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**Research Article**

# Characterization of chitinolytic bacterial strains isolated from local habitat

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## Abstract

Chitinase enzymes have their prospective applications in agro-chemical industry, and the microbes capable of producing chitinases are being used effectively against number of soil-borne fungal pathogens and nematodes. Three potential bacterial strains were isolated from local environment to develop indigenous resources for the production of chitinases or their use as bio-control agents against plant pathogens. Following conventional biochemical tests and microscopy, these strains were preliminary identified as *Serratia* sp. CN-01, *Serratia* sp. CN-07, and *Pseudomonas* sp. CN-05. Chitinolytic activity was determined by incubating these strains on chitin agar plates, which showed the halo of clearing zones of average diameter of 18–23 mm. Growth conditions were characterized as increase in optical density, and fluctuation in pH of growing culture at 28 $^{o}$C and 150 rpm for 72 h in a shaking incubator. Antifungal activity of these isolates was determined against the fungal pathogen *Fusarium oxysporum*, which showed the percentage inhibition ranging from 48% to 73% after seven days of incubation and the radii of inhibition zones varied from 11.0 to 14.7 mm. The results indicated that these bacterial isolates can be used as chitinase producing bio-agents in industry, as well as have potential to be used as effective anti-fungal agents in the field.

**Key Words:** Anti-fungal; bacteria; chitinase; indigenous resource; screening.

## Introduction

Bacteria produce many secondary metabolites such as enzymes, pigments, antibiotics which could be of importance to mankind in many ways (Giri *et al.,* 2004; Van Pee, 2006). Chitinase enzymes are secondary metabolites produced by a number of naturally occurring microbes. Chitin and its derivatives are being used in pharmaceutical and agro-chemical industry extensively. In recent years, the demand for chitinase enzymes with new or desirable properties has increased due to industrial application of chitin, chitosan and chitooligosaccharides. Bacterial chitinases are widely distributed in nature and play an important role in the degradation of chitin (Inbar and Chet, 1991; Zhang and Yuen, 2000).

Chitinolytic bacterial strains are also used in biological control of plant diseases (Minkwitz and Berg, 2001). The suppression of soil-borne and air-borne diseases is due to synergism of chitinases, antibiotics and induction of systemic resistance to the plant. Chitinase have received increased attention due to their potential application in biocontrol of other chitin containing phytopathogenic organisms such as insects and phytopathogenic fungus (Souza *et al*., 2003; Santos *et al*., 2004; Santos *et al*., 2004; Kishore *et al.,* 2005; Chen *et al.,* 2009). In addition to the potential applications as inhibitors and biopesticides, chitinases have been used for the production of single cell protein for animal and aquaculture feed, for the isolation of fungal protoplasts, preparation of bioactive chito-oligosaccharides, and for the development of chemical defense proteins in transgenic plants (Patil *et al.*, 2000; Sharma *et al*., 2011).

Chitinase-producing bacteria are isolated from a variety of natural sources such as soil and aquatic habitats. Such strains in general are found in soil, on plants, on insects and in water systems, as well as in other places where chitin is present. Chitinolytic bacteria can also be isolated from the rhizosphere (the area of the soil that surrounds and is influenced by the plant roots) of a wide variety of plants including sugar beet, cotton, bean or carnation etc. Chitinase producing bacteria can also be obtained from root surfaces, fungal resting structures (sclerotia, chlamydospores), nematode egg masses, insect or arthropod exoskeletons and

irrigation water. However, in Pakistan, most of the chitinase producing strains are provided by culture collection centres at a very high cost. Since chitinase producing bacterial strains can be obtained by known techniques due to their widespread distribution in nature. It is, therefore, need of the hour to exploit our own resources for screening of indigenous bacterial strains in order to meet national needs. This study was planned to investigate the potential of local bacterial isolates for the production of chitinase enzyme, and their antifungal activity.

## Materials and Methods

**Culture media**

Nutrient broth (NB) and nutrient agar (NA) media were used for isolation and maintenance of the bacterial isolates. Potato dextrose broth (PDB) and potato dextrose agar (PDA) were used for the cultivation of fungi.

Chitinase production medium "Chitin broth (CB)" composed of (g $L^{-1}$ of distilled water) $Na_2HPO_4$, 0.65; $KH_2PO$, 1.5; NaCl, 0.25; $NH_4Cl$, 0.5; $MgSO_4$, 0.12; $CaCl_2$, 0.005; colloidal chitin, 10 and pH 6.0. "chitin agar (CA)" medium was developed by adding 10 g $L^{-1}$ agar in the chitin broth medium.

All of the media were autoclaved at 121 °C and 15 lbs pressure for 15-20 minutes.

**Isolation and identification of bacterial strains**

Bacterial strains were isolated locally from rhizoplane of a rice field following standard procedures using spread plate technique on nutrient agar plates. Morphologically distinct colonies were sub-cultured in nutrient broth, and purified to single species level using streak plating repeatedly on nutrient agar plates. Pure isolates were maintained by sub-culturing on nutrient agar slants and stored at 4 °C. Overnight grown cultures (24 h, 28-30 °C) were made in fresh medium before use in the experiment.

The colony morphology and microscopic charafercters for the identification of isolates were examined under a light microscope. Biochemical tests for the identification were carried out according to the Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994).

**Screening of bacterial strains for chitinolytic activity**

Plate and shake flask assays were used for screening of bacterial strains for chitinolytic activity. In plate assay chitin agar medium was used to determine the chitinase production. Freshly grown bacterial culture was spot inoculated at equidistant points on a colloidal chitin agar plate, followed by incubation in an incubator (VWR Scientific) at 30 °C for 2-3 days. Plates were then observed for zone of hydrolysis around the inoculated area. The experiment was conducted in triplicates. The chitinolytic activity of the bacterial strains were scored by their ability to produce a halo of clearing zone on colloidal chitin plate The bacterial strains found to produce chitinase were selected as chitinolyic bacteria because of the usage of chitin as a substrate. These chitinolytic bacterial strains were further evaluated for chitinases production in chitin broth by shake flask assay.

In shake flask assay, growth and production of chitinase in bacterial strains was studied in chitin broth. Approximately 100 mL of CB was taken in 250 mL Erlenmeyer flask and 0.5% (v/v) of fresh inoculum was added to medium and incubated in a shaker (Lab-Line incubator-shaker) at 28 $^{o}$C and 150 rpm for 72 h. Optimum conditions for cultivation such as pH and optical density (OD) were investigated. From the zero hour of inoculation, 3 mL sample was drawn from each growing culture at the interval of every two hours for recording the pH and OD. OD of each sample was measured with a spectrophotometer (CECIL CE 2041) in the range of 550 to 600 nm. OD and pH of the blank (control) were also taken and compared with the cultured ones. The experiment was conducted in triplicate.

**Detection of Antifungal Activity**

Three potential chitinolytic strains were isolated and tested for antifungal activity against fungal pathogens by dual culture technique (Rabindran and Vidyasekaran, 1996). A loopful of overnight culture of bacterial strains was streaked at one side of PDA and CA plates (1 cm away from the edge). A 5 mm mycelial disc from seven days old PDA culture of fungal pathogen was placed on the opposite side in the PDA and CA plates perpendicular to the bacterial streak and plates were incubated at room temperature (28-30 °C) for 3-7 days. At the end of incubation period, the zone of inhibition was recorded by measuring the distance between the edges of the fungal mycelium and the antagonistic bacterium. Plate inoculated with fungus only served as control. Three plates were used as replicates for each experiment. The diameter of the inhibition zone between the bacteria and the fungus was used as an indication of the extent of antagonism. Observations on inhibition zone diameter and

mycelial growth of test pathogens were recorded. The percent inhibition of pathogen growth was calculated as given below.

$$\text{Inhibition (\%)} = \frac{C - T}{C} \times 100$$

where,

C = Growth of pathogen in the control plate (mm)

T = Growth of pathogen in dual cultures (mm).

# Results and Discussion

**Characterization of isolates**

After acclimation and selective enrichment, a total of fifteen bacterial strains with the ability to produce chitinase were obtained. Three of them with best potential of producing chitinase enzyme were selected for further studies. On the basis of distinct morphological and biochemical characteristics, these three strains were identified and designated as *Serratia* sp. CN-01, *Serratia* sp. CN-07 and *Pseudomonas* sp. CN-05. Further characterization of all these strains up to species level using advanced biochemical test kits and sophisticated molecular techniques is foreseen.

**Screening for chitinolytic activity**

Among the 15 isolates, three were found to possess the good chitinolytic activity when grown on solid agar medium with 1% colloidal chitin (Fig. 1). Inoculation of chitinase producing bacteria was made by spotting on chitin agar. These three strains gave clear zones and have the highest chitinase activity. The inhibition zone diameter of these strains ranged from 18-23 mm. Appearance of clear zones indicates the break-down of chitin present within the medium, which is attributed towards the production of chitinase enzymes by these isolates under study. shaker (150 rpm) ranged from OD of 0.021-1.416, 0.044-1.358 and 0.013-1.358, respectively. Gradual increase in OD indicates the growth rate of bacteria, which ultimately shows that these isolates were utilizing chitin as substrate or source of cellular energy. Fig. 2 shows that the difference in initial OD (after 2-(0.093), depicting that these different strains showed different growth rate in the same medium under the same growth conditions, supporting the findings of Santos *et al*. (2011) that *Pseudomonas* sp. have comparatively better growth rate.hours) of *Serratia* sp. CN-01 (0.036), *Serratia* sp. CN-07 (0.042) and *Pseudomonas* sp. CN-05

The comparison of the OD of three bacterial strains grown in chitin broth (CB) medium showed a varied growth pattern (Fig. 2). The minimum OD (0.013) was recorded after 2 hours of incubation which tended to increase till 72 hours (1.488). The chitinolytic activity of *Serratia* sp. CN-01, *Serratia* sp. CN-07 and *Pseudomonas* sp. CN-05, cultured in chitin broth for 72 hon an incubator

The results of comparative study showing the effect of incubation period on the pH of bacterial cultures grown in chitin broth are highlighted in figure 3. The pH of *Serratia* sp. CN-01, *Serratia* sp. CN-07 and *Pseudomonas* sp. CN-05 cultured in chitin broth for 72 h ranged from pH value of 7.64-9.55, 7.40-9.66 and 7.56-9.72, respectively. Fluctuation in pH indicates the production of secondary metabolite i.e. chitinase, as well as the high growth rate of microbes understudy. These attributes are supported by the findings of Harman *et al*. (1993), who reported the purification of chitobiosidase and endochitinase enzymes from *Trichoderma harzianum*.

**Detection of antifungal activity**

*Serratia* sp. CN-01, *Serratia* sp. CN-07 and *Pseudomonas* sp. CN-05 was more effective in inhibiting the growth of fungal pathogens *Fusarium oxysporum* on chitin agar than on PDA (Fig. 4). Clear zones of inhibition were shown in streak method and there were considerable variations in inhibitory activity. Percentage inhibition ranged from 48% to 73% after seven days of incubation and the radii of inhibition zones varied from 11.0 to 14.7 mm. The inhibiting ability of isolates was attributed to the production of chitinases which inhibited the growth of the phytopathogenic fungus *F. oxysporum*.

# Conclusion

Total fifteen bacterial strains were isolated from local environment and screened for their chitinolytic activity. Out of fifteen, three strains showed better capability of producing chitinase enzyme. Two of them were identified as member of genus *Serratia*, while the third one was identified as a *Pseudomonas* species. These strains depicted a good potential of growth and chitinolytic activity both in chitin agar and chitin broth media. The antifungal activity of these strains against a fungal pathogen *F. oxysporum* on potatoes dextrose agar and chiting agar media indicated their potential to be used as an effective bio-control agent. This study will also help the future research to develop indigenous resources for the production of chitinase enzymes and their potential use in industry, as well as in agriculture.

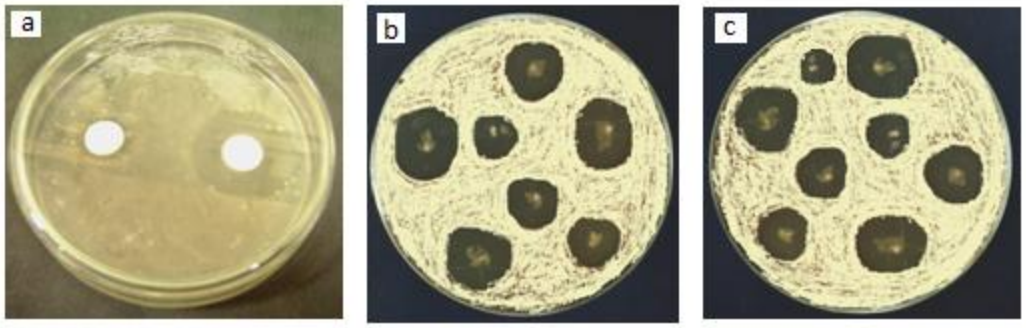


**Fig. 1:** Chitinolytic activity of *Pseudomonas* sp. CN-05 (a), *Serratia* sp. CN-01 (b), and *Serratia* sp. CN-07 (c) on chitin agar.

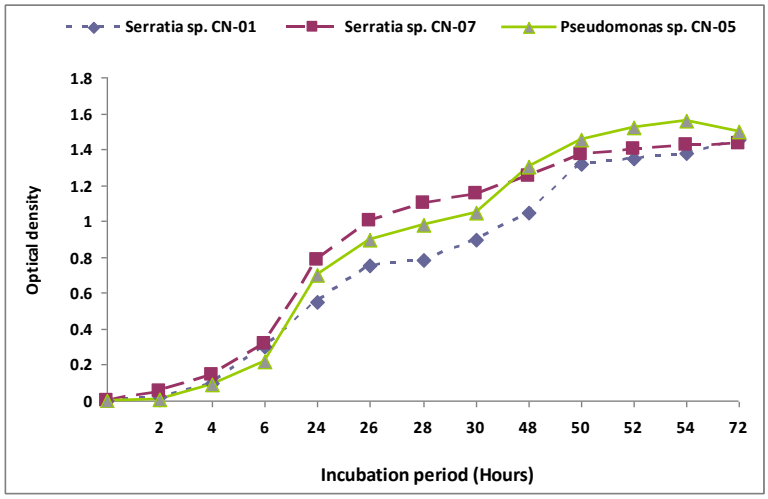


**Fig. 2:** Effect of incubation period on growth (optical density) of bacterial strains in chitin broth.

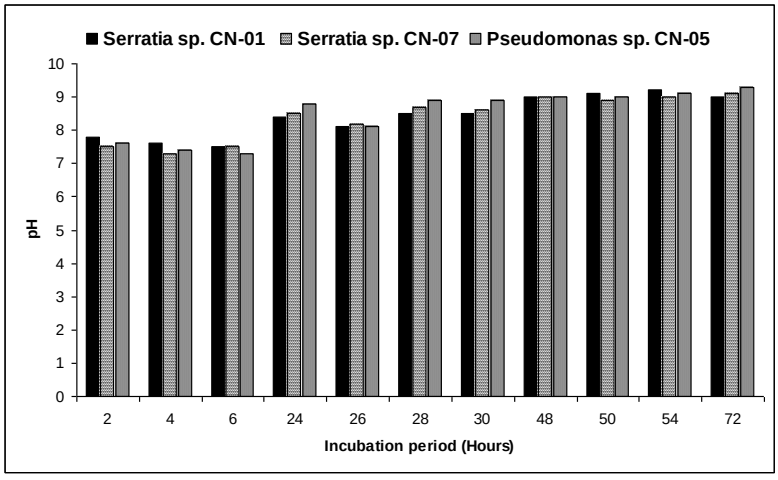


**Fig. 3:** Effect of incubation period on pH of bacterial strains grown in chitin broth.


**Fig. 4:** Antagonistic activity between *Serratia* sp. CN-01 (a), *Serratia* sp. CN-07 (b), *Pseudomonas* sp. CN-05 (c) and fungal pathogen *Fusarium oxysporum* on chitin agar and Potato dextrose agar plates after seven days of incubation

## References


Giri AV, Anandkumar N, Muthukumaran G, Pennathur G, 2004. A novel medium for the enhanced cell growth and production of prodigiosin from *Serratia marcescens* isolated from soil. *Microbiology*, **4**: 11-17.

Inbar J, Chet I, 1991. Evidence that chitinase produced by *Aeromonascaviae* is involved in the biological control of soil-borne plant pathogens by this bacterium. *Soil Biol. Biochem.*, **23**: 973–978.

Minkwitz A, Berg G, 2001. Comparison of antifungal activities and 16S ribosomal DNA sequences of clinical and environmental isolates of *Stenotrophomonas maltophilia*. *J. Clin. Microbiol.*, **39**: 139-145.

Souza C, Lee AM, Bassler BL, Roseman S, 2003. Chitin utilization by marine bacteria: Chemotaxis to chitin oligosaccharides by *Vibrio furnissii. J. Biol. Chem*., **266**: 24268-24275.

Rabindran R, Vidhyasekaran P, 1996. Development of a formulation of *Pseudomonas fluorescens* PfALR2 for management of rice sheath blight. *Crop prot.*, **15**: 715-721.

Harman GE, Hayes CK, lorito M, Broadway RM, Dipietro A, Petetbaur C, Tronsmo A, 1993. Chitinolytic enzymes of *Trichoderma harzianum*: purification of chitobiosidase and endochitinase. *Phytopathology*, **83**: 313-318.

Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST (1994) Bergey's manual of detrerminative bacteriology, 9th edn. Williams and Wilkins, Baltimore

Van Pée KH, Patallo EP, 2006. Flavin-dependent halogenases involved in secondary metabolism in bacteria. *Appl. Microbiol. Biotechnol.*, **70**: 631-641.

Zhang Z, Yuen GY, 2000. The Role of Chitinase Production by *Stenotrophomonas maltophilia* Strain C3 in Biological Control of *Bipolaris sorokiniana*. *Phytopatholgy*, **90**: 384-389.

Chen SC, Liu AR, Wang FH, Ahammed GJ, 2009. Combined over-expression of chitinase and defensin genesin transgenic tomato enhances resistance to *Botrytis cinerea*. *Afr. J. Biotechnol.*, **8**: 5182-5188.

Kishore GK, Pande S, Podile AR, 2005. Biological control of collar rot disease with broad-spectrum antifungal bacteria associated with groundnut. *Can. J. Microbiol.*, **51**: 123-32.

Zhang J, Gratchev A, Riabov V, Mamidi S, Schmuttermaier C, Krusel L, Kremmer E, Workman G, Sage EH, Jalkanen S, Goerdt S, Kzhyshkowska J, 2009. A novel GGA-binding site is required for intracellular sorting mediated by stabilin-1. *Mol. Cell. Biol.*, **29**: 6097-6105.

Santos IS, Cunha MD, Machado OLT, Gomes VM, 2004. A chitinase from *Adenanthera pavonina* L. seeds: purification, characterisation and immunolocali-sation. *Plant Sci.*, **167**:1203-1210.

Santos P, Fortunato A, Ribeiro A, Pawlowski K, 2008. Chitinases in root nodules. *Plant Biotechnol.*, **25**: 299–307.

Patil RS, Ghormadea V, Deshpande MV, 2000. Chitinolytic enzymes: an exploration. *Enzyme Microb. Technol.*, **26**: 473–483.

Sharma N, Sharma KP, Gaur RK, Gupta VK, 2011. Role of chitinase in plant defense. *Asian J. Biochem.*, **6**: 29-37.

Santos SN, Lidiane, Oliveira KX, de Melo IS, Velozo ES, Roque MRA, 2011. Antifungal activity of bacterial strains from the rhizosphere of *Stachytarpheta crassifolia*. *Afr. J. Biotechnol*., **10**: 4996-5000.