Research Article

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**Immobilization and some application of α-amylase purified from Rhizoctonia solani AG-4 strain ZB-34**

Rhizoctonia solani AG-4 strain ZB-34’den Saflaştırılmış α-Amilazın İmmobilizasyonu ve Bazı Uygulamaları

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**Abstract**

**Background:** Aim of the study was to immobilize the α-amylase produced earlier from the mesophilic fungus *Rhizoctonia solani* AG-4 strain ZB-34 by solid-state fermentation and investigate the suitability of immobilized enzymes for some industries.

**Materials and methods:** A novel α-amylase from *R. solani* AG-4 strain ZB-34 was immobilized in chitosan by covalent binding and Ca-alginate by entrapment.

**Results:** The efficiency of chitosan and Ca-alginate immobilization was 67.9% and 59.6%, respectively. The immobilized enzymes showed the highest activity in the presence of starch. Optimum values for chitosan and Ca-alginate immobilized enzymes were pH 4.50 and 40°C and pH 5.50 and 60°C, respectively. It was found that immobilized enzymes were highly stable in terms of thermal and pH stabilities. When the chitosan immobilized enzyme was used with detergents, chocolate stains on dirty laundry was better cleaned. Chitosan immobilized *R. solani* AG-4 strain ZB-34 α-amylase was found to have a higher desizing effect at 40°C in tap water. As a result of Ca-alginate immobilization, the enzyme clarified apple juice more than the free enzyme.

**Conclusion:** The results showed that immobilized enzymes might have potential applications in industry. This is the first report immobilizing an α-amylase produced from the fungus *R. solani*.

**Keywords:** α-Amylase; Desizing; Immobilization; Juice clarification; *Rhizoctonia solani*; Wash performance.

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**Oz**

Amaç: Bu çalışmanın amacı, katı substrat fermentasyonu ile daha önceden mezofilik fungus *Rhizoctonia solani* AG-4 strain ZB-34'den üretilen α-amilaz, kovalent bağlanma ile kitosan ve Ca-alginate immobilize etmek ve immobilize enzimlerin bazı endüstriler için uygunluğunu araştırmaktır.

**Gereç ve Yöntemler:** *Rhizoctonia solani* AG-4 strain ZB-34'den yeni bir α-amilaz, kovalent bağlanma ile kitosan ve Ca-alginate immobilize edildi. Immobilize enzimler en yüksek aktiviteleri nişasta varlığında gösterdi. Kitosan immobilized *R. solani* AG-4 strain ZB-34 α-amylase found to have a higher desizing effect at 40°C in tap water. As a result of Ca-alginate immobilization, the enzyme clarified apple juice more than the free enzyme.

**Sonuç:** Sonuçlar immobilize enzimlerin endüstride potansiyel uygulama alanlarına sahip olabileceğini gösterdi.
Bu çalışma, *R. solani* fungusundan üretilen bir α-amilazın immobilize edildiği ilk çalışmaddir.

**Anahtar Kelimeler:** α-amilaz; hasil alma; immobilizasyon; meyve suyu berraklaştırma; *Rhizoctonia solani*; yıkama performansı.

**Introduction**

α-Amylase (4-α-D-glucan glucanohydrolase, EC 3.2.1.1) is an enzyme used extensively in a range of industries including food and beverages, textiles and detergents, drugs and pharmaceuticals, brewing and fine chemicals, bioconversion of solid waste etc. Due to having so many application areas it is necessary to produce α-amylases at industrial scale. Amylases have been reported to be produced by plant, animal and microbial sources. Microbial amylase production has been reported to be most effective [1, 2]. 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. For this reason, industrial enzymes are frequently immobilized onto solid supports. Immobilization may provide many advantages: efficient recovery, reusability and facile separation of the enzyme from the reaction mixture, increased activity and improvement of some catalytic features such as stability and specificity [3, 4].

Immobilized amylases have generally increased stability compared to free enzymes. Entrapment is one of the most preferable immobilization technique due to it prevents excessive loss of enzyme activity and protects enzyme from microbial contamination. Physical entrapment of α-amylase in calcium alginate beads has shown as a relatively easy, rapid and safe technique. Chitosan can be used for immobilization of enzymes by covalent binding. It has many characteristics like hydrophobicity, biocompatibility, and low biodegradability, high permeability toward water, good adhesion and high affinity towards proteins. It is an inexpensive, inert, non-toxic, high mechanical strength support [1, 5–7].

In this study, a novel α-amylase produced from the mesophilic fungus *Rhizoctonia solani* AG-4 strain ZB-34 and purified with starch affinity technique previously [8] was immobilized in chitosan by covalent binding and Ca-alginate by entrapment. After then, the immobilized enzymes were characterized biochemically and their some industrial applications were investigated.

**Materials and methods**

**Production and purification of the enzyme**

α-Amylase from *R. solani* AG-4 strain ZB-34 was produced previously by solid-state fermentation and purified with starch affinity method [8].

**Covalent immobilization of the enzyme in chitosan**

One gram chitosan was stirred with 100 mL of 0.1 N HCl solution containing glutaraldehyde (%2.5, v/v) for 2 h at 25°C. After the addition of 10 mL of 0.1 M NaOH solution, the precipitate formed was collected by filtration. Then, chitosan molecules were washed several times with purified water for removing the non-binding glutaraldehyde from the mixture. Wet chitosan was mixed appropriate volume of purified enzyme and slowly stirred at 4°C for 1 h. After filtration of the mixture, immobilized enzyme-chitosan molecules were repeatedly washed with distilled water to remove the non-binding enzyme until the absorbance was lower than 0.01 at 280 nm [7, 9].

Total protein concentration in the filtrate was determined and percentage of the amylase binding efficiency (E) was defined as follows [10].

\[
E = \frac{(C_i - C_o)}{C_i} \times 100
\]

\(C_i\): Protein concentration of solution before immobilization

\(C_o\): Protein concentration of solution after immobilization

**Entrapment of the enzyme in Ca-alginate**

Appropriate volume of purified enzyme was added to 5 mL of sodium alginate solution (2%, w/v). The final mixture was dropped using a syringe into 100 mL solution of 1 M CaCl₂. The beads were stirred 15–20 min and left for 24 h at 4°C to get the final hardened form. The final macro beads were removed and washed several times with deionized water for removal of excess CaCl₂. Total protein concentration in the filtrate was determined and the percentage of the binding protein was calculated as mentioned above [11].
Determination of α-amylase activity and protein concentration

One-hundred microliter of 1% soluble starch, 700 μL of phosphate buffer (50 mM, pH 7.00) and 0.1 g immobilized enzyme was mixed in an Eppendorf tube for determination of the enzyme activity. The mixture was incubated at 50°C for 15 min. The immobilized enzyme was separated from the mixture by quick-centrifugation. Eight-hundred microliter of dinitrosalicylic acid (DNS) reagent was added to supernatant and the resulting mixture was boiled in a water bath for 10 min. After cooling to room temperature, the absorbance was recorded at 540 nm and the liberated reducing sugar was calculated from a standard curve using glucose. One unit of enzyme activity was defined as the amount of enzyme producing 1 μmol reducing sugar per minute under the standard assay conditions [12].

Protein concentration was determined by Bradford method, using BSA as a standard [13].

Substrate specificity

Soluble starch, maltose, glycogen, β-cyclodextrin, amylopectin and maltotriose were used to determine the substrate specificity of the immobilized enzymes. All assays were performed in the standard reaction conditions [11]. In all subsequent studies, soluble starch was used as substrate.

Optimum pH and temperature

Enzyme activities were determined in glycine-HCl (pH 3.0), sodium acetate (pH 4.00, 4.50, 5.00, 5.50) and phosphate (pH 6.00, 7.00, 8.00) buffers. The highest enzyme activity was defined as 100%, and others were calculated as relative activities.

The optimum temperature of the each enzyme was determined at optimum pH value by measuring the activity at 10–80°C. The activity was expressed as percent relative activity in relation to the temperature optimum, which was considered as 100%.

pH and thermal stability

α-Amylase activity was determined by incubating the immobilized enzyme with acetate (pH 5.5) and phosphate (pH 7.0–8.0) buffer solutions at 4°C for 24 h. At the end of the incubation, enzyme activity was assayed under optimum reaction conditions. The percentage residual enzyme activity was calculated by comparison with non-incubated enzyme.

To determine the thermal stability, the enzyme solutions were separately incubated at 4, 28, 40, 50 and 60°C. Aliquots were withdrawn at 15, 30, 60, 90 and 120 min, and α-amylase activity was determined at optimum conditions. The activity of non-incubated enzyme was used to determine the 100% activity value.

Effect of some metal ions and detergent on the enzyme activity

The effect of Li⁺, Na⁺, Mn²⁺, Cu²⁺, Zn²⁺, Ni²⁺, Ca²⁺, Co²⁺, Mg²⁺ and EDTA on the enzyme activity was investigated by adding them separately to the standard reaction mixture at a final concentration of 1 mM. The residual enzyme activity (%) was calculated in comparison to that without any additives.

To study the effect of Tween 20, SDS, Triton X-100 and Triton X-114, they were directly added to the standard reaction mixture at the final concentration of 1%. The percentage residual activities were expressed by comparison with standard assay mixture including no detergent.

Determination of salt tolerance

To determine salt tolerance of immobilized enzymes, enzyme activities were assayed in the presence of NaCl with different concentration (0.5–5 M). The enzyme activity in the absence of NaCl was taken as 100% [14].

Reusability of the immobilized enzymes

The effect of repeated usage on the activity of enzyme was determined under optimum conditions. At the end of reaction, the immobilized enzyme beads were separated from the assay mixture by quick centrifugation and activity assay was done in supernatant. The beads were washed three times in distilled water and then a new reaction was repeated under identical conditions [15].

Evaluation of the chitosan immobilized enzyme for use in detergent formulations

Usability of chitosan immobilized enzyme as a detergent additive was examined by using some commercial laundry
and dish washing detergents available at a local market. After the solutions were prepared from solid detergents as 7 mg/mL and liquid detergents as 10% (v/v) to simulate washing conditions, they were pre-heated at 100°C for 60 min to destroy the endogenous enzyme activity. Enzyme activity was determined in the presence of 1 mg/mL and 1% final concentration of solid and liquid detergents, respectively, at 50°C. The activity of the immobilized enzyme assayed in the absence of detergents was taken as 100% [16].

To test the stability of chitosan immobilized enzyme in the presence of protease, 0.1 g immobilized enzyme was mixed with 100 μL of a commercial protease preparation (proteinase K, 0.1 mg/mL) and incubated for up to 150 min at room temperature. Thereafter, the residual activity was determined against the control (amylase without protease treatment) [8].

Wash performance analysis of the chitosan immobilized enzyme

Wash performance analysis of the chitosan immobilized enzyme was evaluated by determining its chocolate stain releasing capacity from cotton fabrics. After the chocolate was liquefied at 70°C, cotton fabrics (5 cm × 5 cm) were stained with 300 μL of the liquefied chocolate and then dried overnight under in a hot air oven. Each piece of stained cloth was dipped in one of the following flasks containing: (a) 25 mL of tap water (control), (b) 25 mL of tap water and 1 g immobilized enzyme, (c) 20 mL of tap water and 5 mL of commercial detergent (Persil®, 1%), and (d) 20 mL of tap water and 5 mL of commercial detergent (Persil®, 1%) containing 1 g immobilized enzyme.

Flasks were stirred at 200 rpm, 40°C for 60 min. Stain removal capabilities of the chitosan immobilized enzyme was examined visually by looking at the pieces of dried cloth. The chocolate stained cloth piece washed with tap water was considered as a control [17].

Desizing of cotton fabrics with the chitosan immobilized α-amylase

Cotton fabrics (5 cm × 5 cm) were weighed and treated with 25 mL of soluble starch solution (1% w/v) at room temperature for 15 min. After then, they were dried and weighed again. The cotton fabrics were desized in 25 mL acetate buffer (50 mM, pH 4.5) containing 1 g immobilized enzyme at 40 and 50°C for 1 h by stirring at 200 rpm. The same procedure was applied by using tap water instead of buffer. Upon the completion of the reaction, the cotton fabrics were washed using tap water, dried at 105°C to a constant weight and weighed. The percent (%) removal of starch was calculated by applying the following formula [18].

\[
\text{Desizing} \% = \left( \frac{\text{Weight of starch removed by enzyme (g)}}{\text{Total starch present on the fabric (g)}} \right) \times 100
\]

The use of Ca-alginate immobilized enzyme in apple juice clarification

Apples (Malus domestica cv. Golden Delicious) were cut into cubes and mashed in a mixer grinder and manually pressed using double layer cheesecloth. After calcium chloride was added to the raw apple juice at a final concentration of 10 mM, aliquots were pasteurized (5 min at 90°C) and immediately cooled to 50°C. A mixture containing 5 mL apple juice and 1 g Ca-alginate immobilized enzyme was incubated at 60°C for 1 and 3 h. After centrifugation at 17136 × g for 10 min, absorbance (440 nm) of the supernatant was determined. Also, the total reducing sugar content was determined by the DNS method [5].

Results

α-Amylase from R. solani AG-4 strain ZB-34 produced previously by solid state fermentation (SSF) and purified with starch affinity method was immobilized in chitosan and Table 1: Substrate specificity of the immobilized α-amylases.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Chitosan immobilized enzyme</th>
<th>Ca-alginate immobilized enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble starch</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Glycogen</td>
<td>61.69±2.5</td>
<td>42.52±0.8</td>
</tr>
<tr>
<td>Amylopectin</td>
<td>90.94±1.2</td>
<td>37.10±1.5</td>
</tr>
<tr>
<td>Maltose</td>
<td>64.51±4.1</td>
<td>75.17±3.0</td>
</tr>
<tr>
<td>Maltotriose</td>
<td>38.90±3.7</td>
<td>55.47±2.5</td>
</tr>
<tr>
<td>β-cyclodextrin</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The enzyme activity was performed in the standard reaction conditions by using soluble starch, maltose, glycogen, β-cyclodextrin, amylopectin and maltotriose as substrates. The highest enzyme activity (0.065 U/mg protein and 0.118 U/mg protein for chitosan and Ca-alginate immobilized enzyme, respectively) was defined as 100%, and other enzyme activities were calculated as relative activities.
Ca-alginate. The binding efficiency of the enzyme to chitosan and Ca-alginate was 67.9% and 59.6%, respectively.

Substrate specificity

Soluble starch, maltose, glycogen, β-cyclodextrin, amylopectin, and maltotriose were used to determine the substrate specificity of the immobilized enzymes. All assays were performed in the standard reaction conditions. Both of the immobilized enzymes showed the highest activity in the presence of soluble starch (Table 1). Although no hydrolytic activity was observed for β-cyclodextrin, the enzymes catalyzed hydrolysis of other substrates used at different ratios.

Optimum pH and temperature

Optimum pH and temperature values were determined as pH 4.50, 40°C and pH 5.50, 60°C for chitosan and Ca-alginate immobilized enzyme, respectively (Figure 1A, B).

pH and thermal stability

As can be seen from Figure 2A, the chitosan immobilized enzyme retained all of its original activity at all tested pH values after 1 day. At the end of 5 days, approximately 70–80% of activity was still present. These results suggest
that the chitosan immobilized enzyme was highly stable at 4°C for tested pH values.

About 90% of the activity of the Ca-alginate immobilized enzyme was maintained at pH 5.5 after 7 days (Figure 2B). At pH 7.00 and 8.00, activity was conserved at the rate of 65% and 50%, respectively, after 7 days.

The activity of chitosan and Ca-alginate immobilized \( \alpha \)-amylases was conserved completely at storage temperature (4°C) and the fungus growth temperature (28°C) for 120 min. They also conserved approximately 60–70% of their original activity at 40°C after 120 min (Figure 3A, B). Ca-alginate immobilized enzyme seems to maintain its activity much more than chitosan immobilized enzyme at 4°C and 50°C. The enzyme had more than about 10% of its activity even after 60 min at 60°C.

**Effect of some metal ions and detergent on the enzyme activity**

When the activity of \( \alpha \)-amylase was examined in the presence of metal ions, it was noted that in the case of some metal ions there were differences between immobilized enzymes (Figure 4). The activation rate was higher in chitosan immobilized enzyme than Ca-alginate immobilized enzyme in the presence of Li\(^{2+}\) and Mn\(^{2+}\). Cu\(^{2+}\) activated the chitosan immobilized enzyme but inhibited Ca-alginate

![Figure 3: Thermal stability of chitosan immobilized enzyme (A) and Ca-alginate immobilized enzyme (B). The enzyme solutions were separately incubated at 4, 28, 40, 50 and 60°C. Aliquots were withdrawn at different times and \( \alpha \)-amylase activity was determined at optimum conditions. 18.8 U/mg protein and 112.51 U/mg protein for chitosan and Ca-alginate immobilized enzyme, respectively, was considered as 100% residual activity.](image)

![Figure 4: Effect of some metal ions, EDTA and detergents on the activity of immobilized enzymes. The effect of some chemicals was investigated by adding them separately to the standard reaction mixture at a final concentration of 1 mM or 1%. The percentage residual activities were expressed by comparison with standard assay mixture with no chemical added. 17.1 U/mg protein and 102.1 U/mg protein for chitosan and Ca-alginate immobilized enzyme, respectively, was considered as 100% residual activity.](image)
α-amylase approximately 10%. In contrast, while Ca\(^{2+}\) inhibited the chitosan immobilized enzyme about 10%, activated the Ca-alginate α-amylase about 30%.

**Determination of salt tolerance**

Whereas no significant change was found in the activity of chitosan immobilized enzyme at salt concentrations of 0.5, 1.0 and 2.0 M, the enzyme was inhibited approximately 20–25% in the presence of 4.0 and 5.0 M NaCl, respectively. The alginate immobilized enzyme was found to be inhibited in a considerable amount at all salt concentration more than 1.0 M (Figure 5).

**Reusability of the immobilized enzymes**

Reusability of immobilized enzymes was determined by performing seven repetitions. For both of the immobilized enzymes, only about 20% of the activity was lost at the end of seven measurements (Figure 6).

**Evaluation of the chitosan immobilized enzyme for use in detergent formulations**

Chitosan immobilized enzyme was tested for its potential to be used as a commercial detergent additive. The highest

<table>
<thead>
<tr>
<th>Detergents</th>
<th>Residual activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Omo® (Solid laundry)</td>
<td>76.9 ± 0.5</td>
</tr>
<tr>
<td>Ariel® (Solid laundry)</td>
<td>74.1 ± 1.2</td>
</tr>
<tr>
<td>Persil® (Solid laundry)</td>
<td>81.1 ± 1.5</td>
</tr>
<tr>
<td>Alo® (Solid laundry)</td>
<td>73.4 ± 0.9</td>
</tr>
<tr>
<td>Etimatik® (Solid laundry)</td>
<td>51.1 ± 2.5</td>
</tr>
<tr>
<td>Perwol® (liquid laundry)</td>
<td>66.2 ± 3.0</td>
</tr>
<tr>
<td>Fairy® (liquid dishwasher)</td>
<td>65.1 ± 0.7</td>
</tr>
</tbody>
</table>

Enzyme activity was determined in the presence of 1 mg/mL and 1% final concentration of solid and liquid detergents, respectively, at 50°C. The activity of the immobilized enzyme assayed in the absence of detergents (19.8 U/mg protein) was taken as 100%.

**Table 2: Testing the compatibility of chitosan immobilized enzyme with various commercial detergents.**
activity of the enzyme was detected in the presence of Persil brand detergent (Table 2).

When the stability of the chitosan immobilized enzyme in the presence of protease was examined, it appeared that there was a loss in enzyme activity about 15% after 150 min (Figure 7).

**Wash performance analysis of the chitosan immobilized enzyme**

The utility of the chitosan immobilized enzyme as a detergent additive was visually tested. For this purpose, the fabric pieces on which the chocolate stain was formed were individually washed in Erlenmeyer flask containing tap water, immobilized enzyme, detergent (Persil®), detergent and immobilized enzyme together. At the end of the washing process, it was observed that the fabric piece washed with detergent and immobilized enzyme together was cleaner than the fabric piece cleaned by only detergent (Figure 8).

**Desizing of cotton fabrics with chitosan immobilized α-amylase**

Chitosan immobilized R. solani AG-4 strain ZB-34 α-amylase was found to have a higher desizing effect at 40°C and in acetate buffer (pH 4.5) (Table 3).

**The use of Ca-alginate immobilized enzyme in the apple juice clarification**

The alteration effect of Ca-alginate immobilized enzyme on some parameters of apple juice was investigated (Figure 9 and Table 4). It has been observed that
Ca-alginate immobilized enzyme affected the change of relevant parameters positively.

**Discussion**

Both of the immobilized enzymes had no hydrolytic activity in the presence of β-cyclodextrin as in the case of free enzyme [8]. This situation can be attributed existence of the cyclic α-(1,4)-glycosidic bounds in β-cyclodextrin.

It was previously reported that the free α-amylase from *R. solani* AG-4 strain ZB-34 had a pH optima at 5.50 [8]. When the literature data are examined, it can be seen that immobilization can change optimum pH of an enzyme. pH is one of the most important parameters with the ability to change the enzyme activity in the reaction mixture. Immobilization may cause optimal pH to shift as it causes conformational changes on the enzyme. This shift occurs when the ionization of the acidic and basic amino acid side groups in the microenvironment around the active site of the enzyme changes. It was reported that optimum pH of the immobilized α-amylases decreased from pH 6.0 to pH 5.75 and from 6.0 to 5.25 in DEAE-cellulose and chitin, respectively [19].

Optimum temperature of the free α-amylase from *R. solani* AG-4 strain ZB-34 was 50°C [8]. Shifting of the optimum temperature from 50°C to 40°C by chitosan immobilization leads to the need to investigate the suitability of the immobilized enzyme for industrial processes or applications that occur at low temperatures. For Ca-alginate immobilized enzyme, the optimum temperature increased from 50°C to 60°C. In a study, commercial pig pancreatic α-amylase was covalently immobilized on glass beads and the optimum temperature of the enzyme was reported to increase from 30°C to 50°C [20]. When the partially purified α-amylase enzyme from *Bacillus subtilis* was immobilized on the chitin surface, the optimum temperature value increased to 65°C with an increase of 20°C [19].

Furthermore, when the pH stability of free and immobilized enzymes was compared, it can easily be seen that the stability increases with immobilization [8]. This will make a positive contribution to the availability of the enzyme in industrial areas. It was reported that commercial *Bacillus* sp. α-amylase was immobilized on acrylic solid support materials and it was stable for 1 h at pH 3.5–8.0 [21]. The pig pancreatic α-amylase covalently immobilized to glass beads was determined to be stable at pH 6.9 and 4°C for 5 days [20].

It has been reported that α-amylase from *B. subtilis* 1 was immobilized to DEAE-cellulose and chitin and immobilized enzymes maintain their activity at 60°C after 1 h [19]. In another study, α-amylase from *B. subtilis* (ITBCCB148) bacterium lost almost all its activity when it was kept at 60°C for 1 h. In the thermal stability of the enzyme immobilized on alginate, an increase of about 10.5 times occurred compared to the free enzyme [22].

Commercial *Bacillus* sp. α-amylase (Sigma, St Louis, MO, USA) was immobilized to N-isopropylacrylamide (NIPAAm) polymer matrix. Free and immobilized enzyme conserved their activity about 33% and 46%, respectively, for 35 min at 70°C [23].

It has been reported that α-amylase from *Aspergillus sclerotiorum* was immobilized on Ca-alginate beads and the immobilized enzyme had a half-life of 164 min at 60°C [24]. Commercial *Aspergillus oryzae* α-amylase from D. Fine-Chem Ltd. (Mumbai, India) was immobilized in Ca-agar microcapsules and the immobilized enzyme was found to have a half-life of 17 min at 75°C [25].

It can be said that immobilized α-amylases from *R. solani* AG-4 strain ZB-34 may be either advantageous or disadvantageous over other α-amylases in terms of thermal stability. However, the most important point here is that the industrial application area of the enzyme to be used and therefore the evaluation must be done accordingly. As a result of immobilization, there was a significant increase in the stability of the *R. solani* AG-4 strain ZB-34 α-amylase, especially at low temperatures, up to 50°C. It is clear that this increase will contribute to the availability of immobilized enzymes in industrial processes especially at low temperatures.

Immobilization is one of the most preferred ways to increase the stability of enzymes under extreme conditions. If the immobilization process is carried out properly, the properties of the enzyme will recover in many ways, especially stability, activity, specificity and reduced inhibition [26]. Immobilization prevents the opening of protein folds leading to decrease in enzyme activity. Thus, the stability and other properties of the enzymes are favorable to develop [22]. The fact that immobilized *R. solani* AG-4 strain ZB-34 α-amylases have higher thermal stability than the free enzyme overlaps these facts.

In the presence of EDTA, immobilized enzymes were found to have higher activity losses than free enzymes [8]. α-Amylase obtained from *Bacillus* isolates and immobilized on polyglycidyl methacrylate beads was found to be inhibited by Mg²⁺, Mn²⁺ and Cu²⁺ ions at different rates [27]. It has also been reported that α-amylase from *Aspergillus niger* immobilized to glutaraldehyde modified polyaniline was inhibited by Ca²⁺, Zn²⁺ and Cu²⁺ ions [28].

The activity of chitosan immobilized enzyme increased about 8% in the presence of Tween 20.
Ca-alginate immobilized enzyme activated 5% in the presence of Triton X-114. Activity of the Ca-alginate immobilized enzyme could not be determined in the presence of SDS because the Ca-alginate beads disintegrated. It can be easily stated that immobilization of the R. solani AG-4 strain ZB-34 α-amylase contributes positively to the availability of the enzyme in some detergents.

The enzyme purified from Marinobacter sp. EMB8 was reported to maintain its activity at 75% as a result of 1 h treatment with SDS and Triton X-100 detergents at 0.1% concentration [29]. The effects of detergents such as Triton X-100 and Tween-20 and surfactants such as SDS on the activity of the α-amylase produced by Bacillus licheniformis AI20 were studied and found that detergents tested at 0.25% concentration did not alter the enzyme activity. Anionic surfactant SDS at the concentration of 1% was found to cause a 35% reduction in enzyme activity [30]. It has been reported that B. licheniformis NH1 α-amylase was treated with Tween-20 and Triton X-100 at 1% concentration at 40°C for 1 h and there was no loss of activity at this time [31].

The increase of amylase activity in the presence of some detergents suggests that these detergents may have positive effects on the enzyme conformation and on the hydrophobic interactions involved in stabilizing the buccal structure of the protein molecule [32]. The decrease in enzyme activity in the presence of certain detergents can also be correlated with a possible deterioration of protein conformation.

It has been reported that having high salt tolerance is a useful feature for amylases used in starch sugar processing and in different applications in the industry [33]. Salt tolerance testing has been reported to be important in the treatment of pollution control mechanisms, cellulose, starch and waste water containing high salt [34, 35]. In our work, the high salt tolerance of chitosan immobilized enzyme supports that it might be used in these industrial applications.

An α-amylase immobilized to N-isopropylacrylamide (NIPAAM) polymer was reported to maintain 54% activity in 12 consecutive measurements [23]. It was reported that α-amylase immobilized on Ca-agar microcapsules lost its activity by 22% after six measuring [26]. α-Amylase produced by SSF from A. sclerotiorum was immobilized on Ca-alginate beads. Beads prepared under optimal immobilization conditions could be used for up to 7 times, losing only 35% of the initial activities [24].

The utility of the chitosan immobilized enzyme as a detergent additive was visually tested. At the end of the washing process, it was observed that the fabric piece washed with detergent and immobilized enzyme together was cleaner than the fabric piece cleaned by only detergent. Also, the stability of the chitosan immobilized enzyme in the presence of protease was higher than the free enzyme [8].

In one study, it was reported that α-amylase (termamylase) obtained from Novozymes Co. (Tehran, Iran) was immobilized on silica nanoparticles and used in detergent formulations to aid the removal of starch stains. Removal of starch stains was been found to be better with the use of detergent containing immobilized enzyme [36].

In this study α-amylase from R. solani AG-4 strain ZB-34 was immobilized covalently on chitosan. In this case immobilized enzyme can interact with the stains on dirty laundry. Because of the enzyme was immobilized in Ca-alginate by entrapment, substrates of the enzyme within laundry stains cannot enter Ca-alginate beads. For this reason Ca-alginate immobilized enzyme cannot be used as detergent additive.

When the effect of Ca-alginate immobilized enzyme on some parameters of apple juice was investigated, it was found that Ca-alginate immobilized enzyme affected the change of relevant parameters positively. This effect was better than free enzyme [8].

In this work, an α-amylase from R. solani AG-4 strain ZB-34 was immobilized for the first time. The immobilized enzymes were characterized biochemically. The suitability of the chitosan immobilized enzyme for detergent industry as an additive and Ca-alginate immobilized enzyme for juice clarification were investigated. Also, chitosan immobilized enzyme was used for desizing of the cotton fabrics. It can be seen from results that some properties of the enzyme changed after immobilization. For example, while the optimum pH and temperature values of the enzyme decreased upon chitosan immobilization, they were increased with Ca-alginate immobilization. Hence, it is necessary to look at where the enzymes are used to decide which method is more advantageous. But in conclusion it can be said that immobilized R. solani AG-4 strain ZB-34 α-amylases might have potential application in different industrial areas.

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**Conflict of interest:** The authors declare no conflicts of interest of any kind.

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