

Research Article



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Purification, immobilization and characterization of thermostable α -amylase from a thermophilic bacterium *Geobacillus* sp. TF14

Termofilik *Geobacillus* sp. TF14'ten α -Amilaz Enziminin Saflaştırılması, İmmobilizasyonu ve Karakterizasyonu

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Abstract

Objective: In this study, α -amylase from a thermophilic bacterium *Geobacillus* sp. TF14 was purified and immobilized on two different supports.

Methods: Ion exchange and hydrophobic interaction chromatography techniques were employed for the purification.

Results: The enzyme was purified as 17.11 fold and determined as a single band of 54 kDa on SDS-PAGE. Purified enzyme showed two pH optimums of pH 5.00 and pH 9.00 and the enzyme is quite stable at these pHs over a period of 48 h. Purified enzyme showed maximal activity at 75°C and stability at this temperature over a period of 72 h. It was observed that Ca^{2+} activated the enzyme at about 70% at 5 mM final concentration. SDS, Triton X100, Triton X114 and Tween 20 caused around 50% loss of initial activity at a final concentration of 1% (w/v). Purified enzyme was immobilized on the surface of Dowex and chitin. Immobilization highly enhanced temperature optima and thermal stability. Dowex immobilized enzyme maintained most of its initial activity in the presence of SDS, Triton X100, Triton X114 and Tween 20 at a concentration of 1%.

Conclusion: It can be concluded that the purified enzyme may find application in many fields of starch based industries.

Keywords: α -Amylase; Purification; Immobilization; Dowex; Chitin; *Geobacillus*.

Özet

Amaç: Bu çalışmada endüstriyel önemi olan α -amilaz enzimi, yeni tanımlanmış bir termofilik bakteri türü olan *Geobacillus* sp. TF14 suşundan saflaştırıldı ve iki farklı katı desteğe immobilize edildi.

Yöntem: Enzim saflaştırılması için amonyum sülfat çöktürmesi, iyon değişim kromatografisi ve hidrofobik etkileşim kromatografisi teknikleri kullanıldı.

Bulgular: α -Amilaz enzimi 17,11 kat saflaştırıldı. Saflaştırılan enzim SDS-PAGE ile analiz edildiğinde 54 kDa molekül ağırlığına sahip tek bir protein bandı tespit edildi. Saflaştırılan enzimin pH 5,00 ve pH 9,00 olan iki pH optimumuna sahip olduğu ve bu pH'larda 48 saat inkübasyon sonunda oldukça kararlı olduğu belirlendi. Saflaştırılan enzimin en yüksek aktiviteye 75°C'de sahip olduğu ve bu sıcaklıkta 72 saat inkübasyon sonunda aktivitesini tamamen koruduğu belirlendi. Ca^{2+} enzim aktivitesini %70 oranında artırdığı tespit edildi. SDS, Triton X100, Triton X114 ve Tween 20 deterjanlarının %1 (a/v)'lik çözeltilerinde enzimin yaklaşık olarak başlangıç aktivitesinin yarısını kaybettiği tespit edildi. Saflaştırılan enzim iyonik etkileşimlerle Dowex'e ve kitin yüzeyine immobilize edildi. Dowex'e immobilize enzimin SDS, Triton X100, Triton X114 ve Tween 20 deterjanları varlığında aktivitesini büyük oranda koruduğu da belirlendi.

Sonuç: Saflaştırılan α -amilaz enziminin nişasta temelli birçok endüstriyel alanda kullanılabileceği sonucuna varılabilir.

Anahtar Kelimeler: α -Amilaz; Saflaştırma; İmmobilizasyon; Dowex; Kitin; *Geobacillus*.

Introduction

Because of their unique properties great effort has been given to the search of microbial enzymes and their applications in various industrial processes. The need for enzymes applicable to industrial processes is on a continuous rise

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and over 500 products are being produced enzymatically [1]. Hydrolases are the major class of enzymes dominating the market because of their industrial importance. They have a major application area in hydrolyzing biopolymers such as starch, pectin, cellulose and protein [2].

α -Amylases (E.C 3.2.1.1) are extracellular, endo-acting enzymes that hydrolyze α -1,4-glycosidic linkages in starch, yielding linear and branched oligosaccharides of different lengths. Thermostable α -amylases are essential for starch industry in “saccharification” and liquefaction process [3].

Nowadays starch hydrolysis is carried out using microbial α -amylases. α -Amylases also have further application in areas such as textile, paper, detergent, fermentation, pharmaceutical and bakery [4]. Each process may need an amylase with different properties such an example would be the starch hydrolysis process which requires highly thermoactive and thermostable α -amylases. Another example may be in the detergent industry which requires α -amylases that are active at a range of alkali pH and stable against oxidants. It is not possible for an α -amylase to meet the requirement of all industrial processes, so investigative research of novel amylases from microorganisms continuously proceed. Amylases from thermophilic microorganisms are especially important because of their superior properties like thermal stability [5, 6].

One of the main problems for enzymes used in industrial areas is their low stability in these environments. Despite their unique properties, the stability of enzymes needs to be improved for industrial applications. Immobilization is one of the most exploited ways for improving their stability. Immobilized enzyme usage also ensures many advantages including possible increase in stability, good catalytic activity, recovery, continuous operation of enzymatic processes, reusability and reduced susceptibility to microbial contamination [7, 8].

In this study, α -amylase from thermophilic bacterium *Geobacillus* sp. TF14 was purified and immobilized onto different solid supports. Biochemical characterization of purified and immobilized enzymes was also carried out. Industrial application potential of the purified enzyme was discussed by comparing biochemical properties of free and immobilized enzymes as well.

Materials and methods

Materials

Soluble starch, amylose, β -cyclodextrin, 3,5 dinitrosalicylic acid (DNS), Trizma base and all the culture media

were purchased from Sigma-Aldrich. Q sepharose and phenyl sepharose were purchased from Merck. All other chemicals were of analytical grade. Bacterial strain was isolated from Ömerli geothermal field of Germencik, Aydın, Turkey, as described previously [9].

Production of α -amylase

For α -amylase production, overnight culture of *Geobacillus* sp. TF14 was inoculated into production medium consisting of peptone (10 g/L), NaCl (5 g/L), yeast extract (5 g/L) and 10 mg/mL soluble starch. After 18 h incubation at 55°C, the turbid culture was centrifuged at 12,857 \times g, 4°C for 10 min and the supernatant was used as crude enzyme extract.

Amylase activity assay and protein determination

Amylase activity assay was performed spectrophotometrically according to DNS method [10]. A reaction mixture containing 300 μ L of 10 mg/mL soluble starch and 300 μ L of enzyme solution was incubated for 30 min at 55°C. For the immobilized enzyme activity, 0.5 g of solid support (enzyme immobilized) was suspended in 350 μ L of buffer and 350 μ L of 10 mg/mL soluble starch solution was added into the suspension. This mixture was incubated for 30 min at 55°C and centrifuged at high speeds, and then a 600 μ L of this supernatant was transferred into new tube. Equal volume of DNS reagent was added into tubes and kept in a boiling water bath to quantify generated reducing sugars. All characterization assays were performed in triplicate. One unit (U) of enzyme activity was defined as the amount of enzyme that liberated 1 μ M of reducing sugar as glucose equivalents in 1 min under the assay conditions [11]. Protein content was measured by the Bradford method using bovine serum albumin as the standard [12].

Purification of α -amylase

Ammonium sulphate precipitation, ion exchange and hydrophobic interaction chromatography in addition with ultra-filtration were employed for enzyme purification. Crude enzyme was precipitated fractionally by adding solid ammonium sulphate [13]. Enzyme solution was brought up to 40% saturation and centrifuged at 4°C, 41,142 \times g for 20 min. Precipitated proteins were removed and the supernatant was brought up to 50% saturation and centrifuged at 4°C, 41,142 \times g for 20 min. Precipitated proteins were

dissolved in 50 mM, pH 8.00 Tris-HCl buffer and passed through Amicon Ultra Centrifugal Filter (cut off mass 30 kDa). Concentrated proteins were loaded in Q Sepharose Fast Flow column (2×30 cm) pre-equilibrated with the same buffer and loaded proteins were eluted with 0–1 M range of NaCl. Protein fractions were collected as 7.5 mL for each tube at a flow rate of 1.5 mL/min. α -Amylase activity was observed at protein active fractions and fractions showing amylase activity were pooled and filtered through Amicon Ultra Centrifugal Filter. Filtrates were loaded into a Phenyl Sepharose column (1.5×15 cm) pre-equilibrated with 0.5 M $(\text{NH}_4)_2\text{SO}_4$ dissolved in 50 mM, pH 8.00 Tris-HCl buffer and loaded proteins were eluted with a 0.3–0 M range of $(\text{NH}_4)_2\text{SO}_4$. Protein fractions were collected as 7.5 mL for each tube at a flow rate of 1 mL/min. Amylase active fractions were pooled and concentrated with Amicon Ultracel membrane.

SDS PAGE and zymogram analysis

Denaturing SDS polyacrylamide gel electrophoresis was performed in Bio-Rad Mini Protean Tetra Cell Electrophoresis Unit. The gel having 10% acrylamide was prepared as described by Laemmli [14] and Coomassie Brilliant Blue R-250 was used for staining. Non-denaturing polyacrylamide gel electrophoresis was performed by using 8% separating gel. After the electrophoresis was run, gel was washed with Tris-HCl buffer and incubated at 55°C for 30 min in the same buffer containing 10 mg/mL soluble starch and then bands were visualized by treating the gel with iodine solution [15].

End product determination

One millilitre of the purified α -amylase and 1 mL of soluble starch solution (10 mg/mL) was incubated at 75°C for 2 h. End products of hydrolysis were analyzed by using Kieselgel Silica gel (TLC-cards) in a solvent system of 2-propanol-ethylacetate-water 3:1:1 (v/v/v). Glucose, maltose, maltotriose and maltotetrose were used as standard. Spots were visualized by treating the cards with 20% H_2SO_4 dissolved in methanol and heating to 110°C [15].

Immobilization of α -amylase on Dowex and chitin

Purified α -amylase was immobilized on Dowex (1×8 Cl⁻ form 200–400 mesh) and chitin by ionic interaction and surface adsorption, respectively. For the immobilization,

5 g of solid support was suspended in 50 mM, pH 8.00 Tris-HCl buffer and 1 mg of purified α -amylase was added into the suspension, and the mixture was allowed to stir gently for 4 h at room temperature, it was filtered and washed several times with 5 mL distilled water. All filtrates were collected and protein contents were quantified. Binding percentage was calculated by subtraction of the remaining enzyme content in the filtrate solution from the initial enzyme concentration. Immobilization yield was calculated from the equation shown below.

Immobilization yield

$$= (\text{mg of bound amylase} / \text{mg of initial amylase}) \times 100$$

Characterization of free and immobilized enzyme

Substrate specificity

In order to determine the substrate specificity of the free enzyme soluble starch, amylose, glycogen, β -cyclodextrin and carboxymethyl cellulose (CMC) were used as substrates and activity assays were performed. The highest activity was considered as 100%.

Optimum pH and stability

For the determination of optimum pH values, free and immobilized enzyme activity assays were carried out by using 50 mM McIlvaine buffers between pH 2.20 to 8.00 and 50 mM Glycine-NaOH buffers in the range of pH 9.00 to 11.00. Relative activity was calculated by considering the highest activity as 100%.

To determine pH stability, free enzyme was mixed with 50 mM, pH 5.00 acetate and 50 mM, pH 9.00 Glycine-NaOH buffers in the ratio of 1:1 (v/v), separately, and then the activity assays were carried out. Remaining mixtures were kept at room temperature up to 48 h and enzyme activity was assayed. Results were calculated as residual activity by comparing with non-incubated enzyme activity. pH stability of the immobilized enzymes was also measured. Immobilized enzymes were mixed with appropriate buffer solutions in the ratio of 1:2 (w/v) and kept at room temperature for up to 48 h. Activity assays were carried out and results were calculated as residual activity by comparing with non-treated enzyme activities.

Optimum temperature and thermal stability

The optimum temperature of the free enzyme was determined by assaying at different temperatures ranging between 55 to 95°C. For immobilized enzymes, optimal temperature was determined by conducting activity assays in the temperature range of 55–100°C. Results were calculated as relative activity by considering the highest activity as 100%.

Thermal stability of free enzyme was determined by incubating enzyme solution at 4, 55, 75 and 90°C temperatures in Eppendorf tubes. Aliquots were withdrawn at the time intervals of 6, 24, 48 and 72 h, rapidly cooled to room temperature and then enzyme activities were determined at standard conditions. A 0.5 g of solid support (immobilized enzyme) was mixed with 1 mL of pH 9.00 and pH 10.00 buffers separately for chitin and Dowex immobilized enzymes respectively. Thermal stability of immobilized enzymes was determined by incubating these mixtures at 4°C and 95°C up to 72 h. Results were calculated as residual activity by comparing with non-incubated enzyme activities.

Determination of kinetic parameters

Kinetic parameters of *Geobacillus* sp. TF14 α -amylase were obtained by measuring the rate of starch hydrolysis in standard assay conditions, at various substrate concentrations ranging from 0.25 to 20 mg/mL, 0.05 to 20 mg/mL and 0.2 to 20 mg/mL for free, Dowex immobilized and Chitin immobilized enzyme, respectively. The Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) values were determined from the Lineweaver–Burk plots [16].

Effect of some chemicals on the enzyme activity

The effect of metal ions on the enzyme activity was separately investigated by adding chloride salt solutions of

Ca²⁺, Co²⁺, Cu²⁺, Fe²⁺, Hg²⁺, Mg²⁺, Mn²⁺, Ni²⁺ and Zn²⁺ ions and EDTA directly to the standard reaction mixture in a final concentration of 5 mM. The effect of SDS, Triton X100, Triton X114 and Tween 20 at a final concentration of 10 mg/mL was also determined. The enzyme activity determined in the absence of chemicals was defined as 100% for the residual activity estimations [17].

Reusability of the immobilized enzymes

The reusability of immobilized amylases was studied for 15 cycles in standard conditions. After each activity measurement, immobilized enzymes were recovered by centrifugation at high speeds and pellets were washed with buffer solution in order to use at other reaction cycles. Results were calculated as residual activity by comparing with the first cycle starch hydrolysis.

Results and discussion

Purification of α -amylase

Ammonium sulphate precipitation, ion exchange chromatography, hydrophobic interaction chromatography and ultrafiltration were employed for purification of α -amylase from *Geobacillus* sp. TF14. All purification steps were summarized in Table 1. Purified α -amylase exhibited 95% of its initial activity which corresponded to an 17.11 fold increase in its specific activity in comparison with the crude enzyme solution.

SDS-PAGE and zymogram analysis

Purified α -amylase from *Geobacillus* sp. TF14 was displayed at a single band both on native and SDS-PAGE gel electrophoresis (Figure 1A and B). Molecular weight of the purified α -amylase was estimated as 54 kDa. Molecular weights of α -amylases from microbial sources

Table 1: Purification steps of *Geobacillus* sp. TF14 α -amylase.

Purification step	Volume (mL)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield	Purification fold
Crude extract	300	1401	37617	26.85	100	1
(NH ₄) ₂ SO ₄ precipitation	15	19.18	1812.6	94.50	4.82	3.52
Q Sepharose column	3	1.67	510.66	305.23	1.36	11.37
Phenyl sepharose column	1	0.26	119.25	459.36	0.32	17.11

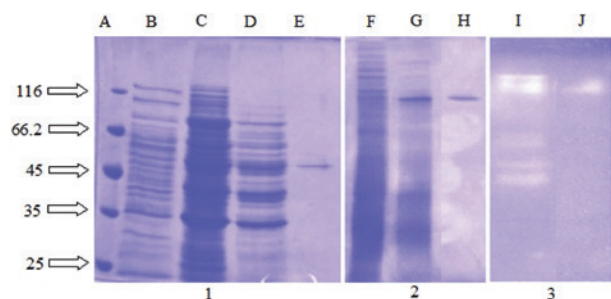


Figure 1: SDS-PAGE and Native Page electrophoresis of purified α -amylase, (40 μ g of protein was loaded each well). **1:** SDS-PAGE, A: Molecular weight markers, B: Crude extract, C: $(\text{NH}_4)_2\text{SO}_4$ precipitate, D: Q Sepharose fraction, E: Phenyl Sepharose fraction (Purified α -amylase). **2:** Native-PAGE stained with Coomassie Brilliant Blue, F: Crude extract, G: Q Sepharose fraction, H: Phenyl Sepharose fraction. **3:** Native-PAGE substrate staining, I: Crude extract, J: Phenyl Sepharose fraction.

vary from 10 kDa to 210 kDa [5]. Three α -amylases with molecular weights of 52 kDa [18], 59 kDa [19] and 63 kDa [20] were reported from different *Geobacillus* strains. Zymogram analysis was also carried out by using 8% separating gel and the gel was stained with iodine solution (Figure 1C). It is clearly seen from the figure that although crude enzyme solution has several amylolytic enzyme activities, only one activity band was achieved after purification.

End product determination

One millilitre of α -amylase and 1 mL of soluble starch solution was incubated at 75°C for 2 h. The end products of starch hydrolysis were analyzed by TLC (Figure 2). At the end of one and two hours of incubation the main end product was maltotetrose and longer chain oligosaccharides. α -Amylases which release oligosaccharides in different lengths as an end product were reported in literature [21, 22]. β -Amylases are exo-hydrolases and they act on the substrate from the nonreducing end by yielding successive amount of maltose [23].

Immobilization of α -amylase on different supports

Dowex and chitin were used as solid support materials for immobilization of α -amylase. After optimization of immobilization conditions, immobilization yields were calculated as 21% and 45% for Dowex and chitin, respectively.

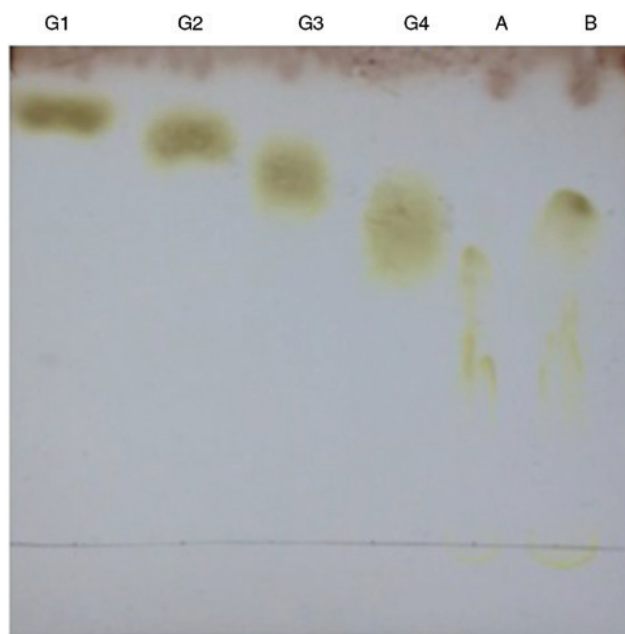


Figure 2: End products of starch hydrolysis G1: Glucose, G2: Maltose, G3: Maltotriose, G4: Maltotetrose. A: End products for 1h of incubation, B: end products for 2h of incubation.

Characterization of free and immobilized α -amylase

Substrate specificity

To determine the substrate specificity of the purified enzyme, soluble starch, amylose, glycogen, β -cyclodextrine and CMC were used. Results showed that purified enzyme had the highest activity for soluble starch and nil activity was detected for CMC (Table 2). It was reported that soluble starch is the best substrate for α -amylases [5]. Another study found that α -amylases from *Aspergillus oryzae* strain S2 and *Aspergillus oryzae* IFO-30103 hydrolyzed starch better than the other substrates tested [24].

Table 2: Substrate specificity of purified α -amylase from *Geobacillus* sp. TF14.

Substrate	Activity (U/mg)	Relative activity (%)
Starch	1983	100
Amylose	242	12
β -cyclodextrine	80	4
Glycogen	68	3
CM-Cellulose	0	0

Effect of pH on the enzyme activity and pH stability

The effect of pH on α -amylase activity was studied by using soluble starch as the substrate at different pH values from 3.00 to 11.00 at 55°C. The pH activity profile of free and immobilized enzymes is given in Figure 3A. It can be seen that the purified enzyme has two optimal pH's; one at pH 5.00 and the other at pH 9.00. Hwang et al. [25] reported a gene encoding a xylanase enzyme having two optimal pH values of 5.50 and 9.50, Ushasree et al. [26] also reported a gene encoding a phytase enzyme having two optimal pH's at 2.50 and 5.50.

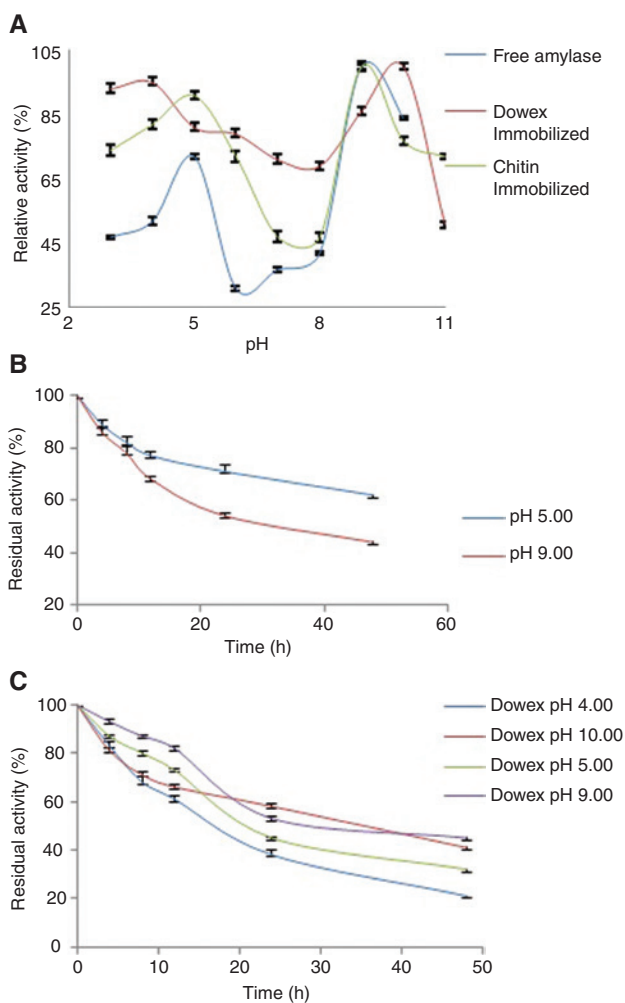


Figure 3: Effect of pH on the enzyme activity.

(A) Effect of pH on free and immobilized α -amylases. Hundred percent activity corresponds to 1110 U/mg, 5585 U/mg and 2723 U/mg for free α -amylase, dowex and chitin immobilized α -amylase activity, respectively. (B) pH stability of free α -amylase. Hundred percent activity corresponds to 1440 U/mg for pH 5.00 and pH 9.00, respectively. (C) pH stability of immobilized α -amylases. Hundred percent activity corresponds to 5063 U/mg for dowex pH 4.00 and dowex pH 10.00 and 2642 U/mg for chitin pH 5.00 and chitin 9.00, respectively.

As shown in Figure 3A immobilized enzymes showed their maximum activity at pH 9.00 and 10.00 for chitin and Dowex immobilized enzyme, respectively. It was reported that the optimum pH may undergo significant shifts, depending on the charge properties of the matrix [27]. Immobilized α -amylases also showed higher activity values for all pH's when compared with free enzyme. This result shows that immobilized α -amylase showed better operational conditions.

The pH stability of purified enzyme was determined by results given in Figure 3B. It was found that purified enzyme was quite stable at its optimal pH values and the enzyme retained about 70 and 50% of its initial activity at pH 5.00 and pH 9.00 respectively at the end of 48 h incubation. α -Amylase from *Bacillus licheniformis* NH1 retained around 90% of initial activity in a pH range from 4.0 to 9.0 after 1 h incubation [22], α -amylase from *Chryseobacterium taeanense* TKU001 retained around 80% of initial activity at pH 9.0 after 30 min incubation [28] and α -amylase from *Bacillus methylotrophicus* strain P11-2 retained over 80% of its original activity in the pH range of 6.0 to 9.0 at 40°C for 1 h incubation [29].

The pH stabilities of immobilized enzymes were also examined and it was found that the Dowex immobilized enzyme was more stable at pH 10.00 than at pH 4.00 and the chitin immobilized enzyme was more stable at pH 9.00 than at pH 5.00 (Figure 3C). Dowex and chitin immobilized α -amylases retained more than 60% of their starting activities after 24 h incubation at pH 10.00 and 9.00, respectively. It is clear from the results that pH stability of the α -amylase was increased significantly at pH 9.00 after immobilization on chitin.

Effect of temperature on the enzyme activity and thermal stability

The effect of temperature on enzyme activity was determined by measuring activity at different temperatures ranging from 55°C to 95°C for free enzymes and 55°C to 100°C for immobilized enzymes. As shown in Figure 4A free and immobilized enzymes were active at broad temperature ranges, maximally at 75°C for free and 95°C for Dowex and chitin immobilized α -amylases respectively. It was stated that temperature optima of α -amylases obtained from thermophilic organisms were between 55°C to 85°C [5]. Optimum temperatures of α -amylases from *Bacillus* sp. ANT-6 [30], *Bacillus* sp. A3-15 [31] and *Geobacillus* sp. NMS2 [32] were reported as 80, 70 and 50°C, respectively. Immobilization enhances operational conditions of enzymes [33]. Kahraman et al. [34] reported that

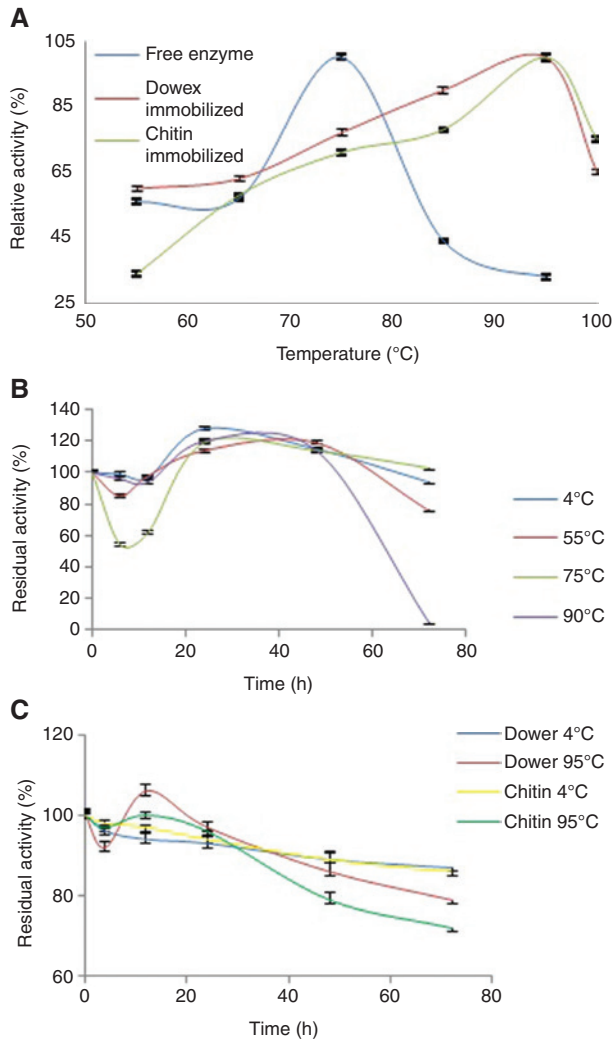


Figure 4: Effect of temperature on the enzyme activity. (A) Effect of temperature on free and immobilized α -amylases. Hundred percent activity corresponds to 2935 U/mg, 5519 U/mg and 2697 U/mg for free α -amylase, dowex immobilized and chitin immobilized α -amylase, respectively. (B) Heat stability of free α -amylase. Hundred percent activity corresponds to 1789 U/mg. (C) Heat stability of immobilized α -amylases. Hundred percent activity corresponds to 4960 U/mg for dowex 4°C and dowex 95°C and 2715 U/mg for chitin 4°C and chitin 95°C, respectively.

temperature optima of α -amylase shifted from 30°C to 50°C after immobilization on glass beads. El-Banna et al. [35] reported 20°C increment of temperature optima for Dowex and Ca-Alginate immobilized α -amylase. It was stated that temperature optimum of chitin immobilized α -amylase shifted from 45 to 65°C [36]. Temperature optima of α -amylase from *Geobacillus* sp. TF14 shifted from 75 to 95°C after immobilization which represents a better operational condition for starch liquefaction process.

Thermal stability of *Geobacillus* sp. TF14 α -amylase was examined and results were shown in Figure 4B, it

is seen that free enzyme conserved almost nearly all its original activity after 48 h of incubation at tested temperatures. While free α -amylase retained most of its original activity after incubation at 4 and 75°C whereas 80% of its activity was conserved after incubation at 55°C and nearly all activity was lost after incubation at 90°C for 72 h. α -Amylase from *Geobacillus thermoleovorans* conserved 50% of initial activity after incubation for 5 h at 60 and 70°C [19]. Thermostable α -amylase from *Bacillus* sp. A3-15 conserved nearly all its activity after 30 min pre-incubation at 100°C [31]. It was reported that α -amylase from *Bacillus subtilis* JS-2004 conserved all initial activity after incubation at 80°C for 1 h [37]. Our results clearly show that *Geobacillus* sp. TF14 α -amylase is highly thermostable and this property makes it very important for industrial starch liquefaction processes.

Thermal stability of the immobilized enzymes was also tested and results were given in Figure 4C. It was found that α -amylases, immobilized both on Dowex and chitin, preserved over 75% of their initial activities after 72 h of incubation at 4 and 95°C. It is clearly seen that in both of these immobilization processes, thermal stability of the purified α -amylase was enhanced. It was reported that α -amylase from *Bacillus subtilis* immobilized on Dowex and chitin lost more than half of its activity after incubation at 60°C for 1 h [35]. Chen et al. [38] reported that α -amylase immobilized on NIPAAm matrix retained 46% of initial activity after incubation at 70°C for 35 min. It was reported that α -amylase immobilized on CELBEADS was stable at 55°C after incubation of 24 h [39]. It can be concluded from these results that immobilization of purified α -amylase on Dowex and chitin enhanced the heat stability of the enzyme and both of these immobilized enzymes were much better than most of the α -amylases reported in literature in terms of heat stability.

Determination of kinetic parameters

Substrate saturation graphics of free and immobilized α -amylases represent typical Michaelis-Menten reaction rate for the hydrolysis of soluble starch. K_m and V_{max} values for free and immobilized enzymes on Dowex and chitin were determined from the Lineweaver-Burk plots as 3.5 mg/mL, 0.67 mg/mL, 5.75 mg/mL and 5000 U/mg, 3333.33 U/mg, 2500 U/mg, respectively (Figure 5A–C). Different K_m values for α -amylases from various microorganisms changing from 0.315 mg/mL [19] to 8.03 mg/mL [35] have been reported. After immobilization the V_{max} value was decreased; this may be due to the steric hindrance of solid support.

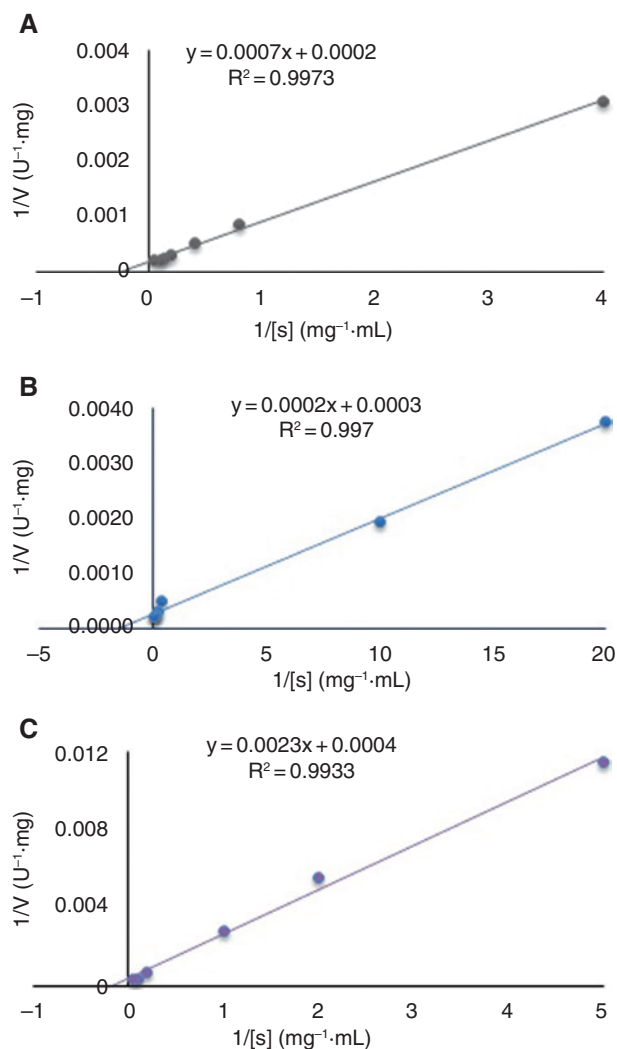


Figure 5: Lineweaver–Burk plots of the enzymes. (A) Free α -amylase. (B) Dowex immobilized α -amylase. (C) Chitin immobilized α -amylase.

Effect of some chemicals on enzyme activity

The effects of various metal ions and some detergents on free and immobilized enzymes were investigated. It was found that Mn^{2+} and Cu^{2+} ions almost completely inhibited free enzyme, but other tested metal ions showed inhibitory effects at different ratios (Table 3). Mehta and Asgher reported inhibitory effect of Mn^{2+} and Cu^{2+} on α -amylase activity [19–37]. As mentioned in the literature α -amylases are calcium containing enzymes [5], we found that Ca^{2+} increased the free enzyme activity nearly two fold. In the presence of EDTA free enzyme activity was decreased and this result corresponded with the fact that α -amylases are metallo-enzymes [20]. The effect of some detergents on enzyme activity was also studied. It found that the free enzyme preserved around 50% of its original activity

Table 3: Effect of various metal ions and some detergents on α -amylase enzyme activity.

Metal ion	Free α -amylase	Dowex immobilized	Chitin immobilized
Control	100±1.245	100±1.692	100±0.687
Ca^{2+}	173±1.613	103±1.542	88±1.834
Co^{2+}	47±1.939	107±1.955	56±0.812
Cu^{2+}	4±0.872	5±0.795	47±1.925
Fe^{2+}	58±1.278	112±1.562	60±2.655
Hg^{2+}	49±1.197	106±0.918	110±4.213
Mg^{2+}	37±2.665	107±1.310	45±1.270
Mn^{2+}	2±0.809	1±0.459	1±0.308
Ni^{2+}	65±1.919	102±1.655	73±1.877
Zn^{2+}	52±0.780	107±1.138	56±0.933
EDTA	90±1.116	71±0.966	59±3.908
Triton X100	48±0.813	96±1.377	37±1.227
Triton X114	45±0.831	83±1.355	14±1.059
Tween 20	52±1.902	61±1.828	12±0.542
SDS	55±1.499	112±1.215	24±1.822

Control activity accepted as 100% corresponds to 3227 U/mg, 3163 U/mg and 2593 U/mg for free enzyme, Dowex and Chitin immobilized enzyme activity respectively.

in the presence of detergents. Although immobilization on Dowex prevented inhibitory effect of all tested metal ions on enzyme activity, Mn^{2+} and Cu^{2+} still inhibited both Dowex and chitin immobilized α -amylase. It was also found that Dowex immobilized α -amylase retained nearly all of its original activity in the presence of detergents.

Reusability of immobilized enzyme

Applications of enzymes in industrial areas are possible only if they are stabilized against harsh reaction conditions. Immobilization is one of the ways to stabilize enzymes [33]. The reusability of immobilized amylase was studied for 15 cycles in standard assay conditions. After 15 cycles both of the immobilized enzymes conserved their initial activities (Figure 6). Chen et al. [38] reported 46%

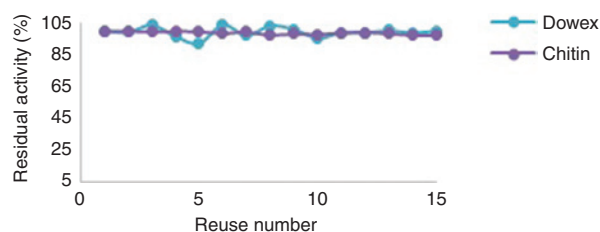


Figure 6: Reusability of immobilized α -amylases. First cycle was defined as 100% and corresponds 2870 U/mg and 2410 U/mg for Dowex and chitin immobilized α -amylase activity, respectively.

decrease of initial activity after 12 cycles and Sharma et al. [40] reported 22% decrease of initial activity after 6 runs. It is clear that both immobilized enzymes were quite stable in terms of reuse and this result indicates that both of the immobilized enzymes might be favorable for their use in continuous processes.

Conclusion

In this study, α -amylase was purified and immobilized on two different supports. Biochemical characterization of purified and immobilized α -amylase was also reported. It is clearly seen from our results that the purified enzyme showed maximum activity at pH 5.00 and pH 9.00. The liquefaction process of starch was carried out at in the pH range 5.00–6.50. It is also apparent that thermal stability of the purified α -amylase is very high when compared to commercial amylase obtained from *Bacillus licheniformis* or *Bacillus amyloliquefaciens* strains. This property is very important for starch liquefaction processes; so the purified α -amylase may be a candidate for starch liquefaction processes. After immobilization on Dowex, enhanced pH-activity profile was achieved. Immobilization also contributed to thermal stability of the enzyme. After 15 reuses, the immobilized α -amylases conserved nearly their initial activities. These properties are very important for continuous processes and immobilized α -amylase may be applicable to continuous processes.

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