue color intensity of starch-iodine solution at 40℃ in 10 min. The starch concentration was determined spectrophotometrically by using iodine solution. Growth was estimated in terms of dry weight of cell. Iodine color decreasing rate measurement: 5 ml of 4% starch was added to the enzyme solution at 60℃. The variety of iodine color and viscosity was determined at different reaction time.

2.5. Crude enzyme extraction

Spray-drying method was follows: culture broth was clarified using spray drier Lab S1 (ANHYDRO COPENHAGEN, DENMARK)]. Salting out method was follows: The cells were separated by centrifugation at 10000 rpm for 10 min. The clarified culture broth was added to saturated ammonium sulfate and was placed steadily. The crude enzyme is filter cake. Organic solvent precipitationmethod was follows: the fermentation filtrate without cells was added to some organic solvent such as ethanol or acetone. It was putted in refrigerator. The sediment through filtration was crude enzyme.

2.6. Properties of Enzyme

The alkaline α-amylase was kept at various temperatures for 30 min. The effect of temperature on enzyme activity was determined by assay activity of remainder amylase. The effect of Ca^{2+} ion and EDTA on enzyme activity was determined by altering their concentration. The effect of pH on enzyme activity was studied by measuring enzyme activity at pH values from 6 to 10.

III. RESULTS AND DISCUSSION

3.1. Identification of Organism

To obtain the strain for effective production of alkaline α-amylase at alkaline medium (pH 11), a number of bacteria were isolated from different soils, and ten strains were screened for effective production of alkaline α -amylase. Among ten strains, the strain-L112 was selected, because it

exhibited the high alkaline α-amylase yield. In order to identify the isolated strain, the Biochemical and physiological characteristics according to Bergey's manual of systematic bacteriology were determined. The results are shown in Table 1.

Table 1. Biochemical and physiological characteristics of strain-L122.

Characteristics	Strain -L112
1) Gram reaction	positive rods
2) Spore morphology	
a) Shape	ellipsoidal
b) Position	central or partial central
3) Motility	motile
4) Catalase	positive
5) Sugar fermentation	
a) glucose	positive
b) lactose	positive
c) xylose	positive
d) sucrose	positive
e) mannose	positive
f) maltose	positive
6) Growth in	
a) Media at pH10-11	positive
b) 7% NaCl	positive
7) Temperature for growth,℃	
a) maximum	45
b) minimum	10
8) Starch hydrolysis	positive
9) VP test	negative
10) Reduction of nitrate to nitrite	negative
11) Use of citric acid	positive
12) Liquefaction of nutrient gelatin	positive
13) Hydrolyzation of fatty acid	positive
14) Methylene blue reaction	positive
15) Cell magnitude	1.0~1.2×3.0~3.6μm

The cell is ellipsoidal, about 1.0-1.2 × 3.0-3.6 μm in diameter, Gram- positive, motile, and central partial central. Growth occurs in media containing 7% of NaCl and in the temperature ranging from 10 to 45℃. Catalase is positive. This strain could utilize D-glucose, lactose, xylose, sucrose, mannose, and maltose, and but it does not utilize D-fructose, and glycogen. In addition, VP test and reduction of nitrate to nitrite were negative. However, in the case of use of citric acid, liquefaction of nutrient gelatin, hydrolyzation of fatty acid, and methylene blue reaction, they were positive. Therefore, this strain was identified as Bacillus megaterium based on the morphological and physiological properties.

3.2. Extract of enzyme and properties of Enzyme

In order to extract the alkaline α-amylase from culture broth, various methods using spray-drying, salt out, and organic solventwere investigated. The results are shown in Table 2.

Table 2. Comparison ofvarious methods of alkaline α-amylase extraction.

Method	Spray-drying	Salt out				Organic solvent	
		Concentration of saturated ammonium sulfate (%)				Ethanol	Acetone
		60	70	80	100		
Retractation ratio (%)	4.2	75.3	93.6	94.6	93.3	89.1	98.7

When the spray-drying method was used, the retractation ratio was only 4.2%. Among the various concentrations of saturated ammonium sulfate, the retractation ratio in range of 70 to 100% was about 93%. However, when below 60% of saturated ammonium sulfatewas used, it was decreased. In the case of organic solvent such as ethanol and acetone, when acetone was used, the retractation ratio was about 98.7%. Although organic solvent method cost a lot, it is benefit to alkaline α-amylase recovery whereas salting out shows high recovery ratio but filter cake forms hardly after salting out process. Spray-drying shows low recovery ratio

but can be manipulated easily. To study effect of Ca^{2+} and EDTA concentration on alkaline α-amylase activity, a range of 0.1 to 0.6% of Ca^{2+} and 0.125 to 1.25% of EDTA were investigated. The results are shown in Figure 1.

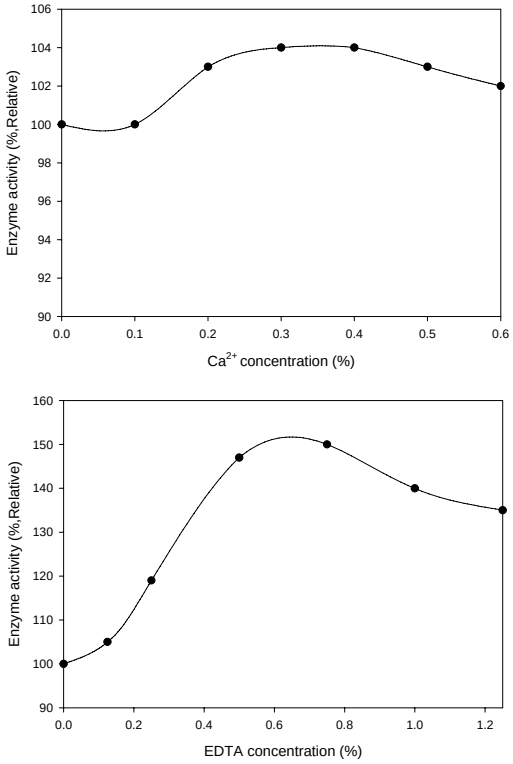

Fig. 1. Effect of Ca^{2+} and EDTA concentration on alkaline α-amylase activity.

When the concentration of Ca^{2+} was a range of 0.2 to 0.5 %, the alkaline α-amylase activity ranged from 103 to 104%. However, below 0.2 % of Ca2+ or above 0.5 % of Ca^{2+}, they were decreased. On the other hand, in the case of EDTA, when the concentration of EDTA was a range of 0.5 to 1.0 %, it ranged from 140 to 145%. However, below 0.5% of EDTA or above 1.0% of EDTA, they were decreased. The results show that EDTA has activating effect and Ca^{2+} has no effect on alkaline α-amylase activity.

3.3. Thermal stability of alkaline α-amylaseand optimum temperature

To investigate the heat stability of alkaline α-amylase, a range of 20 to 80℃ were studied. Experimental conditions are follows: pH 9.0 of alkaline α-amylase 30 min of heat preservation time, and 30 min of reaction time. The results are shown in Figure 2.

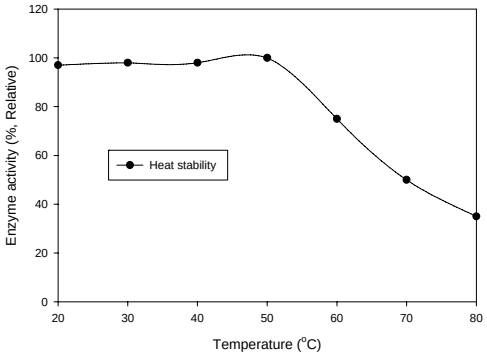

Fig. 2. Effect of temperature on stability of alkaline α-amylase.

When it was carried out at the range of 20 to 50℃ the alkaline α-amylase activity ranged from 97.0 to 99.4%. However, in the case of below 60℃ it was sharply decreased.

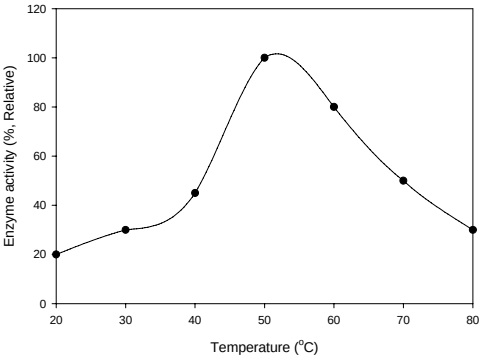

Fig. 3. Effect of temperature on alkaline α-amylase activity.

Figure 3 was effect of temperature on α-amylase activity at pH 9.0 for 10 min of reaction. The residuary alkaline α-amylase activity was lower than 30% after being maintained for 10 min at 70℃ This shows that the alkaline α-amylase has low thermal stability. The optimal temperature for reaction is 50℃

3.4. Effect of pH on enzyme activity

To study the effect of pH on stability of alkaline α-amylase, range of pH 2.0 to 12.0 was investigated. Experimental conditions are as follows: 50℃of treatment temperature, 30 min of treatment time, 50℃of reaction temperature, and 10min of reaction time. The results are shown in Figure 4.

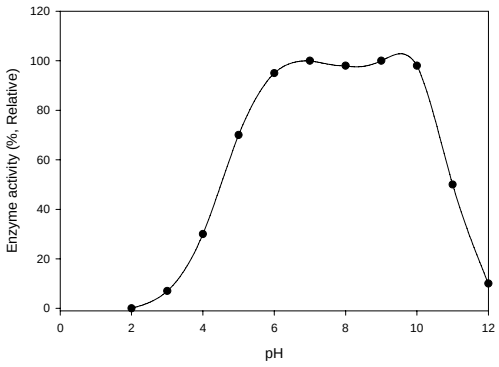

Fig. 4. Effect of pH on stability of alkaline α-amylase.

When pH was increased from 2.0 to 6.0, the stability of alkaline α-amylase was increased from 1.2 to 98%. Especially, it was stable in range of pH 6.0 to 10. However, in the case of above pH 11, it was sharply decreased. Finally, it was about 7.2% at pH 12. Figure 5 was effect of pH on alkaline α-amylase activity for 10 min at 50℃ of reaction. Figure 5 was effect of pH on alkaline α-amylase activity for 10 min at 50℃of reaction. When pH was increased from 4.0 to 6.0, α-amylase activity was increased from 40.4 to 98.5%. Especially, itwas stable in range of pH 6.0 to 11. However, in the case of above pH 12, it was sharply decreased. Finally, it was about 23.2 % at pH 12. These results show that the alkaline α-amylase activity maintained stabilizing at pH 6-11 and the optimal pH for reaction was 9-105.

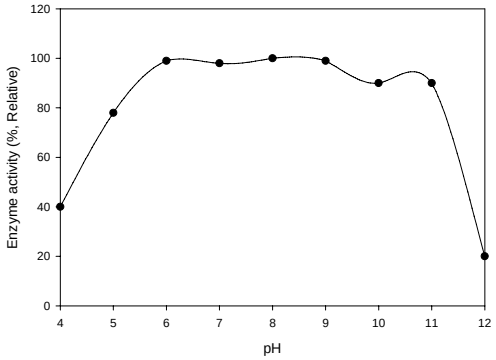

Fig. 5. Transformation of rate of iodine color fade and viscosity.

To study the effect of transformation of rate of iodine color fade and viscosity, various reaction timeswere investigated. When reaction time was increased 0 to 15hr, the viscosity transformation was decreased from 100 to 43%.

However, after 20 hr of reaction, it was not decreased. In the case of transformation of rate of iodine color fade, when reaction time was increased 0 to 10 hr, it was sharply decreased by about 1.9%. However, after 15 hr of reaction, it was not decreased. These results show that it can be concluded partially that enzyme produced from strain is alkaline α-amylases.

IV. CONCLUSION

Alkaline α-amylase produced from Bacillus megaterium L-49 was stabilized in the broad pH range 6.0~11.0. Ca^{2+} had no effect on enzyme activity. Activating effect of EDTA on enzyme activity indicated that this enzyme can be used in alkaline scour and was resistant to high calcic water. When reaction time was increased 0 to 10 hr, transformation of rate of iodine color fade of alkaline α-amylases was sharply decreased by about 1.9%. However, after 15 hr of reaction, it was not decreased. These results show that it can be concluded partially that enzyme produced from strain is alkaline α-amylases.

REFERENCES

1. Pratima, B. and Pramod, K. P.: High-temperature alkaline alpha-amylase from Bacillus licheniformis TCRDC-B13. Biotech. Bioeng., 33: 72-78, 1989.
2. Buchanan, K. E. and Gibbons, E. S.: Bergey's Manual of Determinative Bacteriology, 8th Ed., Baltimore, 1974.
3. Medda, S. and Chandra, A. K.: New strains of Bacillus licheniformis and Bacillus coagulans producing thermostable alpha-amylase active at alkaline pH. J. Appl.Bacteriol., 48: 47-58, 1980.
4. Kazumasa, H., Yamane, K. and Maruo, B.: Stepwise introduction of regulatory genes stimulating production of alpha-amylase into Bacillus. Agr. Biol. Chem., 43: 2343-2349, 1979.