Short Scientific Report

doi:10.19071/jpc.2015.v43.i3.2859
Journal of Plantation Crops, 2015, 43(3): 236-239

Detection of chitinase activity and its characterization from *Pseudomonas fluorescens* of tea rhizosphere

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(Manuscript Received:03-01-2015, Revised:14-09-2015, Accepted:12-10-2015)

Keywords: Biocontrol agent, chitin, gel-filtration chromatography, integrated management, microbial chitinase

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 $(C_8H_{13}O_5N)_n$, an insoluble, most abundant polysaccharide is composed of linear chains of β1,4-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 *et al.* (1996) isolated chitinase from *Myrothecium verrucaria* bacterium and studied its activity against *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 *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 (*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

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Table 1. Classification of bacterial strains based on chitinase assay

High chitinase producer		Moderate chitinase producer		Low chitinase producer	
Bacterial strain	**Units mL^{-1}**	**Bacterial strain**	**Units mL^{-1}**	**Bacterial strain**	**Units mL^{-1}**
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. amyloliquefacience*	6.2	J-16 *B. subtilis*	0.1
C-13 *B. cereus*	26.3	BCM-1 *B. amyloliquefacience*	5.4	J-18 *B. subtilis*	0.2
BC-15 *B. thuringiensis*	18.7	MP-18 *B. amyloliquefacience*	6.6	BC-9 *B. subtilis*	0.9

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).

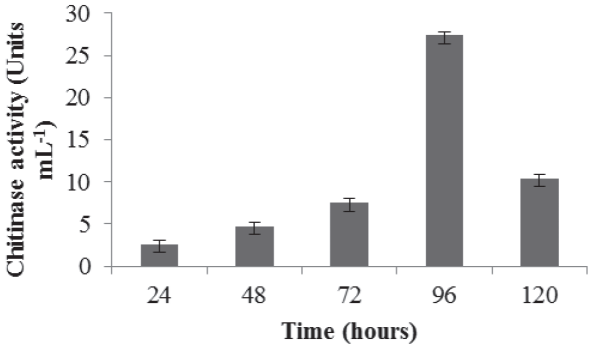

Fig. 1. Time course of chitinase production by *P. fluorescens* in a medium supplemented with colloidal chitin as a sole carbon source. (Values represents mean of five replications ± SE)

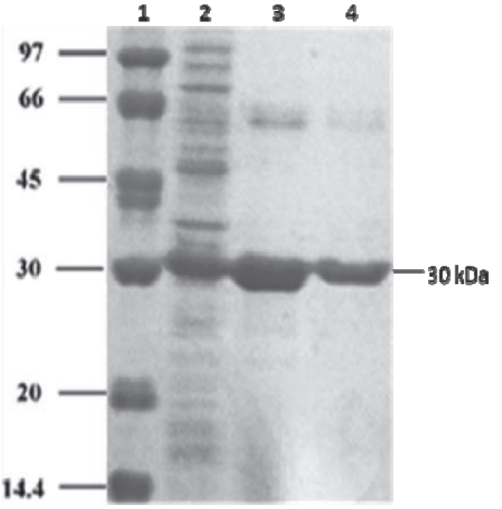

Fig. 2. SDS-PAGE analysis of chitinase from *P. fluorescens* (MP-13)
Lane 1-Protein marker; Lane 2- culture supernatant; Lane 3- enzyme after 70% ammonium sulphate precipitation; Lane 4 - chitinase purified by gel-filtration chromatography

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 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

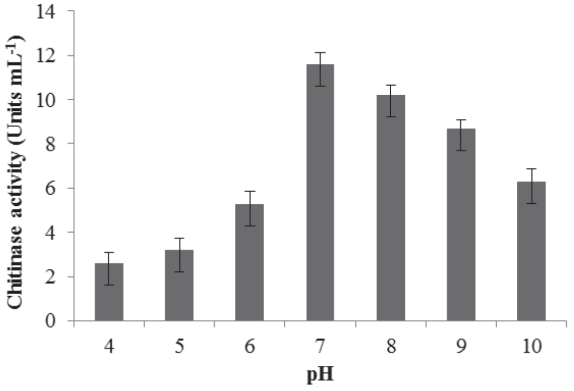

Fig. 3. Effect of different pH on chitinase production by *P. fluorescens* (Values represents mean of five replications ± SE)

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).

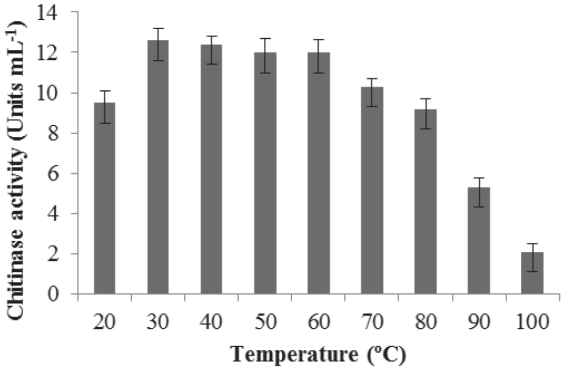

Fig. 4. Effect of different temperature on chitinase production by *P. fluorescens* (Values represents mean of five replications ± SE)

The stability of chitinase varied from 20 to 100 ^{0}C. Chitinase activity was reduced considerably at 20 ^{0}C (9.5 U mL^{-1}) and increased upto 10.3 U mL^{-1} (70 ^{0}C). Chitinase activity remained stable after 30 min at 30-60 ^{0}C (Fig. 4). After that, chitinase activity declined rapidly (2.1 U mL^{-1} at 100 ^{0}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

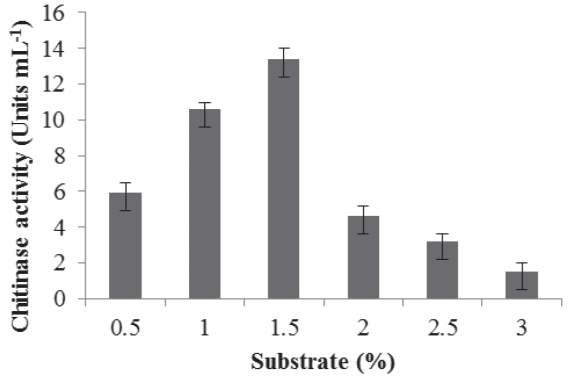

Fig. 5. Production of chitinase by *P. fluorescens* in different concentrations of colloidal chitin (Values represents mean of five replications ± SE)

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.

Acknowledgements

The authors are thankful to Dr. R. Premkumar, Deputy Director & HOD, Plant Pathology Division for providing biocontrol agents. Encouragements and support extended by Dr. B. Radhakrishnan,

Director, UPASI Tea Research Institute are gratefully acknowledged. Thanks are due to my colleagues in Plant Pathology division and Plant Physiology division for their technical support.

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