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# PURIFICATION OF AN ENDO-BETA 1,4-MANNANASE FROM *CLITOCYBE GEOTROPA* AND IMMOBILIZATION ON CHITOSAN-COATED MAGNETITE NANOPARTICLES: APPLICATION FOR FRUIT JUICES

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In this study, β-mannanase enzyme obtained from *Clitocybe geotropa* fungus was immobilized on magnetite ($Fe_3O_4$) nanoparticles and its usability for the clarification of some fruit juices has been investigated. For this purpose, β-mannanase enzyme was purified 178.03 fold from *Clitocybe geotropa* fungus by using ammonium sulfate precipitation, ion exchange chromatography (DEAE-Sephadex) and gel filtration chromatography (SephacrylS 200) techniques. Then, it was modified with chitosan (CS) $Fe_3O_4$ nanoparticles and immobilized on the surface of purified mannanase chitosan coated magnetic nanoparticles ($Fe_3O_4$-CS) using adsorption method. The morphology of immobilized mannanase on $Fe_3O_4$-CS system was studied by employing the field emission scanning electron microscope (SEM), FT-IR spectroscopy and X-ray diffraction analysis (XRD) methods. The optimum pH and temperature values and kinetic parameters such as pH stability, storage stability and reusability were investigated and the change of the activity against repeated use of immobilized systems were also identified for free and immobilized β-mannanase systems. According to these analyses, all immobilized systems retained more than 73% of their initial activity at the end of 10 batch uses. At the last phase of the study, the effectiveness of immobilized mannanase enzyme on clarification of some fruit juices such as grape, peach, orange and pomegranate juices were investigated. In the comparison of immobilized mannanase and free mannanase enzymes, immobilized mannanase enzyme was found to be much more effective in clarification.

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*Keywords:* Mannanase, *Clitocybe geotropa*, Purification, Immobilization, Chitosan coated magnetic nanoparticles ($Fe_3O_4$-CTS)

## 1. Introduction

According to the latest Enzymes Market Analysis, the amount spent for enzymes in the global market will reach up to $7,652.0 million and the increased catalyzed demand for enzyme are mostly for food and beverage industries (Grand View Research 59 Inc., 2014; ISBN Code: 978-1-68038-022-4).

There are many advantages of immobilizing enzymes into support materials in biotechnological applications; they are more resistant to environmental conditions, they can be easily removed at the end of the reaction, the product is not contaminated with the enzyme, enzymes can be used repeatedly easily. In addition; product formation can be taken under control, they can show more activity compared to free enzyme and they are more stable than the free enzyme [1,2].

In the last decade, nano sized magnetic particles (magnetite, $Fe_3O_4$) are widely used in food, biology and medicine fields for purposes such as magnetic resonance imaging, controlled

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delivery of anticancer drugs, RNA and DNA purification and immobilization of enzyme and protein. In addition, the nanomagnetites found use possibilities in a wide range with its features such as high surface area, less diffusion problems of enzymes, high adhesion, low swelling ability, mechanical resistance and low cost [3,4].

Hemicellulose located in plant cell walls are the most abundant polymeric carbohydrates in nature. 1.4- β -D-xylans and 1.4- β -D-mannans are the most important heteropolymers. 1.4-N-beta-D contained in Endo- β -D-mannanase (EC:3.2.1.78, mannan endo-1.4- β -D- mannosidase), mannans, galactomannans, glucomannans and galactoglucomannans hydrolyzes the mannosidic linkages [5]. Mannanases play important role in the processes of degradation of the hemicelluloses structure containing lignin, cellulose and mannans in fungi. Mannanases are used in various biotechnological applications in industries such as pulp bleaching, reducing the viscosity of coffee and clarification of fruit juice and wine [6-11]. In addition, mannanases are used in the production of oligosaccharides, which have physiological importance in the pharmaceutical industry [10].

Mannanase enzyme was characterized from many bacteria including *Bacillus circulans* [12], *Vibrio* sp. strain MA-138 [13], *B. circulans* K-1 [14] and *Saccharo phagus* degradans [15]. β–mannanase was isolated from fungi such as *Aspergillus niger* [16] and some plant species such as tomato [17].

In the literature, there are some studies related to immobilization of β-D-mannanase enzyme on various types of support materials [18-20]. This is the first study in the literature on characterization, purification and immobilization of β-D-mannanase enzyme from *Clitocybe geotropa*. In this study, a thermostable β-D-mannanase was isolated, purified and characterized from a *Clitocybe geotropa.* Then, the purified enzyme was immobilized onto chitosan coated magnetic nanoparticles ($Fe_3O_4$-CS). The effect of pH on free and immobilized β-D-mannanase enzyme activity was investigated as well as the optimum temperature, kinetic parameters and reusability of the enzyme in clarification processes of some fruit juices.

## 2. Experimental details

### 2.1 Chemicals

Bovine serum albumin (BSA), Locust bean gum, Na-phytate, xylan, chitin, starch, gelatin, DEAE-sephedex, and Sephacryl S-200, dithioerythritol, β-mercaptoethanol and agents for SDS-PAGE were purchased from Sigma (USA). Ethanol, sodium acetate ($CH_3COONa$), ammonium sulphate ($(NH_4)_2SO_4$), sodium chloride (NaCl) and sodium hydrogen phosphate monohydrate ($Na_2HPO_4$ x $H_2O$) were purchased from Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

### 2.2 Plant material and storage conditions

*Clitocybe geotropa* fungus were collected from Karabuk and surrounding areas around May-July and stored at -40$^{o}$C until they have been analyzed. It was identified by a botanist.

### 2.3 Purification of mannanase enzyme from *Clitocybe geotropa* mushroom

Mannanase enzyme was purified from fungus in two steps. In the first step, the homogenate was precipitates by ammonium sulfate precipitation within the ranges of 0-20, 20-40, 40-60, 60-80, 80-100% and the range with the highest activity was determined by checking the mannanase activity of supernatant and precipitate. Then, precipitate was dissolved in 20 mM Na-citrate buffer with pH 6.0 and dialyzed against the same buffer [21].

In the second step, the homogenate was equilibrated with 20 mM Na-citrate buffer (pH 6.0) and applied to a DEAE-sephadex column and the same column and buffer were washed. Mannanase enzyme was eluted from the column with increasing ionic strength gradient using 20 mM Na-citrate buffer (pH: 6.0) containing 0 to 1 M NaCl concentration. The mannanase enzyme activity in eluates obtained from the columns of DEAE-Sephadex was checked by Locust bean gum substrate and the eluates showing enzyme activity were combined [21].

### 2.4 SDS-PAGE Electrophoresis

Sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) was used in order to determine the number of sub-units of mannanase enzyme purified from *Clitocybe geotropa* mushrooms. The number of sub-units of mannanase enzyme was determined by using the method developed by Laemmli [22]. According to this method, SDS-PAGE electrophoresis was in two different concentrations as follows; 3% for stacking gel and 10% separating gel, respectively.

### 2.5 Immobilization of purified enzyme

#### *2.5.1. Preparation of chitosan particles*

Chitosan particles were prepared in accordance with a method developed by Shao-Huaet al [23]. First, the chitosan molecules were stirred for 3 hours by treating them with acetic acid. Then, the chitosan molecules were neutralized by addition of 1 N NaOH in ethanol medium and washed with distilled water. Chitosan molecules were treated with solution of 5% glutaraldehyde for 4 hours in order to bind the amine group to chitosan molecules. Finally, non-binding glutaraldehydes were removed from medium by washing chitosan molecules with distilled water.

#### *2.5.2. Gaining nano magnetic properties to chitosan molecules and immobilization of purified mannanase enzyme*

First, chitosan particles were treated with $Fe_3O_4$, which was dispersed in distilled water, and chitosan surface was allowed to gain magnetic properties. After treatment of chitosan with $Fe_3O_4$ nanoparticles, non-binding nanoparticles were removed from medium by washing with distilled water. Then, the wet precipitate was dried at 40 °C during 72 hours and then stored at +4 °C for using in experimental studies.

The mannanase enzyme (68 EU/mL; 1.08 mg protein/mL), which was purified from *Clitocybe geotropa* fungus and 0.25 M $NaCNBH_3$ in the Na-citrate buffer solution (pH 6.0) as well as support material activated by glutaraldehyde and modified (surface) by Nano $Fe_3O_4$, was immobilized. The amount of enzyme binding to the constant material with time was determined by following the amount of protein in the reaction medium by Warburg and Bradford methods in different time intervals [24,25].

### 2.6 Determination of the optimal conditions for immobilization of mannanase enzyme purified to chitosan molecules, which gained nano magnetic properties

The following optimization processes were performed in all immobilization studies in order to determine the best conditions, in which the mannanase enzyme was immobilized to nano magnetic chitosan ideally:

Immobilization was performed at pH 5.0, 6.0, 7.0 and 8.0 values to determine the optimum pH for immobilization.

Immobilizations were performed at 10, 15 and 20 $^oC$ in order to determine the optimum temperature for immobilization. The mannanase binding efficiency (E) is defined as follows:

$$E = (C_1-C_o)/C_1$$

Where $C_1$ and $C_o$ are the amounts of mannanase protein in the solution before and after immobilization, respectively. The activity amount of the immobilized phytase was calculated as follows:

$$\text{Immobilized mannanase} = A/(A_1-A_o)$$

Where A is the activity of the immobilized phytase and $A_1$ and $A_o$ are the activities of the free phytase in solution before and after immobilization, respectively.

### 2.7 Determination of mannanase activity

All mannanase activity measurements were performed usingthe locust bean gum as substrate. To this end; after hydrolization of Locust bean gum with mannanase enzyme, they have created a colorful complex with DNSA (3.5-dinitrosalisilic acid) and activity was determined by measuring the change of absorbance at 540 nm. 0.1 g of immobilized enzyme was added to the

reaction mixture for immobilizedenzyme measurement and the blind sample was prepared by using distilled water rather than enzyme. One unit of mannanase activity was defined as amount of enzyme producing 1 micromole of mannose per minute under the experimental conditions [26,27].

**2.8 Kinetic parameters**

Cellulose, Na-phytate, pectin, casain, starch, gelatin, xylene and locust bean gum solutions were prepared at different concentrations (0.05, 0.15, 0.25, 0.35 and 0.45 mg $mL^{-1}$) in order to investigate the substrate specificity of these solutions to free and immobilized mannanase activity and activity measurements were performed on these solutions.

Lineweaver-Burk graphs of free and immobilized enzyme were plotted by using activity measurements and $V_{max}$ and $K_m$ constants of free and immobilized mannanase were found for each substrate using these graphs.

**2.9 Protein Determination**

Using the Bradford method, protein concentration of all samples was determined spectrophotometrically [24]. Bovine serum albumin (BSA) was used as a standard in these processes.

**2.10. Determination of the optimal pH for activity and stability**

The optimum pH value was found at 50 ◦C by using different buffers as follows: 50 mM citric acid buffer for pH values of 3.0-5.0, whereas 50 mM MES buffer was found for pH 5.0-7.0 and 50 mM Tris buffer was determined for pH 7.0-9.0. In order to give an activity of 30 U/mL, the free form and immobilized phytase was suspended in each buffer at different pH values. The stability of pH was tested in 100mM buffer, and then incubated at 4 ◦C for 4 hours.

**2.11. Determination of temperature optima and thermal stability**

In order to find the optimal temperature for the enzyme reaction, different temperature values were tested in the range of 20-90 ◦C in 50mM Na-citrate buffer (pH 6.0). Furthermore, thermal challenges were performed in 50 mM Na-citrate buffer (pH 6.0) at different temperatures (10-90 $^{o}$C) for 1 hour to determine the thermostability of the enzyme. Then, the heated and treated enzymes were placed on ice. As described before, residual phytase activity was measured at 50 $^{o}$C, which was the optimum temperature, with a pH value 6.0 previously [26,27]. Each process was repeated at least three times.

**2.12. Molecular weight determination by gel filtration:**

Sephadex G100 column (3.0 x 50 cm) gel filtration column was used in order to determine the molecular weight of mannanase enzyme purified from *Clitocybe geotropa* mushrooms. The column was equilibrated with 0.05 M $Na_2HPO_4$, 1 mM dithioerythretol pH 7.0-buffer solution. First, standard protein marker with a known molecular weight was passed through the column (bovine serum albumin, 66 kDa; egg ovalbumin, 45 kDa; pepsin, 34 kDa; trypsinogen, 24 kDa; β-lactoglobulin and lysozyme, 14 kDa) and the same buffer was eluted from protein column by using the solution. Then, the mannanase enzyme purified from *Clitocybe geotropa* fungus of the column was eluted under same conditions. The molecular weight of purified mannanase enzyme was calculated by comparing the elution volume with standard protein marker [28].

**2.13. Effect of some metal ions**

The effect of various metal ions such as $Cu^{2+}$, $Fe^{2+}$, $Ca^{2+}$, $Mg^{2+}$, $Zn^{2+}$ and $Hg^{2+}$ at various concentrations (1 to 5 mM) on free and immobilized mannanase activity was investigated by preincubating the free and immobilized mannanase with different compounds for 10 minutes at the room temperature. Then, residual activity was calculated against the control value.

The activity measurement of immobilized mannanase enzyme was performed at least 10 times with in order to determine its reusability. Immobilized mannanase was added to the medium containing substrate and standard mannanase activity measurements were performed. Immobilized mannanase was washed with buffer solution after each activity measurement and reused for

activity measurements. A graph of number of cycles against % activity was plotted to analyze the reusability of immobilized mannanase.

### 2.14. Fruit juice clarification

Some fruits were selected and carefully washed, dried and homogenized in order to prepare the fruit juice. These fruits are peach, orange, pomegranate, grape, kiwi and apple. 2 mL of enzyme solution (17.6±1.6 EU/mL) and 10 g of apple homogenate was added (in control experiment, 2 mL distilled water was added). The same experiment was also conducted with 2 mL of crude homogenate. Fruit homogenates were treated at a natural pH value and 50 $^{o}$C for 4 hours. A certain amount of fruit juice was filtered through a filter paper for 15 minutes and the dried material remained on the filter and the volume of filtered fruit juice were determined [28,29].

### 2.15. Statistical analysis

All tests were repeated three times to determine the mannanase activity. Statistical analyses were performed on SPSS 20.0 software (SPSS Inc, Chicago, IL., USA) and data were expressed as mean ± standard error. According to Tukey's test, significant differences were determined at 95% confidence intervals ($p < 0.05$).

**ABBREVIATIONS**

| | |
|---|---|
| CTS | chitosan |
| MGT | magnetite |
| MGT-CTS | magnetite citosan |
| NPs | nanoparticles |

## 3. Results and discussion

Mannanase enzyme was purified from *Clitocybe geotropa* mushroom at 3 steps using ammonium sulfate precipitation, DEAE-sephadex and Sephacryl S200 gel filtration chromatography techniques. The all obtained results were given in Table 1.

*Table 1.The purification process of purifiedβ-mannanase enzyme from Clitocybe geotropa*

| Enzyme Fraction | Volume mL | Activity EU/mL | Total Activity (EU) | Yield % | Protein (mg/ml) | Specific EU/mg | Purification Fold |
|---|---|---|---|---|---|---|---|
| Crude extract | 30 | 69.0±0.17 | $2.07x10^3$ | 100 | 145.3±0.12 | 0.475 | - |
| $(NH_4)_2SO_4$ (40-80%) | 25 | 53.7±0.13 | $1.34 x10^3$ | 64.7 | 8.1±0.23 | 6.63 | 13.96 |
| DEAE-Sephadex | 25 | 28.3±0.09 | $7.08 x10^2$ | 34.2 | 1.07±0.7 | 26.45 | 55.7 |
| Sephacryl S 200 | 25 | 17.6±1.6 | $4.40 x10^2$ | 21.3 | 0. 21±0.6 | 83.81 | 176.4 |

In the first stage, mannanase enzyme was collapsed the ammonium sulfate saturation between the range 0-100% and it was observed the highest percentage of precipitation of purified mannanase enzyme at 40-80% range. It was shown 13.96 purification-folds and 64.7% yield in this first purification step (Table 1). In the second step, the dialyzed enzyme sample was applied to DEAE-sephadex ion exchange column and it was determined that mannanase enzyme was purified 55.7-fold with 34.2% yield from column. In the last step, the obtained fractions from ion-exchange column were concentrated by lyophilized and it was applied to a Sephacryl S 200 column. A

single peak was obtained from this column. The enzyme was purified 176.4- -fold with 21.3% yield and specific activity was calculated as 83.81 EU / mg protein (Table 1).

The purification profiles of purified mannanase enzyme from *Clitocybe geotropa* mushroom using gel filtration chromatography and anion exchange chromatography was given in Fig. 1 and Fig. 2.

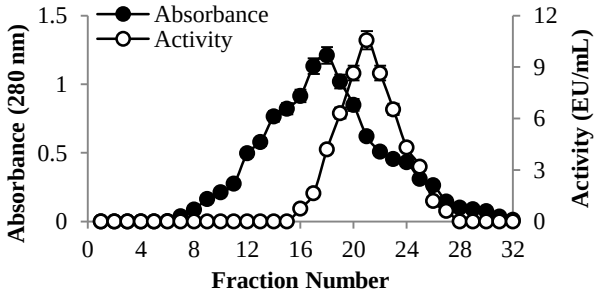


*Fig. 1. Purification of mannanase by ion exchange chromatography using DEAE-cellulose.*

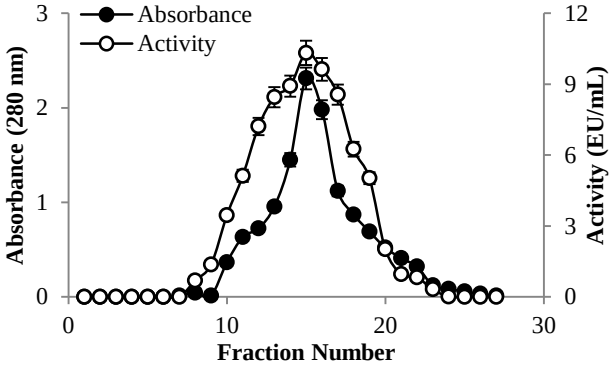


*Fig. 2.Elution profile of protein and mannanase activity on Sephacryl S200.*

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)'s results of the purified mannanase enzyme have seen that it was consist of single subunit as 39.5 kDa (Fig. 3). To find the molecular weights of the active form of the enzyme, the Sephadex G-100 gel filtration chromatography was applied and the graph was drawn as log MA-Kav.

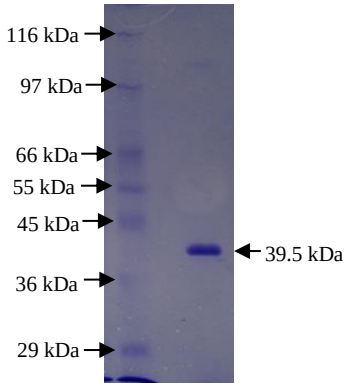


*Fig. 3.SDS-PAGE electrophoretic pattern of mannanase [Lane (I); high protein marker (Sigma), bands marked: 205 kDa, myosin, 116 kDa, β-galactosidase, 97 kDa, phosporylase b; 66 kDa, bovine serum albumin; 55 kDa, glutamic dehydrogenase, 45 kDa, ovoalbumin; 36 kDa, glyceraldehyde-3-phosphate dehydrogenase; 29 kDa; carbonicanhydrase) line (II); purified mannanase enzyme from Clitocybe geotropa].*

In this study the beta-mannanase gene was amplified from *Aspergillus aculeatus* to *Aspergillus oryzae* were placed and the molecular weight of the resultant recombinant enzyme was found to be 45 kDa [30]. Also, molecular weight of the mannanase enzyme of *Aspergillus niger* was obtained as 40 kDa [31].

Firstly, chitosan was activated by binding the glutaraldehyde to the amino group ($-NH_2$) located chitosan for connecting the purified mannanase enzyme to the modified chitosan molecules with $\gamma$-$F_3O_4$ nanoparticles. Then, it was provided binding of purified mannanase by creating schift base to glutaraldehyde. $NaCNBH_3$ was used for reducting base of schift formed in both of two steps. After synthesized the support material, its surface was coated with $Fe_3O_4$ nanoparticles for purpose gain magnetic feature of chitosan [32].

The optimum conditions of covalent immobilization were determined. For this purpose, pH and temperature was primarily determined. Purified mannanase enzyme was immobilized by using appropriate buffers among pH 4-8, and it was calculated relative activity(%) of immobilized phytase. Accordingly, it was determined that the pure mannanase enzyme was connected to support material at the high level at pH 5 and 20 °C. Then, the immobilization was monitored for 6 hours to determine the most appropriate immobilization time at pH 5 and 20 °C. It was determined that purified mannanase enzyme was connected to MG-CTS after 1 hour at the rate of 85.6% (Fig. 4).

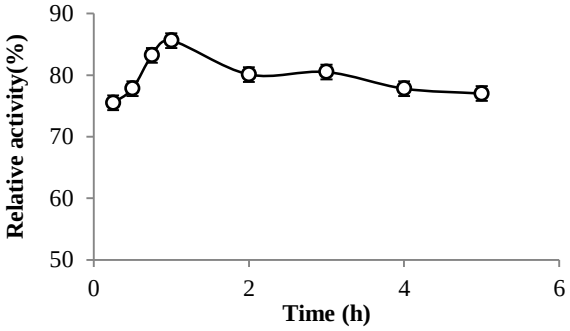


*Fig. 4. The effect of time to the immobilization of Mannanase enzyme onto modified chitosan with nanomagnetite.*

Structure of CTS, MGT-CTS NPs; III: purified mannanase immobilized MGT-CTS NPs at SEM images were shown in Fig. 5 I-III. The thickness of the wall is about 20 ~ 30 nm, the pore size is about 0.5 ~ 1 μm. From the SEM image, it was obseved that the support material was fine-pore structure and its structure was appropriated to modify with nanomaterials and to immobilization with mannanase enzyme. In addition, it had large-sized support and structural stability.

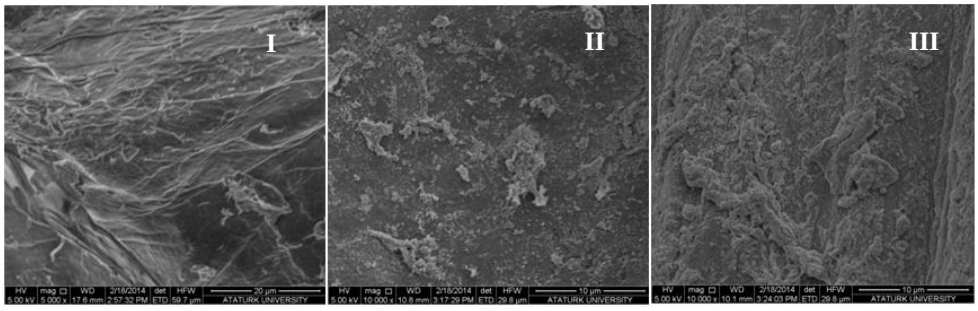


*Fig. 5. SEM images I: nature CTS; II: MGT-CTS NPs; III: immobilized mannanase onto MGT-CTS NPs*

Fig. 6 showed XRD patterns of nature CTS; MGT-CTS; purified mannanase immobilized to the surface of MGT-CTS. XRD patterns chitosans are illustrated in Fig. 6. The XRD pattern of chitosan exhibits broad diffraction peaks at 2θ = 9.5º and 20.5º which are typical fingerprints of crystal chitosan [33]. In the XRD pattern of prepared $Fe_3O_4$ modified chitosan NPs and purified phytase immobilized modified chitosan with nano magnetite, the peak corresponding to chitosan could be seen at around 2θ = 20.5°, but it became much lower and wider. Also, five diffraction peaks were observed at 2θ = 34.0°, 42.5°, 53.5°, 57.0°, 63.0°. These peaks are belonging to typical fingerprints of $Fe_3O_4$ NPs [34].

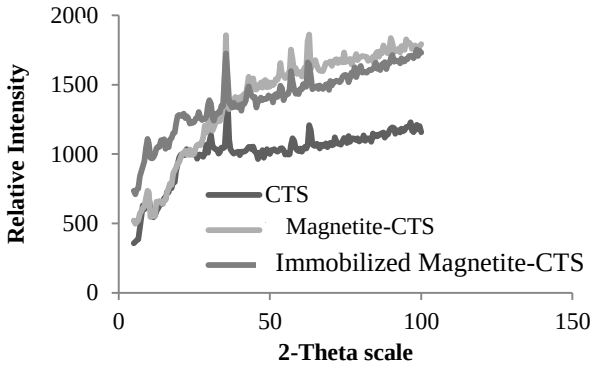


*Fig. 6.XRD photographs for nature chitosan; MGT-CTS NPs; mannanase MGT-CTS NPs*

The structure of the support material of nature CTS (a), MGT-CTSNPs (b), immobilized MGT-CTS NPs with purified mannanase from *Clitocybe geotropa* mushroom (c) was confirmed by FTIR analysis. The spectra of chitosan showed a broad absorbance at 3650.60–2879.6 $cm^{-1}$ (O-H, hydroxyls and -NH stretching vibrations of free amino groups), at 2879.63 $cm^{-1}$ and 2122 $cm^{-1}$ (-$CH_3$ and -$CH_2$ in chitosan structure), at 1655.58-1030.58 $cm^{-1}$ (-C=O stretching of amide, -C=O secondary amide, -N-H bounds stretching vibrations) and at 895.1 $cm^{-1}$ and 695.4 $cm^{-1}$ (-C-H bending of amide bonds stretching vibrations) in curve Fig 6a-c. In the Fig. 6b and 6c, the peaks at 557.92 $cm^{-1}$ and 440.23 $cm^{-1}$ related to Fe-O group of $Fe_3O_4$ nanoparticles. When the Fig 6a compared with Fig 6b, a new sharp peak appeared at the 1661.5 $cm^{-1}$. It indicated that at the 2121.6 $cm^{-1}$ peak of -N–H bending vibration shifted to 1589.68 $cm^{-1}$ and chitosan react with glutaraldehyde to form *Schiff base* [35, 36]. It was shown that there was no significant change in the functional biomass groups of magnetic $Fe_3O_4$-chitosan nanoparticles after immobilization of phytase enzyme. The results of FT-IR spectrums indicated that chitosan was coated to the magnetic $Fe_3O_4$ nanoparticles and phytase enzyme was immobilized onto the magnetic $Fe_3O_4$-chitosan nanoparticles successfully.

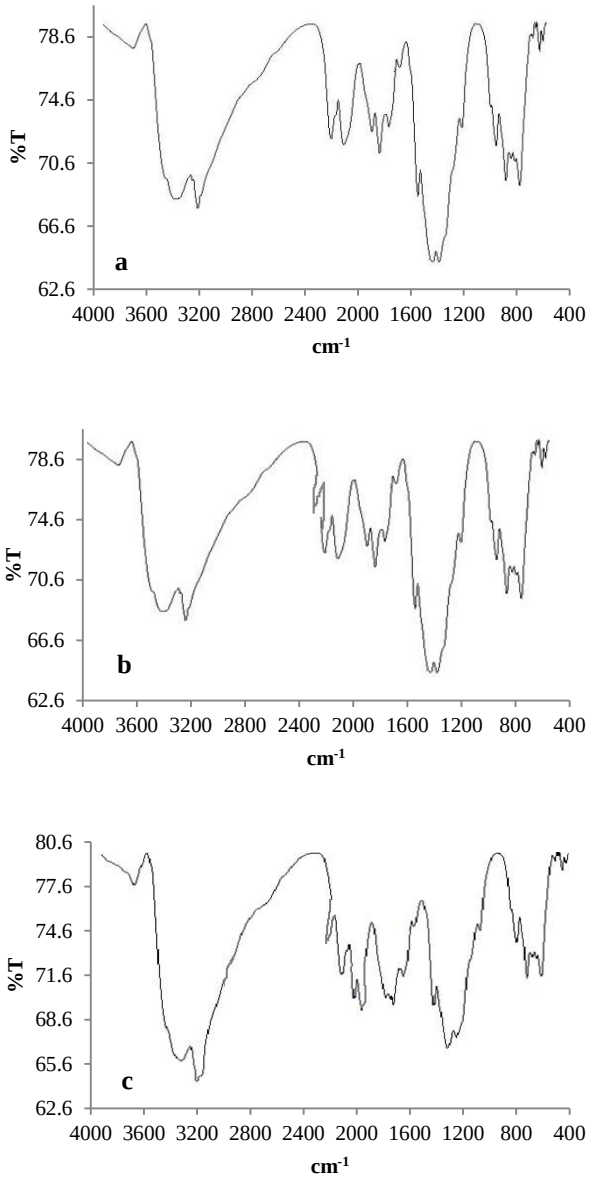


*Fig. 7. FT-IR spectrum of nature CTS (a), MGT-CTS (b), immobilized mannanase MGT-CTS (c)*

Measurements of activity were performed incrementing with one unit at range of pH 3.0-11.0 using Locust bean gum as a substrate and results were given as Relative activity (%) - pH graph at Fig. 8 for free and immobilized β-mannanase. Optimum pH values of free and immobilized β-mannanase were determined as 5.0.

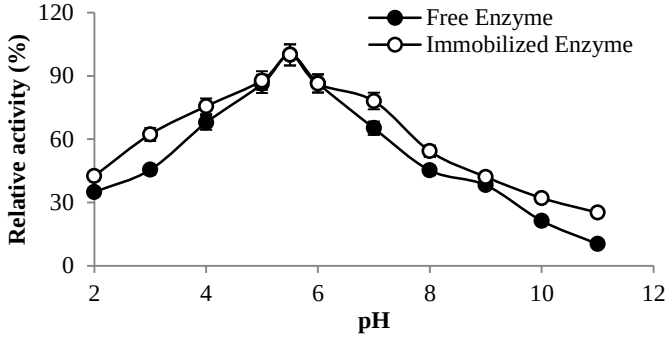


*Fig. 8.Effect of pH on theactivity of the purified mananase.*

Optimum temperatures of free and immobilized β-mannanase enzyme were obtained as 60 °C (Fig. 9). Optimum temperature of β-mannanase wasn't changed by immobilization. As seeen from Fig. 9, immobilized β-mannanase was more stable at all tested temperatures acording to free β-mannanase. These results indicated that immobilization of the purified β-mannanase on magnetit-CTS surface was increased enzyme stability against temperature changes.

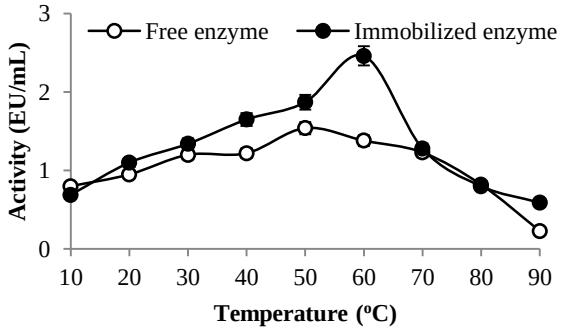


*Fig. 9.The effect of pH on the activity of purified β-mannanase from Clitocybegeotropa*

Then enzymes were immobilized on a support, its kinetics differs due to factors such as nature of support, heterogeneity of system and accessibility of the substrate to the enzyme.

In this research, Lineweaver-Burk graphics were drawn for free and immobilized β-mannanase using substrates such as cellulose, Na-phytate, pectin, casain, gelatin, starch and locust bean gum. $K_m$ and $V_{max}$ values of free and immobilized β-mannanase were given in Table 2, respectively.

*Table 2. Substrates pecificity of purifiedβ-mannanase from Clitocybe geotropa*

| | **Free Mannanase** | | **Immobilized Mannanase** | |
|---|---|---|---|---|
| **Substrates** | **$V_{max}$ (EU/mg)** | **$K_m$ (mg/mL)** | **$V_{max}$ (EU/mg)** | **$K_m$ (mg/mL)** |
| **Celulose** | 0.74 | 0.209 | 1.49 | 0.52 |
| **Na-phytate** | 3.69 | 0.199 | 4.0 | 0.244 |
| **Pectin** | 3.105 | 0.16 | 2.034 | 0.102 |
| **Casain** | 2.502 | 0.149 | 2.512 | 0.173 |
| **Gelatin** | 3.66 | 0.208 | 3.03 | 0.170 |
| **Starch** | 2.99 | 0.167 | 5.23 | 0.309 |
| **Locust bean gum** | 10.194 | 0.588 | 14.35 | 0.122 |

$V_{max}$ values of immobilized β-mannanase enzyme were increased according to free β-mannanase enzyme against for all used the substrate, but $K_M$ values were decreased for some substrates (Pectin, starch and locust bean gum). The highest Vmax value was obtained for locust bean gum substrate.$V_{max}$ values were measured as 10.2 EU/mg and 14.35 EU/mg for free and immobilized β-mannanase enzymes, respectively. Their $K_M$ values were also measured as 0.588 mg/mL and 0.122 mg/mL, respectively.

$K_M$ value had indicated the relevance of an enzyme to substrate [37] and it was determined that $K_M$ value was generally increased after immobilization at previous studies [37]. In this study, increasing in $V_{max}$ values and decreasing in $K_M$ values of immobilized mannanase enzyme were indicated that the changes at three-dimensional structure of enzyme after immobilization of enzyme were showed a favorable effect on enzyme activity [38-40].

The reusability of the immobilized enzymes is extremely important in terms of assess the effectiveness of the selected carrier or method. Immobilized enzymes are more advantageous than the free enzymesin term of reusability, stability and economic [41]. In this study, reusability of the immobilized β-mannanase enzyme was determined doing successively activity measurements and activity of immobilized β-mannanase enzyme was only lost 21.4% after 10 cycles (Fig. 10). It was shown that the reusability of immobilized β-mannanase enzyme without important reduction activity, so this immobilized mannanase enzyme can be easily used in food industry.

In order to determine the resistance of free and immobilized β-mannanase enzyme againist metal ions, the effects of some metal ions such as $Cu^{2+}$, $Fe^{2+}$, $Zn^{2+}$ and $Hg^{2+}$ was investigated on both free and immobilized enzyme activity. The inhibitory / activator results of metal ions which were tested the effects at 1 mM and 5 mM concentrations were given in Table 3. While 5 mM $Mg^{2+}$ ion was inhibited the free mannanase enzyme about 10% rate, both 1 mM and 5 mM concentrations of $Zn^{2+}$ ion were inhibited at about 10% degree. It was determined that immobilized mannanase enzyme was inhibited by $Ca^{2+}$ and $Mg^{2+}$ ions. In generally, it was showed that almost tested all metal ions increased both free and immobilized mannanase enzyme activities. It was determined that $Cu^{2+}$, $Fe^{2+}$, $Zn^{2+}$ and $Hg^{2+}$ metal ions were increased the activity of mannanase enzyme. Especially, 1 mM and 5 mM of $Zn^{2+}$ ions were activated the free and immobilized enzyme at 363.3% and 430.0%, respectively. Actived the enzyme by almost all metal ions was shown that it could be suitable to use in several industrial purposes. Purified mannanase enzymes from oakbug milkcap (*Lactarius quietus*) was activated by metal ions in our previously studies, and these findings were supported our data [32].

*Table 3. The effect of some chemical compounds on free and immobilized β-mannanase activity*

| **Free Enzyme** | | | | |
|---|---|---|---|---|
| **Chemical Compounds** | **Concentration (mM)** | **Relative Activity (%)** | **Concentration (mM)** | **Relative Activity (%)** |
| None | - | 100 ± 0.0 | - | 100 ± 0.0 |
| $Cu^{2+}$ | 1 | 187.9 ± 0.03 | 5 | 151.5 ± 0.15 |
| $Fe^{2+}$ | 1 | 103.03 ± 1.2 | 5 | 118.2 ± 1.1 |
| $Ca^{2+}$ | 1 | 107.6 ± 0.45 | 5 | 133.4 ± 0.31 |
| $Mg^{2+}$ | 1 | 109.0 ± 0.32 | 5 | 89.7 ± 0.04 |
| $Zn^{2+}$ | 1 | 93.9 ± 0.25 | 5 | 90.9 ± 0.09 |
| $Hg^{2+}$ | 1 | 169.7 ± 0.07 | 5 | 454.6 ± 0.19 |
| **Immobilized enzyme** | | | | |
| None | - | 100 ± 0.0 | - | 100 ± 0.0 |
| $Cu^{2+}$ | 1 | 100.4 ± 0.36 | 5 | 153.4 ± 0.44 |
| $Fe^{2+}$ | 1 | 163.4 ± 0.06 | 5 | 176.7 ± 0.14 |
| $Ca^{2+}$ | 1 | 63.4 ± 0.32 | 5 | 40.5 ± 0.28 |
| $Mg^{2+}$ | 1 | 103.3 ± 0.03 | 5 | 83.4 ± 1.05 |
| $Zn^{2+}$ | 1 | 363.3 ± 0.6 | 5 | 430.0 ± 0.01 |
| $Hg^{2+}$ | 1 | 143.2 ± 0.5 | 5 | 156.8 ± 0.18 |

All metal ions were used as chloride salt ($CuCl_2$, $FeCl_2$, $CaCl_2$, $MgCl_2$, $ZnCl_2$, $HgCl_2$)

The elimination of the high viscosity and turbidity in juice production requires high cost. The use of mannanase enzyme in fruit juice production helps mannans to be hydrolyzed; thus, viscosity of the medium decreases; and since release of the water held with in the same structure occurs, the amount of fruit juice also increases, so use of enzyme was reduced of total cost of process.

Removing solid substance and improving productivity was separately determined with free and immobilized mannanase and the results were presented in Table 4 and 5. The volume of juice of orange, pomegranate, strawberry, kiwi, rosehip and apple was increased, compared to the control, by the treatment of fruit pulps with free and immobilized mannanase enzyme (Table 4). According to the obtained results, it was determined that free and immobilized mannanase enzyme have effective on rosehip production with a yield of 146.2% and 223.1%, respectively.

In the literature, there are several studies indicating that fruit juice clarification process can be performed by using the mannanase enzyme. So, our results are consistent with the findings of these studies in the literature [42].

The material was pressed more easily than the control and the residual dry weight of solid residue decreased to 36.0 % (Table 5). As a result, the productivity yield of fruit juice was increased. The juice obtained by enzymatic treatment had lower viscosity compared to those non-treated, possibly due to reduction of mannan content [21, 29].

## 4. Conclusions

From the obtained data, the mannanase enzyme which was purified from *Clitocybe geotropa* mushroom and immobilized on MG-CTS NPs had very high catalytic activity, resistance against metal ions and high stablity against temperature and different pH. We also determined the action of immobilized mannanase in fruit juice production. It was concluded that immobilized mannanase can be useful in the food industry.

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## References

[1] S.F.D. Souza, Curr. Sci. **77**, 69 (1998).
[2] J. Turkova, J. Chromatogr. B **722**, 11 (1999).
[3] C.C. Yu, Y.Y. Kuo, C.F. Liang, W.T. Chien, H.T. Wu, T.C. Chang, F.D. Jan, C.C. Lin, Bioconjugate Chem. **23**, 714 (2012).
[4] C. Xu, K. Xu, H. Gu, R. Zheng, H. Liu, X. Zhang, Z. Guo, & B. Xu, J. Am. Chem. Soc. **126**, 9938 (2004).
[5] M.R.S. Chandra, Y.S. Lee, I.H. Park, Y. Zhou, K.K. Kim, & Y.L. Choi, Korean Soc. Appl. Biol. Chem. **54**(3), 325 (2011).
[6] G. Li, Y. Jiang, K. Huang, P. Ding, & J. Chen, J. Alloys Compd. **466**, 451 (2008).
[7] C.M. Tang, L.D. Waterman, M.H. Smith, C.F. Thurston, Appl. Environ. Microbiol. **67**(5), 2298 (2001).
[8] J. Buchert, J. Salminen, M. Sikaaho, M. Ranua, & L. Viikari, Holzforschung **47**, 473 (1993).
[9] W.A. Cuevas, A. Kantelinen, P. Tanner, B. Bodie, & S. Leskinen, Purification and characterization of novel mannanases used in pulp bleaching. In Biotechnology in the Pulp and Paper Industry.Srebotnik E and Mesner K (eds.), pp. 123-126, Facultas-Universitätsverlag, Vienna, Austria (1996).
[10] G.M. Gubitz, M. Hayn, G. Urbanz, W. Steiner, J. Biotech. **45**, 165 (1996).
[11] A. Sachslehner, G. Foildl, N. Foidl, G. Gübitz, & D. Haltrich, J. Biotech. **80**, 127 (2000).

[12] L. Yanan, P. Yang, K. Meng, Y. Wang, H. Luo, N. Wu, Y. Fan, & B. Yao, J. Microbiol. Biotech. **18**, 160 (2008).
[13] M. Tanaka, Y. Umemoto, H. Okamura, D. Nakano, Y. Tamaru, & T. Araki, Biosci. Biotech. Biochem. **73**, 109 (2009).
[14] S. Yoshida, Y. Sako, & A. Uchida, Biosci.Biotech.Biochem. **62**, 514 (1998).
[15] S.W. Hutcheson, H. Zhang, & M. Suvorov, Marine Drugs **9**, 645 (2011).
[16] K. Naganagouda, P.V. Salimath, & V.H. Mulimani, J. Microbiol. Biotech.**19**, 1184 (2009).
[17] R. Prakash, S.L. Johston, H.L. Boldingh, R.J. Redgwell, R.G. Atkinson, L.D. Melton, D.A. Brummell, R. Schröder, J. Plant Physiol. **169**, 1125 (2012).
[18] M. Xu, W. Yang, L. Ke,. Shipin Gongye Keji **28**(2), 160-162 (2007).
[19] I. Mazeaud, B.R. Poulsen Poul, M.W. Christensen, J. Brask, Immobilization of enzymes by adsorption on porous carrier with subsequent crosslinking in the presence of a polyfunctional for use in organic synthesis. PCT International Application, WO 2007036235 A1 20070405 (2007).
[20] Z. Liang, D. Sun, Q. Lin, X. Huang, & F.Z. Shenqing, Method for immobilizing mannanase on macroporous resin. CN 102586219 A 20120718 (2012).
[21] H. Nadaroglu, A. Adiguzel, & G. Adiguzel, Int. J. Food Sci. Tech. **50** (5), 1158 (2015).
[22] U.K.Laemmli, Nature **22**, 680 (1970).
[23] S.H. Chiu, T.W. Chung, R. Giridhar, & W.T. Wu, Food Res. Int. **37**(3), 217 (2004).
[24] M. Bradford, Anal. Chem. **72**, 248 (1976).
[25] O. Warburg, & W. Christian, Biochem. Z. **310**, 384 (1942).
[26] G.L. Miller, Anal Chem. **31**(3), 426 (1959).
[27] M.J. Bailey, P. Biely, & K. Poutanen, J. Biotechnol. **23**, 257 (1992).
[28] J. R.Whitaker, Anal.Chem. **35**, 1950 (1963).
[29] A. Adiguzel, H. Nadaroglu, G. Adiguzel, J. Food Sci. Technol. **52,** 5292 (2015).
[30] S. Christgau, S. Kauppinen, J. Vind, L.V. Kofod, & H. Dalboge, Biochem.Mol. Biol. Int. **l33** (5), 917 (1994).
[31] P. Ademark, A. Varga, J. Medve, V. Harjunpaa, T. Drakenberg, F. Tjerneld, H. Stalbrand, J. Biotechnol. **63**, 199 (1998).
[32] H. Onem, H. Nadaroglu, J. Food Nutr. Res. **2**(12), 938 (2014).
[33] K. Ogawa, S. Hirano, T. Miyanishi, T. Yui, & T. Watanabe, Macromol. **17**, 973 (1984).
[34] C. Cao, L. Xiao, C. Chen, X. Shi,Q. Cao, & L. Gao, Powder Technol. **260**, 90 (2014).
[35] M. Guo, P. Diao, & S. Cai, J. Solid State Chem. **178**(6), 1864 (2005).
[36] M. Miya, R. Lwamoto, S. Yoshikawa, & S. Mima, Int. J. Biol. Macromol. **2**, 323 (1980).
[37] D. Park, S. Haam, K. Jang, I.S. Ahn, & W.S. Kim,Process Biochem. **40**, 53 (2005).
[38] G. Demirel, G. Ozcetin, F. Sahin, H. Tumturk, S. Aksoy, & N. Hasirci, React. Funct. Poly. **66**, 389 (2006).
[39] F. Kara, G. Demirel, H. Tumturk, Bioprocess Biosyst. Eng. **29**, 207 (2006).
[40] M. Uygun, D.A. Uygun, & A.A. Karagozler, Tralleis Elektronik Dergisi, **1**, 45 (2013).
[41] T.C. Hung, R. Giridhar, S.H Chiou,.& W.T. Wu, J. Mol. Catal. B: Enzym. **26,** 69 (2003).
[42] G. Adiguzel, Z. Sonmez, A. Adiguzel, H. Nadaroglu, Eur. Food Res. **242,** 769-776 (2016).