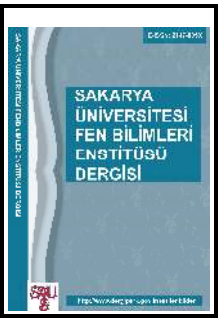
SAKARYA ÜNİVERSİTESİ FEN BİLİMLERİ ENSTİTÜSÜ DERGİSİ
SAKARYA UNIVERSITY JOURNAL OF SCIENCE
e-ISSN: 2147-835X
Dergi sayfası: http://dergipark.gov.tr/saufenbilder

Geliş/Received	Doi
22-02-2017	
Kabul/Accepted	
01-08-2017	10.16984/saufenbilder.293313



Covalent Immobilization of α-Amylase from Thermophilic *Geobacillus sp.* TF14 on Chitosan Beads

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ABSTRACT

In this study, α-amylase formerly purified from *Geobacillus sp.* TF14 strain was covalently immobilized onto chitosan beads. Chitosan beads were prepared by dissolving chitosan powder in 5% acetic acid solution and by addition dropwise to 1 M NaOH solution. The consisted beads were washed to remove excessive amount of NaOH. Immobilization was carried out in two steps. Firstly, chitosan beads were activated by reacting with 2.5% Glutaraldehyde solution. Next, activated chitosan beads were mixed with enzyme solution to complete immobilization. Biochemical characterization of immobilized α-amylase was also carried out. It was found that immobilized α-amylase achieved maximum activity at pH 9.00 and the enzyme was quite stable at this pH over a period of 48 h. Temperature optimum of the enzyme was determined as 95 °C. It was also determined that the enzyme protected 50% of its initial activity after incubation of 48 h at this temperature. While Mn^{2+}, Co^{2+} and EDTA almost completely inhibited the enzyme, other metal ions showed inhibitory effects at different ratio. In the presence of some detergents the enzyme conserved its initial activity. It can be concluded that the immobilized α-amylase may find application in many fields of starch based industries.

Keywords: α-Amylase, Immobilization, Chitosan, Characterization, *Geobacillus sp.*

Termofilik Geobacillus sp. TF14'ten saflaştırılan α-Amilaz enziminin Kitosan boncuklara kovalent immobilizasyonu

ÖZ

Bu çalışmada, daha önce *Geobacillus sp.* TF14den saflaştırılmış α-amilaz enzimi, kitosan boncuklara kovalent olarak immobilize edildi. Kitosan boncuklar, toz haldeki kitosanın % 5'lik asetik asit çözeltisinde çözülmesi ve 1 M NaOH çözeltisine damla damla eklenmesiyle elde edildi. Daha sonra boncuklar NaOH'in fazlasının giderilmesi için ard arda saf su ile yıkandı. İmmobilizasyon iki aşamada gerçekleştirildi. Öncelikle, % 2,5 Gluteraldehit çözeltisi ile reaksiyona sokularak kitosan boncuklar aktive edildi. Aktive edilmiş kitosan boncuklar immobilizasyonun tamamlanması için enzim çözeltisi ile karıştırıldı. İmmobilize edilen α-amilazın biyokimyasal karakterizasyonu da gerçekleştirildi. İmmobilize α-amilazın pH 9,00'da maksimum aktiviteye ulaştığı ve enzimin 48 saatlik bir sürede bu pH'da oldukça kararlı olduğu tespit edildi. İmmobilize enzimin optimum sıcaklık değeri 95 °C olarak belirlendi. Enzimin, bu sıcaklıkta 48 saat inkübasyon işleminden sonra başlangıçtaki aktivitesinin % 50'sini koruduğu tespit edildi. Mn^{2+}, Co^{2+} ve EDTA'nın immobilize enzim aktivitesini neredeyse tamamen inhibe ettiği, diğer metal iyonlarının farklı oranlarda inhibisyona neden olduğu belirlendi. Bazı deterjanlar varlığında enzimin aktivitesini koruduğu tespit edildi. İmmobilize edilen α-amilazın nişasta esaslı birçok sanayi alanında kullanılabileceği sonucuna varılabilir.

Anahtar Kelimeler: α-Amylase, İmmobilizasyon, Kitosan, Karakterizasyon, *Geobacillus sp.*

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1. INTRODUCTION

Microbial enzymes and their applications in various industrial processes are off great importance due to their unique properties as higher reaction rates, reaction specifity and regulation capacity [1]. Hydrolases are the major class of enzymes dominated the market because of their industrial importance. They have a major application area of hydrolyzing biopolymers such as starch, pectin, cellulose and proteins [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 in different length. Thermo-stable α-amylases are essential for starch industry in starch saccharification and liquefaction process. α-Amylases have application not only in starch hydrolysis but also in several industrial process such as textile, paper, detergent, fermentation, pharmaceutical, sugar and bakery [3].

One of the main problems of enzyme usage in industry is low stability of enzymes at harsh reaction conditions. Despite of their unique catalytic properties, their stabilities need to be improved for industrial applications. Immobilization is one of the most exploited ways for improving the stability of enzymes. It is stated that immobilized enzymes may exhibit much better functional properties than the corresponding soluble forms including possible increase in stability, good catalytic activity, easier product and enzyme recovery, continuous operation of enzymatic processes, convenience in handling, reusability and reduced susceptibility to microbial contamination [4, 5].

Main scope of this study was the immobilization of formerly purified α-amylase [6] onto Chitosan beads. Biochemical characterization of immobilized enzyme was also carried out. Industrial application potential of immobilized enzyme was also discussed by compering biochemical properties of free and immobilized enzyme.

2. MATERIAL AND METHODS

2.1. Materials

Chitosan powder, NaOH, Acetic acid, Soluble starch, DNS (3,5 dinitrosalycilic acid), Glutaraldehyde, disodium hydrogen phosphate, Tris and Glycine were purchased from Sigma Aldrich. Ultra pure water was suplied by using Milipore pure water system. α-Amylase was formerly purified from *Geobacillus sp.* TF14. All other chemicals were of analitical grade.

2.2. Preparation of Chitosan beads

Chitosan beads were prepared according to Rodrigues and co-worker's method [7]. Briefly, 2 g of chitosan powder was dissolved in 5% acetic acid solution. This solution was transferred into a chromatography column and added dropwise into the 1M NaOH solution. The composed beads were filtered and washed with pure water to remove excess amount of NaOH.

2.3. Modifying Chitosan Beads by Glutaraldehyde

Prepared chitosan beads were suspended into 50 mM pH 8.00 Tris-HCl buffer including 2.5% glutaraldehyde solution. The suspension was gently mixed for 4 h at room temperature to complete the reaction between chitosan beads and glutaraldehyde. At the end of the period chitosan beads were filtered and washed with pure water to remove excessive amount of glutaraldehyde [8].

2.4. Immobilization of α-Amylase onto Chitosan Beads

Formerly purified α-amylase from *Geobacillus sp.* TF14 [6] was added on to glutaraldehyde activated chitosan beads suspended in 50 mM pH 8.00 Tris-HCl buffer. Immobilization was carried out at room temperature for 12 h. At the end of the reaction period beads were filtered and washed with 50 mM pH 8.00 Tris-HCl buffer. Filtrate (washing solution) was collected and the amount of unbound protein was determined by using Bradford's dye binding method [9]. The yield of immobilization was calculated with the fallowing equation;

Immobilization Yield = [(A-B)/A]*100,

Where A is the initial protein (mg), B is the total unbound protein (mg) [10].

2.5. Characterization of Immobilizel-Amylase

Characterization assays were given by means of three separate experiments. ± Deviations are given on the graphs. Amylase activity assay was performed spectrophotometrically according to DNS method [10]. One unit (U) of enzyme activity was defined as the amount of enzyme that liberated 1 µM of reducing sugar as glucose equivalents in 1 min under the assay conditions.

2.5.1. The Effect of pH on Enzyme Activity and pH Stability

In order to determine the optimum pH, activity assays were conducted with immobilized enzyme by using 50 Mm Mcilvaine buffers between pH 3.00 to pH 8.00 and 50 mM Glycine-NaOH buffers between pH 9.00 to pH 11.00. Relative activity was calculated by considering the highest activity as 100% [7]. pH stability of immobilized enzyme was determined for pH 6.00 and 9.00 by the time course. For this, immobilized enzyme was separately mixed with pH 6.00 and 9.00 buffers in five different Eppendorf tubes and kept at room temperature. Activity assays were carried out after incubation of 6, 12, 24 and 48 h and results were calculated by comparing with non-incubated enzyme activities [5].

2.5.2. Optimum Temperature and Thermal Stability

To determine optimum temperature, activity assays were carried out at different temperatures ranging from 65 to 105 ºC at optimal conditions. Results were calculated as relative activity by considering the highest activity as 100 %. Thermal stability of immobilized α-amylase was determined by incubating the enzyme immobilized beads at 75 ºC and 95 ºC in Eppendorf tubes separately. After the incubation of 6, 12, 24 and 48 h, the tubes were rapidly cooled to room temperature and enzyme activities were determined at standard assay conditions. Results were calculated by comparing with non-incubated enzyme activity [5].

2.5.3. Determination of Kinetic Parameters

Kinetic parameters of immobilized α-amylase were obtained by measuring the rate of starch hydrolysis at various substrate concentrations in the standard reaction mixture ranging from 0.25 mg/mL to 25 mg/mL. The Michaelis–Menten constant (*K*m) and maximum velocity (*V*max) values were determined from the Lineweaver–Burk plot [11].

2.5.4. Effect of Some Chemicals on Enzyme Activity

The effect of metal ions on the enzyme activity was separately investigated by adding Cl^- salt solutions of Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+} and Zn^{2+} 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 on enzyme activity at a final concentration of 1% was also determined. Enzyme activity determined in the absence of chemicals was defined as 100% [6].

2.5.5. Reusability of Immobilized Enzyme

The reusability of immobilized amylase was studied for 8 cycles in standard assay conditions with 10 mg/mL of starch as the substrate. After each activity measurement, the immobilized enzymes were recovered by fast centrifugation and washed with buffer solution for use in second cycle and so on. Results were calculated as relative activity by comparing with the first cycle starch hydrolysis [5].

3. RESULTS AND DISCUSSION

3.1. Immobilization of α-Amylase on Chitosan Beads

Chitosan beads were used as solid support for covalent immobilization of α-amylase. Purified α-amylase (4.36 mg) was added into chitosan beads suspended in 50 mM pH 8.00 Tris-HCl buffer. After 12 h of reaction beads were filtered and washed with the same buffer. Filtrate was collected and total unbound protein content was determined as 2.63 mg. Immobilization yield was calculated as 39%.

3.2. Effect of pH on Enzyme Activity and pH Stability

To determine the optimum pH of immobilized enzyme, activity assays were carried out at different pHs ranging from 3.00 to 11.00. Results were calculated as relative activity. The pH activity profile of immobilized enzyme was given

in Figure 1A. It can be seen from the figure that optimum pH of the immobilized enzyme remained the same but the activity of the enzyme around neutral pHs was highly increased. Similar results were reported in literature [12, 13]. It is stated that the optimum pH may undergo apparent shifts after the immobilization as the alteration of side chain ionization around the active site [14, 15].

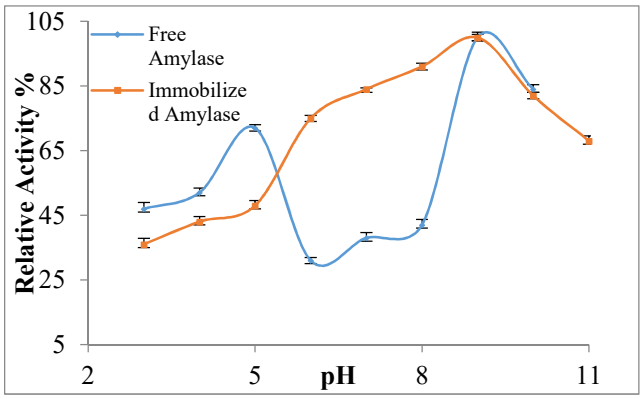

Figure 1 A: Effect of pH on Enzyme Activity

The pH stability of immobilized enzyme was also examined and it was found that immobilized enzyme was more stable at pH 9.00 than at pH 6.00 (Figure 1B). Chitosan immobilized α-amylase retained 85% and 65% of its starting activity at pH 9.00 after 12 and 48 h of incubation respectively. Free enzyme retained only 50% of its initial activity after incubation of 48 h at pH 9.00 [6]. These results clearly show that immobilization enhanced the pH stability of the enzyme.

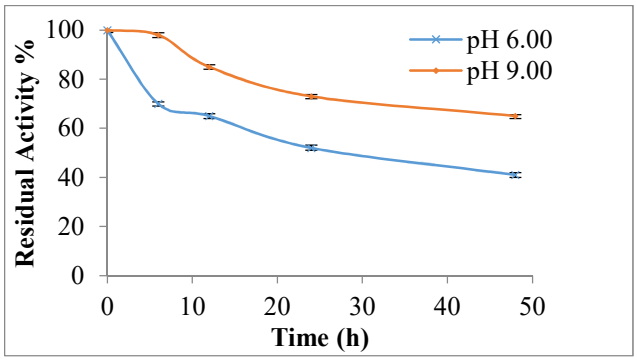

Figure 1B: pH Stability of Immobilized α-Amylase

3.3. Optimum Temperature and Thermal Stability

The effect of temperature on the immobilized enzyme activity was determined by measuring its activity at different temperatures ranging 65 to 105 °C. Results were calculated as relative activity and given in Figure 2A. It was found that temperature optimum of the α-amylase was shifted from 75 °C to 95 °C after the immobilization. This result quite consistent with the fact that immobilization is the one way of improving the operational conditions of the enzymes [16]. Kahraman *et al.* reported that temperature optima of α-amylase shifted from 30 to 50 °C after immobilization on glass beads [17]. El-Banna *et al*. reported that 20 °C increment of tempera-ture optima was achieved both for Dowex and Ca-Alginate immobilized α-amylases [18].

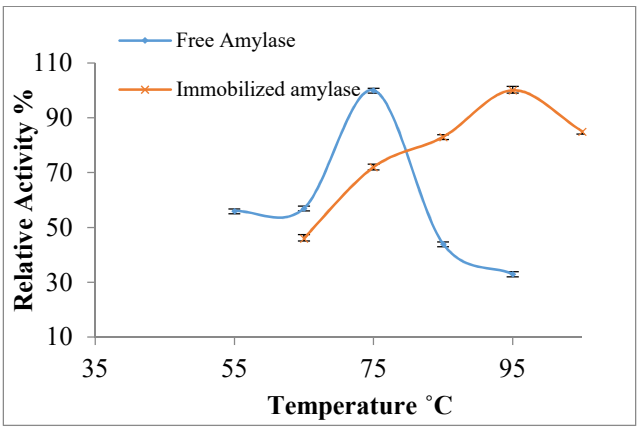

Figure 2 A: Effect of Temperature on Enzyme Activity

Thermal stability of the immobilized enzyme was also tested and results were given in Figure 2B. It was found that the immobilized α-amylase conserved around 50% of its initial activities after 48 h of incubation at 75 and 95 °C. It was previously found that purified α-amylase was highly thermostable and preserved nearly all its starting activity at 90 °C for 48 h [6]. It was reported that α-amylase from *Bacillus subtilis* separately immobilized on Dowex and chitin lost more than half of its initial activity after incubation at 60 °C for 1 h [18]. Chen *et al.* reported that α-amylase immobilized on NIPAAm matrix retained 46% of initial activity after incubation at 70 °C for 35 min [19]. It can be concluded from these results that immobilization of purified α-amylase on chitosan beads barely enhanced the heat stability of the enzyme and this result was much better than some of the other results reported in literature in terms of heat stability.

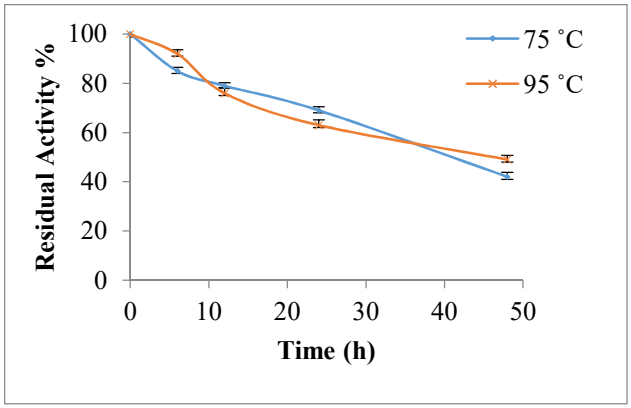

Figure 2 B: Thermal Stability of the Immobilized α-Amylase

3.4. Determination of Kinetic Parameters

Kinetic parameters of immobilized α-amylase were determined by conducting activity assays at different soluble starch concentrations ranging from 0.25 mg/mL to 25 mg/mL. Substrate saturation graphic of immobilized α-amylase represents typical Michaelis-Menten reaction rate for the hydrolysis of soluble starch. K_m and V_{max} values for immobilized enzyme were determined from the Lineweaver–Burk plot as 0.526 mg/mL and 526.316 U/mg respectively (Figure 3). V_{max} and K_m values of free enzyme were calculated formerly as 5000 U/mg and 3.5 mg/mL [6]. Decreasing V_{max} value with immobilization may be due to the steric hindrance of solid support.

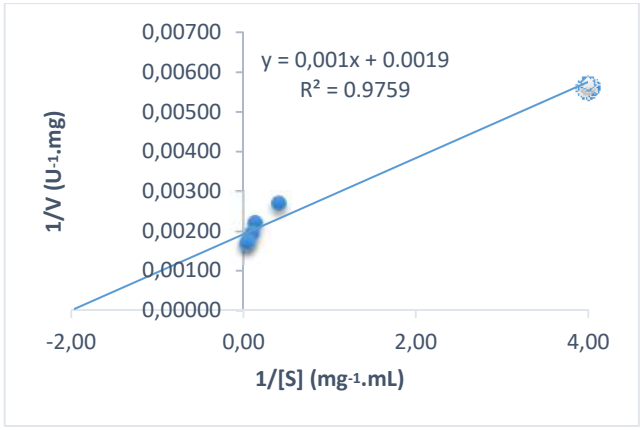

Figure 3: Lineweaver–Burk Plots of Starch Hydrolysis

3.5. Effect of Some Chemicals on Enzyme Activity

The effect of various metal ions and some detergents on immobilized α-amylase was determined. It was found that Mn^{2+} and Co^{2+} completely inhibited immobilized α-amylase whereas all other tested metal ions showed lower inhibitory effect. It was also found that only Fe^{3+} activated immobilized α-amylase (Table 1). The effect of some detergents was also tested and it was found that immobilized α-amylase conserved its initial activity in the presence of 1 % detergent concentrations. These results clearly showed that immobilization enhanced the stability of the enzyme in the presence of detergents.

Table 1: Effect of Some Metal Ions and Detergent on Immobilized α-Amylase

Chemical	Relative Activity % (Immobilized enzyme)	Relative Activity %* (Free Enzyme)
None (Control)	100	100
Ca^{2+}	70	173
Co^{2+}	6	47
Cu^{2+}	59	4
Fe^{3+}	130	58
Hg^{2+}	60	49
Mg^{2+}	25	37
Mn^{2+}	2	2
Ni^{2+}	41	65
Zn^{2+}	95	52
EDTA	7	90
Triton X100	108	48
Triton X114	124	45
Tween 20	103	52
SDS	83	55

*Relative activity % for free enzyme was obtained from the reference 6.

3.6. Reusability of Immobilized Enzyme

Applications of enzymes at industrial scale are possible in case they are stabilized against harsh reaction conditions. Immobilization is the one way of stabilization of enzymes [16] and enables using enzymes several times. The reusability of immobilized amylase was studied for 8 cycles in standard assay conditions with 10 mg/mL of starch as the substrate. The activity observed after each cycle was compared with initial activity,

considering that it was 100%. After 6 cycles the immobilized α-amylase conserved nearly all of its initial activity (Figure 4). At the end of 8 cycle 50% of initial activity was conserved. Chen *et al.* reported 46% decrease of initial activity after 12 cycles [19] and Sharma *et al.* reported 22% decrease of initial activity after 6 run [20]. *Singh et al.* reported 40% decrease of initial activity after 30 cycles for commercial α-amylase immobilized on NIPAAm film [5]. It is clear that immobilized α-amylase is quite stable in terms of reuse and this result indicates that immobilized α-amylase may be favorable for using continuous process.

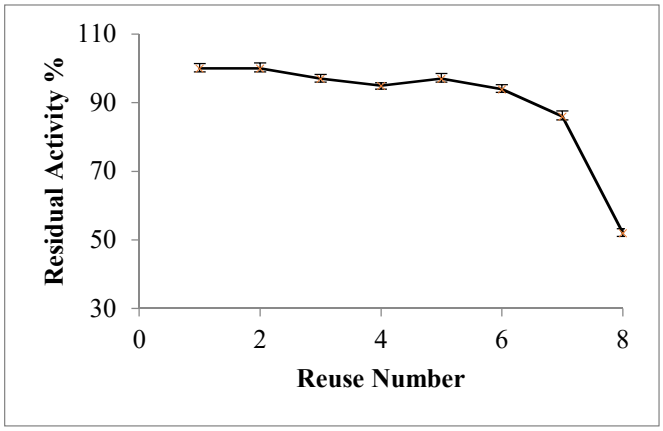

Figure 4: Reusability of Immobilized α-Amylase

4. CONCLUSION

In this study covalent immobilization of α-amylase purified from *Geobacillus sp.* TF14 on chitosan beads and biochemical characterization of immobilized enzyme was reported. Immobilized enzyme was active and stable at basic pHs. Additionally, it was highly active in the presence of some surfactants. These results shows that immobilized α-amylase may be a candidate for detergent industry. Thermal stability of the enzyme was highly enhanced after immobilization. This property is very important in starch hydrolysis processes. Immobilization also contributed to reuse of enzyme for several times making it possible to use in continuous processes for starch hydrolysis.

Acknowledgment

We gratefully appreciate the financial support of this work with project code of 11549 by KTÜ-BAP.

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