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Comparison of *in vitro* Antifungal Activity Methods Using Extract of Chitinase-producing *Aeromonas* sp. BHC02

Bilge Hilal Cadirci[1] · **Gulesme Yilmaz**[2,3]

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Abstract
Biological control to prevent fungal plant diseases offers an alternative approach to facilitate sustainable agriculture. Since the chitin in fungal cell walls is a target for biocontrol agents, chitinases are one of the important antifungal molecules. In this study, the aim was to investigate a new chitinase isolated from a fluvial soil bacterium and to show the antifungal activity of the characterized chitinase by comparing the three common methods. The bacterium with the highest chitinase activity was identified as *Aeromonas* sp. by 16 S rRNA sequence analysis. Following the determination of the optimum enzyme production time, the enzyme was partially purified, and the physicochemical parameters of the enzyme were investigated. In the antifungal studies, direct *Aeromonas* sp. BHC02 cells or partially purified chitinase were used. As a result, in the first method in which the *Aeromonas* sp. BHC02 cells were spread on the surface of petri dishes, no zone formation was observed around the test fungi spotted on the surface. However, zone formation was observed in the methods in which the antifungal activity was investigated using the partially purified chitinase enzyme. For example, in the second method, the enzyme was spread on the surface of PDA, and zone formation was observed only around *Penicillum* species among the test fungi spotted on the surface. In the third method, in which the necessary time was given for the formation of mycelium of the test fungi, it was observed that the growth of *Fusarium solani*, *Alternaria alternata* and *Botrytis cinerea* was inhibited by the partially purified chitinase. This study concludes that the results of the antifungal activities depend on the method used and all fungal chitins cannot be degraded with one strain's chitinase. Depending on the variety of chitin, some fungi can be more resistant.

Keywords Chitinase · Antifungal activity · *Aeromanas* sp. · Biocontrol

1 Introduction

It is known that more than 8000 fungal species cause plant diseases [1]. Due to the fungal attack on agricultural and horticultural crops, economy and biological productions affected in 5–25% in developed countries and 20–50% in emerging countries [2]. Chemical fungicides may cause soil contaminations and may affect human health. Therefore, there is a need for cheaper and safer methods to protect plants from fungal attack. An ecofriendly approach as an alternative to agrochemicals is biocontrol, which means to use living organisms producing mycolytic enzymes, like chitinases and glucanases, which are able to degrade fungal cell-wall components .

Chitin, is one of the most abundant polysaccharide polymer occurring in nature, is a highly insoluble β-(1,4) bonded polymer composed of NAG. Chitinases are enzymes that can hydrolyze chitin into its oligomeric, dimeric and monomeric components. Chitinases have a wide distribution in nature, including bacteria, fungi, plants, insects, protozoa, humans, and yeasts. [3]. The roles of chitinases in these organisms are diverse. In bacteria, chitinases are often involved in nutrition and parasite mineralization. Fungal chitinases play a physiological role in cell division and differentiation.

✉ Bilge Hilal Cadirci
bilgehilal.cadirci@gop.edu.tr

Gulesme Yilmaz
gulesmeyilmaz@gmail.com

[1] Department of Crop Protection, Tokat Gaziosmanpasa University, Tokat, Turkey

[2] Department of Bioengineering, Hacettepe University, Ankara, Turkey

[3] Vocational School of Health Services, Medical Laboratory Techniques Fenerbahce University, Istanbul, Turkey

In plants, chitinases are part of plant defense mechanisms against fungal pathogens. In insects, it has been found to be involved in the molting process during their development. Chitinases have also been found in human gastric juices [4].

Extensive studies over the past two decades on chitinases have been done by many laboratories. This is mostly due to the antifungal property of chitinases. Since there are a lot of methodologies to measure in vitro antifungal activity, varying results have been obtained. In the antifungal tests generally test fungi were inseminated in the center of the Petri plates containing PDA and when the diameter of the colony was almost 2 cm, enzyme impregnated with sterile blank paper discs locates the center of the plates [5]. Thakur et al., [6] purified and characterized extracellular chitinase from *B. cereus* NK91 and then tested the partially purified enzyme for its antifungal activity against some phytopathogens. They added different concentrations of partially purified chitinase into warm molten PDA and after solidification, 5-day old agar mycelial plugs of fungi were plated on enzyme-including media. After incubation together, antifungal activity of *B. cereus* strain NK91 chitinase was determined.

Two different well diffusion assays were also determined in measuring antifungal activity. In the study of Ekundayo et al. [7], the mycelium of the test fungi was placed in the center of the petri plates containing PDA and wells bored with a cork borer filled with enzyme and the plates incubated together to observe zones of inhibition. Cd et al. [2] also checked the antifungal activity by well diffusion method. In their study, the antifungal activity of cell free supernatants of chitinase producing bacteria against fungal phytopathogens by well diffusion method. They spread the fungi onto PDA plates and bored wells were filled with cell-free supernatant of *Bacillus* chitinase extract. In the end of the incubation at 30 °C for 120 h, they observed Translucent type (complete inhibition of fungal growth) and Opaque type halos (possible alteration of the mycelial development).

In this study, the antifungal effects were compared for (1) using chitin producing bacteria, (2) covering the plate by enzyme and (3) adding chitinase on the plates after visible mycelium formation by the test fungi.

Considering the huge size of microbial and environmental diversity, the isolation of wild type microorganisms is still important because it is possible to find in those environments the desired characteristics of microorganisms. In this study, it is also aimed to isolate chitinase producing microorganisms and to investigate the enzymes' physicochemical properties.

2 Materials and Methods

2.1 Bacteria Isolation and Culture Conditions

10 gr fluvial soil from Yeşilirmak River, Tokat was added in 90 mL of water and shaken vigorously at 30 °C for 1 h. One ml of the supernatant of the sample was inoculated into 100 mL of LB medium in 500 mL Erlenmeyer and was incubated at 37 ºC for 24 h at 200 rpm. After the decimal dilutions, CHD Agar media [(g/L): Na_2HPO_4 (0.65), NaCl (0.25), KH_2PO_4 (1.5), NH_4Cl (0.5), yeast extract (0.12 g), colloidal chitin (10.0 g) and agar (20.0 g) at pH 7.0)] was used to spread on the samples. Colloidal chitin was prepared by mixing continuously chitin from shrimp shells (Sigma C7170) with concentrated hydrochloric acid with a stirrer and precipitating with distilled water [8]. The plates were incubated at 37ºC for 36 h, and the colonies showing clear halos were selected.

Colonies forming the hydrolysis zone were picked individually and further purified by subculturing. To determine the microorganism producing the chitinase enzyme with the highest efficiency, production was carried out again CHD media at 37 ºC for 48 h and continued with the organism showing maximum halo-zone. Isolates were stored as a glycerol stock at -80 C. Among these strains, the one exhibiting highest enzyme activity was selected for further investigations.

2.2 Identification and Phylogenetic Analysis

After determination of the Gram properties of the best chitinolytic bacteria, total genomic DNA was extracted from the cells by using genomic DNA isolation kit (Geneall, Korea). 16s rRNA region of the genome was amplified with the 27 F and 907R primers. The PCR amplicon was processed for sequencing by Refgen Biotechnology, Turkey. The chromatograms of the sequences were analyzed by Chromas (Technelysium Pty Ltd., Austria, Version 2.6.6) and the sequences were compared with the reference sequences by BLAST program in NCBI. After identifying the closest phylotypes, their sequences were aligned in MEGA X program [9] and the evolutionary history was inferred using the Neighbor-Joining method [10]. All ambiguous positions were removed for each sequence pair (pairwise deletion option). The evolutionary distances were computed using the Maximum Composite Likelihood method [11].

2.3 Enzyme Assays

The chitinase activity was assayed by measuring reducing sugar released from colloidal chitin as the substrate according to the modified method of Toharisman et al. [12]. Briefly,

100 µL of the culture supernatant and 900 µL of substrate (50 mM pH 7.0 phosphate buffer containing 1% colloidal chitin) was mixed and incubated at 37 ºC for 60 min. Following the centrifugation at 10,000 g for 10 min, 1 mL of supernatant and 1 mL of DNS reagent were mixed and boiled for 15 min. After cooling, measurements were carried out in a spectrophotometer at 540 nm.

1 unit of enzyme activity was defined as the amount of enzyme required to release 1 µmol of NAG in 1 min [13]. The standard curve of NAG solution with a slope equation of y = 2,993x-0,007 and correlation coefficient (R^2) of 0,996 was used.

2.4 Generation of Crude Extract by Partial Purification of the Enzyme

The chitinase producing strain *Aeromonas* sp. was activated twice at 30 ºC for 18 h. 2 mL of the preactivated culture was inoculated into 200 mL CHD media containing 1% of colloidal chitin and was incubated at 30 ºC for 30 h at 200 rpm. The cell-free supernatant obtained by centrifugation at 6000 g for 20 min was called as crude enzyme extracts. Proteins of the crude enzyme extracts were concentrated by 20–60% of ammonium sulfate precipitation at + 4 ºC for 8 h. After each gradient, proteins in the supernatant were obtained by centrifugation at 10,000 g at + 4 ºC for 10 min. Desalting of the protein fractions were performed for overnight in a phosphate buffer of pH 7.0 and 50 mM at + 4 ºC by a dialysis membrane with a cut-off value of 10,000 Da [14]. The dialysis buffer was changed at appropriate intervals. After dialysis, enzymatic activities of the crude extracts were determined. Then, the fraction with high specific chitinase activity was stored at + 4 ºC for following physicochemical characterizations.

The partially purified chitinase fractions were analyzed by SDS-PAGE on a 12% (v/v) polyacrylamide gel [15]. Following the electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 for 1 h, and then the gel image was obtained after destaining overnight [16].

Protein concentrations were measured by Bradford method [17]. Briefly, 1 mL of Bradford reagent was added to 50 µL of purified enzyme sample and after it was kept in the dark for 10 min at room temperature, absorbance was measured at 595 nm in a UV spectrophotometer. BSA was used as a standard [18].

2.5 Effect of Temperature and pH on Enzyme Activity

To determine the optimum reaction temperature of the partially purified chitinase enzyme, activity experiments were carried out with 1% colloidal chitin substrate prepared with 50 mM pH 7.0 phosphate buffer at 20, 30, 40, 50, 60ºC, respectively. For the activity determination, standard activity determination was made by mixing 100 µL of enzyme and 900 µL of substrate.

To find the optimum reaction pH of the chitinase enzyme, substrate solutions containing 1% chitin were prepared by using various buffer solutions (50 mM): citrate buffer (pH 2.0–4.0), sodium phosphate buffer (pH 5.0–8.0), and glycine-NaOH buffer (pH 9.0) (citrate, phosphate, glycine-NaOH). Standard DNS method was used for the activity determination.

2.6 Determination of Substrate Specificity and Amylase and Cellulase Activity of the Crude Extract

The substrate specificity of the crude extract was determined by measuring the activity with colloidal chitin, crystal chitin, starch, and cellulose as substrates. The 1% substrates were prepared in 50 mM phosphate buffer (pH 7.0). After 60 min of incubation, reducing sugar concentrations were measured by standard DNS method.

2.7 Determination of Chitinase Enzyme Kinetics

Kinetic constants of the chitinase were calculated by measuring the enzyme activity with colloidal chitin substrate with various concentrations (0.2-2 mg/L). K_M and V_{max} constants were calculated by using the slope of the Lineweaver-Burke graph in the Michaelis Menten equation [19].

2.8 Detection of Antifungal Activity

The fungal pathogens (*Aspergillus niger, Fusarium solani, Alternaria alternate, Botrytis cinerea, Phytophthora sp., Sclerotinia sp., Penicillium sp.*) are kind present from the culture collection of Prof. Dr. Yusuf Yanar, Plant Protection Department of Faculty of Agriculture, Tokat Gaziosmanpasa University [20]. The cultures were activated and cultivated in PDA medium at 30 ºC for 3–5 days [5].

2.8.1 Antifungal Study of Chitinase Producing Bacteria on Fungal Phytopathogens

To investigate the effect of chitinase-producing *Aeromonas* sp. BHC02 cells on fungal pathogens, test fungi were inoculated on one end of the petri dishes containing PDA medium by spot cultivation. After 48 h of incubation at 30 ºC, sufficient micelle formation was observed. Then, *Aeromonas* sp. BHC02 cells were cultivated in the other part of the petri dish. Incubation was then continued for another 24 h. Subsequent inhibition zones were observed.

Fig. 1 Selection of chitinase producing organisms based on activity zones on CHD agar. The colony 10 showed the largest inhibition zone and quantitively the highest chitinase activity by DNS method. According to 16srRNA sequence analysis, colony 10 was identified as Aeromonas sp. and called *Aeromonas* sp. BHC02 strain

2.8.2 Antifungal Study of Partially Purified Chitinase on Fungal Phytopathogens

The effect of the partially purified chitinase enzyme on the test fungi was investigated by two different methods. With the first method, the entire surface of the PDA medium was coated with the enzyme. In the second method, fungus was inoculated at one end of the petri dish and enzyme was added to the other end.

In the first method [21]; 100 µL of partially purified chitinase enzyme was spread on the surface of the petri dish in PDA medium. In the experiment, the surfaces of the control groups were not coated with the enzyme. Test fungi were inoculated in three spots and incubated for seven days.

As a second method [22], test fungi were cultivated on one end of the petri dish containing PDA medium. After incubation at 30 ºC for 48 h, after sufficient micelle formation was observed, 30 µL of partially purified chitinase enzyme was added to the other part of the petri dish and the zones formed during five days were observed.

3 Results

3.1 Detection and Identification of Chitinase Producing Microorganisms on CHD Agar

Enzyme producing bacteria were detected according to the halo-zone formation around the colony in the media with substrate. Chitinase halo-zone was observed in the CHD agar medium petri dishes incubated at 30 ºC for 36 h [23]. Morphologically different 10 colonies forming halo-zones were selected randomly and after purification by subculturing, one single point of the active bacteria was planting on new petri dishes CHD agar medium. Colony 4, 7 and 10 formed the large zone diameters (Fig. 1). But the colony 10 showed the highest activity by DNS method. So further studies were carried out with colony 10.

Chitinase production time of the colony 10 in CHD broth media (pH: 7.0) at 37 ºC was determined spectrophotometrically by DNS method. As shown in Fig. 2 the highest enzyme activity was recorded at 30th h of the growth.

According to the Gram staining procedure [24], it was observed that the colony 10 was gram negative (data not shown). After analysis of the homology of the 16srRNA gene sequence by BLAST algorithm, the isolated strain has maximum homology (99.76%) with *Aeromonas* sp. Evolutionary analyses were conducted in MEGA X. The optimal tree is shown next to the branches. The evolutionary distances are in the units of the number of base substitutions per site. In Fig. 3, it was seen that there was a lot of crossing between *Aeromonas* species, and *A. media* and *A. caviae* species were close to our isolate. Based on the phylogenetic analysis, the strain isolated from colony 10 was named as *Aeromonas* sp. BHC02.

4 Partial Purification and Characterization of Chitinase

The purification profile of the partially purified chitinase enzyme is summarized in Table 1. The activity of the enzyme, which was partially purified after 60% ammonium sulfate precipitation, was determined as 0.18 ± 0.024 U/mL with a yield of 38.04%. The molecular size on 12% SDS gel was determined for the enzyme mixture, which was partially purified with ammonium sulfate. SDS image of chitinase enzyme is given in Fig. 4. In this mixture, 3 bands with sizes of ~ 25 kDa, ~ 37 kDa, ~ 110 kDa were determined.

In Fig. 5, the activity of the chitinase enzyme at pH 7.0 in different temperature is shown graphically. It was observed that the optimum temperature value is 30 ºC, and the enzyme retained close to maximum activity also at 40–50ºC. It was determined that the enzyme retained 85%

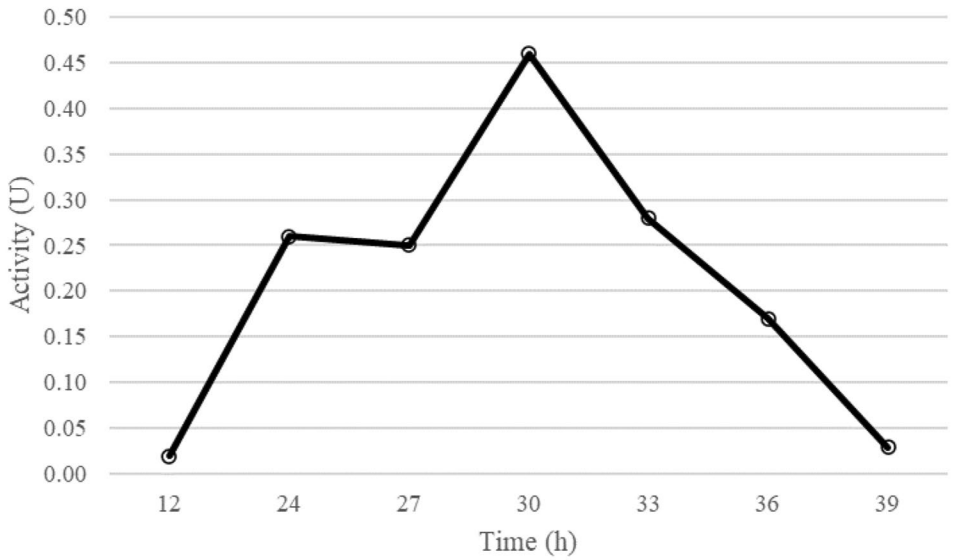

Fig. 2 Time course of chitinase production of colony 10 at 37 ºC in CHD broth

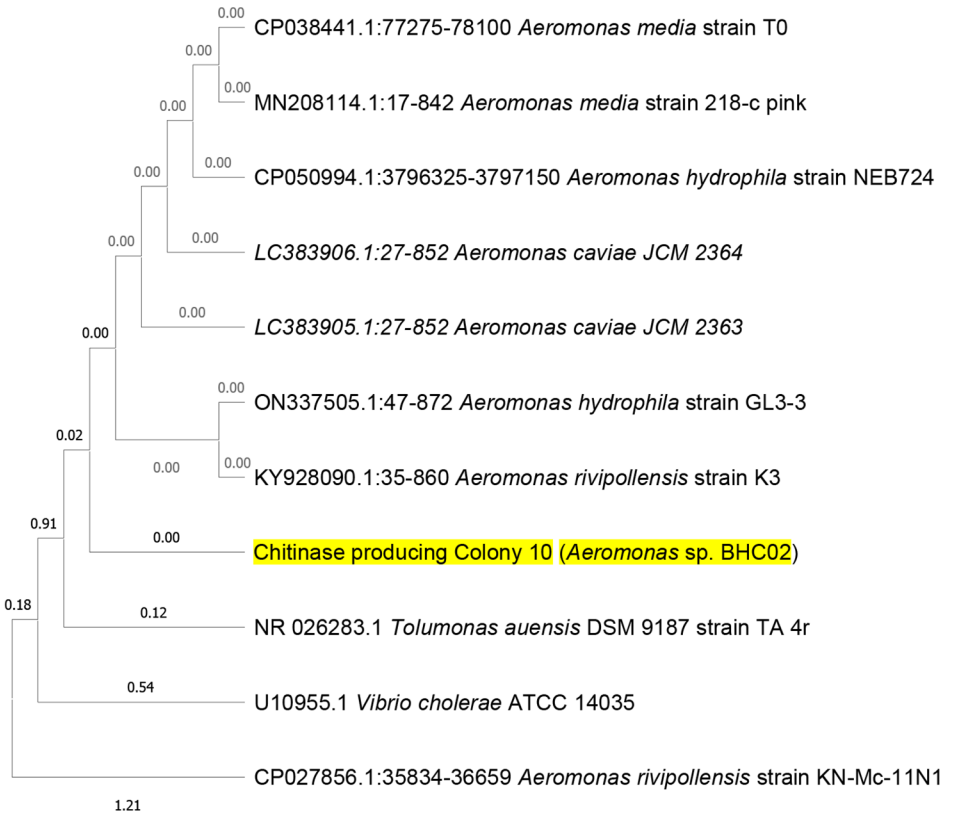

Fig. 3 Neighbor-joining phylogenetic tree of the chitinase producing colony 10

Table 1 Purification profile of partially purified chitinase enzyme

	Activity (U/mL)	Protein (mg/mL)	Specific Activity (U/mg protein)	Purification Coefficient	Purification Yield (%)
Supernatant	0.46	0.25	1.84	1	100
$(NH_4)_2SO_4$ precipitation (60%)	0.18	0.075	2,4	1.3	38.04

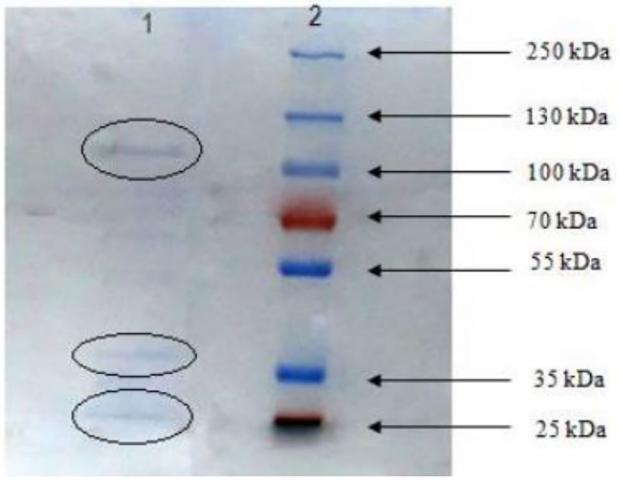

Fig. 4 SDS-PAGE image of partially purified *Aeromonas* sp. BHC02 chitinase Lane 1: After 60% ammonium sulfate precipitation Lane 2: Marker

Table 2 Enzymatic activity of partially purified enzyme mixture with various substrates

	Substrates			
	Colloidal Chitin	Chitin	Filter-paper cellulose	Soluble starch
Activity (U/mL)	0.38	0.22	0.13	0.17

of its activity when stored in the refrigerator for 2 months (data not shown). In the experiment carried out to find the optimum pH range of the enzyme reaction, it was observed that the maximum activity occurred at pH 7.0 in the phosphate buffer. However, the enzyme was found to be active at pH range 4–9 (Fig. 5).

Although cellulase and amylase activities were observed in the partially purified enzyme mixture in small amounts (0.13 U/mL and 0,17 U/mL), it was found that the highest activity was obtained from colloidal chitin as a substrate. It was also shown that BHC02 chitinase can hydrolyze crystalline chitin with 57.9% activity when compared to colloidal chitin (Table 2). The kinetics of BH02 chitinase with colloidal chitin substrate was also investigated. By calculating from the Lineweaver-Burk graph, the kinetic parameters were determines as K_M = 2.45 mM and V_{max} = 2.94 mM/min [25].

Table 3 Effect of partially purified *Aeromonas* sp. BHC02 chitinase enzyme on fungi

Pathogen	Pathogen for	Result	Working Anti-fungal Activity Method
Aspergillus niger	Onion [26]	No Zone	None
Fusarium solani	Tomatoes [27]	Zone formation	After mycelial formation occurs
Alternaria alternata	Apple[28]	Zone formation	After mycelial formation occurs
Botrytis cinerea	Strawberry [29]	Zone formation	After mycelial formation occurs
Phytophthora sp.	Apple [30]	No zone	None
Sclerotinia sp.	Soybean [31]	No zone	None
Penicillium sp.	Potatoes [32]	No growth	Enzyme spread onto PDA plates

5 Antifungal Activity Tests

The results of the total experiments are given in Table 3. The antifungal activity experiment in which *Aeromonas* sp BHC02 was inoculated into the petri dish and incubated together with the test fungi, no antagonistic activity was detected. The effect of the partially purified chitinase enzyme on the test fungi was investigated in two different ways. In the experiment, in which the surface of the PDA medium was coated with enzyme, it was observed in the end of seven days of incubation, the enzyme did not cause any inhibition on the growth of other fungi except *Penicillium sp*. The result of the experiment, in which the chitinase enzyme was added after the mycelial formation of the test

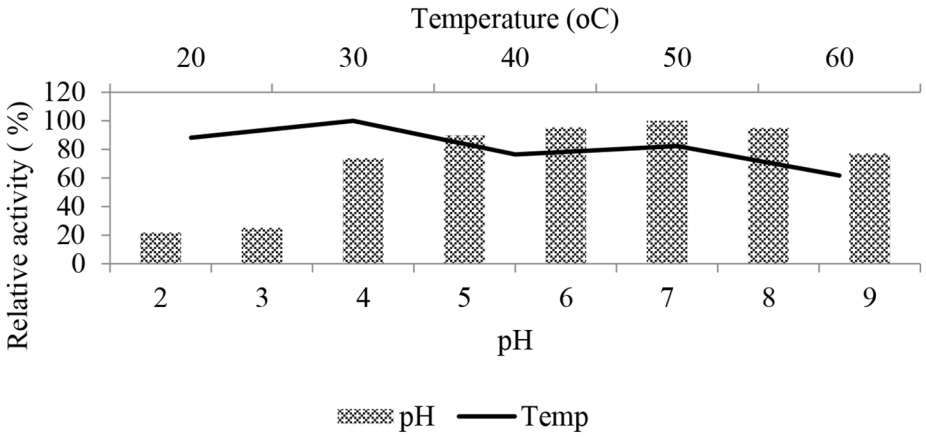

Fig. 5 Temperature- and pH-dependent activities of *Aeromonas* sp. BHC02 chitinase. The enzyme assays were performed with colloidal chitin as substrate. The temperature graph was done at 30ºC. The pH graph was done at pH 7.0

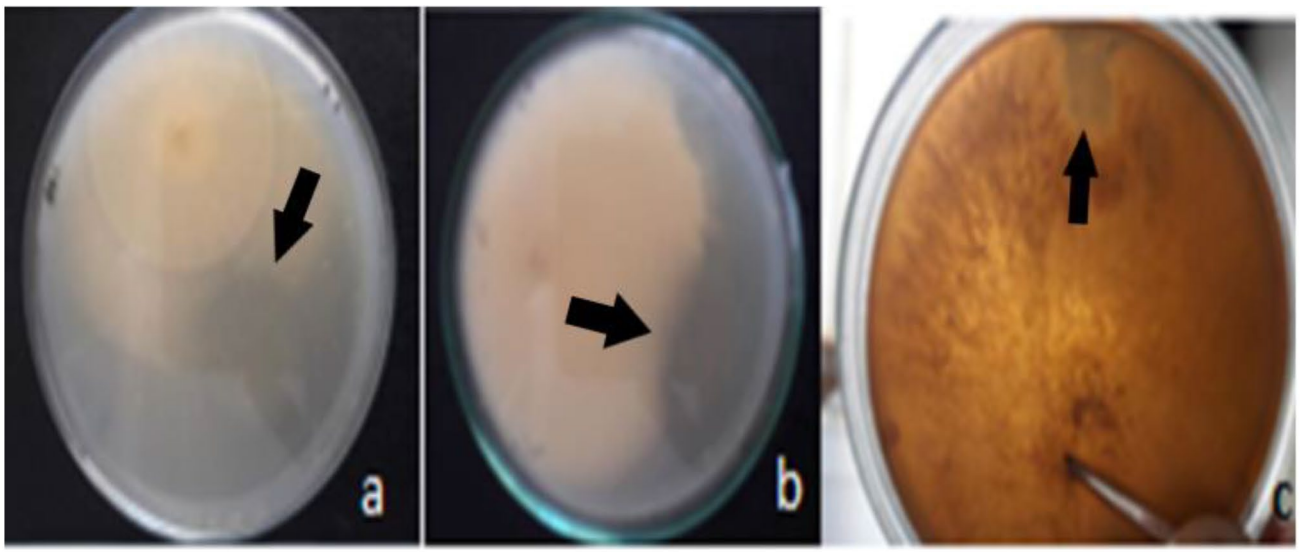

Fig. 6 Inhibition zone of *Aeromonas* sp. BHC02 chitinase enzyme on *Alternaria alternate* (a), *Fusarium solani* (b), *Botrytis cinerea* (c) fungi on PDA agar

fungi, inhibition zones were determined against *(A) alternata, F. solani, (B) cinerea* fungi as seen in the Fig. 6.

6 Discussion

In this study, it was aimed to investigate chitinase producing bacteria from the microbial flora of river Yesilirmak in Tokat, as potential source for fungicides. Chitinase producing bacteria were explored according to the halo-zone formation around the colony in the media with colloidal chitin as a substrate. Just 10 colonies showed inhibition zones. But the colony with the largest halo-zone was determined by 16 S rRNA analysis as *Aeromonas* and the strain is called *Aeromonas* sp. BHC02.

Aeromonas genus is found very commonly in aquatic environment that's the reason why they can secrete multiple enzymes, including chitinases. *A. caviae*, a mesophilic species of the genus *Aeromonas*, was also discovered to be a potential biological control agent against fungal pathogens with its chitinolytic activity [33]. *A. caviae* CB101 was isolated as an efficient degrader with four chitinases of different molecular weights into the culture's supernatant. Mehmood et al. studied the Chi1 chitinase and according to their results, *A. caviae* CB101 produces 0.68 U/mg protein. [34].

Cardozo et al., [35] demonstrates the ten marine-derived *A. caviae* isolates potential to produce NAG from α-chitin at 37 °C with 2% (w/v) colloidal chitin with the activity of 0.5 U/mL by all the isolates with yields from 14 to 85% at 6 h, 17–89% at 12 h and 19–93% after 24 h.

A.hydrophila HS04 is one of the best chitinase source in *Aeromonas* genus. Saima et al., [36] showed 6 of 58 isolates hydrolyze chitin in petri dishes. The isolates identified as *A. hydrophila* HS4 and *A. punctata* HS6 have the highest chitinolytic activity in 0.1% colloidal chitin at 37ºC after 24 h of incubation with 5946 U/mL and 6072 U/mL, respectively. Stumpf et al. [37] also studied on *A.hydrophila* called strain AH1-N which was isolated by enrichment with *Aspergillus tubingensis* mycelia, showed maximum activity 67.93 ± 1.51 $\mu mol \cdot h^{-1}$at 45ºC with 0.1% colloidal chitin. It is obvious that even there is a confliction in produced enzyme activity between *A. hydrophila* strains, our results with others conclude that, chitinase activity of *Aeromonas* sp. BHC02 is around 0.5 U/mL at 30ºC in neutral pH with 1% colloidal chitin.

Zhang et al. [38] worked with the chitinase of *A. veroni* with 30 U/mL activity and found the optimum pH as 5.0 and the optimum temperature as 50 ºC. When they compared their chitinase with the commercial one, they concluded that even chitinases degrade colloidal chitin, untreated chitin of the shrimp shell is not a substrate for both enzymes. To increase the yields, they transferred the chitinase gene of *A. veroni* to *Pichia pastoris* and obtained high yields. Therefore, the recombinant expression e.g., in *Pichia i*s likely to give higher yields also for *Aeromonas* sp. BHC02 chitinase.

Because of the structural similarity of chitin and cellulose and the chitinolytic and cellulolytic pathways follow parallel steps, cellulase and amylase activities of the crude extract were investigated. As expected, the results showed that, chitinase activity on colloidal chitin is the highest one. But there were some amylase and cellulase activities. This result suggested that the presence of chitin in the medium

may also have an inducing effect on *Aeromonas* sp. BHC02 amylase and cellulase enzymes. It is recommended that this issue be explored and worked on later.

In the antifungal studies, direct *Aeromonas* sp. BHC02 cells or partially purified chitinase were used. Seven fungal species (*A. niger*, *F. solani*, *(A) alternata*, *(B) cinerea, Phytophthora* sp., *Sclerotinia* sp., *Penicillium* sp.) were used in antifungal trials. As a result, in the first method in which the *Aeromonas* sp. BHC02 cells were spread on the surface of petri dishes, no zone formation was observed around the test fungi spotted on the surface. This result indicates that *Aeromonas* sp. BHC02 cells produces so low amount of chitinase (and/or other antifungal factors) that concentrating is needed. The minimum inhibition concentration of the chitinase is not known.

However, zone formation was observed in the methods in which the antifungal activity was investigated using the partially purified chitinase enzyme. For example, in the second method, the enzyme was spread on the surface of PDA, and zone formation was observed only around *Penicillum* species among the test fungi spotted on the surface. And in the third method, in which the chitinase enzyme was added after the mycelial formation of the test fungi, inhibition zones were determined against *(A) alternata*, *F. solani*, *(B) cinerea*. Chang et al. [39] also used a similar method to our second method but they add the enzyme into the molten PDA with a 1:1 (v/v) ratio. Even plant pathogenic fungi *Fusarium oxysporum* and *F.solani* were inhibited by *B. cereus* chitinase, *Pythium ultimum* CCRC32725 was not inhibited. This may be related to the concentration of the enzyme. In our study, 100 µL of the chitinase was spread onto PDA and the spores of test fungi were also spotted into the media. Because of the restricted diffusion ability of the chitinase in PDA, during incubation, fungal micelle may be able to grow inside the media. Probably the enzyme couldn't inhibit the fungal growth due to the low water activity caused by the long incubation period. It is also possible the enzyme may bind to agar. These conclusions must be proved by appropriate experiments.

In the investigations of Cd et al. [2] test fungi were spread onto PDA plates cell-free supernatant of *Bacillus* chitinase extract were filled into bored wells. After incubation together, it is resulted that, fungi *Alternaria* and *Colletotrichum acutatum* were the most affected by all the cell-free supernatants from four chitinolytic *Bacillus* sp., whereas fungi of the genus *Fusarium* were the most resistant. Thakur et al. [6] also showed that, after incubation together, antifungal activity of *B. cereus* strain NK91 chitinase was determined against *F. oxysporum*, *R. solani*, and *C. gloeosporioides*. Pandya and Saraf [40] studied chitinase of *Bacillus safensis* MBCU6 by incubating them with the fungal spores together on the PDA media. They showed that, although *Macrophomina phaseolina* and *Rhizoctonia solani* were inhibited strongly both by crude and purified enzymes, *Fusarium oxysporum*, *Sclerotium rolfsii* and *Sclerotinia sclerotiorum* were not inhibited. The comparison of the antifungal test methods used by Cd [2], Thakur [6] and Pandya and Saraf [40] can support our hypothesis. Even though the species may be different in those investigations, the results showed that *Fusarium* sp. is the most resistant test fungi if the cell-free supernatants of *Bacillus* were added to the petri dishes after the mycelial growth occurs. But when the chitinase-producing *B. cereus* cells are incubated together at the same time with fungi, *F. oxysporum* becomes one of the sensitive test fungi. It is obvious that if the chitinase-producing microorganism change, the resistance of the fungi changes because of the differences in the chemical structures of the enzymes. However, after surveying the literature, the reason for the result is thought to be related to the method too. It is estimated that the micelle of *Fusarium* sp. secretes proteases which may hydrolyze the chitinases of *Bacillus* sp. So, if the chitinases are added onto the plate after an appropriate time, the occurred fungal micelle will have time to secrete a wide variety of proteases or such antifungal inhibitors. But when the spores of *Fusarium* incubate together with chitinases, chitinases will inhibit the emerging fungal cell wall. But this hypothesis must be proved.

Also, other concentrated factors in the medium could be involved in antifungal activity. It is obvious that antifungal activity exists, and it may be likely (but not proven by this experiment) that growth on chitin induces antifungal property. This may confirm by growing the cells on mere sugar or some other fiber medium and check the antifungal activity. It is also necessary to check if secreting antifungal factors exist into ammonium sulfate fractionated fractions. The expected situation based on the literature is that chitinase is critical for antifungal activity. This situation needs improved by using commercial purified enzymes as controls.

While investigating the effect of purified chitinase enzyme on fungi in antifungal studies, it was observed that none of the method we tried with *Aeromonas* sp. BHC02 cause an inhibition on *A. niger, Phytophthora sp., Sclerotinia sp*. When we searched for articles that would be compatible with the results we obtained, we saw that no study used exactly similar microorganisms. But Zarei et al. [5] test the partially purified chitinase from isolated *Serratia marcescens* B4A on the mycelium of seven phytopathogenic fungi including: *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, *Bipolaris* sp, *Fusarium graminearum*, *Trichoderma reesei*, *Alternaria raphani*, *A. brassicicola*. In their antifungal activity method, the test fungi were inseminated in the center of the petri plates containing PDA and when the diameter of the colony was almost two cm, enzyme impregnated with sterile blank paper discs locates the center of the

plates. This enzyme exhibited antifungal activity against *R. solani*, *Bipolaris* sp, *A. raphani*, *A. brassicicola*, But not to *Sclerotinia* sp. like our results. 5-min-boiled partially purified chitinase was used as control. Their results indicate the inhibition is not related with the fungal growth but due to with antifungal chitinase. Pentekhina et al. [41] were cloned and expressed GH18 and GH19 chitinase genes of *A. salmonicida* and then they used purified and lyophilized chitinases to determine the antifungal activity on *T. reesei* by inhibition of mycelial extension method. They conclude that GH18 chitinases did not exhibit any activity against fungi, while GH19 chitinases suppressed the hyphal growth of *T. reesei*.

The results of this investigation showed that, before concluding the inactivity of the enzyme against a fungus, more than one method must be used. If the antifungal activity cannot be measured despite all the methods, this will be related to the chitin and its structure in the cell wall of that fungus.

7 Conclusion

Protecting plants from diseases produced by phytopathogenic fungi is one of the most important challenges in agriculture. Total loss because of plant diseases reaches almost 50% of the yield in developing countries [42]. A third of this is a result of fungal infections. For this reason, it is very important in agriculture to find biological products that can be used in biological control. Recent studies have shown that chitinase from plants and microorganisms can inhibit fungal growth [43].

Since chitin is an important structural component of the fungal cell wall, it was thought that chitinase-producing microorganisms could be used as a biocontrol agent for different fungal diseases of plants [44]. Using chitinolytic microorganisms for biological control offers an alternative strategy to control agricultural phytopathogens.

In this study, a natural chitinase with antifungal activity against various phytopathogens was produced. In addition, the chitinase obtained in this study may have important effects on agriculture, such as the biological control of insects, which are plant pests. With the developing technology, the usage areas and importance of enzymes obtained from microorganisms have increased to a great extent [43]. Among the reasons for the use of microbial enzymes in many areas are easy to obtain, rapid growth, development in more economical ways, high activity, and abundant enzyme synthesis. Considering the industrial importance of the results we obtained; Chitinase enzyme, which has antifungal activity against *Alternaria alternata, Fusarium solani, Penicillium, Botrytis cinerea* can be used in agriculture as a biocontrol agent.

This study also conclude that the results of the antifungal activities depend on the method used. And all fungal chitins cannot be degraded with a chitinase. Depending on the variety of chitin, some fungi can be more resistant. In this context, it is necessary to conduct a detailed study on the chitins in the cell wall of the fungi.

Abbreviations

SDS-PAGE	Sodium dodecyl sulfate- polyacrylamide gel electrophoresis
NAG	N-acetylglucosamine
CHD	Chitinase-detection
NCBI	National Centre for Biotechnology Information
DNS	3,5-Dinitrosalicylic acid
BSA	Bovine serum albumin
V_{max}	Maximum rate of reaction
K_M	Michaelis Constant
PCR	Polymerase chain reaction
PDA	Potato dextrose agar

Author Contribution Bilge Hilal CADIRCI established the hypothesis of the research, wrote and edit the manuscript. Gulesme Yilmaz did the experiments and prepared almost most of the figures.

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