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Purification of Chitinase enzymes from *Bacillus subtilis* bacteria TV-125, investigation of kinetic properties and antifungal activity against *Fusarium culmorum*

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Abstract

Background: Chitin is the main structural component of cell walls of fungi, exoskeletons of insects and other arthropods and shells of crustaceans. Chitinase enzyme is capable of degrading chitin, and this enzyme can be used as a biological fungicide against phytopathogenic fungi, as well as an insecticide against insect pests.

Methods: In this study, 158 isolates, which were derived from bacteria cultures isolated from leaves and root rhizospheres of certain plants in Turkey, were selected after confirming that they are not phytopathogenic based on the hypersensitivity test performed on tobacco; and antifungal activity test was performed against *Fusarium culmorum*, which is a pathogenic fungi that cause decomposition of roots of vegetables. Accordingly, chitinase enzyme activity assay was performed on 31 isolates that have an antifungal activity, and among them the isolate of *Bacillus subtilis* TV-125 was selected, which has demonstrated the highest activity.

Results: Chitinase enzyme was purified by using ammonium sulphate and DEAE-sephadex ion exchange chromatography. Ammonium sulphate precipitation of chitinase enzyme from *Bacillus subtilis* TV-125 isolate was performed at maximum range of 0-20%, and 28.4-fold purification was obtained with a 13.4% of yield. Optimum activity of the purified enzyme was observed at pH 4.0 and at 50°C of temperature. In addition, it was identified that *Bacillus subtilis* TV-125A isolate retains 42% of its activity at 80°C temperature.

Conclusion: In the last phase of the study, chitinase enzyme purified from *Bacillus subtilis* TV-125A was tested on four fungal agents, although all the results were positive, it was particularly effective on *F. culmorum* according to the findings.

Keywords: Chitinase, Purification, Antifungal activity, *Fusarium culmorum*, *Bacillus subtilis*

Background

Chitin ((1,4)-2-acetamide-2-deoxy-beta-D-glucan) is a polysaccharide created by beta,1-4 bonding of N-acetyl glucosamine residues. Functional properties and physiological activities of chitin oligomers depend on molecular weight and chain length mostly [1]. Although most of the natural polysaccharides are either neutral or acidic, chitin is basic [2,3].

Chitin is the main component of most insects' cuticles and peritrophic membranes that protect intestines of insects. Pathogenic bacteria that infect intestines are required to pass this chitin-rich barrier first [4-6]. At the same time, chitin is present in tracheal tubes of insects, on their upper skin and muscles along with protein and other components [7].

Chitin is hydrolyzed by two types of enzymes, namely chitinase (E.C. 3.2.1.14) and beta-N-acetyl hexosaminidase (E.C. 3.2.1.52) [8].

Bacterial chitinase enzymes have been implicated in protection against parasites in fungi, protozoa and invertebrates, and especially in breakdown of micelles of

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fungal pathogens. In addition, chitinase are involved in the defense mechanisms of plants and vertebrates as well. And, plants produce chitinase enzyme to defend themselves against fungal pathogens [9]. The baculoviruses used in biological control of insect pests produce chitinase for pathogenicity [10]. Chitoooligosaccharides are produced via chitinase enzyme. Chitoooligosaccharides are substances produced in the plant defense and has a substantial use in medicine. For example, chitohexose and chitoheptose have an antitumor effect. Chitinase obtained from *Vibrio alginolyticus* produces chitopentose and chitotriose from colloidal chitin [11].

In recent years, it was done some researches related to leaving shellfish to decay. For this purpose, new some products were obtained for recycling waste by utilizing chitinase enzyme with chemical or biological methods. Particularly, the pollution emerged at the bottom of sea and oceans as a result of the death shellfishes is prevented by using microorganisms (*Vibrio furnis* etc.) that have chitinase enzyme, which decomposes chitin. Moreover, chitinase enzyme has wide range of applications in the food industry, feed industry, cosmetic industry, medical and fertilizer production areas [12].

In this study, first it was aimed to determine antifungal activities of 100 different microorganisms against *F. culmorum* isolated from cucumber that cause root rot of vegetables as a pathogenic fungi, and also characterization of chitinase enzyme was intended by selecting the strains that have the highest chitinase production capability. In the 2nd phase of the study, purification and characterization of the chitinase enzyme produced extracellularly by using *Bacillus subtilis* TV-125a bacteria, which was found to have the highest chitinase activity, were aimed; and in the last phase of the study, its antifungal activity was investigated against *F. culmorum*.

Methods

Bacteria isolates used in the study

In this study, a total of 158 bacteria isolates were used. These isolates are from the *Bacillus megaterium*, *Bacillus subtilis*, *Bacillus pumilus*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Bacillus thuringiensis*, *Paenibacillus macerans*, *Paenibacillus polymyxa* and *Pantoea agglomerans* species. These bacteria were provided by Assoc. Prof. Dr. Recep Kotan (Atatürk University, Faculty of Agriculture, Department of Crop Protection). Most of the bacterial isolates used have been selected because of their bioagent properties against various plant-pathogenic bacteria and fungi. Bacteria species used and total number of isolates from these species are shown in Table 1.

Table 1 The used bacterial strains in this study and the total numbers of isolates of these species

The used bacterial strains	Total isolates numbers
<i>Bacillus megaterium</i>	46
<i>Bacillus subtilis</i>	21
<i>Bacillus pumilus</i>	19
<i>Pseudomonas fluorescens</i>	15
<i>Pseudomonas putida</i>	15
<i>Bacillus thuringiensis</i>	11
<i>Paenibacillus macerans</i>	11
<i>Paenibacillus polymyxa</i>	10
<i>Pantoea agglomerans</i>	10
Total	158

Purification of chitinase enzyme from *Bacillus subtilis* TV-125A

Chitinase enzyme was partially purified by ammonium sulfate precipitation of *Bacillus subtilis* TV-125A bacteria. For this purpose, the bacteria homogenate was precipitated by ammonium sulfate precipitation at 0-100% ranges and chitinase activity of the precipitate and the supernatant was examined to identify the range with the highest activity. The precipitate was dissolved in 0.1 M phosphate buffer (pH:7.0) and dialyzed against the same buffer [13]. The chitinase enzyme was allowed to stand at -25°C in 0.1 M Tris-HCl buffer (pH 7.0) for further processing.

The dialysed suspension was applied to previously equilibrated DEAE-sephadex ion exchange column (2.5 × 30 cm) with 20 mM sodium citrate (pH: 6.0). The column was washed with the same buffer. Then, bound proteins were eluted by applying a gradient to the column from 0 to 1 M NaCl. The fractions were collected as 3 mL, with a 3 mL/min flow rate. Protein elution absorbance was spectrophotometrically measured at 280 nm. Involved activities of chitin were measured for all fractions. The fractions with chitinase activity were pooled and it was allowed to stand at 4°C.

Determining chitinase enzyme activity

Chitinase enzyme activity was determined using colloidal chitin substrate. After transferring substrate into the medium of the enzyme solution, it was subjected to reaction by incubation at 37°C for 30 minutes. Subsequently, after adding staining solutions into the reaction mixture, it was allowed to stand at 80°C for 10 minutes, and a measurement with spectrophotometer (PG-T80 Instrument UV-VIS Spectrophotometer) was performed against blind sample that was created by putting distilled, and activity was determined by monitoring absorbance change at 540 nm.

Table 2 Purification steps of the enzyme chitinase from *Bacillus subtilis* TV-125A

Purification steps	Volume (mL)	Activity (EU/mL)	Total activity		Protein amount (mg/mL)	Specific activity (EU/mg)	Purificationfold
			EU	%			
Crude extrat	50	44.95	8155.0	100	6.46	6.95	-
Ammonium sulphate precipitation (%0-20)	5	255.5	1277.5	15.7	1.64	155.79	22.4
DEAE Sephadex column	20	55.2	1104.0	13.4	0.28	197.14	28.4

Determining optimum pH value of chitinase enzyme

Using sodium acetate (pH 2.0-3.0), sodium citrate (pH 4.0-6.0), Tris-HCl (pH 7.0-9.0) and Na-carbonate (pH 10-11) buffers, chitinase enzyme activity assay was performed at different pH values (pH 2.0-11) to determine the optimum pH of the chitinase enzyme. Colloidal chitin was used as substrate in the standard activity assay method to perform the measurements.

Determining optimum temperature value of chitinase enzyme

For determination of optimum temperature of enzyme activity, activity measurements were performed at temperatures of 10-90°C. For this purpose, standard activity assay method was used. Each experiment was repeated for three times and data were averaged.

Determining protein amount

Bradford method was used to determine the protein amount. Results were compared to values of standard graphics plotted by using bovine serum albumin, and the total protein values in the samples were calculated. The sensitivity of this method is 1 to 100 µg [14].

SDS-PAGE electrophoresis

In order to determine the number of sub-units of the chitinase enzyme partially purified from *Bacillus subtilis* TV-125a bacteria, discontinuous sodium dodecyl

sulfate gel electrophoresis (SDS-PAGE) was performed by a method stated by Laemmli (1970), in two different acrylamide concentrations with a 3% stacking gel and 10% separating gel [15]. Obtained gel image was photographed.

Determination of the antifungal activity of the bacterial isolates in petri dish

F. culmorum causes rot to roots in vegetables and this disease is commonly seen in all vegetables in Turkey. So, we choose this fungus for antifungal activity. Determination of antifungal properties of 158 bacterial isolates against pathogenic fungi of *F. culmorum* was performed in Potato Dextrose Agar (PDA-Oxoid) nutrient media at 24°C under 15 hours of light and 9 hours of dark conditions.

The discs 6 mm in diameter taken from the pathogenic fungus culture growing on PDA were placed in the center of Petri dishes containing PDA nutrient media. And, bacterial cultures of 1×10^8 cells/mL concentration were drawn around these pathogen-inoculated petri dishes. Each bacterium was studied in 3 replications, and the average inhibition zone created by bioagent was identified with the help of the values obtained [16].

Results

Chitinase (poly-beta-1, 4-[2-acetamido-2-deoxy]-δ-glucoside glycanohydrolase, EC 3.2.1.14), which is the first

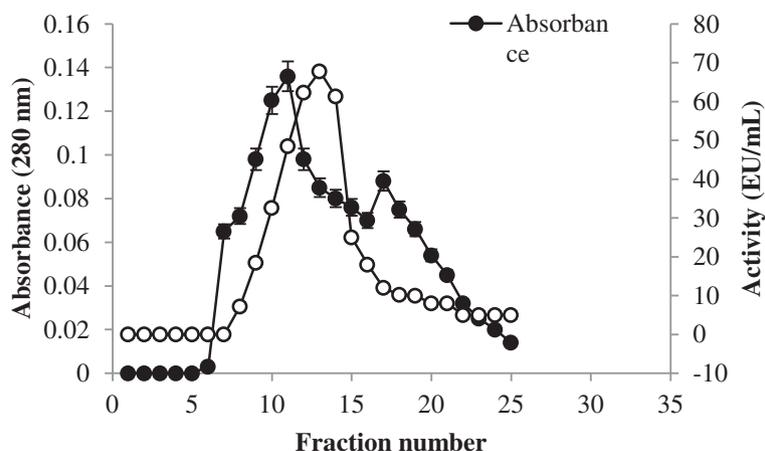


Figure 1 Purification of chitinase enzyme from *Bacillus subtilis* TV-125A by ion exchange chromatography using DEAE-Sephadex.

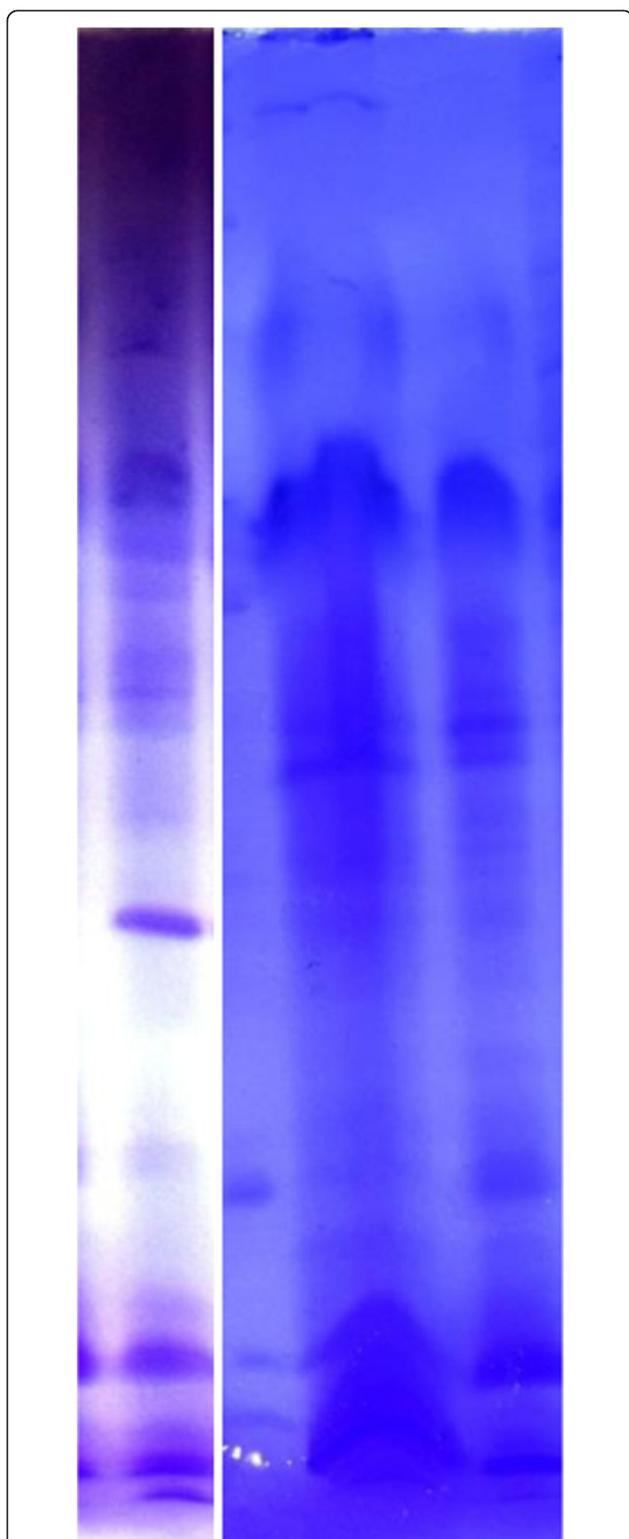


Figure 2 SDS-PAGE electrophoretic pattern of chitinase purified chitinase enzyme from *Bacillus subtilis* TV-125A]. Standart protein (β -Amylase, sweet potato, 200 kDa; alcohol dehydrogenase, yeast, 150 kDa bovine serum albumin, 97.4 kDa; rabbit muscle phosphorylase A, 66 kDa; egg ovalbumin, 45 kDa; pepsin, 29 kDa; carbonic anhydrase); cytochrome c, horse heart 12.4 kDa (II).

important enzyme of the two types of enzymes, plays a role in hydrolysis of chitin [17].

After centrifugation of chitinase crude extract of the isolate of *Bacillus subtilis* TV-125, the supernatant was precipitated by $(\text{NH}_4)_2\text{SO}_4$ precipitation at 0–20, 20–40, 40–60, 60–80, 80–100% ranges. The amount of protein, enzyme activity (EU/mL) and specific activity values determined for samples obtained for each method and step of partial purification steps of the chitinase enzyme derived from *Bacillus subtilis* TV-125 strains are presented in Table 2. Chitinase enzyme was extracellularly produced from *Bacillus subtilis* TV-125 and it was purified by using two steps. According to the results, the enzyme obtained from the *Bacillus subtilis* TV-125 using 0–20% $(\text{NH}_4)_2\text{SO}_4$ saturation with 22.4-fold purification and 15.7% recovery, was subjected to ion-exchange chromatography, which gave one peak with chitinase activity. Passage from DEAE-Sephadex column further purified the enzyme to 28.4-fold with a recovery of 13.4% and specific activity of 197.14 EU/mg. Chitinase enzyme from *Bacillus subtilis* TV-125, bacteria elution profiles with anion exchange chromatography was purified, and they were presented in Figure 1.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)'s results were presented (Figure 2).

The pH 2.0–11.0 range was tested in order to find optimum pH value of chitinase enzyme derived and purified from *Bacillus subtilis* TV-125A. Sodium acetate (pH 2.0 to 3.0), sodium citrate (pH 4.0–6.0), Tris (pH 7.0 to 9.0) and Sodium carbonate (pH 10.0–11.0) buffers were used in order to create different pH media (Figure 3).

As a result of the tests, optimum pH value of chitinase enzyme partially purified from *Bacillus subtilis* TV-125A was determined as 4.0, as shown in Figure 3. Not much decrease was observed in the enzyme activity assay performed for pure enzyme in the range of pH 4.0 to 11.0. It was found that activity of the enzyme was retained by 71% at pH 11.0. The optimum pH-value for chitinase enzymes isolated from *Gliocladium catenulatum*, *Stenotrophomonas maltophilia* C3 and *Alcaligenes oxyloxydans* bacteria are found to be pH 4.0. These studies support the findings obtained in our study. These characteristics are important, because most enzymes are catalytically unstable at acidic pH values, thus limiting their usefulness in the food industry [18–20].

Standard activity assay was performed at different temperature ranges in order to determine optimum temperature of the chitinase enzyme obtained from isolate of *Bacillus subtilis* TV-125A. Based on the results obtained, the activity (EU/mL) was calculated, and the relationship between temperature and activity are given in Figure 4. Optimum temperature value of chitinase enzyme derived from *Bacillus subtilis* TV-125A was found

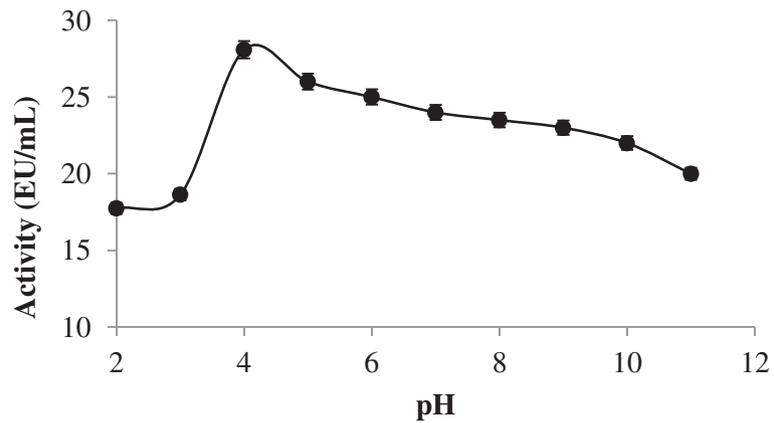


Figure 3 The effect of pH on purified chitinase enzyme activity from *Bacillus subtilis* TV-125A.

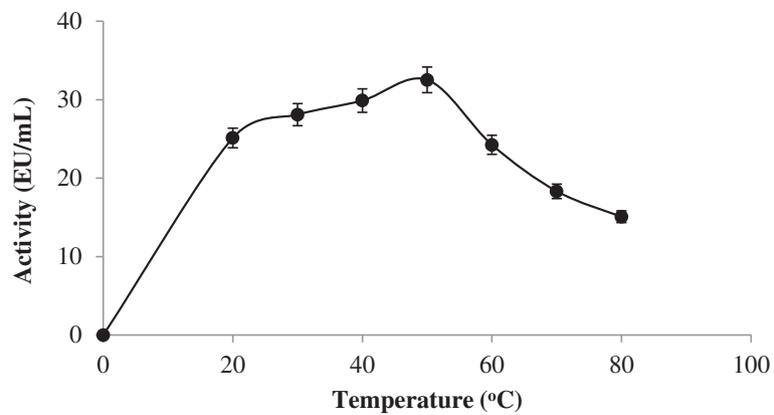


Figure 4 The effect of temperature on purified chitinase enzyme activity from *Bacillus subtilis* TV-125A.

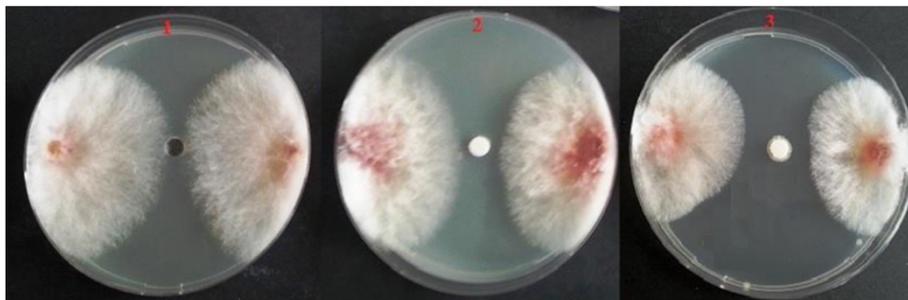


Figure 5 The inhibition effect of purified chitinase enzyme activity from *Bacillus subtilis* TV-125A on the *Fusarium culmorum* (1: Control, 2: Homogenate 3: Purified chitinase).

as 50°C. At elevated temperature, it was identified that isolates of *Bacillus subtilis* TV-125A retains its extracellular chitinase enzyme activity to a great extent by 42% without inhibition (Figure 2).

Yan et al. [21] have reported that *Bacillus subtilis* SL-13 chitinase enzyme has an optimum activity at 50°C, Vaidya et al. [20] have reported that *Alcaligenes xylosoxydans* chitinase enzyme has an optimum activity at 50°C, Bhushan & Hoondal [22] have reported an optimum activity of *Bacillus* chitinase enzyme at 45-55°C, Ghasemi et al. [23] have reported that ChiS chitinase enzyme by the bacterium *Bacillus pumilus* SG2 has an optimum activity at 50°C, and Zhang et al. [19] have reported that *Stenotrophomonas maltophilia* C3 chitinase enzyme showed an optimum activity at 45-50°C. These results support the findings obtained in our study.

Effective number of isolates and minimum-maximum inhibition zone values of 158 isolates from 9 different species tested *in vitro* for antifungal properties of the chitinase enzyme partially purified from *Bacillus subtilis* TV-125A extracellularly in a petri medium against pathogenic fungi are given in Table 1. According to these results, the total 158 isolates were found to form inhibition zones in the range of 12 to 37 mm. It was determined that 10 isolates of *Bacillus megaterium* species, 8 isolates of *Bacillus subtilis* species, 6 isolates of *Bacillus pumilus* species, 2 isolates of *Pseudomonas fluorescens* species, 3 isolates of *Pseudomonas putida* species, 1 isolate of *Paenibacillus macerans* species and 1 isolate of *Pantoea agglomerans* species were effective against the pathogen and prevented development of fungal disc in Petri dish substantially. And, 11 isolates of *Bacillus thuringiensis* species and 10 isolates of *Paenibacillus polymyxa* species were found to be ineffective [21,22]. The inhibition effect of purified chitinase enzyme activity from *Bacillus subtilis* TV-125A on the *F. culmorum* was shown in Figure 5.

Discussion

Produced chitinase enzyme from *Bacillus subtilis* TV-125 which has antifungal activity against *F. culmorum* is expected to be used in the medical field against fungal infections and in agriculture as biocontrol agents. Purified chitinase from *Bacillus subtilis* TV-125, which is thermostable and active in acidic pH, with a high purification factor and yield, could be an excellent choice in application of food, biotechnology and pharmacology industries.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

This work was carried out in collaboration between all authors. HN designed the study. MS and RK managed the analyses of the study. HN and ND performed the statistical analysis, wrote the protocol, and wrote the first

draft of the manuscript, managed the literature searches. All authors read and approved the final manuscript.

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