PURIFICATION OF α-AMYLASE FROM PENIBACILLUS SP

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ABSTRACT

The bacterial strain Penibacillus sp was shown to produce extracellular α-amylases activity. The enzyme was purified to homogeneity with an overall recovery of 24 % and specific activity of 57.1 U/mg. The native protein showed a molecular mass of 160 kDa composed of a homodimmer of 82 kDa polypeptide by SDS-PAGE. The optimum pH and temperature of the amylase were 5.5 and 45ºC, respectively. The purified enzyme was stable from pH 7.5 to 9.0 and able to prolong its thermal stability up to 50ºC. The purified amylase shows interesting properties useful for industrial applications.

Keyword: Amylase, purification, Penibacillus sp.

Introduction

Starch is a major carbohydrate reserve in plants. Variations in the extent of starch hydrolysis create a range of important products with different physical properties for food and pharmaceuticals (Khire, 1994). Enzymatic conversion of starch to dextrins and sugars take place by the enzymes obtained from plants, animals, bacteria, and fungi (Fogarty, 1983). Amylase has a wider application in the biotechnological-based food, detergent, and pharmaceutical industries (Mohapatra et al., 1998). Amylases are extracellular enzymes which hydrolyze starch into a range of products such as glucose and maltose (Messaoud et al., 2004; Hashim et al., 2005). Although amylases can be derived from several sources, the enzymes from microbial are preferred in industrial sector and a large number of them are available commercially (Kathiresan and Manivannan, 2006). Microbial amylases have successfully replaced chemical hydrolysis of starch in starch processing industries. Amylase has been derived from several microorganisms however; enzymes from fungal and bacterial sources have dominated applications in industrial sectors (Haska and Ohta, 1994; Pandey et al., 2000). The bacterial α-amylases were derived from Bacillus subtilis, Bacillus myocodes, Bacillus polymyxa and Bacillus aterrimus (Bessler et al., 2003). Bacillus species are employed for commercial applications to produce α-amylase (Kikuchi et al., 1993; Siedenberg et al., 1997; Mamo and Gessesse, 1997; Abe, 1998). Fungal and bacterial systems are widely used for the production of α-amylases but bacterial system is preferred (Pandey et al., 2000). Therefore, the present investigation was undertaken to study the purification of extracelular α-amylase from Penibacillus sp.

Materials and Methods

Screening for amylase producers

The isolated colonies were streaked onto starch medium containing (g/L), soluble starch, 20; casein enzyme hydrolysate, 10; K_2HPO_4, 0.8; $MgSO_4$, 6; NaCl, 5 and agar, 20 with pH 7.0. Inoculated plates were incubated overnight at 40°C to obtain colonial growth. The zone of hydrolysis was visualized by flooding the plates with Gram's iodine solution. The colonies with clear zones were evaluated as amylase producers. Microbiological properties of the isolated strain were determined according to the methods described in Bergey's Manual of Determinative Bacteriology (Holt et al. 1994).

Identification of potent amylase producer

The isolate was identified as Penibacillus sp according to the methods described in "Bergey's Manual of Determinative Bacteriology" and on the basis of the 16S rDNA sequence analysis. The genomic DNA of the pure bacteria was extracted using the Genejet™ Genomic DNA purification kit according to the manufacture's instructions. A polymerase

chain reaction (PCR) was performed using ITS1 and ITS4 primers. A single discrete PCR amplicon band was observed when resolved on agarose gel. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 27F/1492R primers for bacteria, and then performs 35 amplification cycles. Sequencing was performed by using Big Dye terminator cycle sequencing kit. Sequencing products were resolved on an Applied Bio-systems model 3730XL automated DNA sequencing system. Sequences were submitted to GenBank on the NCBI website (http://www.ncbi.nlm.nih.gov). Sequences obtained in this study were compared to the Gen Bank database using BLAST software on the NCBI website (http://ncbi.nlm.nih.gov/BLAST/). Fifteen sequences including our SD5 were selected and aligned using multiple alignment software program Clustal W and the phylogenetic tree was constructed using MEGA 4 (Tamura et al., 2007).

Production of amylase

For amylase production, the bacteria suspension 1ml was inoculated in 250 ml Erlenmeyer flasks containing 50 ml starch medium without agar. The flasks were incubated on a rotary shaker 150 rpm at 40°C for 72 h. Ten-milliliter samples were taken at 2 h intervals, centrifuged at 5000 rpm for 20 min at 4°C, and supernatants considered for enzyme activity and protein content.

Preparation of the crude enzyme

At the end of incubation period, the culture was centrifuged at 5000 rpm for 20 min at 4°C. The obtained filtrate was then estimated for both protein content and amylolytic activity.

Amylolytic activity

This was done according to Bergmann et al. (1988) by estimating the released reducing sugars from 1% starch in 0.5 M citrate-phosphate buffer (pH 6.0) at 40 ºC. Amylase activity was determined by measuring released reducing sugars using the DNS method (Miller 1959). One unit of enzyme activity (U) was defied as the amount of protein that produced 1 mg of reducing sugar per ml of enzyme solution at 40 ºC in 30 min.

Protein determination

Protein was determined either by measuring the absorbance at 280 nm (Warburg and Christian 1942) or by the method of Bradford (1976) using bovine serum albumin as a standard.

Purification of α-amylase production from Penibacillus sp

The purification of α-amylase was carried out in three steps. Grounded ammonium sulfate was gradually added to the chilled enzyme solution while stirring until 80 % saturation was obtained. The solution was the stirred at 0 ºC for 2 h. The precipitate was collected by centrifugation at 5000 rpm at 4 ºC for 30 min (Green and Hughes 1955). The protein pellet was dissolved in a minimal volume of 0.5 M citrate-phosphate buffer pH 6. The enzyme solution was dialyzed overnight against the same buffer at 4 ºC. The dialyzed fraction (60 % saturation ammonium sulfate) was then applied directly to ion exchanger DEAE-cellulose column (2.5 X 60 cm). The adsorbed amylase was eluted by a linear gradient of NaCl from 0 to 0.3 M in the same buffer. Fractions in 5 ml volume were collected at a flow rate of 0.7 ml/min. The eluted fractions were monitored at 280 nm for protein and assayed for enzyme activity (Bergmann et al 1988; Warburg & Christian 1942). In final step the active fractions were pooled, concentrated and dialyzed against the same buffer and loaded onto a gel filtration column (2.5 X 90 cm) Sephadex G-200 and flow rate was maintained at 0.5 ml/min. The eluted fractions were monitored at 280 nm for protein and assayed for enzyme activity

Polyacrylamide gel electrophoresis

Electrophoresis under non-denaturing conditions was performed in 10 % (w/v) acrylamide stab gel according to the method of Davis (1964) using a Tris-glycine buffer, pH 8.3. Protein bands were located by stained with Coomassie Brilliant Blue R-250.

Molecular weight determination

Molecular weight was determined by gel filtration technique using a Sephadex G-200 (Andrews 1964 and 1965). The column (2.4X80 cm) was calibrated with pepsin (35 KDa), egg albumin (45 KDa) and phosphorylase b (97 KDa). Dextran blue (2,000,000) was used to determine the void volume. Subunit molecular weight was determined by SDS-polyacrylamide gel electrophoresis (Laemmli 1970). SDS-denatured phosphorylase b (97 KDa), bovine serum albumin (67 KDa), ovalbumin (45 KDa) carbonic anhydrase (30 KDa), Trysin inhibitor (20 KDa) and α-Lactalbumin (14 KDa) were used as standard protein molecular weight markers.

Enzyme characterization

The purified enzyme was characterized with respect to its optimum pH, temperature, stability at different temperature and pH values on activity and stability. Amylase activity was assayed at different pH values (pH 4.5-10) using different buffers 0.5 M such as citrate-phosphate buffer (pH, 4.5-7), sodium phosphate buffer (pH, 6.5-8), tri-HCl buffer (pH, 7.0-8.5) and glycin NaOH buffer (pH, 8.6-10). To determine pH stability, amylase preparations in buffer at different pH ranging from 4.5-10 were activity assayed under standard conditions. Amylase activity was assayed at different temperatures ranging from 20-60 ºC at pH 6.0 in citrate-phosphate buffer (0.5 M). To determined thermo stability, amylase preparation was incubated at temperature ranging from 20-80 ºC.

Results and discussion

Screening of microorganisms

The bacterial strain exhibited large clear zone around the colony on starch agar plate was tested of the characters according the methods described in Bargey's Manual, and 16S rDNA gene product with approximately 1418 bp was sequenced for the isolated microorganism. This sequence, containing at least 1100 bp constituted by nucleotides with Phred scores ≥20, was used for the database query (Figure 1). The organism selected was identified and designated as Penibacillus sp.

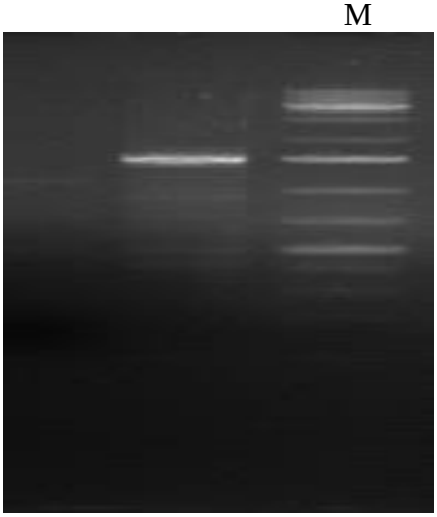

Figure (1): the recovered PCR product after gel purification

Purification of α-amylase

The culture supernatant was used as a starting material for the purification of amylase from Penibacillus sp. The enzyme was purified by a three-step strategy including ammonium sulphate precipitation and dialysis, ion exchange chromatography, and gel filtration (Figure 2

and 3). The recovery of dialysed enzyme was 85% followed by 76% in ion-exchange chromatography and 24% in gel filtration (Table 1). The overall purification strategy attained 21-fold purification of amylase with specific activity of 57.1 U/mg. The molecular weight of the enzyme was about 149 kDa protein using gel filtration techniques. This value was confirmed by SDS-PAGE (Figure 4), where subunit molecular weight of α-amylase was estimated to be 82 kDa protein as dimmer subunit. The result obtained from the column was similar to the molecular weight estimated using to SDS-PAGE. Therefore, it can be concluded that α-amylase is a dimmeric protein. Other molecular masses for different amylases have been reported: 55 and 65 kDa protein for Acinetobacter sp. amylase I and amylase II, respectively (Onishi and Hidaka 1978); 56 kDa protein for Halothermothrix orenii (Mijts and Patel 2002); 58 kDa protein for Haloferax mediterranei (Perez-Pomares et al. 2003); 74 kDa protein for Natronococcus sp. (Kobayashi et al. 1992); and 89 kDa protein for Micrococcus halobius (Onishi and Sonada 1979).

Table 1 Purification of α-amylase from Penibacillus sp

Purification steps	Activity (U)	Protein (mg)	Specific activity (U/mg)	Purification fold	Recovery (%)
Supernatant	200	75	2.66	1	100
$(NH_4)_2SO_4$ (60 %)	170	34	4.90	1.8	85
DEAE-cellulose					
A1	27. 4	23	1.20	0.45	13.5
A2	153.3	3.8	6.67	2.5	76
Sephadex G-200					
A2	48.5	0.8	57.1	21.2	24

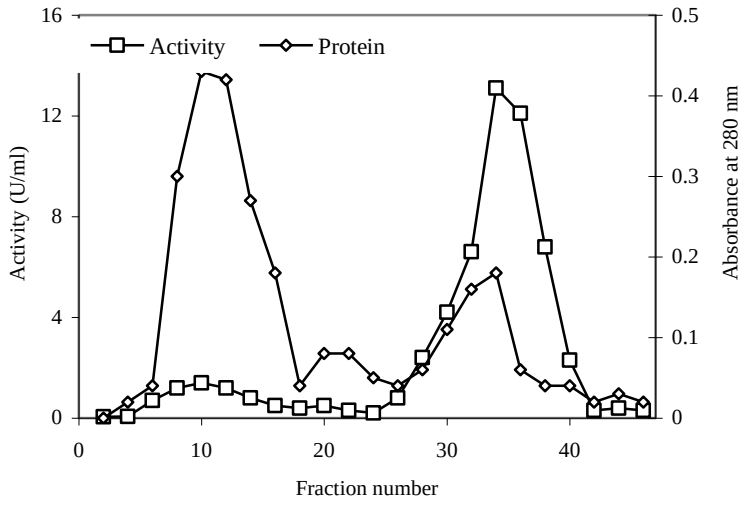

Figure (2). Typical elution profile for the chromatography of amylase on DEAE-cellulose column

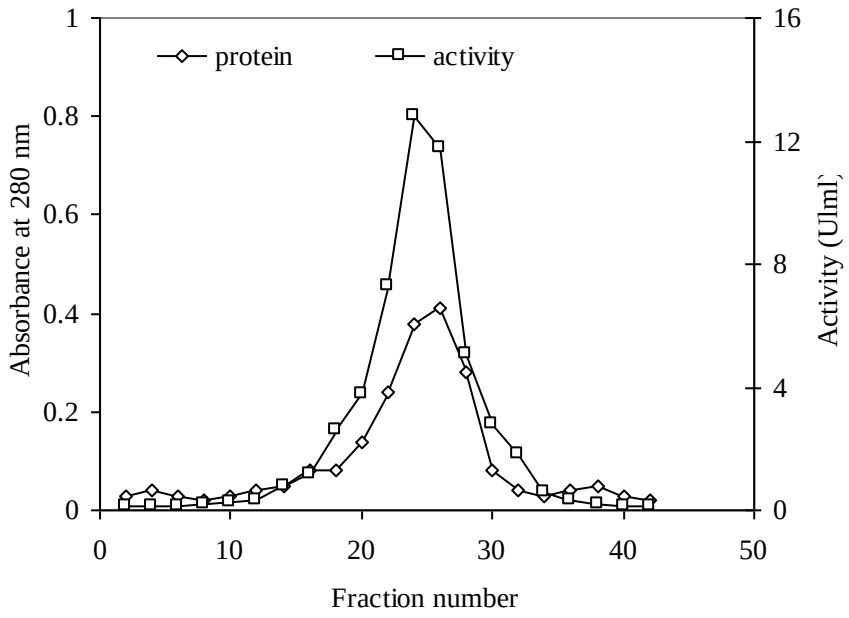

Figure (3). Gel filtration for the chromatography of Amylase DEAE-cellulose faction on Sephadex G-200 column

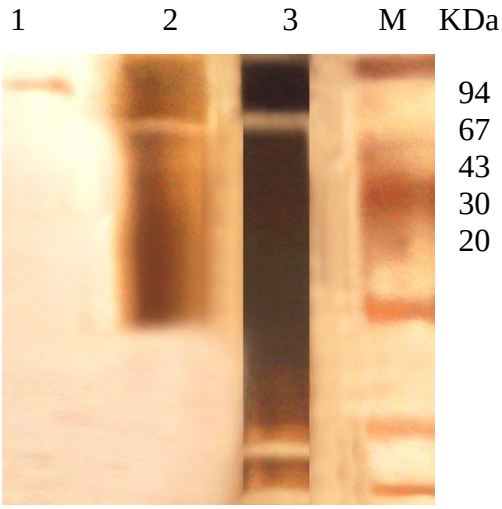

Figure (3). Polyacrylamide gel electrophoresis for Penibacillus sp during purification steps.1-Crude enzyme; 2-DEAE-cellulose; 3-Sephadex G-200 and M-Standard protein

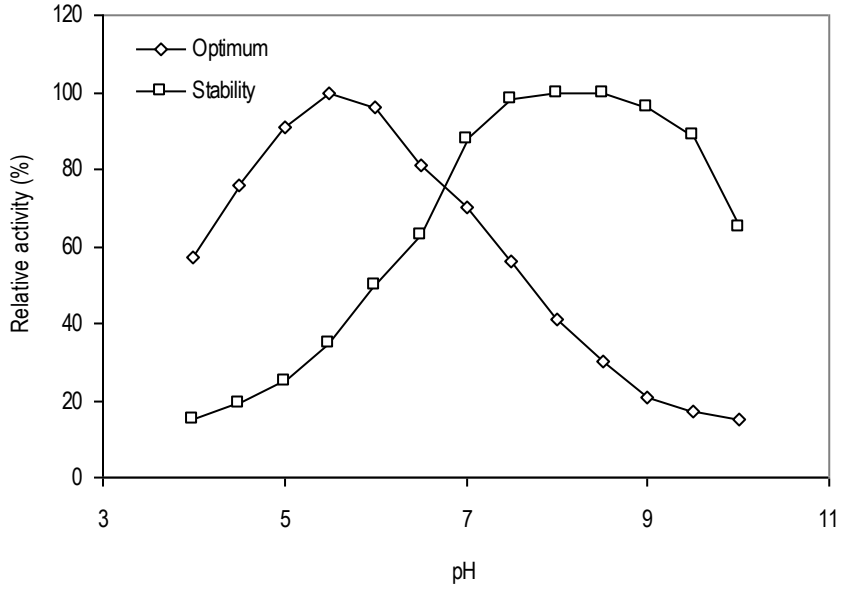

Figure (4). Optimum pH and stability of Penibacillus spamylase

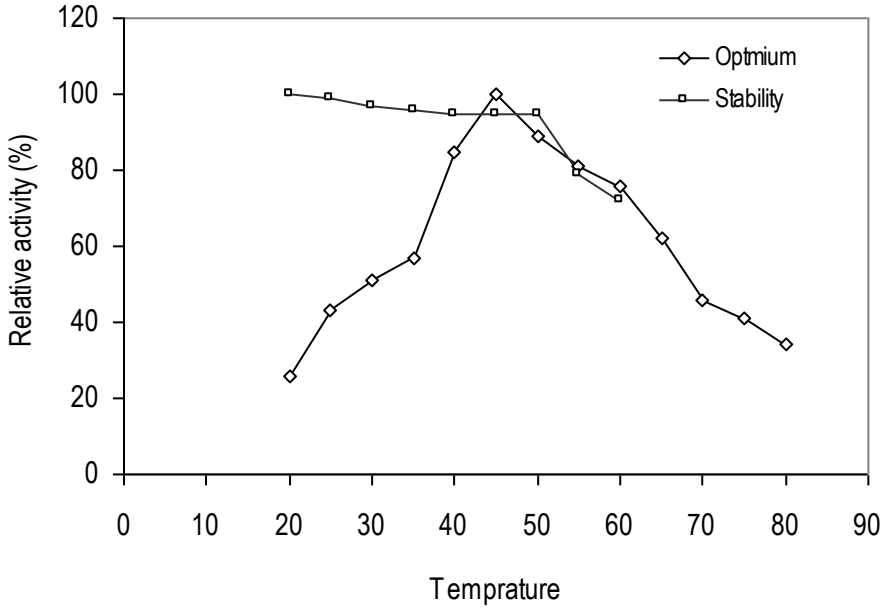

Figure (5). Optimum temperature and stability of Penibacillus sp amylase

Characterization of the purified α-amylase

Effect of pH on enzyme activity and stability

Enzyme activity was measured using the standard assay method by varying the pH values ranging from pH 4.0 to 10. The optimum pH of the purified α-amylase was determined as pH 5.5 in 10 mM sodium phosphate buffer (Figure 5), where the enzyme retained 20 % of its activity below pH 7. Meanwhile, the purified α-amylase was stable from pH 7.5 to 9.0 (Figure 5). The results showed that the enzyme was very stable at the pH 9.0 and retained 37 % and 11 % of its activity at pH 7.0 and 9.0, respectively. Therefore the present amylase can have potential applications for hydrolyzlyzing starch under high pH conditions in starch and textile industries and as ingredients in detergents for automatic dishwashers and laundries (Grant and Horikoshi 1989; Ozaki & Tanaka 1990).

Effect of temperature on enzyme activity and stability

The activity of the purified α-amylase was measured at different temperatures at pH 5.5 by the standard assay method. The optimum temperature for α-amylase from Penibacillus sp was 45 °C (Figure 6). The activity was still retained up to 50 °C at pH 5.5. However, it began to lose 54 % of its total activity at around 70°C and was only able to retain 25 % of its remaining activity at 80°C (Figure 6). The optimal temperature for the α-amylases from Penibacillus sp was in the same range as the optimal temperature 5 °C reported for the enzyme from Natronococcus sp. (Kobayaski et al. 1992).

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الملخص العربي

تنقية انزيم الالفا-اميليز من بكتريا بينبسللس

للسادة الدكاترة

خالد الشيباوى محمد- ابتسام مختار القاضى

مـــــــــــــن

قسم التكنولوجيا الحيوية الميكروبية، المركز القومي للبحوث، الدقي، القاهرة، مصر

وقد أظهرت السلالة البكتيرية بينبسللس قدرة على إنتاج انزيم الالفا-اميليز خارج الخلية. تم تنقية انزيم الالفا-اميليز بالطرق المختلفه مثل الترسيب بكبريتات الامونيوم والفصل على الاعمده الكروماتوجرافيه حيث امكن الحصول على انويم متجانس بنسبه 24%. أظهر البروتين الأصلي كتلة الجزيئية من 160 كيلو دالتون على الجل كلروماتوجرافى بينما كان 82 كيلو دالتون بواسطة PAGE-SDS مما تؤكد انه عباره عن وحدتين كلا منها وزنه الجزيئى 82 كيلو دالتون. كانت درجة الحموضة ودرجة الحرارة المثلى للأميليز 5.5 و 45 درجة مئوية، على التوالي. كان الانزيم المنقى ثابت على درجة الحموضة 7.5-9.0 ودرجه حراره 50 درجة مئوية. يظهر الأميليز المنقى امكانيه استخدامه فى عديد من للتطبيقات الصناعية.