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Comparison of in vitro Antifungal Activity Methods Using Aeromonas sp. BHC02 Chitinase, Whose Physicochemical Properties were Determined as Antifungal Agent Candidate

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Abstract

Biological control to prevent fungal plant diseases offers and alternative approach to faciliate sustainable agriculture. Since inhibition of chitinolytic fungal cell walls synthesis is a target for antifungal agents, chitinases are one of the biocontrol agents. This study, it was aimed to investigate isolating a new bacterium from fluvial soil as a chitinase source and the antifungal activity of the characterized chitinase. During planning the in vitro antifungal activity, three common methods were preferred and compared. The bacterium with the highest chitinase activity was identified as *Aeromanas caviae* by 16S 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. It was determined that the partially purified chitinase showed antifungal activity against *Alternaria alternata, Fusarium solani, Botrytis cinerea, Penicillium* sp. 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.

1 Introduction

It is known that more than 8000 fungal species cause plant diseases. 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 [1]. 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 or their products like chitinases, glucanases which could degrade fungal cell-wall components.

Chitin, the most abundant aminopolysaccharide polymer occurring in nature, is a highly insoluble β-(1,4) bonded polymer composed of GlcNAc. 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. [2]. 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. 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 [3].

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. But there are a lot of methodology to measure in vitro antifungal activity.

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 [4].

Thakur et al., [5] purify and characterize extracellular chitinase from *B. cereus* NK91 and then to test the partially purified enzyme for its antifungal activity against some phytopathogens. They add different concentrations of partially purified chitinase into warm molten PDA and after solidified, 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 assay was also determined in antifungal activity. In the study of Ekundayo et al. [6], 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. Delfini et al. [1] 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 crude chitin 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, a comparison was carried out in three antifungal activity methods. The antifungal effects of 1) using chitin producing bacteria, 2) covering the plate by enzyme and 3) after a visible mycelial growth were compared. As a result, with the first method, no antagonistic activity was detected. But while *Penicillum* was inhibited with the second method, *Fusarium solani*, *Alternaria alternata* and *Botrytis cinerea* were inhibited by the third method.

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 Yeşilırmak River, Tokat was added in 90 ml of water and shaken vigorously at 30°C for 1 hour. One ml of the supernatant of the sample was inoculated into 100 ml of LB medium in 500 mL erlenmayer and was incubated at 37 ºC for 24 hours at 200 rpm. After the decimal dilutions, CHDA media ((g/L): Na2HPO4 (0.65), NaCl (0.25), KH2PO4 (1.5), NH4Cl (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 [7]. The plates were incubated at 37 C for 36 hours, and the colonies showing clear halos were selected.

Colonies formed 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 CHDA media at 37 ºC for 48 hours and continued with the organism showing maximum halo-zone. Isolates were stored as a glycerol stock at -80 C. Among these strains, one exhibited high 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 27F 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 identify the closest phylotypes, their sequences were aligned in MEGA 10 program and the phylogenetic tree was constructed with Neighbour joining method [8, 9].

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. [10]. Briefly, 100 µl of the culture supernatant and 900 µl of substrate (0.05 M pH 7.0 phosphate buffer containing 1% colloidal chitin) was mixed and incubated at 35 ^{o}C for 60 minutes. Following the centrifugation at 10000 rpm for 10 minutes, 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 minute [11]. 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 crude enzyme extracts were obtained by inoculating 1% of 18-hours activated culture into 200 ml media containing colloidal chitin and incubated at 30 ^{o}C for 30 h at 200 rpm. The cell pellet was removed by centrifugation at 6000 rpm for 20 minutes. Proteins in the culture supernatant was concentrated by 20–60% of ammonium sulfate precipitation in + 4 C for 8 hours. After each gradient, proteins in the supernatant were obtained by centrifugation at 10000 rpm at + 4 ^{o}C for 10 minutes. Desalting of the protein fractions were performed for 1 night in a phosphate buffer of pH 7.0 and 0.05 M at + 4 ^{o}C by a

dialysis membrane with a cut-off value of 10000 Da [12]. 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 ^{o}C for following physico-chemical characterizations.

The partially purified chitinase fractions were analyzed by SDS-PAGE on a 12% (v/v) polyacrylamide gel [13]. Following the electrophoresis, the gel was stained with Coomassie Brilliant Blue R- 250 for 1 hour, and then the gel image was obtained after destaining overnight [14].

Protein concentrations were measured by Bradford method [15]. 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 minutes at room temperature, absorbance was measured at 595 nm in a UV spectrophotometer. Bovine serum albumin (BSA) was used as a standard [16].

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 0.05 M pH 7.0 phosphate buffer at 20, 30, 40, 50, 60^{o}C, 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 in colloidal chitin and crystal chitin, starch, and cellulose as substrates. The substrates were prepared at a rate of 1% and dissolved in 0.05M phosphate buffer (pH 7.0). After 60 minutes of incubation, reductive 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). Km and Vmax constants were calculated by using the slope of the Lineweaver-Burke graph in the Michaelis Menten equation [17].

2.8 Detection of Antifungal Activity

The fungal pathogens *Aspergillus niger, Fusarium solani, Alternaria alternate, Botrytis cinerea, Phytophthora sp., Sclerotinia sp., Penicillium sp.* were cultivated in PDA medium [18].

2.8.1 Antifungal study of chitinase producing bacteria on fungal phytopathogens.

To investigate the effect of chitinase-producing microorganism on fungal pathogens; Test fungi were inoculated on one end of the petri dishes containing PDA medium by spot cultivation. After 48 hours of incubation at 30 C, sufficient micelle formation was observed, and chitinase-producing bacteria were cultivated in the other part of the petri dish. Incubation was then continued for another 24 hours. Subsequent zones were observed.

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 2 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.

2.8.2.1 In the first method [19]; 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 7 days.
2.8.2.2 As a second method [20], test fungi were cultivated on one end of the petri dish containing PDA medium. After incubation at 30 ^{o}C for 48 hours, 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 for 5 days were observed.

3 Results

3.1 Detection and Identification of Chitinase Producing Microorganisms on CHDA

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 CHDA medium petri dishes incubated at 30 C for 36 hours [21]. 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 CHDA medium. Colony 4, 7 and 10 formed the large zone diameters but the colony 10 formed the largest one (**Fig. 1**).

Chitinase production time of the microorganism was determined by 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 [22], it was observed that the isolated colony was gram negative. After analysis of the homology of the 16srRNA gene sequence by BLAST algorithm, the isolated strain has maximum homology (99%) with *Aeromonas* sp. In the phylogenetic tree (**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. In order to avoid any confusion, our isolate was identified as *Aeromonas sp.* BHC02.

3.2 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 of the 12% run gel was determined by SDS of 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 ~25kDa, ~37kDa, ~110 kDa were determined.

In **Fig. 5**, the activity of the chitinase enzyme at pH 7.0 in different temperature ranges is shown graphically. It has been observed that the optimum temperature value is 30 ^{o}C. It was determined that the enzyme retained 85% of its activity when stored in the refrigerator for 2 months (data not shown).

In the study carried out to find the optimum pH range of the substrate 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 over a wide pH range (**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. But it was also showed that, BHC02 chitinase can hydrolyze crystalline chitin 57.9 % in relative activity to colloidal chitin.

The kinetics of BH02 chitinase with colloidal chitin substrate was also investigated. The accuracy of the Lineweaver-Burk Graph was R^2 = 0.9894 with the equation y=0.8332x+0.3395. The calculations from the slope of the graph in the Michaelis Menten equation, Km = 2.45 mM and Vmax = 2.94 mM/min were found [23].

3.3 Antifungal Activity Tests

The results of the total experiments are given in **Table 2.** 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 2 different ways. In the study in which the surface of the PDA medium was coated with enzyme, it was observed in the end of 7 days of incubation, the enzyme did not cause any inhibition

on the growth of other fungi except *Penicillium sp*. The result of the study 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* fungi as seen in the F**ig 6.**

Discussion

In this study, it was aimed to investigate presence of the chitinase producing bacteria in the microbial flora of a river Yesilirmak in Tokat, as their potential use as 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 16S rRNA analysis as *A. caviae* and the stain is called *Aeromonas* sp. BHC02.

Aeromonas genus found very common 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 [31]. *A. caviae* CB101 was isolated as an efficient degrader with four chitinases of different molecular weights into the cultures supernatant. Mehmood et al. study the Chi1 chitinase and according to their results, *A. caviae* CB101 produce 0.68 U/mg protein. [32].

Inbar et al. [33], in their study with the chitinase of *Aeromonas caviae,* got three bands in SDS PAGE analysis; one strong band with a molecular weight of 80 kDa and two weak bands with a molecular weight of 48 and 59 kDa. In our study, even just a partial purification with gradient precipitation was done, there were three bands of ~25kDa, ~37kDa and ~110 kDa. Mehmood et al. [32] found four enzymes with molecular sizes of 92, 82, 70 and 55 kDa in the culture supernatant in their work with *A. caviae* CB101.

Cardozo et al., [34] demonstrates the ten marine-derived *A. caviae* isolates potential to produce GlcNAc 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., [35] 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 37 C after 24h of incubation with 5946 U/mL and 6072 U/mL, respectively. Stumpf et al. [36] 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 μmol·h^{-1}at 45 C 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 *A. caviae* is around 0.5 U at 30 C in neutral pH with 1% colloidal chitin.

Zhang et al. [37] worked with the chitinase of *Aeromonas veroni* with 30 U activity and found the optimum pH as 5.0 and the optimum temperature as 50 ^{o}C. When they compared their chitinase with the commercial one, it was found that the shrimp shell did not break down the chitin. To increase the yields,

they transfer the chitinase gene of *Aeromonas veroni* to *Pichia pastoris* and obtained high yields. So, it is also possible for *A. caviae* BHC02.

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. But the results showed that, amylase and cellulase activities are very low but chitinase activity on colloidal chitin is the highest one.

Seven fungal species were used in antifungal trials. Chitinase enzyme showed antagonistic effect on 4 of them. No antifungal activity was detected with *A. caviae* BHC02 cells. During the planning the method, it was expected the *Aeromonas* cells will grow faster and to produce chitinase with the induction effect of growing fungal mycelium. But the results showed the opposite. The reason is probably the growth of the mycelium didn't overlap the active *Aeromonas* cells. Especially when considering of PDA media is not very suitable for bacteria, this explanation is meaningful.

While investigating the effect of chitinase enzyme on fungi in antifungal studies, it was observed that none of the method we tried with *A. caviae* 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. [4] 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*. This enzyme exhibited antifungal activity against *R. solani*, *Bipolaris* sp, *A. raphani*, *A. brassicicola*, But not to *Sclerotinia sp.*like our results.

The investigations of Delfini et al. [1] resulted that, fungi *Alternaria* and *Colletotrichum acutatum* were the most affected by all the cell-free supernatants, whereas fungi of the genus *Fusarium* were the most resistant.

Culture filtrate of *Streptomyces albus* was able to inhibit the growth of all the selected pathogenic fungi namely *Magnaporthe oryzae*, *Fusarium graminearum*, *Rhizoctonia solani*, *Puccinia* species and *Botyrtis cinerea* whereas *Streptomyces rimosus* has the least inhibitory activity [6].

Thakur et al. [5] also showed that, after incubation together, antifungal activity of *B. cereus* strain NK91 chitinase was determined against *F. oxysporum*, *R. solani*, and *C. gloeosporioides*.

All the results of those studies conclude that, because of the differences of the chemical structures of the fungal chitins, chitinolytic activities also varies as a result of the subsite structure in the enzyme binding cleft. This is the reason why not all the fungi affected by the enzyme.

One of our results is, dependent on the method, the growth of different fungal strain is inhibited. By spreading the enzyme onto the petri dish method, the enzyme did not cause any inhibition on the growth of other fungi except *Penicillium* sp. But as a result of the study 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*. Even Delfini et al. [1] concluded that *Fusarium* genus was the most resistant phytopathogen fungi, but our results by using active mycelium of the cell method, it is possible to inhibit it.

In a study, researchers stated that chitinase from *B. subtilis* has antifungal activity against plant pathogens, *A. niger, A. flavus* and *P. chrysogenum*. Purified chitinase from *Streptomyces sp*. showed antifungal activity against the cell wall of *Botrytis cinerea*, a fungus that affects many plant species. Purified chitinase from *B. cereus* was found to have inhibitory activity on the germination of *B. elliptica*, the major fungal pathogen of lily leaf blight. Extracellular chitinases from the supernatant of *B. pumilus* strain showed antifungal activity against plant pathogens such as *Rhizoctonia solani, Verticillium sp., Nigrospora sp., Stemphyllium botryosum* [39].

In 2013, Okay et al. determined the antifungal activities of chitinase against *Fusarium graminearum* and *Rhizoctonia solani* phytopathogens. They found the optimum temperature of their enzymes at 60C and pH of 7.0, and the molecular weight of the enzyme 42 kDa [40]. Bhushan et al. (1998) compared *Bacillus sp* chitinase with commonly used fungicides and pesticides. They found that chitinase can increase the activity of insecticides and also minimize the concentrations of essential chemicals [41].

All those results can be extended. But our results 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.

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 [40].

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 [41]. 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 [40]. 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
CHDA	Chitinase-detection agar
NCBI	National Centre for Biotechnology Information
DNS	3,5-Dinitrosalicylic acid
BSA	Bovine serum albumin
Vmax	Maximum rate of reaction
Km	Michaelis Constant
PCR	Polymerase chain reaction
PDA	Potato dextrose agar

References

1. Delfini CD, Villegas LB, Martínez MA, Baigorí MD (2021) Extracellular Antifungal Activity of Chitinase-Producing Bacteria Isolated from Guano of Insectivorous Bats. Curr Microbiol 78:2787–2798
2. Elieh A, Kom D, Sharma L, Dela Cruz CS (2018) Chitin and Its Effects on Inflammatory and Immune Responses. Clin Rev Allerg Immunol 54:213–223
3. Basu D, Chaudhuri A (2013) Purification and Characterization of chitinase from Thermophilic *Staphylococcu*s sp. Internatıonal J Envıronmental Scıences 4:4
4. Zarei M, Aminzadeh S, Zolgharnein H, Safahieh A, Daliri M, Noghabi KA, Ghoroghi A, Motallebi A (2011) Characterization of a chitinase with antifungal activity from a native *Serratia marcescens* B4A. Braz J Microbiol 42(3):1017–1029
5. Thakur N, Nath AK, Chauhan A, Gupta R (2021) Purification, characterization, and antifungal activity of *Bacillus cereus* strain NK91 chitinase from rhizospheric soil samples of Himachal Pradesh, India. Biotechnol. Appl. Biochem

6. Ekundayo FO, Folorunsho AE, Ibisanmi TA (2022) Antifungal activity of chitinase produced by Streptomyces species isolated from grassland soils in Futa Area, Akure. Bull Natl Res Cent 46:95
7. Hueso-Rodríguez JA, Jimeno ML, Rodríguez B, Savona G, Bruno M (1983) Abietane diterpenoids from the root of *Salvia phlomoides*. Phytochemistry 22(9):2005–2009
8. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28(10):2731–2739
9. Meena S, Gothwal RK, Saxena JM, Mohan K, Ghosh P (2014) Chitinase production by a newly isolated thermotolerant Paenibacillus sp. BISR-047. Ann Microbiol 64:787–797
10. Toharisman A, Suhartono MT, Spindler-Barth M, Hwang JK, Pyun YR (2005) Purification and characterization of a thermostable chitinase from *Bacillus licheniformis* Mb-2. World J Microbiol Biotechnol 21:733–738
11. Wang D, Li A, Han H, Liu T, Yang Q (2018) A potent chitinase from *Bacillus subtilis* for the efficient bioconversion of chitin-containing wastes. Int J Biol Macromol 116:863–868
12. Wang D, Shih L, Liang W, Wangn CH (2002) Purification and Characterization of Two Antifungal Chitinases Extracellularly Produced by *Bacillus amyloliquefaciens* V656
13. Stoscheck CM (1990) Quantitation of protein. Methods Enzymol 182:50–68
14. Smith BJ (1994) SDS polyacrylamide gel electrophoresis of proteins. Basic protein and peptide protocols 23–34
15. Ernst O, Zor T (2010) Linearization of the Bradford Protein Assay. J. Vis. Exp 38:1918
16. Yuli PE, Suhartono TM, Rukayadi Y, Hwang JK, Pyun YR (2004) Characteristics of thermostable chitinase enzymes from the indonesian *Bacillus* sp. Enzym Microb Technol 35:147–115
17. Choi B, Rempala GA, Kim JK (2017) Beyond the Michaelis-Menten equation: Accurate and efficient estimation of enzyme kinetic parameters. Sci Rep 7(1):1–11
18. Zarei M, Aminzadeh S, Zolgharnein H, Safahieh A, Noghabi K, Ghoroghi A (2011) Characterization of a chitinase with antifungal activity from a native *Serratia marcescens* B4A. J Microbiol 42:1017–1029
19. Vaidya RJ, Shah IM, Vyas PR, Chhatpar HS (2001) Production of chitinase and its optimization from a novel isolate *Alcaligenes xylosoxydans*: potential in antifungal biocontrol. World J Microbiol Biotechnol 17:691–696
20. Kirubakaran SI, Sakthivel N (2007) Cloning and overexpression of antifungal barley chitinase gene in *Escherichia coli*. Protein Exp Purific 52:159–166
21. Sarker KN, Das Mohapatra PK, Dutta S (2019) Use of low cost natural resources for enhanced chitinase production and optimization using CCD and RSM: A new initiative for bio-control of plant pathogen. Indian Phytopathol 72(2):281–300
22. Smith AC, Hussey M (2005) Gram stain protocols. Am Soc Microbiol 1:14

23. English BP, Min W, Van OAM, Lee KT, Luo G, Sun H, Xie XS (2006) Ever-fluctuating single enzyme molecules: Michaelis-Menten equation revisited. Nat Chem Biol 2(2):87–94
24. Vijayanandraj VR, Nagendra Prasad D, Mohan N, Gunasekaran M (2006) Effect of ozone on Aspergillus niger causing black rot disease in onion. Ozone: Sci Eng 28(5):347–350
25. Karima HEH, Nadia GE (2012) In vitro study on *Fusarium solani* and *Rhizoctonia solani* isolates causing the damping off and root rot diseases in tomatoes. Nat Sci 10(11):16–25
26. Zhu L, Ni W, Liu S, Cai B, Xing H, Wang S (2017) Transcriptomics analysis of apple leaves in response to *Alternaria alternata* apple pathotype infection. Front Plant Sci 8:22
27. Petrasch S, Knapp SJ, Van Kan JA, Blanco-Ulate B (2019) Grey mould of strawberry, a devastating disease caused by the ubiquitous necrotrophic fungal pathogen Botrytis cinerea. Mol Plant Pathol 20(6):877–892
28. Latorre BA, Rioja ME, Wilcox WF (2001) Phytophthora species associated with crown and root rot of apple in Chile. Plant Dis 85(6):603–606
29. Bajpai A, Agnihotri R, Prakash A, Johri BN (2022) Biosurfactant from Bacillus sp. A5F Reduces Disease Incidence of *Sclerotinia sclerotiorum* in Soybean Crop. Curr Microbiol 79(7):1–11
30. Donegan KK, Schaller DL, Stone JK, Ganio LM, Reed G, Hamm PB, Seidler RJ (1996) Microbial populations, fungal species diversity and plant pathogen levels in field plots of potato plants expressing the Bacillus thuringiensis var. tenebrionis endotoxin. Transgenic Res 5(1):25–35
31. Lim YL, Ee R, Yin WF, Chan KG (2014) Quorum sensing activity of Aeromonas caviae strain YL12, a bacterium isolated from compost. Sensors 14(4):7026–7040
32. Mehmood MA, Gai Y, Zhuang Q, Wang F, Xiao X, Wang F (2010) *Aeromonas caviae* CB101 contains four chitinases encoded by a single gene chi1. Mol Biotechnol 44(3):213–220
33. Inbar J, Chet I (1991) Evidence that chitinase produced by *Aeromonas caviae* is involved in the biological control of soil-borne plant pathogens by this bacterium. Soil Biol Biochem 23:973–978
34. Cardozo FA, Gonzalez JM, Feitosa VA, Pessoa A, Rivera ING (2017) Bioconversion of α-chitin into N-acetyl-glucosamine using chitinases produced by marine-derived *Aeromonas caviae* isolates. World J Microbiol Biotechnol 33(11):1–11
35. Saima MK, Roohi IZA (2013) Isolation of novel chitinolytic bacteria and production optimization of extracellular chitinase. J Genetic Eng Biotechnol 11(1):39–46
36. Stumpf AK, Vortmann M, Dirks-Hofmeister ME, Moerschbacher BM, Philipp B (2019) Identification of a novel chitinase from *Aeromonas hydrophila* AH-1N for the degradation of chitin within fungal mycelium. FEMS Microbiol Lett 366(1):fny294
37. Zhang Y, Zhou Z, Liu Y, Cao Y, He S, Huo F, Qin C, Yao B, Ringo E (2014) High-yield production of a chitinase from *Aeromonas veronii* B565 as a potential feed supplement for warm-water aquaculture. Appl Microbiol Biotechnology 98:1651–1662
38. Watanabe T, Ito Y, YamadaT, Hashımoto M, Sekıne S, Tanak H (1994) The Roles of the C-Terminal Domain and Type III Domains of Chitinase Al from Bacillus circulans WL-12 in Chitin Degradation. J

Bacteriol 176:4465–4472

39. Sandhu JS, Sidhu MK, Yadav IS (2017) Control of fungal diseases in agricultural crops by chitinase and glucanase transgenes. In Sustainable agriculture reviews (pp. 163–212)
40. Okay S, Ozdal M, Kurbanoglu EB (2013) Characterization, antifungal activity, and cell immobilization of a chitinase from Serratia marcescens MO-1. Turkish J Biology 37:639–644
41. Bhushan B (1998) Isolation, purification, characterization and scale up production of a thermostable chitinase from an alkalophilic microorganism. Ph.D. thesis, Department of Microbiology Chandigarh: Punjab University
42. Zhang W, Ma J, Yan Q, Jiang Z, Yang S (2021) Biochemical characterization of a novel acidic chitinase with antifungal activity from Paenibacillus xylanexedens Z2–4. Int J Biol Macromol 182:1528–1536

Tables

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
(NH4)2SO4 precipitation (60%)	0.18	0.075	2,4	1.3	38.04

Table 2 Effect of partially purified chitinase enzyme on fungi

Pathogen	Pathogen for	Result	Working Antifungal Activity Method
Aspergillus niger	Onion[24]	No Zone	None
Fusarium solani	Tomatoes[25]	Zone formation	After mycelial formation occurs
Alternaria alternata	Apple[26]	Zone formation	After mycelial formation occurs
Botrytis cinerea	Strawberry[27]	Zone formation	After mycelial formation occurs
Phytophthora sp.	Apple[28]	No zone	None
Sclerotinia sp.	Soybean[29]	No zone	None
Penicillium sp.	Potatoes[30]	No growth	Enzyme spread onto PDA plates

Figures

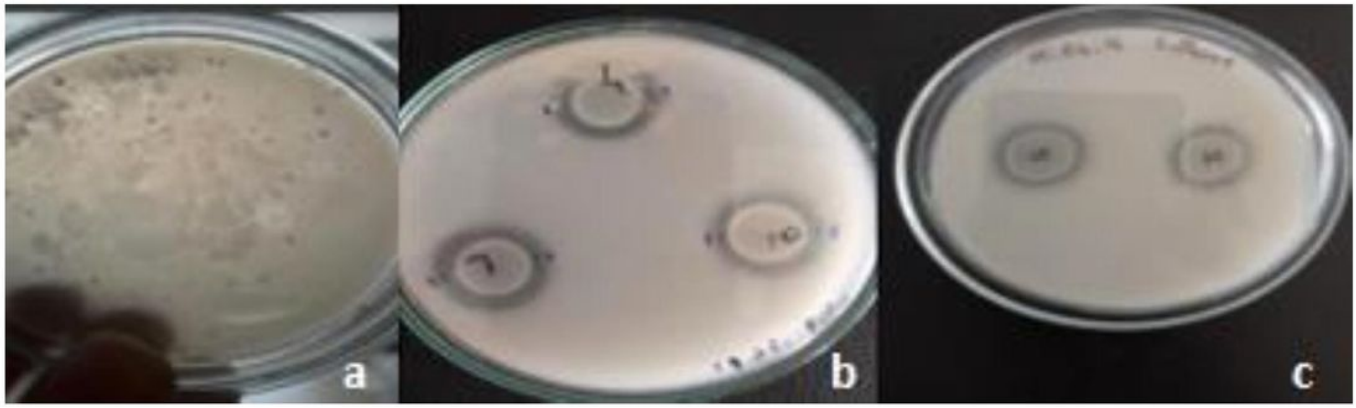


Fig. 1 (a), Selection of chitinase producing organisms from zones on CHDA agar (b), Zones formed by 3 chitinase producing organisms on CHDA agar (c), The zone of chitinase producing organism number 10 which forms the largest zone on CHDA agar.

Figure 1

See image above for figure legend.

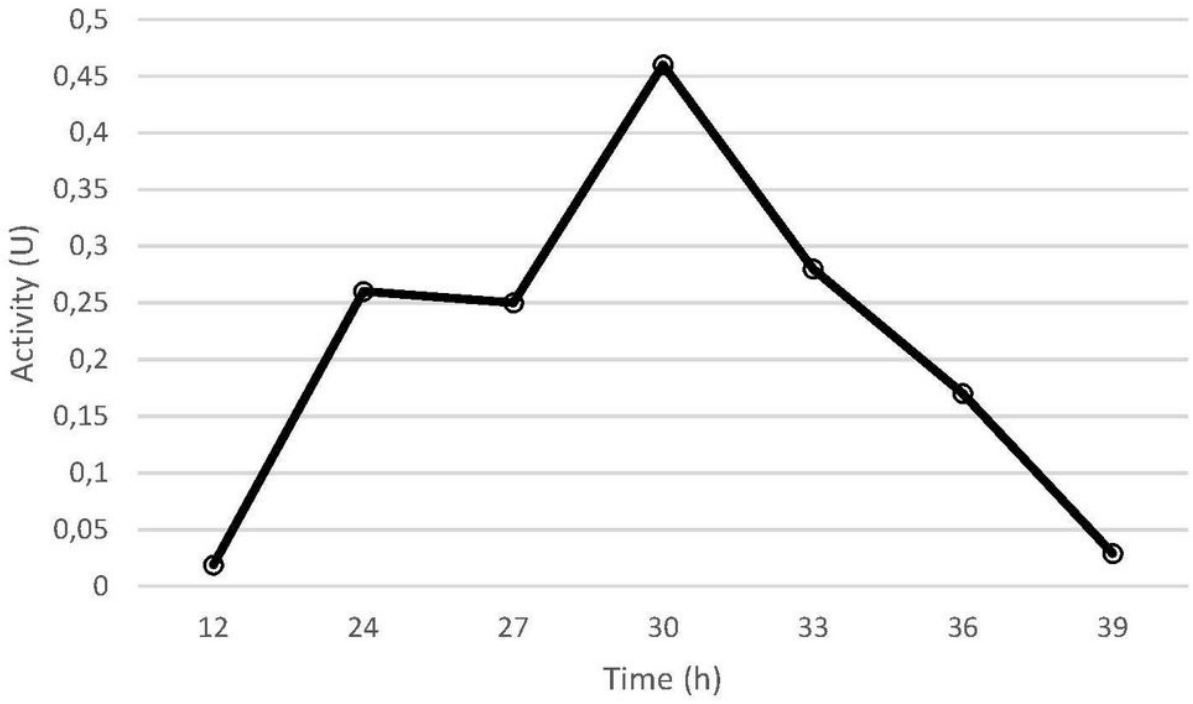


Fig.2. Time course of enzyme production

Figure 2

See image above for figure legend.

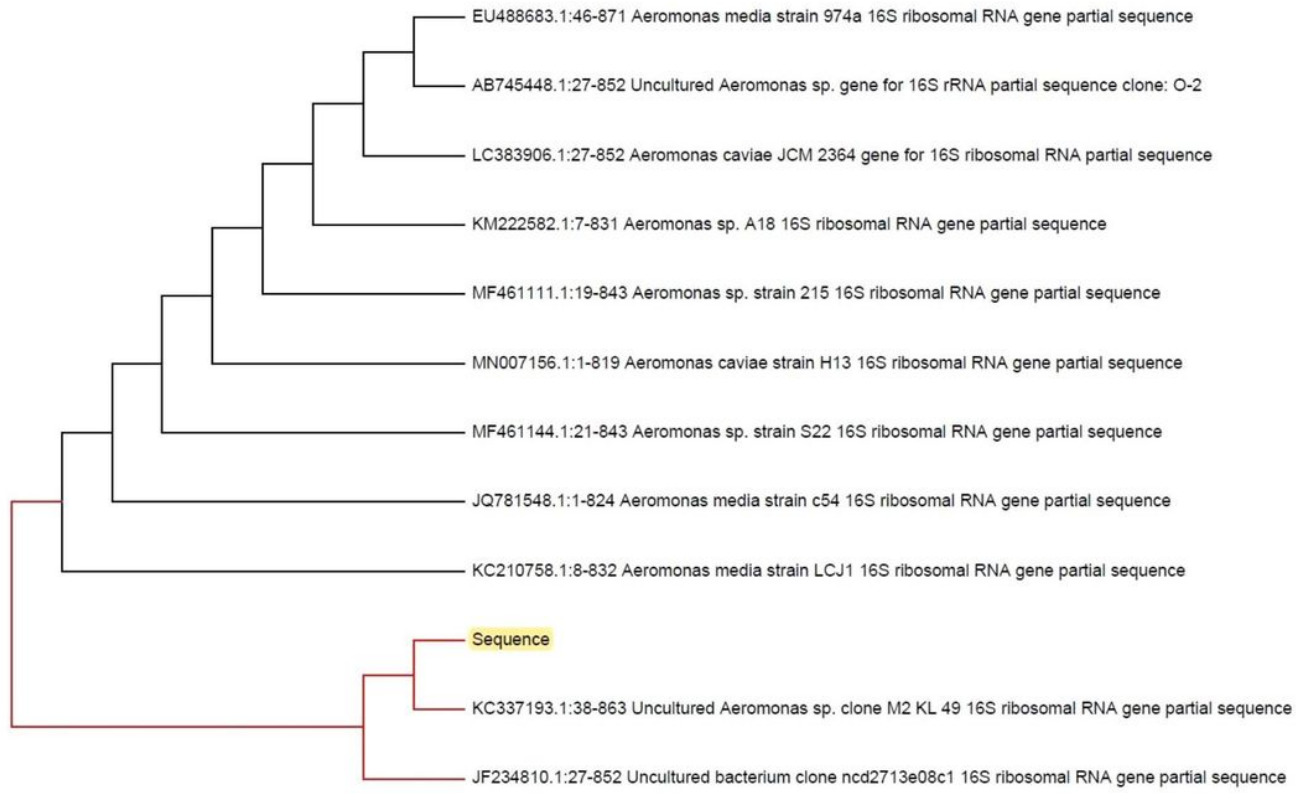


Fig. 3 Phylogenetic tree of Aeromonas sp. BHC02.

Figure 3

See image above for figure legend.

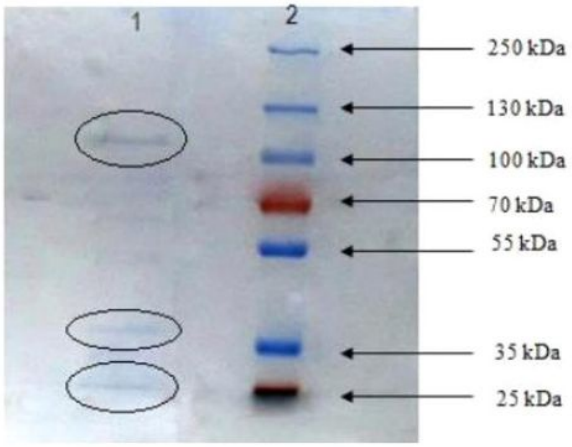


Fig. 4 SDS-PAGE image of partially purified chitinase 1.Lane: After 60% ammonium sulfate precipitation 2. Lane: Marker

Figure 4

See image above for figure legend.

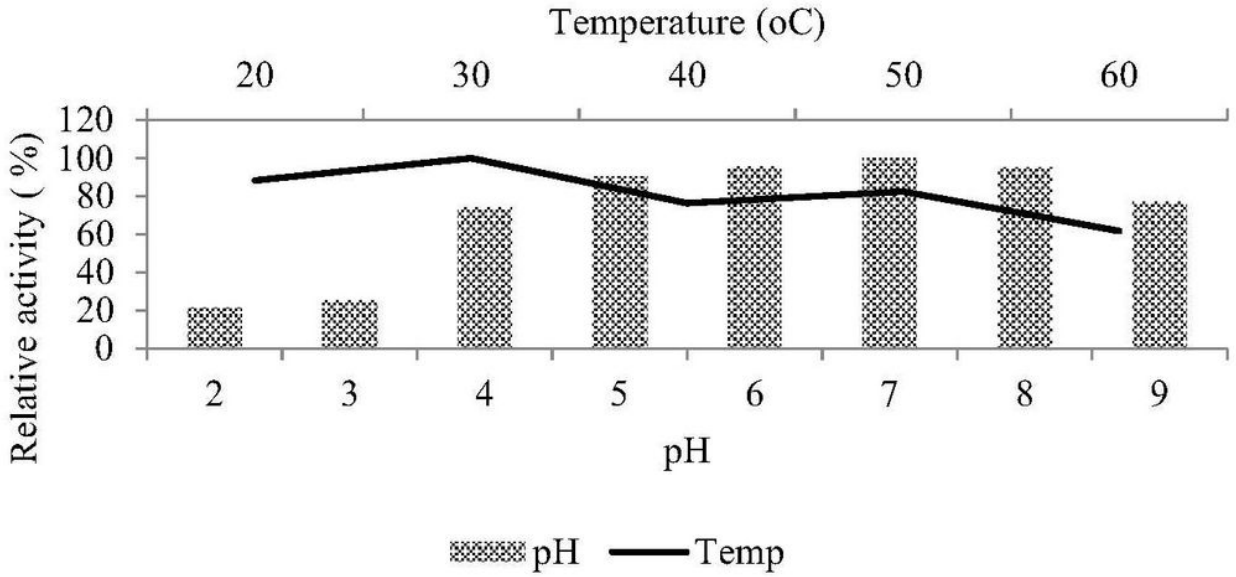


Fig. 5 Optimum temperature and pH of chitinase

Figure 5

See image above for figure legend.

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

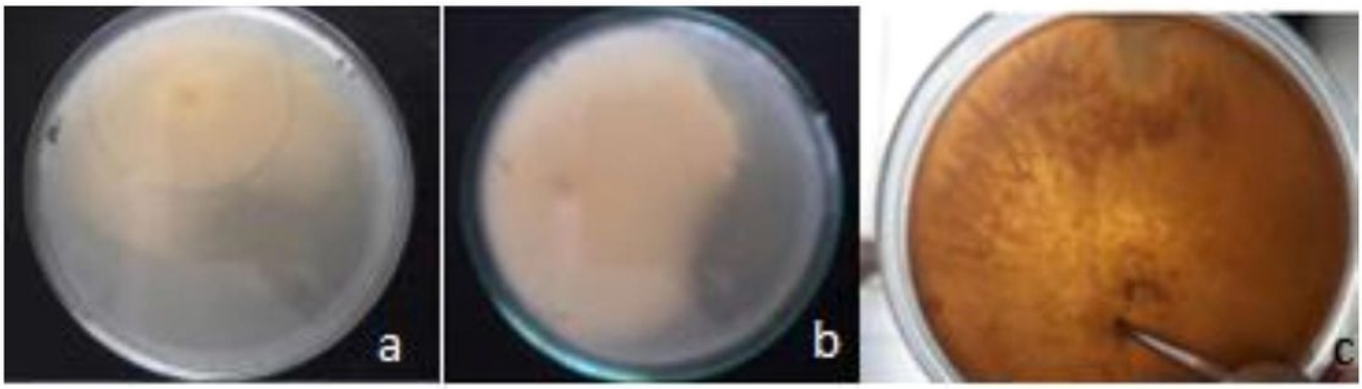


Figure 6

See image above for figure legend.