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Purification and Immobilization of Amylase Enzyme Produced from Local Isolate of *Bacillus subtilis*

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Abstract

Amylase enzyme produced from *B. subtilis* was purified by two main steps included precipitation with ammonium sulfate 40-80% saturation and gel filtration chromatography on Sephadex column (G-100). The obtained purification fold and recovery were (67.92) and (43.9) % respectively.

The enzyme was immobilized by entrapment in calcium alginate beads. The enzyme retained 53% of it's original activity after a month of storage at 4°C. On the other hand, free enzyme lost its activity completely in less than 20 days. These results were revealed the possibility of using purified amylase in industry fields. Results also indicate the efficiency of immobilization technique by calcium alginate beads and preserving of the enzyme activity for a relatively long period of time.

Keywords: Amylase, *B. subtilis*, Purification, Immobilization.

Introduction

Amylases are among the most important enzymes used for industrial purposes, and now in the light of biotechnology they are considered useful for biopharmaceutical applications. They are useful tools in medicinal and clinical chemistry. They are most widely used enzymes in the industry for starch

hydrolysis (α-amylase [EC 3.2.1.1, α-1,4-glucan-4-glucanohydrolase] which catalyses the undo cleavage of α-1,4-glycosidic linkages and releases short oligosaccharides and α-limit dextrins) [1,2].

Alfa-amylase was purified from cotyledons of mungbean seedlings after five days of germination using ammonium sulfate precipitation, DEAD cellulose and Sephadex G-150 column chromatography [3].

To a homogenous state ,Rahman *et al.* [4], purified amylase from juice of healthy and diseased sugar cane, Ishurdi (Isd)-20 by successive chromatographies on DEAE - Sephadex A-50 and Sephadex G-150.

Traditionally the purification of amylases from fermentation media has been done in several steps which include centrifugation of the culture (a step of extraction may be required for solid media), selective precipitation of the enzyme by ammonium sulfate or organic solvents such as ethanol in the cold. Then the crude enzyme is subjected to chromatography (usually affinity or ion exchange chromatography) and gel filtration [5]. As bacterial α-amylases have generally been produced from the strain belonging to genus *Bacillus*, several attempts have been made at their purification and characterizations, from both mesophilic and thermophilic strains [6]..

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A study described a thermostable extracellular α-amylase from *B. subtilis*, which was purified 24-fold to a specific activity of 2200 units/mg portein per liter, while another study purified an α-amylase to homogeneity using a combination of ammonium sulfate precipitation, ion-exchange chromatography and gel filtration [6].

Immobilized enzymes are used in food technology, biotechnology and analytical chemistry because of their various advantages. Immobilization of enzymes facilitates the purification of the reaction systems (separation of the reactants and products easily from the reaction media) and recovery of enzyme and make it possible to use the enzyme repeatedly or continuously [7]. There are many different procedures for the immobilization of an enzyme. Covalent binding to an activated support, copolymerization of the enzyme molecules with the polymers, cross linking between the enzyme onto a solid support and entrapment of the enzyme molecules in polymeric structures, are some of the ways of immobilization [7, 8]. Immobilization of enzyme on insoluble supports has been a topic of active research in enzyme technology and is essential for their application to industrial processes. Several methods have been developed for the preparation of immobilized α-amylase with each having its own advantages and disadvantages specific to the methods [9], α-amylase has been commonly immobilized by entrapment within organic gels, such as acryl amide copolymers [7]. Calcium alginate a non toxic polyanion, is widely used as an entrapping agent [10]. Micro capsules of calcium alginate coated with a polycation have been widely investigated for applications like immunoprotective containers in cell transplantation [11], enzyme immobilization [12] and drug release systems [13].

The present study describes the purification and immobilization of amylase enzyme produced from local isolate of *B. subtilis* as a first step towards understanding its properties.

Material and Methods

Organism and Culture Conditions

Bacillus subtilis strain was isolated and identified in a published paper by Hamza and Mhmood [14]. It was cultured in a fermentation medium contains wheat bran moisted with a nutrient broth (solid state fermentation) . A 250 ml Erlenmoyer flasks contain 10 gm wheat bran (1 : 4 w/v) were used. The medium was adjusted to a pH (7.0) with (1N NaOH) before sterilization. After inoculation with 34×10^9 cell/ml broth culture of *Bacillus subtilis* , the flasks were incubated at 37°C for 72 hrs. [14].

Enzyme assay

Amylase activity was determined by two methods:

a) Semi-quantitative method using starch agar plates [15]. The starch agar used consists of a mixture of soluble starch (0.5%) and agar (2.5%) to set it into jelly. The starch agar was mixed, heated to sterilize it, then whilst still molten it was poured in a thick layer into sterile Petri dishes. After it set, it had holes (wells) cut in the center of plate using a heat – sterilized cork bores, and the discs were removed aseptically. Various suspected enzyme – containing substances were added to the wells (about two drops) very careful not to overflow the well.

Allow the starch-agar plate with enzyme to incubate at 37°C for 18 hr, after incubation the diameter of each clear zone (indicate of amylase activities) in millimeters was measured after flood the top of starch agar plates with few drops of Gram's iodine solution.

b)Quantitative method according to Somogyi's method [16]. The assay mixture contained (1ml) of buffered starch substrate (0.04%, pH 7.0), after 3 min. in water bath at 37°C, (0.1 ml) of sample was added. The tubes containing reaction mixture were incubated for exactly (15 min) in water bath at 37°C. After that added (0.4 ml) of working iodine solution (0.01 N) and (8.5 ml) of water, then the mixture was measured at 660 nm in spectrophotometer (type UV-9100). One unit of α-amylase activity (unit/ml) was defined as the amount of enzyme digesting (5 mg) of starch in these conditions.

Total protein assay

Protein concentration was determined by the absolute method [17]. The protein in the samples was precipitated by (7% V/V) perchloric acid, after centrifugation at 5000 rpm/15min, the precipitated protein was dissolved in minimum amount of (0.05 N) NaOH. The absorbency was measured at 235 and 280 nm respectively using (0.05 N) NaOH solution as blank. The protein concentration was estimated according to the following equation:

$$Protein Conc.(mg/ml) = \frac{A_{235} - A_{280}}{2.51}$$

Enzyme Purification

The amylase enzyme was extracted from the fermentation medium by adding (50 ml) distilled water to the fermentation flasks with mixing for about 20 min in ice bath. The mixture was centrifuged in a centrifuge (type NUVE NF 615) at 5000 rpm/15 min to remove the cells and the residues medium. The supernatant (crude enzyme) was clarified by filtration through glass wool, and was used as a source of enzyme for further works. Partial purification of amylase from the crude culture filtrate was done by adding appropriate quantities of ammonium sulfate at 4°C [18] to obtain different fractions (i. e. 20% , 40%, 60%, 80%, 100%). The precipitated enzyme were dissolved in the minimum amount of phosphate buffer (0.005 M, pH 7.0). The protein fraction so precipitated, possessing amylase activity, was loaded on Sephadex G-100 column (1 × 25 cm), previously equilibrated with (0.01 M) phosphate buffer (pH 7.0) and eluted with the same buffer at flow rate of 30 ml/hr. Fractions of (5 ml) were collected and monitored for protein (A_{280} nm) and amylase activity.

Enzyme Immobilization

Amylase enzyme was immobilized by gel entrapment using alginate gel. The purified amylase (about 100 units/ml) was added to 10 ml of (3%) sodium alginate solution. After mixing very well for about 10 min, the mixture was dripped from a height of approximately 20 cm into excess (100 ml) of stirred (0.2 M $CaCl_2$) solution with a sterile syringe at room temperature with diameter about 2 mm. Immerse about (1gm) of the gel beads prepared in (10 ml) of buffered starch solution (0.04%, pH 7.0) in a sterile flask. The flask was incubated at 37°C for 18 hr, then the amylase activity was determined in the reacted starch solution.

Result and Discussion

In the present work, initially, an attempt was made to partially purify the amylase present in the culture filtrate obtained from fermentative action by *B. subtilis.* Figure 1 shows the specific activity of amylase recovered at different ammonium sulfate saturation fractions. Ammonium sulfate was used to concentrate the enzyme and to obtain a degree of purification. It was recovered in 40-80% ammonium sulfate fraction. At 40-60% saturation fractions, the specific activity was (3.968) u/mg, whereas at 60-80% saturation fraction, it was (4.78 u/mg).

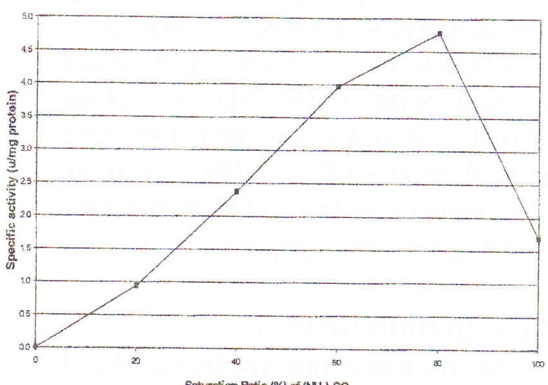

Figure 1: Specific activity of concentrated amylase enzyme with various saturation(%) of ammonium sulfate.

The purification protocol developed for amylase of *B. subtilis* is summarized in Table 1. The Ammonium sulfate removed about 35% of the contaminating proteins and it gave a 52.2% yield with 1.47 fold. The commonly used compound for precipitation of proteins in solution is ammonium sulfate because of its high solubility in water and it does not denature proteins [1].

Three protein peaks were found after gel filtration of amylase enzyme in Sephadex G-100 column, but only one peak (2) had amylase activity (figure 2).

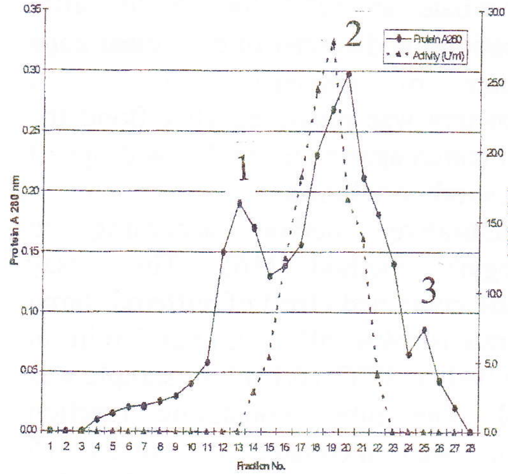

Figure2: Elution profile of amylase enzyme using 1×25 cm Sephadex G-100 column.

This peak (2) was found in the fractions of (16-21), and it is confirmed by another test of amylase activity through starch agar plates because amylase enzyme can actively digest starch and it will create a starchless area around the well [15] (figure 3).

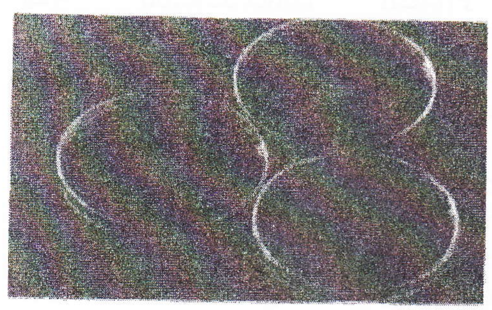

Figure 3: Amylase enzyme activity using starch agar plate:

a) *Culture filtrate*

b) *Redissolved ppt. (40-80%)$(NH_4)_2$ So_4)*

c) *Gel-filtration eluent (peak no.2)*

Amylase activity purification techniques, the data obtained reflects peak purity which is confirmed by the close related protein content obtained

Table 1: Summary of purification of amylase from local isolate Bacillus subtilis

Step	Volume (ml)	Activity (u/ml)	Protein (mg/ml)	Specific Activity (u/mg)	Total Activity (units)	Yield (%)	Purification (fold)
Culture Supernatant	90	7.38	2.3	3.208	664.2	100	1
Redissolved ppt. (40-80%) $(NH_4)_2SO_4$	8	43.4	9.2	4.717	347.2	52.2	1.47
Gel filtration eluate	10	29.2	0.134	217.9	292	43.9	67.92

during the partial purification. Very high purification of enzyme is usually recommended only when the enzyme has special uses like therapeutic and medicinal applications. In most other applications, partial purification is sufficient [19].

Enzymes in solution are not stable and during storage their activities decrease gradually over time. The immobilization process was applied by gel entrapment using calcium alginate (figure 4).

Figure 4: Amylase enzyme immobilized in calcium alginate beads.

The storage stabilities of amylase at 4°C in calcium alginate, were investigated by measuring the enzyme activities at certain time intervals and the results are given in figure 5.

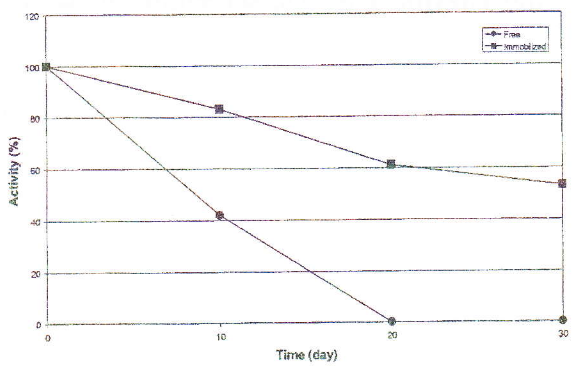

Figure 5: Effect of storage on the activity of free and immobilized amylase

For free enzyme, it lost its activity completely in about 15 days of storage at 4°C. The retained activities were found to be 53% for calcium alginate immobilized enzyme, upon 30 days of storage at 4°C.

Investigating the stability of the enzyme, the data obtained from immobilization process, reflects a high stability of the entrapped enzyme in comparison with the free enzyme. This retention of the original activity may be due to presence of Ca^{2+} ions found in the beads since most of thermostable amylases require additional Ca^{2+} for their thermostability [20], and for increase amylase activity [4]. Enzyme activity was found to be 38.9% for ECH – activated P(HEMA) bound enzyme after storage for 1 month, on the other hand it was 90.7% in the presence of Ca^{2+} ions [7].

Conclusion

The results of this study suggest the possibilities of purification of bacterial amylases by using gel-filtration chromatography and the immobilization results indicate promoted enzyme stability and activity. This study may provide a valuable source of experimental materials for further investigations, such as study of the enzyme kinetics, screening of natural substrates and enzyme applications. Further studies on the purified and immobilized enzyme are currently in progress because the system may provide a good substitution for practical.use in the continuous reactors in biotechnological applications.

References

1-Whitaker, J., R.; Principles of enzymology for the food science; 1972; New York; 80, 84, 443.

2-Lidia, M. P., Spartaco, A. F., and Cirano, J. U.; Purification and some properties of an α-amylase glucoamylase fusion protein from *Saccharomyces cerevisiae*; *world J. of Microbio. & Biotech.*; 1999; **15**: 561-564.

3-Kaur, N., Kaur, P., and Gupta, A. K.; Inter organ comparison of amylases and starch content in mungbean seedling; *Acta physio. Planta.*; 2001; **23** (3).

4-Rahman, M. H., Uddin, Md. S., Islam, N., Nikkon, F., and Hasan, M. F.; Comparative analysis on purified amylases from health and diseased sugarcane juice; *Pakistan J. of Biologi. Science.*; 2001; **4** (6): 728-732.

5-Volesky, B., and Luong, I.; Microbial enzymes productions, purification & isolation; *Critical Rev. in Biotech.*; 1985; **2**: 119-146.

6-Ashok, P., Pooram, N., Carlos, R. S., Vanete, T. S., Dalel, S., and Radjiskumar, M.; Advances in microbial amylases; *biotech. Appl. Biochem.*; 2000; **31**: 135-152.

7-Tümtürk, H., Aksoy, S., and Hasirci, N.; Covalent immobilization of α-amylase onto poly (2-hydroxyethyl methacrylate) and poly (styrene-2-hydroxythyl methacrylate) microspheres and the effect of Ca^{2+} ions on the enzyme activity; *Food Chem.*; 2000; **68**: 259-266.

8-Paul, S. and Diana, S.; Dictionary of microbiology and molecular biology; 1995; **1**: 444; A wiley – inter-science publication.

9-Bagai, R. and Madamwar, D.; Continuous production of halophilic α-amylase through whole cell immobilization of *Halobacterium salinarium*; *Appl. Biochem. & Biotech.*; 1997; **62**: 213-218.

10-Nussinoritch, A., Nussinovitch, M., Shapira, R., and Gershon, Z.; Influence of immobilization of bacteria, yeast and fungal spores on mechanical properties of agar and alginate gels; *Food Hydrocollo.*; 1994; **8**: 361-371.

11-Lim, F., and Sun, A. M.; Microencapsulated islets as bioartificial endocrine pancreas; *Science*; 1980; **210**: 908-910.

12-Pammersheim, R., Schrezenmeir, J., and Vogt, W.; Immobilization of enzymes by multilayer microcapsules; *Macromol. Chem. Phys.*; 1994; **195**: 1557-1567.

13-Hari, P. R., Chandy, T., and Sharma, C. P.; Chitosan / calcium alginate microcapsules for intestinal delivery of nitrofurantion; *J. Microencapsulation*; 1996; **13**: 319-329.

14-Hamza, H. M., and Mhmood, Sh., A.; Semi-Quantitative screening of Sulaimani local isolate of *Bacillus subtilis* producing amylase enzyme; *Kurdi. Academ. J.*; 2001; **1A** (1):1-8.

15-Davis, B., D.; Determining enzyme activity by radial diffusion; *the American Biology Teacher*; 1977: 217-226.

16-Wootton, I. D.; Micro-analysis in medical biochemistry; 1964; printed in Great Britain; 36, 138-139.

17-Whitaker, J. R., and Granum, P. E.; An absolute method for protein determination based on deference in absorbance at 235 and 280 nm; *Analytical Biochem.*; 1980; **109**: 156-159.

18-Scopes, R.; Protein purification, principles and practice; 1987; New York; 302.

19-Rasouli, I., and Kulkarni, P. R.; Partial purification and characterization of β-Galactosidase from *Aspergillus niger* UV-S; *J. Science. I. R. Iran*; 1995; **6**(1) : 14-21.

20-Long-Liulin, Chamg-Cherng Chyau, and Wen-Hwei Hsu; Production and Properties of a raw-starch-degrading amylase from the thermophilic and alkaliphilic *Bacillus sp.* TS-23; *Biotech. Appl. Biochem.*; 1998; **28**:61-68.

پوختەکردنو پاراستنی ئەنزیمی ئەمیلێزی بەرهەم هاتوو لە لایەن جیاکراوەی ناوخۆی *Bacillus subtilis* ەوە

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پوختە

پوختەکردنی ئەنزیمی ئەمیلێز کە لەلایەن جیاکراوەی ناوخۆیی *Bacillus subtilis* بەرهەم دەهێنرێت توانرا بە دوو هەنگاو کە بریتی بوون لە پەیت کردنەوە بە بەکارهێنانی کبریتاتی ئەمۆنیۆم بە ڕێژەی تێرکردن ٤٠-٨٠٪ ، و پاشان بەکارهێنانی کرۆماتۆگرافی پالاوتنی جێلی Gel-filtration chromatography لە کۆلۆمی جێلی سیفادیکس SephadexG-100 ئەنزیمی ئەمیلێزی بەرهەم هاتوو لە لایەن جیاکراوەی ناوخۆیی *Bacillus subtilis* ەوە پوختە بکرێت . ژمارەی پوختەکردنەکانی ٦٧,٩٢ کەرەت بوون و بە ئەنجامێکی ئەنزیمی بەبڕی ٤٣,٩٪ .

بە ڕێگەگرتن "Entrapment " بە هۆی ئەلجیناتی کالسیۆمەوە دەریا نخست کە ئەم ئەنزیمە دوای پاراستن بۆ ماوەی مانگێک لە پلەی "٤"ی سەدی دا ، بە ڕێژەی ٥٣٪ چالاکی دەمێنێت . لە لایەکی ترەوە هەمان ئەنزیم بە بێ بەکارهێنانی ڕێگای قەتیس کردن پاش هەلگرتنی بۆ ماوەی ٢٠ ڕۆژ لە هەمان پلەی سەدیدا دەرکەوت کە چالاکیە ئەنزیمیەکەی وون دەکات .

ئەم توێژینەوەیە ئاماژە بەوە دەدات کە دەکرێت تەنها بە دوو هەنگاو ئەنزیمی ئەمیلێزی بەرهەم هاتوو لە جیاکراوەی ناوخۆیی *Bacillus subtilis* ەوە پوختە بکرێت بۆ بەکارهێنانی پیشەسازی بە زیادکردنی کبریتاتی ئەمۆنیۆم بۆ پوختەی ئەنزیمەکە ، پاشان بەکارهێنانی کرۆماتۆگرافی پالاوتنی جێلی بە هۆی کۆلۆمی جێلی سیفادیکس SephadexG-100 ەوە ، هەروەکو چۆن ئەم توێژینەوەیە ئاماژە بە چوستی تەکنیکی پاراستنی Immobilization دەدات ، بە بەکارهێنانی .

تنقية أنزيم الأميليز المنتج من العزلة المحلية *Bacillus subtilis* وتقييده

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الخلاصة

نقي انزيم الأميليز المنتج من العزلة المحلية *B. subtilis* بخطوتين رئيسيتين شملت الترسيب بأستخدام كبريتات الأمونيوم بنسبة اشباع ٤٠ – ٨٠٪، ثم أستخدام كروماتوغرافيا الترشيح الهلامي Gel – filtration chromatography في عمود هلام السيفادكس Sephadex G-100. وكان عدد مرات التنقية ٦٧,٩٢ مرة وبحصيلة انزيمية مقدارها ٤٣,٩٪. قيد الأنزيم بطريقة الأقتناص Entrapment بأستخدام ألجينات الكالسيوم، مع أحتفاظه بـ ٥٣٪ من فعاليته الأصلية بعد خزنه لمدة شهر بدرجة ٤ درجة مئوية. ومن ناحية أخرى، فأن الأنزيم غير المقيد قد فقد فعاليته بشكل كامل في أقل من ٢٠ يوم وبنفس الظروف.

تشير هذه الدراسة الى امكانيةاستخدام أنزيم الأميليز المنقى في مجالات الصناعة .كما أشارت هذه الدراسة الى كفاءة تقنية التقييد Immobilization بأستخدام ألجينات الكالسيوم والحفاظ على فعالية الأنزيم لمدة طويلة نسبياً.

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