ABSTRACT

Fungal diseases of plants are one of the major concerns in cultivation world wide resulting in loss of billions of dollars. Vascular wilt of carnation caused by *Fusarium oxysporum* f.sp. *dianthi* (Prill. & Delacr.) Synder, W.C. & Hans, H.N. inflicts substantial yield and quality loss to the crop. Microbes are potential sources of antifungal enzymes that are either absent in plants or more potent in biological activity than their plant equivalent. Antagonistic microbes with chitinolytic activity could be useful candidate for biological control of fungi, nematodes and insects. This study was therefore aimed to elucidate the role of chitinases in the antifungal activity of the antagonistic fluorescent pseudomonads displaying chitinolysis using soil reaction and substrate specificilty as indicators. A glycosyl hydrolase family 5 (*GH5*) gene specific for chitin and chitosan was cloned and overexpressed in *Escherichia coli* and the recombinant *GH5* was characterized for biochemical and antifungal properties.

The diversity and antifungal activity of fluorescent pseudomonads isolated from rhizospheres of tea, gladiolus, carnation and black-gram grown in acidic soils with similar texture and climatic conditions were studied. Biochemical characterisation including antibiotic resistance assay, RAPD and PCR–RFLP studies revealed a largely homogenous population. At soil pH (5.2), the isolates exhibited growth with varying levels of siderophore production, irrespective of crop rhizospheres. Two isolates with maximum chitinase production showed antagonism. The bacterial populations in general lacked the ability to produce deleterious traits such as cellulase, pectinase and hydrogen cyanide. However, increased pH levels beyond 5.2 caused reduction in metabolite production with reduced antifungal activity. The homogeneity of the bacterial population irrespective of crop rhizospheres together with decreased secondary metabolite production at higher pH levels reinstated the importance of soil over host plant in influencing rhizosphere populations. In another study, fluorescent pseudomonads antagonistic to the vascular wilt pathogen were studied for their ability to grow and produce chitinases on different substrates. Bacterial cells grown on chitin-containing media showed enhanced growth and enzyme production with increased anti-fungal activity against the pathogen. Furthermore, the cell-free bacterial culture filtrate from chitin-containing media also significantly inhibited the mycelial growth. Extracellular proteins of highly antagonistic bacterial strain were isolated from cell-free extracts of media amended with chitin and fungal cell wall. These cell-free conditioned media
contained one to seven polypeptides. Western blot analysis revealed two isoforms of chitinase with molecular masses of 43 and 18.5 kDa.

A chitinolytic and antagonistic *P. putida* strain P3(4) of pea rhizosphere soil was used to clone the GH5 gene specific for chitin and chitosan and overexpress in *Escherichia coli* for biochemical and functional characterization of the recombinant protein. Standard bacteriological tests and sequencing of the 16S rRNA indicated the taxonomic affiliation of the isolate to *Pseudomonas putida*, a member of fluorescent pseudomonads. PCR primers specific for glycosyl hydrolase family 5 (GH5) of *Pseudomonas putida* isolate KT2440 amplified a 947bp fragment of the GH5 gene from P3(4). Cloning of this gene into *Escherichia coli* M15 using an expression vector pQE-30UA and screening on chitin and chitosan detection agar identified one positive clone (Pchi^+^). Sequence analysis of the cloned insert revealed an open reading frame of 947 nucleotides corresponding to a protein of 315 amino acids with a predicted molecular mass of 38.0 kDa. The deduced amino acid sequence of the open reading frame (gene product/GH) showed 83-84% homology to the GH5 of *P. putida* strains F1 and KT2440, respectively. The purified enzyme was homogenous, as examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and was visualized as single fluorescent band in native gel assay with 4-methylumbelliferyl-β-D-glucosaminide and glycol chitosan, respectively. For hydrolysis of 4-nitrophenyl-β-D-glucosaminide (pNP-(GlcNAc) and colloidal chitosan, the enzyme had an optimal temperature of 40°C, and was stable within the temperature range of 10°C to 40°C. The enzyme showed an optimal pH of 3.5, with maximum stabilities at 5.0 and 5.5 for hydrolysis of pNP-(GlcNAc) and colloidal chitosan, respectively. Fe^{3+} and Cu^{2+} stimulated chitinase and chitosanase activities by 74.20 and 51.38%, respectively. The purified glycosyl hydrolase displayed 70 and 45% inhibition of spore germination of the pathogenic fungi, *Fusarium oxysporum* f.sp. *dianthi* and *Alternaria solani*.

In conclusion, this study indicates that the bifunctional 38.0 kDa GH5 is a hitherto unexplored bifunctional enzyme exhibiting antifungal activity. Therefore, this enzyme is a good candidate for biotechnological application to produce biopesticide and generate oligosaccharide elicitors from chitin or chitosan. Further, GH5 gene can be utilized (candidate) to design appropriate strategies for transgenic resistance in crop plants to combat fungal pathogens and to improve biocontrol strains producing secondary metabolites, although further characterization in this regard remains to be done.