Detection of chitinase activity and its characterization from \textit{Pseudomonas fluorescens} of tea rhizosphere

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Rhizosphere soil was used for the isolation of chitin-degrading bacteria that produce chitinase enzyme with pesticidal activity. The use of chitinase from bacterial biocontrol agents instead of spraying whole organism to control insect pests is an attractive field of biotechnological research. Chitin (C\textsubscript{13}H\textsubscript{28}O\textsubscript{5}N\textsubscript{2}O\textsubscript{5})\textsubscript{n}, an insoluble, most abundant polysaccharide is composed of linear chains of \(\beta\text{1,4-N-acetylglucosamine (GlcNAc)}\) residues that are highly cross-linked by hydrogen bonds. Chitin is widely distributed in nature and it is the principle structural component of outer skeleton (50% of the cuticle made up of chitin), foregut, hindgut and midgut lining of peritrophic membrane and essential for structural integrity of many insects, nematodes and most of the fungi (Bhattachrya et al., 2007; Park et al., 2011). Chitinase degrade chitin into its monomeric or oligomeric components, hence, it might be speculated that if applied on to the insect, either it enters the gut of insect larvae causing significant damage to the peritrophic membrane structure, which restricts feeding activity, leading to its death.

Application of bio-pesticides is an alternate pest control approach, reducing environmental pollution and insect resistance developed by the use of inorganic pesticides. The use of selective metabolites like chitinase which are produced by antagonistic microorganisms is advantageous than the use of whole living microorganisms as foliar application (Shternshis et al., 2002). Chitinase act as both contact and systemic molecule to kill the insects (Broadway et al., 1998).

Mendonsa \textit{et al.} (1996) isolated chitinase from \textit{Myrothecium verrucaria} bacterium and studied its activity against \textit{Aedes aegypti} mosquito. The fluorescent pseudomonads are known for active biocontrol agent against many pests and the strain has been reported to control the red spider mite in tea (Roobakkumar et al., 2011). Production of chitinase is considered to be the major antagonistic activity of pseudomonads; hence, utilization of this specific enzyme against pest is very effective and economical than the inorganic chemicals. In the present study, chitin degrading bacterial biocontrol agents were isolated from soils collected from different tea growing regions of south India viz., The Nilgiris, The Anamallais, High Ranges, Central Travancore, Wayanad and Karnataka. Optimization of chitinase production and characterization of the same were studied with \textit{Pseudomonas fluorescens} Meppadi-13 (Pf MP-13) with an intention of inclusion of pest management strategies, in general, and in particular, to control tea mosquito (\textit{Helopeltis theivora} Waterhouse).

A total of 113 bacterial strains were received from Plant Pathology Division, UPASI Tea Research Institute, Valparai which were isolated from the soils collected from different tea growing regions of south India. This study was conducted during summer season of 2012. Each bacterial strain was revived on nutrient agar (NA) medium. All bacterial strains were screened for chitinase activity colorimetrically.
enzyme activity was detected at declining growth phase (Priya et al., 2011). One step purification of chitinase was already reported in *Serratia marcescens* NK1 (Nawani and Kapadnis, 2001). In the present study, chitinase purified from *Pf* MP-13 strain using gel-filtration chromatography (sephacryl CL-250 column) exhibited a molecular weight of 30 kDa on SDS-PAGE (Fig. 2). Its activity was confirmed through chitinase activity assay using chitin as a substrate. Earlier, chitinase isolated and purified from *P. fluorescens* had a molecular weight of 50 kDa (Park et al., 2011). Watanabe et al. (1990) found that *Bacillus circulans* strain secretes five kinds of chitinases and its molecular weight varied from 38 to 60 kDa. Using 96 h old cultures to identify the potential chitinase producer. Among 113 bacterial strains, 6 strains were regarded as low chitinase producers (0.1 to 0.9 U mL⁻¹), 6 strains as moderate producers (5.0 to 7.7 U mL⁻¹) and 6 strains were found to produce higher levels of chitinase (13.7 to 27.4 U mL⁻¹) (Table 1). Among 18 strains, *Pf* MP-13 recorded the highest chitinolytic activity (27.4 U mL⁻¹). Nawani and Kapadnis (2003) reported the presence of a large number of chitin degrading bacteria in agricultural fields.

The time course of the study revealed that the initial chitinase production was very less (2.6 U mL⁻¹) in 24 h old culture. Whereas the chitinase production was maximum (27.4 U mL⁻¹) at 96 h and at 120 h it declined to 10.4 U mL⁻¹ (Fig. 1). Microorganism requires prolonged time to decompose chitin and produce chitinase due to its higher molecular weight. In *Streptomyces hygroscopicus* VMCH2 strain, there was no chitinase production until 49 h of incubation and it increased from 96 to 240 h while maximum enzyme activity was detected at declining growth phase (Priya et al., 2011).

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### Table 1. Classification of bacterial strains based on chitinase assay

| Bacterial strain | Units mL⁻¹ | Bacterial strain | Units mL⁻¹ | Bacterial strain | Units mL⁻¹ |
|------------------|------------|------------------|------------|------------------|------------|
| High chitinase producer | | Moderate chitinase producer | | Low chitinase producer | |
| MP-13 *P. fluorescens* | 27.4 | BC-10 *B. cereus* | 7.7 | MP-35 *B. subtilis* | 0.6 |
| AWRH40 *P. putida* | 19.1 | BC-7 *B. cereus* | 5.0 | C-6 *B. subtilis* | 0.6 |
| J-6 *P. putida* | 13.7 | BCM-8 *P. monteilii* | 6.3 | J-7 *B. subtilis* | 0.8 |
| J-11 *P. putida* | 15.2 | MP-11 *B. amyloliquefaciace* | 6.2 | J-16 *B. subtilis* | 0.1 |
| C-13 *B. cereus* | 26.3 | BCM-1 *B. amyloliquefaciace* | 5.4 | J-18 *B. subtilis* | 0.2 |
| BC-15 *B. thuringiensis* | 18.7 | MP-18 *B. amyloliquefaciace* | 6.6 | BC-9 *B. subtilis* | 0.9 |

**Fig. 1.** Time course of chitinase production by *P. fluorescens* in a medium supplemented with colloidal chitin as a sole carbon source. (Values represents mean of five replications ± SE)

**Fig. 2.** SDS-PAGE analysis of chitinase from *P. fluorescens* (MP-13)
Lane 1-Protein marker; Lane 2- culture supernatant; Lane 3- enzyme after 70% ammonium sulphate precipitation; Lane 4 - chitinase purified by gel-filtration chromatography

Chitinase from *P. fluorescens* of tea soils
to 69 kDa. A significant difference in the size of chitinase among different microorganisms is reported.

The partially purified chitinase isolated from Pf MP-13 was characterized in terms of pH, temperature and substrate specificity. Even though chitinase was found to be highly active within a wide range of pH (4 to 10), its activity was considerably higher at pH 7.0 (Fig. 3). Chitinase activity was relatively higher in alkaline pH than that of acidic pH and hence, chitinase with an alkaline pH can effectively exert its stability in alkaline insect gut. Mostly all bacteria are reported to secrete chitinase at pH 7.0 or at slightly alkaline pH (Annamalai et al., 2010, Gomma, 2012).

The stability of chitinase varied from 20 to 100 °C. Chitinase activity was reduced considerably at 20 °C (9.5 U mL⁻¹) and increased up to 10.3 U mL⁻¹ (70 °C). Chitinase activity remained stable after 30 min at 30-60 °C (Fig. 4). After that, chitinase activity declined rapidly (2.1 U mL⁻¹ at 100 °C). Concentration of colloidal chitin is yet another parameter which should be standardized to enhance the production of chitinase. In the present study, chitinase activity (13.4 U mL⁻¹) was substantially higher at 1.5 per cent colloidal chitin compared to other concentrations (Fig. 5) and similar results were reported by Gomma (2012). Considering the thermostability of chitinase, it can be used under field conditions to control insect pests. Contrarily, Pseudomonads screened for chitinase production exhibited higher activity at 1 per cent chitin containing Kings B medium (Viswanathan and Samiyappan, 2000). Colloidal chitin was easily utilized by the organism due to its colloidal nature compared to chitin and degrades the chitin into monomers and oligomers.

This study will help in the developing new avenue in pest management in tea plantations besides opening a new approach in formulating chitinase based biopesticides in near future.

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References

Annamalai, N., Giji, S., Arumugam, M. and Balasubramanian, T. 2010. Purification and characterization of chitinase from *Micrococcus* sp., AG84 isolated from marine environment. *African Journal of Microbiological Research* 4(24): 2822-2827.

Bhattacharya, D., Nagpure, A. and Gupta, R.K. 2007. Bacterial chitinases: properties and potential. *Critical Review in Biotechnology* 27(1): 21-28.

Broadway, R.M., Gongora, C., Kain, W.C., Sanderson, J.P., Monroy, J.A., Bennett, K.C., Warner, J.P. and Hoffmann, M.P. 1998. Novel chitinolytic enzymes with biological activity against herbivorous insects. *Journal of Chemical Ecology* 24(6): 985-998.

Gomaa, Z.G. 2012. Chitinase production by *Bacillus thuringiensis* and *Bacillus licheniformis*: Their potential in antifungal biocontrol. *Journal of Microbiology* 50(1): 103-111.

Mendonsa, E.S., Vartak, P.H., Rao, J.U. and Deshpande, M.V. 1996. An enzyme from *Myrothecium verrucaria* that degrades insect cuticles for biocontrol of *Aedes aegypti* mosquito. *Biotechnology Letters* 18(4): 373-376.

Nawani, N.N. and Kapadnis, B.P. 2001. One-step purification of chitinase from *Serratia marcescens* NK1, a soil isolate. *Journal of Applied Microbiology* 90(5): 803-808.

Nawani, N.N. and Kapadnis, B.P. 2003. Chitin degrading potential of bacteria from extreme and moderate environment. *Indian Journal of Experimental Biology* 41(3): 248-254.

Park, J.K., Kim, W.J. and Park, Y.I. 2011. Purification and characterization of an exo-type α-N-acetyl glucosaminidase from *Pseudomonas fluorescens* JK-0412. *Journal of Applied Microbiology* 110(1): 277-286.

Priya, C.S., Jagannathan, N. and Kalaichelvan, P. 2011. Production of chitinase by *Streptomyces hygroscopicus* vmch2 by optimization of cultural conditions. *International Journal of Pharma and Bio Science* 2(2): 210-219.

Roobakkumar, A., Babu, A., Vasantha Kumar, D., Jasim Rahman, V. and Sarkar, S. 2011. *Pseudomonas fluorescens* as an efficient entomopathogen against *Oligonychus coffeae* Nietner (Acari:Tetranychidae) infesting tea. *Journal of Entomology and Nematology* 3(5): 73-77.

Shternshis, M.V., Beljaev, A.A., Shpatova, T.V., Bokova, J.V. and Duzhak, A.B. 2002. Field testing of bacticide, phytoverm and chitinase for control of the raspberry midge blight in Siberia. *Biocontrol* 47: 697-706.

Viswanathan, R. and Samiyappan, R. 2000. Antifungal activity of chitinases produced by some fluorescent pseudomonas against *Colletotrichum falcatum* Went causing rot disease in sugarcane. *Microbiological Research* 155: 1-6.

Watanabe, T., Oyanagi, W., Suzuki, K. and Tanaka, H. 1990. Chitinase system of *Bacillus circulans* WL-12 and importance of chitinase A1 in chitin degradation. *Journal of Bacteriology* 171: 4017-4022.