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Activity and stability of immobilized alpha-amylase produced by *Bacillus acidocaldarius*

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Abstract

An isolated strain (from rice), *Bacillus acidocaldarius* was able to produce extracellular α-amylase.The enzyme was partially purified with 50% acetone and showed 4.3-fold purification. Amylase was immobilized on different carriers by different methods and its specific activity for starch hydrolysis studied. Immobilized α -amylase on glass beads (covalent binding) and cation exchange resin (ionic binding) had the highest immobilization yield (85.6 and 84.3%), respectively. It was further observed that, thermal and pH stabilities of immobilized enzymes were higher compared to free enzyme. The immobilized enzymes had higher K (Michaelis constant) and lower V (Maximum A rate of reaction) than the free enzyme. Activation energy (E) of free enzyme was 2.37 Kcal/mol which was higher than the immobilized enzyme by covalent binding or by ionic binding (1.05 and 1/2 1.59 Kcal/mol), respectively. Half life time (t) of immobilized enzyme on glass beads was 83 min which was higher than that of immobilized enzyme on cation exchange resin (61 min). Immobilized enzyme on glass beads showed the highest operational stability for up to 8 reuses with 70% residual activity. On the other hand, α-amylase immobilized on cation exchange resin retained 66.2% of its original activity after 8 cycles.

Key-Words: α-amylase, *Bacillus acidocaldarius,* immobilization, production

Introduction

Amylases are enzymes, which hydrolyze starch molecules to give diverse products including dextrins and progressively smaller polymers composed of glucose units (Windish and Mhatre, 1965). These enzymes are of great significance in present day biotechnology with applications ranging from food, baking, brewing, fermentation, detergent applications, textile desizing and paper industries to analysis in medicinal and clinical chemistry (Pandya *et al*., 2005 and Alva *et al*., 2007). Microbial amylases are available commercially and they have almost completely replaced chemical hydrolysis of starch in starch processing industry (Gupta *et al.*, 2003). One of the main directions of investigation in applied enzymology is to study the stability of the enzyme molecule (El-Batal *et al*., 2005).

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Recently, a number of workers reported that the addition of various compounds to the enzyme led to increase its catalytic activity and stability by preventing spontaneous or heat induced denaturation of the enzymes (Mozhaev *et al*., 1989). Industrial development of enzymic reactors requires the use of immobilized enzymes in order to reduce the cost of the biocatalyst. To a large extent this procedure prevents enzyme losses due to washout and at the same time maintains biocatalyst at high concentrations (Bladino *et al*., 2001). Effective enzyme immobilization can be achieved using several techniques including adsorption to insoluble materials, entrapment in polymeric matrix encapsulation, cross linking with a bifunctional reagent, or covalent linking to an insoluble carrier (Kara *et al*., 2005).

The most important advantages of immobilization methods are the stability of enzyme activity after immobilization and reuse of the enzyme and support material for different purposes because of reversibility of the method.

Reversible enzyme immobilization is a very powerful tool that may be considered to solve this cost problem

and saving time (Akgoll and Denizli, 2004 and Hamilton *et al*., 1999). In the present study, α-amylase was produced by *B. acidocalarius* and partially purified. The enzyme was immobilized on different carriers by different methods.

Material and Methods

Microorganism

Bacterial strains (*B. licheniformis, B. subtilis, B. circulans and B. megaterium*) were obtained from the Department of Microbiology V.B.S. Purvanchal University, Jaunpur uttar pradesh. The isolated strain was obtained by Department of Microbiology, V.B.S.Purvanchal University and Department of Biotechnology V.B.S. Purvanchal University. It was identified according to Bergey's manual.

Carriers for Enzyme Immobilization

The strongly acidic cation exchange resin(H+) was obtained from Merck, total capacity 1.8 mmol/ml particle size (0.3-1.1 mm) 90%. The strongly basic anion exchanger (Cl-) was obtained from Merck, total capacity 1.3 mmol/ml, particle size (0.3-1.18 mm) 90%. Glass, wool, glass wool, sponge and ceramics were obtained from local market.

Starch Hydrolysis Methods

Amylase activity was detected on plates by incorporating starch agar medium containing (g/l): peptone, 5.0; soluble starch, 2.0; meat extract, 3.0; agar, 15.0 and subsequently visualizing starch degradation holes by staining with iodine vapours according to Hols *et al*. (1997).

Growth Medium and Cultivation

2 Basal medium for liquid culture consists of (g/l): starch, 10; nutrient broth, 2.5; CaCl , 0.5 and the pH was adjusted to 7.0 before autoclaving. The same medium was also used for inoculum preparation. Cultivation was in 250 ml Erlenmeyer flasks containing 50 ml of sterile medium. The flasks were inoculated with 1 ml of a 24 h old culture and incubated at 40°C for 42h with shaking at 200 rpm. Culture broth was centrifuged in a refrigerated centrifuge (K70; Janektzhi, Germany) at 6000xg for 10 min, and the supernatant was assayed for enzyme activity.

Enzyme Assay

α-amylase activity was determined according to Apar and Ozbek (2005). 200 µl of the enzyme preparation was incubated with 1ml of 0.2% soluble starch in acetate buffer (50 mM; pH 5.9) at 40°C for 10 min. 200 µl of the reaction mixture was added to 5 ml of iodine solution to stop the reaction. The degradation of the starch by the enzyme was measured at 620 nm. One unit of the α-amylase activity was defined as the quantity of enzyme required to hydrolysis of 0.1 mg starch under assay conditions.

Determination of Protein

Protein content was estimated by the method of Lowry *et al*. (1951).

Fractional Precipitation with Acetone

The crude enzyme prepared as described above was added slowly to two fold cold acetone (v/v) with constant stirring. The mixture was allowed to stand for 1h at 4°C and the enzyme fraction was dried over anhydrous calcium chloride under decreased pressure at room temperature. The fraction tested for enzyme activity and was used for enzyme immobilization.

Immobilization Methods

Entrapment

An equal volume of enzyme solution and sodium alginate or agar solution were used to obtain final concentration range of 2-3% (w/v). The mixture obtained by sodium alginate was extruded drop wise 2 through a Pasteur pipette (1 mm diameter) into a gently stirred 2% CaCl solution for 2h as reported by Dey *et al.* (2003).

Physical Adsorption

One gram of each carrier was incubated with one ml enzyme solution in acetate buffer (50 mM, pH 5.9) overnight at 4°C.

Covalent Binding

One gram of each carrier was covered with 5 ml of acetate buffer (50 mM, pH 5.9) containing 2.5% (v/v) glutaraldehyde (GA) and left for 2h at 30°C. The carriers were washed with distilled water to remove excess GA and incubated with enzyme solution as reported by Abdel-Naby *et al*. (1998).

Ionic Binding

One gram of each carrier was equilibrated with 0.01 M HCl or NaOH and washed with distilled water to remove the excess HCl or NaOH. Then the carriers were incubated with enzyme solution overnight at 4°C as reported by Ahmed *et al*. (2007).

Result and Discussion

Production α-amylase

The first step in this search aimed at evaluating the ability of various bacterial strains (*B. licheniformis,B. subtilis, B. circulans and B. megaterium*) to hydrolyse starch and gave a zone. The results indicated that *B. acidocaldarius* gave the largest zone of starch hydrolysis, and it was the potent strain for α-amylase production.

The addition of starch to the fermentation medium increased the production of α-amylase, so in the present study the soluble potato starch (powder) was replaced with the naturally occurring low cost starchy

substrates such as agricultural raw (corn flour, wheat flour, soy bean, sweet potato, potato and rice).

Among the substrates evaluated, rice (1.4%) was found to be the best substrate for highest production of α-amylase (61.4 U/ml). U-Haq *et al*. (2005) suggested that pearl millet starch increased the production of α-amylase by *B. licheniformis.* The addition of lactose as carbon source (2.5%) to the production media beside rice increased the enzyme production by 18.6% Hamilton *et al.* (1999) found that lactose (4%) only as carbon source gave the highest level of activity (26 U/ml) by *Bacillus sp.* IMD435 which lower than our search (70.1 U/ml).

Maximum production of α-amylase was obtained at pH 5.0 (91.4 U/ml). This result agreed with that of Sajedi *et al.* (2005) on the production of α-amylase from *Bacillus sp.* KR-8104. The optimum conditions for 2 maximum α-amylase production were, rice 1.4%, lactose 0.5%, nutrient broth 0.25%, CaCl 0.05%, initial pH (5.0) incubation temperature 40°C, incubation time 42 h and 200 rpm.

α-amylase produced by *B. acidocaldarius* was partially purified by fractional precipitation with ammonium sulphate, acetone and ethanol. The most active fractions listed in Table (1) showed that of all fractions, acetone at 50% and ethanol at 60% were most active (759.1 U/mg protein) and showed 4.3-fold purification (compared to the culture filtrate). Due to the less amount of acetone compared with ethanol, fraction precipitated at 50% acetone was used for immobilization process.

Enzyme Immobilization:

Immobilization of *B. acidocaldarius α* -amylase was attempted in order to assess the activity retained upon immobilization in comparison to the free α-amylase and to investigate the operational stability of the immobilized enzyme. On the other hand, industrial development of the enzyme reactors requires the use of immobilized enzyme in order to reduce the cost of the biocatalyst. To large extent this procedure prevents enzyme losses and at the same time maintains biocatalyst at high concentration (Bladino *et al*., 2002). α-amylase was immobilized on different carriers by different method (entrapment, physical adsorption, covalent binding and ionic binding). The efficiency of enzyme immobilization was evaluated according to different parameters including the retained catalytic activity, the specific activity of the immobilized enzyme, and the loading efficiency (immobilized activity/gram carrier) and the immobilization yield is the key parameter.

Immobilization by Entrapment

Immobilization of α-amylase by entrapment was recorded in Table (2) and showed that the highest immobilization yield (61.4%) was obtained with sodium alginate 2%. Dey *et al.* (2003) reported that sodium alginate 4% gave the highest immobilization yield 75% of *B. circulans* α-amylase. Decreasing immobilization yield with increasing in carrier concentration have been due to the decrease in the porosity of the gel matrix, which caused diffusion limitation of the substrate. The lower immobilization yield in case of lower percentage of sodium alginate or agar solutions might be due to larger pore size and consequently greater leakage of the enzyme from matrix (Dey *et al*., 2003).

Immobilization by Physical Adsorption

Immobilization of α-amylase by physical adsorption indicated that the highest loading efficiency (1552 U/g carrier) and immobilization yield (55.3%) was found with centered glass G-35 (Table 3). On the other hand, the lowest loading efficiency (433.8 /Ug carrier) was detected with the enzyme immobilized on glass wool.

In physical adsorption the binding forces between the enzyme and the matrix are weak in comparison with covalent or ionic binding (Bickerstaff, 1997).

Immobilization by Covalent Binding

Different carriers were used for immobilization by covalent binding (Table 4) through a spacer group (glutaraldehyde). Glass beads showed the highest loading efficiency (1824.1 U/g carriers) and immobilization yield (85.6%). This result is higher than that obtained by Bryjak (2003) on immobilized α-amylase by covalent binding (60.2% immobilization yield) and Varavinit *et al*.(2002) on immobilized thermostable α-amylase covalently on cellulose fiber (44% immobilization yield). Good loading efficiency might have been due to the formation of stable cross linking between the carrier and the enzyme through a space group (Abdel-Naby *et al.,* 1998b).

Immobilization by Ionic Binding

A series of ion exchangers was used for α-amylase immobilization by ionic binding (Table 5). Cation exchange resin was the most suitable for enzyme immobilization gave the highest immobilization yield (84.3%) with the highest loading efficiency (1626.2 U/g carriers).

Properties of Immobilized α-amylase

In the following experiments, the enzyme immobilized on cation exchange resin (by ionic binding) and glass beads (by covalent binding) were used for studying its properties (Table 6). The immobilized enzyme retained 67.4% and 72.6% of specific activity (in cation resin

and glass beads), respectively. This drop in the specific activity can be attributed to steric hindrance in the immediate vicinity of the enzyme molecules. The hindrances are probably caused by the shielding effect of the substrate and by the excessive packing of the enzyme, which render their active sites less accessible to the substrate (Abdel-Naby 1993). Other search reported the decrease in the specific activity after immobilization of α-amylase with mesoporous silicas which retained 80% of the specific activity of free enzyme (Pandya *et al.,* 2005).

The optimum pH of reaction was not affected by immobilization process (in case of covalent binding and ionic binding). El-Batal *et al.* (2005) suggested that the immobilized α-amylase by ionic binding had the same pH optima as the free enzyme.

Immobilization procedure contributed to improvement of the enzyme stability. The results in (Table 6) showed that the temperature optima of immobilized α-amylase activity shifted toward higher temperature from 50°C to 60°C. A similar increase in temperature optima had been found in immobilized α-amylase by Pandya *et al.* (2005), Konsoula and Kyriakides (2006). A The activation energy (E) of free enzyme (2.37 Kcal/ mol) was higher than that of the immobilized A enzyme on cation exchange resin and glass beads (1.05 and 1.59 Kcal/ mol), respectively. Decreasing of (E) after enzyme immobilization due to the internal diffusion limitation is the rate limiting step (Abdel-Naby *et al*., 1998a).

Thermal stability of immobilized α-amylase compared with the free enzyme. The results showed that the immobilization process protected the enzyme against heat in activation. The residual activity of free enzyme after heating at 60°C for 60 min (38%), which lower than the immobilized enzyme (50 and 68%) for 1/2 cation exchange resin and glass beads. The calculated half-life values (t) at 60°C for the immobilized enzyme on cation exchange resin and glass beads were 61 and 83 min, respectively which higher than the free enzyme 47 min. It is well-known that the activity of the immobilized enzymes, especially in a covalently binding system is more resistant against heat than the free enzyme (El-Batal *et al.,* 2005). Deactivation rate constant for free enzyme at 60°C was (6.6X10 -3/min) which is higher than those reported for the immobilized enzyme on cation exchange resin (5.1X10-3/min) and glass beads (3.7X10-3/ min). m max The calculated values of kinetic parameters K and V for the immobilized enzyme are listed in Table (6). Immobilized enzymes exhibited K values higher than the free enzyme due to the lower accessibility of the substrate to the active site of the immobilized enzyme. This result is similar to that obtained by Kara *et al.* (2005) and El-Batal *et al*. (2005).The maximum rate of the reaction catalyzed by the immobilized max enzymes were lower than the free enzyme. Decreasing V value of α-amylase after immobilization covalently on plastic supports was reported by Roig *et al*. (1993). The operational stability of immobilized enzyme is one of the most important factors affecting the utilization of an immobilized enzyme system. The results indicated that on repeated use of the immobilized α-amylase on cation exchange resin and glass beads retained 70.0 and 73.4% from the initial activity up to 6 cycles. After that, the activity decreased which may due to enzyme denaturation and physical loss of enzyme from the carriers. The enzyme immobilized in this study is operationally more stable than the α-amylase immobilized on nitro cellulose membrane which retained only 65% of the initial activity after 7 runs (Tanyolac *et al.*, (23)). On the hand, Dey *et al.* (10) reported that *B, circulans* α-amylase immobilized by entrapment in calcium alginate beads retained 83% of the initial activity after 7 cycles.

pH Stability

The pH stability of α-amylase was determined by pre-incubation at different pH values for 1h at 30°C. The results indicated that there was a significant improvement in pH stability after immobilization process. Immobilized enzyme by covalent binding (on glass beads) showed highest pH stability. El-Batal *et al*. (2005) reported that enzyme immobilization especially by covalent binding increased its stability.

Conclusion

The overall performance of the immobilized *B. acidocaldarius* α-amylase indicated that catalytic activity increased optimal reaction temperature, thermal stability, and durability of the catalytic activity in repeated use are rather promising than that of the free enzyme. All these criteria can therefore, be successfully utilized in practical application.

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Table 1: Partial purification of *B. acidocaldarius* α-amylase

Purification	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Recovered activity (%)	fold purification (- fold)
Crude enzyme	259.1	1446.7	173.3	100.0	1.0
Ethanol (60%)	24.9	5986.7	759.1	41.40	4.2
Acetone (50%)	23.7	5681.6	759.1	39.32	4.3
Ammonium sulphate	4.81	252.1	156.5	1.74	0.93

Table 2: Immobilization of *B. acidocaldarius* α-amylase by entrapment

Carrier	Concentration (%)	Unbounded enzyme: Protein content (mg/g carrier)	Unbounded enzyme: Activity (U/g carrier) (B)	Immobilized enzyme: Protein content (mg/g carrier)	Immobilized enzyme: Activity (U/g carrier) (I)	Immobilization yield I / (A-B) %
Sodium alginate	1.0%	0.56	4500.6	0.51	1445.0	56.7
	2.0%	0.628	4400.7	0.582	1566.6	61.4
	3.0%	0.739	3974.7	0.473	1453.8	49.2
Agar	1.0%	0.62	5704.1	0.58	650.1	50.2
	2.0%	0.614	5643.2	0.599	713.6	52.4
	3.0%	0.569	4887.8	0.641	769.5	36.4

Added enzyme contains 1.372mg protein with activity 7000U (A)

Table 3: Immobilization of *B. acidocaldarius* α-amylase by physical binding

Carrier	Unbounded enzyme: Protein content (mg/g carrier)	Unbounded enzyme: Activity (U/g carrier) (B)	Immobilized enzyme: Protein content (mg/g carrier)	Immobilized enzyme: Activity (U/g carrier) (I)	Immobilization yield I / (A-B) %
Ceramics	1.142	5294.3	0.068	610.1	34.8
Wool	0.331	3787.1	0.876	626.8	19.5
Glass wool	0.533	6072.9	0.673	433.8	48.0
Glass	1.184	4831.8	0.028	862.5	38.8
Polystyrene	1.182	4613.8	0.028	1160.6	48.6
Sponge	1.121	5167.7	0.082	851.0	47.0
Centered glass (G 35)	1.184	4087.0	0.027	1552.4	55.3

Added enzymes contain 1.372mg protein with activity 7000U (A)

Table 4: Immobilization of *B. acidocaldarius* α-amylase by covalent binding

Carrier	Unbounded enzyme		Immobilized enzyme		Immobilization yieldI / (A-B) %
	Protein content (mg/g carrier)	Activity (U/g carrier) (B)	Protein content (mg/g carrier)	Activity (U/g carrier) (I)	
Ceramics	1.115	4164.1	0.096	472.8	16.7
Wool	0.707	3492.8	0.505	698.9	19.93
Glass wool	0.069	4007.2	1.143	199.2	6.6
Glass	0.665	4888.8	0.546	1824.1	85.6
Polystyrene	1.133	4943.8	0.078	589.3	28.6
Sponge	0.817	3700.1	0.395	185.4	5.6
Centered glass (G 35)	1.078	3843.9	0.133	359.8	11.4

Added enzyme contain 1.372mg protein with activity 7000U (A)

Table 5: Immobilization of *B. acidocaldarius* α-amylase by ionic binding

Carrier	Unbounded enzyme		Immobilized enzyme		Immobilization yield I / (A-B) %
	Protein content (mg/g carrier)	Activity (U/g carrier) (B)	Protein content (mg/g carrier)	Activity (U/g carrier) (I)	
Sephadex (G 100)	0.601	6102.4	0.610	320.7	51.05
Cation Exchange resin	0.656	5093.5	0.555	1626.2	84.3
Anion exchange resin	1.046	992.4	0.165	305.3	5.1

Added enzymes contain 1.372mg protein with activity 7000U (A)

Table 6: Properties of immobilized and free α-amylase

Properties	Free enzyme	Immobilized Enzyme	
		Cation	Glass
Specific activity (U mg/ protein)	5100.1	3435.5	3704.0
Optimum pH	7.25	7.25	7.25
Optimum temperature (°C)	50	60	60
A Activation energy E (Kcal/ mol)	2.37	1.05	1.59
Thermal stability at 60°C for 60min (residual activity %)	38	50	68
1/2 Half Life time at 60°C t (min)	47	61	83
Deactivation rate constant at 60°C (min-1)	6.6X10-3	5.1 X10-3	3.7X10-3
m K (mg/ ml)	0.86	0.91	1.05
max V (U/mg protein)	144.1	125.8	127.6