MATERIALS AND METHODS

3.1 FUNGAL PATHOGENS

The fungal pathogen, *Fusarium oxysporum* f.sp. *dianthi* (FOD) (Prill. & Delacr.) W.C Synder & H.N. Hans isolate CAFO-IHBT inflicting vascular wilt of carnation was used as the test pathogen. To validate the antifungal activity of the purified glycosyl hydrolase, *Alternaria solani* (AS) Sorauer isolate TOAS-IHBT (HM484353) causing early blight of tomato and available in the culture collections of Floriculture Pathology Laboratory of the host institute was used.

3.1.1 Sample collection

Vascular wilt infected carnation samples were collected from commercial polyhouses of different locations in Himachal Pradesh (H.P).

3.1.2 Media preparation

Potato dextrose agar (PDA) (Riker and Riker, 1936), carnation leaf agar (CLA) (Snyder and Hansen, 1947); synthetic nutrient-poor agar (SNA) (Nirenberg, 1976) and potato carrot agar (PCA) media were used for isolation and morphological characterisation. PDA, SNA and PCA were prepared as described in Appendix II. To prepare CLA, the carnation leaf pieces were prepared from fresh carnation leaves free from fungicide or insecticide residue. Immediately after collection, the leaves were cut into 5-8 mm pieces and dried in a forced-air oven at approximately 70°C for 3-4 h until brittle. The dried leaf pieces were packaged in aluminium or polycarbonate containers and sterilized by gamma irradiation (92.5 megarads). Sterilised leaf pieces were stored at 2-5°C before their use. CLA was prepared by placing sterile carnation leaf pieces of approximately 1 piece per 2 ml agar on a Petri plate and then by adding sterile 2% water agar (20 g agar in 1 L of distilled water).
3.1.3 Isolation

FOD was isolated from the infected stem tissue by employing standard isolation methods (Riker and Riker, 1936). The infected tissues were cut into small pieces and surface sterilized with % sodium hypochlorite for 30 s. They were then washed in three changes of sterile distilled water (SDW) and dried between two layers of blotting papers. The bits were further plated on Potato Dextrose Agar (PDA) or Carnation Leaf Agar (CLA) medium containing 100 ppm streptomycin in sterile Petri dishes and incubated at room temperature (28±2°C). The fungal growth appearing around the bits were sub-cultured on plates containing PDA, synthetic nutrient-poor agar (SNA) (Nirenberg, 1976) and Potato Carrot Agar (PCA) media.

3.1.4 Pathogenicity

Plants grown in pots containing sterilized soil were used as sources of planting materials. Prior infection of the cuttings was examined on 10 randomly selected samples. The cuttings were cut into pieces, and the pieces were surface sterilized with NaOCl (1%), washed in SDW, inoculated on PDA plates and incubated at 28±2°C for 10 days and observed for the growth of the pathogen.

Pathogenicity test for the fungus on carnation was carried out under artificial inoculated conditions. Carnation (cv. Irlamda) cuttings of 2 nos were planted in individual 30 cm diameter earthen pots containing 5 kg steam sterilized soil. The experiments were carried out in completely randomized design with 10 replicates of 20 cuttings each per treatment.

The cuttings were infected after 15 days of planting by incorporating FOD multiplied earlier in sand corn meal medium (10 g corn meal+90 g sand+20 ml distilled water) at a ratio of 1:19 (sand-maize inoculum:soil) i.e., 250 g (5%) per pot around the planting materials. Control plants without inoculation were also maintained.

3.1.5 Morphological characterisation

Monoconidial cultures of CAFO-IHBT were obtained through single spore isolation technique (Riker and Riker, 1936). Briefly, a spore suspension was prepared from 7-day-old
culture, dispersed on 2% water agar Petri plates and incubated at 28±2°C. Single spore was marked under a microscope, picked with sterilized cork borer and transferred to PDA slants for growth. To study the morphological characteristics, a portion of actively growing culture discs of the fungi from PCA was picked up with a sterile needle and teased out in a drop of lactophenol cotton blue placed on a microscopic slide. The slide was covered with a clean cover slip taking care to exclude air bubbles. The morphological characters of conidiophores, microconidia, macroconidia and chlamydospores (Nelson et al., 1983) were studied by examination under an Olympus research microscope with maximum possible magnification (100X).

3.1.6 Molecular characterisation

The identities of six pathogenic cultures, viz., CAFO-IHBT, CAFO-Chambi (S. nagar) 2; CAFJ-Ghaneta; CAXX-5B Chail; CIFU-Tissa (1) (Chamba) and CAFU-Tissa (2) (Chamba) were also established by PCR amplification and sequencing of either 18S rRNA or ITS gene sequences as follows.

3.1.6.1 Fungal mycelium preparation

Five days old submerged culture mycelium was used for DNA isolation. For submerged culture, well-isolated colonies of each of the Fusarium spp. were inoculated separately in 50 ml of PDB. The inoculated flasks were incubated on a rotary shaker at 180 rpm and 28±2°C. The mycelium was filtered out on a sterile Whatman No. 1 filter paper and washed thrice with sterile distilled water to remove traces of the medium. The fungal mass was air dried to remove excess of moisture and frozen immediately in liquid nitrogen. The dried mats were ground with a mortar and pestle in liquid nitrogen and stored immediately in -80 °C.

3.1.6.2 DNA isolation

Genomic DNA was isolated from the fungal mycelium following standard procedure with certain modifications, Saghai-Marooft et al. (1984) as follows:
i. About 0.5 g of fresh mycelium was ground in liquid nitrogen.

ii. The powdered mycelium was suspended in CTAB extraction buffer (0.1 M Tris pH 7.5; 1% CTAB; 0.7 M NaCl; 10 mM EDTA; 1% β-mercaptoethanol; proteinase K to a final conc. of 0.3 mg/ml prior to use) and mixed well.

iii. The mixture was then incubated at 65°C in a water bath for 30 min.

iv. After cooling on ice for 10-15 min, equal volume of chloroform:isoamyl mix (24:1) was added and mixed well.

v. The mix was then centrifuged at 12,000 rpm for 20 min at 4°C.

vi. The aqueous layer was transferred to a fresh tube and re-extracted with equal volume of phenol:chloroform:isoamyl alcohol (25:24:1).

vii. The supernatant was transferred to a fresh tube and extracted once again with chloroform:isoamyl mix.

viii. To the aqueous layer, 0.6 volume of isopropanol was added and mixed gently, micro centrifuged for 15 min at 4°C.

ix. The pellet was washed twice with 70% ethanol and air-dried.

x. Finally, the pellet was resuspended in 50 μl of nuclease free water.

xi. The DNA was then treated with RNase (10 mg/ml) at 37°C for 1 h to avoid RNA contamination.

xii. Equal volume of chloroform:isoamyl mix (24:1) was added to the solution and mixed well.

xiii. The mix was then centrifuged at 12,000 rpm for 20 min at 4°C.

xiv. The aqueous layer was transferred to a fresh tube and precipitated with 0.6 volume of isopropanol.

xv. The solution was mixed gently, micro centrifuged for 15 min at 4°C.

xvi. The pellet was washed twice with 70% ethanol and air-dried.

xvii. Finally, the pellet was resuspended in 50 μl of nuclease free water.

3.1.6.3 PCR amplification

To characterize the unknown fungal pathogen, a region of the nuclear rRNA gene containing the ITS regions 1 and 2 and the 5.8s rRNA gene was amplified from the genomic DNA by
PCR using the primer combinations ITS1 (5’ TCC GTA GGT GAA CCT GCG G 3’) and ITS4 (5’ TCC TCC GCT TAT TGA TAT GC 3’) to get an amplicon size of 630 bp (White et al., 1990). PCR amplification of 18S rRNA was done using the primer combinations nu-SSU-0817 (5’- TTAGCATGGGAATAATRRAATAGGA- 3’) and nu-SSU-1536 (5’- ATTGCAATGNCYCTATCCCCA- 3’) to get an amplicon size of 762 bp (Borneman and Hartin, 2000). Amplifications were carried out in 50 µl reaction volume consisting of 10X buffer, 5.0 µl; 2 mM dNTPs, 5.0 µl; 3 U/µl Taq DNA polymerase, 0.33 µl; 100 ng/µl of each primer, 2 µl; 50-100 ng template DNA, 1 µl and H2O 34.67 µl in a Biorad (USA) thermalcycler using the PCR conditions 95ºC for 5 min (initial denaturation), 94ºC for 30 sec (denaturation), 56ºC for 10 sec (annealing) and 72ºC for 1 min (extension). The total number of cycles was 35, with the final extension of 72ºC for 7 min. The amplified products (50 µl) were size separated on 1.0% agarose gel containing 0.5 µg ml⁻¹ ethidium bromide and photographed with the gel documentation system (Alpha Imager 2200). A 100 bp DNA ladder (MBI, Fermentas) was used as molecular weight size markers.

3.1.6.4 Purification of the PCR product

The PCR products, 630 and 762 bp for ITS and 18S rRNA, respectively were purified from contaminating products by column purification of the gel slice containing the excised desired fragment with Qiaquick gel extraction kit (Qiagen, USA) as described below:

i. The DNA fragment was excised from the agarose gel with a clean and sharp scalpel.

ii. The gel slice was weighed in a colorless microcentrifuge tube. About 3 volumes of buffer QG was added to 1 volume of gel.

iii. The tube was then incubated in a water bath at 50ºC for 10 min. The tube was vortexed for every 2 min during this incubation step.

iv. After the gel slice has dissolved completely, the mixture was checked for yellow colour

v. One volume of isopropanol was added to the sample and mixed by vortexing.

vi. A QIAquick spin column was placed in a 2 ml collection tube.
vii. The sample mixture was pipetted into the column and centrifuged at 13,000 rpm for 1 min.

viii. The flowthrough was discarded and the column was placed back in the same collection tube.

ix. Buffer QG (0.5 ml) was added to the column and centrifuged at 13,000 rpm for 1 min.

x. Buffer PE (0.75 ml) was added to the column and centrifuged at 13,000 rpm for 1 min.

xi. The flow-through was discarded and the column was centrifuged for an additional 1 min at 13,000 rpm.

xii. The column was placed into a clean 1.5 ml microcentrifuge tube.

xiii. Deionized water (30 µl) was added to the centre of the column membrane and allowed to stand for 1 min. The column was centrifuged and the purified DNA was collected in the microcentrifuge tube.

3.1.6.5 Cloning of the PCR product

3.1.6.5.1 Ligation

The gel eluted amplicon of the 18S rRNA gene was ligated in pGEM-T easy vector (Promega, USA) following the manufacturer’s protocols (Promega, USA) as follows:

i. The vector and control insert DNA tubes were briefly centrifuged to collect contents at the bottom of the tubes.

<table>
<thead>
<tr>
<th>Component</th>
<th>Standard Reaction</th>
<th>Positive Control</th>
<th>Background Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X Rapid ligation buffer, T4 DNA ligase (vortexed vigorously before use)</td>
<td>5 µl</td>
<td>5 µl</td>
<td>5 µl</td>
</tr>
<tr>
<td>pGEM®- Vector (50 ng)</td>
<td>1 µl</td>
<td>1 µl</td>
<td>1 µl</td>
</tr>
<tr>
<td>PCR product</td>
<td>3 µl</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Control insert DNA</td>
<td>-</td>
<td>2 µl</td>
<td>-</td>
</tr>
<tr>
<td>T4 DNA ligase (3 Weiss U/ µl)</td>
<td>1 µl</td>
<td>1 µl</td>
<td>1 µl</td>
</tr>
<tr>
<td>Deionized water to a final volume of</td>
<td>10 µl</td>
<td>10 µl</td>
<td>10 µl</td>
</tr>
</tbody>
</table>
ii. The ligation reaction was set up as follows in a 0.5 ml PCR tube
iii. The reaction was mixed by pipetting and incubated overnight at 4°C in a circulatory water bath.

3.1.6.5.2 Preparation of competent *Escherichia coli* cells

Any one of the following strains DH5α or M15 [pREP4] cells (Qiagen, USA) were used for preparing competent cells before proceeding for transformation.

i. A trace of cells (Qiagen, USA) was removed and streaked it out on LB agar containing 25 µg/ml kanamycin
ii. The plate was incubated at 37°C overnight
iii. A single colony was picked, inoculated in 10 ml of LB-kanamycin (25 µg/ml) and grown overnight at 37°C
iv. One ml of overnight grown culture was added to 100 ml of prewarmed LB containing 25 µg/ml kanamycin and incubated at 37°C on a rotary shaker until an OD₆₀₀ of 0.5 was reached, which was approximately 90-120 min.
v. The culture was cooled on ice for 5 min, and the cells were harvested by centrifugation at 4000 x g for 5 min at 4°C.
vi. The supernatant was discarded carefully by keeping the cells on ice.
vii. The cells were resuspended gently in 30 ml of cold TFB I buffer (Appendix IV) and the suspension was incubated on ice for an additional 90 min.
viii. The cells were recovered by centrifugation as above (4000 x g for 5 min at 4°C), and resuspended in 4 ml of ice-cold TFB II buffer (Appendix IV)
ix. Aliquots of 200 µl were prepared in sterile microcentrifuge tubes and freezed in liquid nitrogen before storing at −70°C.

These competent cells were prepared either fresh for each use or pre-made and stored at -70°C.
3.1.6.5.3 Transformation

Transformation of the recombinant plasmids was carried onto commercially available competent cells; *E. coli* strain DH5α by heat shock method following the manufacturer’s protocols (Promega, USA) as follows:

i. The tubes containing the ligation reactions were centrifuged to collect the contents at the bottom of the tube.

ii. Each ligation reaction (2 µl) was added to a sterile 1.5 ml microcentrifuge tube on ice.

iii. Competent cells (50 µl) were added to the microfuge tube containing the ligation mixture; the tubes were gently flicked to mix the contents and placed on ice for 20 min.

iv. The cells were placed in a water bath at exactly 42°C for 45 s.

v. The tubes were transferred immediately on ice for 2 min to reduce damage to the *E. coli* cells.

vi. One ml of LB (with no antibiotics) was added and the tubes were incubated for 1.5 h at 37°C in a shaking incubator (150 rpm). About 100 µl of the resulting culture was spread on predried LB plates with 100 µg/ml ampicillin, X-gal 40 µl (20 mg/ml in dimethyl formamide) and IPTG, and incubated overnight at 37°C.

vii. Colonies containing plasmids with inserts were identified by blue-white selection on agar plate (Sambrook *et al.*, 1989).

viii. The positive colonies were picked about 14 h later.

3.1.6.5.4 Plasmid isolation

The recombinant plasmid DNA was randomly isolated from individual white colony by boiling prep method (Holmes and Quingley, 1981).

i. Three ml of overnight grown (37°C) culture were pelleted by centrifuging at 14,000 rpm for 5 min.

ii. After decanting off the supernatant, the pellet was washed with 500 µl of nuclease free water.

iii. The cells were centrifuged at 14,000 rpm for 2 min and the supernatant was discarded.
iv. To the pelleted cells, 110 µl of STET buffer (sucrose 8%; Triton X 100 0.5%; Tris HCl (pH 8.0) 50 mM; EDTA (pH 8) 50 mM) was added and the cells were ruptured by dragging on a microfuge tube stand for 10 min.

v. Ten µl of lysozyme (10 mg/ml) was added to the resuspended cells; the contents were mixed by vortexing and incubated at 28±2°C for 5 min.

vi. The tubes were transferred to a boiling water bath for 40 s and centrifuged at 14,000 rpm for 20 min at room temperature; the debris was removed with a sterile toothpick.

vii. Ribonuclease (1 µl) was added to the tubes and the tubes were then incubated in a water bath for 30 min at 37°C.

viii. Isopropanol (110 µl) was added to precipitate the DNA in the supernatant and centrifuged at 14,000 rpm for 20 min to collect the DNA.

ix. The supernatant was removed and the pellet was washed with 70% alcohol at 14,000 rpm for 15 min.

x. The pellet was air dried and dissolved in 40 µl of nuclease free water.

xi. The isolated plasmid DNA was subjected to restriction digestion by EcoRI, the sites flanking the cloned insert to ensure the presence of correct insert size in each clone as follows: Template (plasmid)-10 µl i.e., 1 µg; 10X EcoRI buffer (Fermentas)-3 µl; EcoRI (10 U/µl)-1 µl and double distilled water-16 µl.

### 3.1.6.6 Nucleotide sequencing and analysis of the amplicon

The recombinant plasmid DNA was isolated and purified for sequencing using the plasmid extraction kit (Qiagen, USA). About 300-350 ng of plasmid (double stranded) DNA was used as template and diluted in deionized water to a total volume of 5 µl. The primers were diluted in deionized water. For each reaction, the sequencing reagent premix was combined with the diluted DNA template as follows: ready reaction mix (Applied Biosystems, USA)-4 µl; primer (100 ng/µl)-1 µl; DNA template-5 µl to have a total volume of 10 µl. PCR reaction was carried out for the samples in a Biorad (USA) thermalcycler using the PCR conditions 94°C for 10 s (denaturation), 50°C for 40 s (annealing) and 60°C for 4 min (extension). The total number of cycles was 25, with a final holding temperature of 4°C (∞).
In order to remove the contaminating salts and unincorporated dye terminators from DNA sequencing reactions, the reaction mixture was subjected to the following sequencing reaction clean-up protocol using Montage SEQ96 sequencing reaction cleanup Kit (Millipore).

i. The sequencing PCR reactions were diluted by adding 20 μl of injection solution and gently mixed by pipetting up and down for 5 times.

ii. The diluted reactions were transferred onto the bottom of SEQ96 plate wells.

iii. The SEQ96 plate was then placed on the vacuum manifold.

iv. The vacuum pump was set to 23–25" Hg and vacuum was applied until the solution was completely removed from the wells. Application of vacuum was continued for 15 to 30 s after the last well was emptied.

v. The vacuum source was shut off and the SEQ96 plate was removed from the manifold.

vi. The excess liquid was removed from the bottom of the SEQ96 plate by briefly pressing against a paper towel.

vii. Injection solution (20 μl) was added into the bottom of each well.

viii. The SEQ96 plate was placed on the vacuum manifold and vacuum was applied until the solution was completely removed from the wells. Application of vacuum was continued for 15 to 30 sec after the last well was emptied.

ix. The vacuum source was shut off and the SEQ96 plate was removed from the manifold.

x. The excess liquid was removed from the bottom of the SEQ96 plate by briefly pressing against a paper towel as earlier.

xi. Injection solution (20 μl) was added into the bottom of each well.

xii. The purified sequencing products were re-suspended in the injection solution by pipetting up and down for 30 times.

xiii. The purified sequencing products were transferred to an appropriate sequencing plate.

Sequencing was then performed using an automated sequencer (ABI PRISM 310, Applied Biosystems, USA). Both strands of the cloned DNA were sequenced by the dideoxy chain termination method, Sanger et al. (1977) with the Big Dye™ (Terminator version 3.0) Ready Reaction Cycle Sequencing Kit (Applied Biosystems, USA) using an automated sequencer (ABI PRISM 310, Applied Biosystems, USA). The primers used for sequencing was T7 that
correspond to the DNA flanking the cloned insert (Promega, USA). The reaction products were analysed by capillary electrophoresis on an ABI 310 Genetic Analyzer (Applied Biosystems).

3.1.6.7 Phylogenetic analysis

A sequence similarity search was done using GenBank BLASTN (Altschul et al., 1997). The nucleotide sequence of 18S rRNA was deposited in GenBank. Phylogenetic analysis was performed using TREETOP software (http://www.genebee.msu.su/services/phtree_reduced.html).

3.2 FLUORESCENT PSEUDOMONADS

3.2.1 Sample collection and isolation

Soil samples (100-200 g) were collected from the rhizospheres of cultivable crops including carnation and forest trees and used for isolation. Rhizosphere-colonising fluorescent pseudomonads were isolated from fresh intact root systems of the host plants by direct plating and enrichment culture. The root samples collected from the gardens were vigorously shaken to remove the soil loosely adhered to the roots. A soil suspension was obtained by shaking 10 g of root plus lightly adhering soil in 90 ml of SDW for 30 min at 180 rpm in a rotary shaker.

For direct plating, the suspension was diluted up to $10^4$ and one ml of the suspension was plated onto King’s B (KB) agar (King et al., 1954). Single colonies that fluoresced under UV light (366 nm) after incubation at $28 \pm 2^\circ$C for 2 days were selected and further cultured on the same medium to establish pure cultures.

For enrichment culture, the suspension was used as an inoculum in KB broth (HiMedia) supplemented with 1% colloidal chitin. Colloidal chitin was prepared from crab shell chitin (Sigma) as described (Berger and Reynolds, 1958). The crab shells were powdered and digested overnight with concentrated HCl at 4°C. The digested chitin was washed repeatedly with distilled water to adjust the pH to 7.0. The chitin suspension in water was centrifuged and the pellet was collected, dried and used at 1% in the medium. Fungal cell wall preparation was obtained from the mycelial mats (Chet and Huttermann, 1980) grown on
potato dextrose broth (PDB) for 8 days at room temperature (28±2°C). The mycelium was then collected by filtration through Whatman No.1 filter paper, washed with SDW and homogenized in chloroform and methanol (1:1). The resulting suspension was filtered and homogenized in acetone. The mycelium was washed with distilled water repeatedly to remove excess acetone and dried at 45°C. The dried cell wall material was used at 0.3% in the medium. Soil suspension (3 ml) was inoculated in 20 ml broth and incubated by shaking at 180 rpm and 28°C. Following four transfers (30 µl into 20 ml of fresh broth) after every 24 h, the cultures were diluted (1 ml culture in 9 ml of distilled water) and plated on chitinase detection agar (CHDA, water agar medium incorporated with 0.4% colloidal chitin) plates. After incubation for 24–48 h at 28°C, morphologically different colonies appearing on the plates were isolated and subjected to further purification by streaking on same medium.

3.2.2 Screening of fluorescent pseudomonads for antagonism

The antifungal activity of the bacteria was assessed against the test pathogen (FOD) by dual culture technique (Dennis and Webster, 1971a) on PDA with or without supplementation of FeCl₃ in triplicate. FeCl₃ was added to the concentration of 100 µg/ml at the time of pouring the plates. PDA medium was allowed to settle for 1 h in sterile Petri dishes. After solidification of the media, an agar plug (5 mm diameter) taken from an actively growing zone of the test pathogen on PDA was separately removed by a sterile cork borer and transferred to near periphery of one half of Petri dish. After 48 h, the fluorescent pseudomonads were streaked perpendicular to the agar plug on the opposite side towards the edge of plates. Plate inoculated with fungal agar plugs alone was used as control. The plates were incubated at 28±2°C until the fungal mycelia completely covered the agar surface in control plate. Strains that inhibited the mycelial growth of the pathogen were identified by measuring the inhibition zone from the edge of mycelium to the bacterial streak.
3.2.3 Characterisation of antagonistic fluorescent pseudomonads

The characteristics of the fluorescent pseudomonads were established according to Bergey’s Manual of Systematic Bacteriology (Holt et al., 1994) as follows. All the assays were replicated thrice for each of the strains.

3.2.3.1 Morphological characterisation

3.2.3.1.1 Gram staining

A thin smear of each of the bacteria was prepared on a clean glass slide and the smear was covered with crystal violet for 20 s. The stain was then briefly washed off with distilled water and the excess water was drained off. Later, the smear was covered with Gram’s iodine solution for one min and after draining the same, the smear was flooded with 95% ethyl alcohol for 20 s. The action of alcohol was stopped by washing the slide under running distilled water for a few seconds and the smear was covered with safranin for 20 s. Finally, the stain was washed off and dried at room temperature (28±2°C) and immediately observed under a microscope.

3.2.3.2 Physiological characterisation

3.2.3.2.1 Effect of temperature

Each of the bacterial strains was streaked on KB plates and incubated aerobically at -20, 4, 24, 28 and 37 and 41°C. The effect of temperature on the growth was assessed after 7 days of incubation.

3.2.3.2.2 Effect of pH of medium

KB plates of varying pH viz., 4.5, 5.2, 6.0, 7.0, 8.5, 9.0, 9.5 and 11.0 were prepared and the bacterial strains were streaked. The plates were incubated at 28±2°C for 7 days and the growth was assessed.
3.2.3.3.3 Effect of salt concentration

KB plates of 5 different concentrations of sodium chloride *viz.*, 0% (control), 2.5%, 5.0%, 7.5%, and 10% were prepared. The plates were streaked with the bacterial strains and incubated at 28±2°C for 7 days to assess the growth.

3.2.3.3 Biochemical characterisation

The strains were phenotypically characterized for the following reactions in triplicate and results of these tests were scored as either positive or negative (Stanier *et al.*, 1966; Holt *et al.*, 1994; Bossis *et al.*, 2000). Uninoculated tubes or plates served as control in all the experiments.

3.2.3.3.1 Catalase and oxidase activity

Hydrogen peroxide solution (3%) was prepared from normally available concentration (30%). Bacterial smears were prepared on clean glass slides and one drop of 3% solution was added over the smears to observe for effervescence. Commercially available oxidase discs (HiMedia) were used to assess oxidase activity.

3.2.3.3.2 Gelatin hydrolysis

Gelatin-agar medium (peptone 5 g; beef extract 3 g and gelatin 120 g dissolved in 1 L distilled water) was prepared and 10 ml was dispensed into 30 ml culture tubes. The culture tubes were inoculated with the fluorescent pseudomonads by stab inoculation (i.e. punctured the agar column from top to bottom with withdrawal of the needle through the same path). The tubes were incubated at 37°C for 5 days. After incubation, the tubes were placed in a refrigerator at 4°C for 15 min. Deep gelatin inoculated tubes that remained liquefied produced gelatinase and showed positive test for gelatin hydrolysis. Those tubes that remained solid demonstrated negative reaction for gelatin hydrolysis.
3.2.3.3.3 Starch hydrolysis

Starch agar (beef extract 3 g; soluble starch 10 g and agar 20 g dissolved in 1 L distilled water) plates were prepared and streaked with the bacterial strains. After incubation at 28±2°C for 2 days, the surface was flooded with Gram’s iodine and observed for either colorless zone or blue/purple colour around the colonies to record positive or negative reactions, respectively.

3.2.3.3.4 Casein hydrolysis

Skim milk agar (skim milk powder 100 g; peptone 5 g and agar 20 g dissolved in 1 L distilled water; pH 7.2) plates were prepared and streaked with the bacterial strains. After incubation at 28±2°C for 2 days, appearance of any clearing around the line of growth was checked for proteolytic activity.

3.2.3.3.5 Urea hydrolysis

Urea base medium (peptone 1 g; NaCl 5 g; KH₂PO₄ 2 g and agar 20 g dissolved in 1 L distilled water; glucose 1 g and phenol red (0.2%) 6 ml were added to the autoclaved molten base; after steaming (1 h) and cooling, filter sterilized aqueous solution of urea (20%) 100 ml was added aseptically to the basal medium) was prepared and dispensed into 30 ml tubes to prepare slants. Each of the bacterial strains was inoculated and incubated aerobically at 28±2°C for 3 days. The change in colour of the medium from yellow to red or pink red was examined for urease activity.

3.2.3.3.6 Oxidation-Fermentation (O-F) test

O-F glucose agar medium (pancreatic digest of casein 2 g; NaCl 5 g; dipotassium phosphate 0.3 g; bromothymol blue 0.08 g and agar 20 g dissolved in 1 L distilled water) was prepared and dispensed in 30 ml sterile glass vials. Each of the bacterial strains was stab inoculated in duplicate. In one of the tubes of each pair, liquid paraffin was poured over the medium to form a layer of about one cm. The inoculated vials were incubated at 28±2°C for 3 days. Whilst, fermentative reaction produced acid from glucose and change the colour of the
medium from blue to yellow, oxidative reaction produced a deep blue colour indicating an alkaline reaction.

### 3.2.3.3.7 Indole production

Tryptone broth (1% i.e. 10 g peptone dissolved in 1 L distilled water) was prepared and 5 ml was distributed in 30 ml sterile glass vials. Each of the strains was inoculated and the vials were incubated at 28±2°C aerobically for 2 days. One ml of Kovac’s reagent (HiMedia) was added to each tube including control and the vials were shaked gently after intervals for 10-15 min. A positive reaction was indicated by a deep golden red ring.

### 3.2.3.3.8 Methyl-Red (MR) and voges Proskauer (VP) tests

MRVP broth (pancreatic digest of casein 3.5 g; peptic digest of animal tissue 3.5 g; potassium phosphate 5 g and dextrose 5 g dissolved in 1 L distilled water) was prepared and 5 ml was distributed in 30 ml sterile glass vials. Each of the strains was inoculated in two sets of three vials each and the vials were incubated aerobically at 28±2°C for 2 days. MR test was carried out by adding 2-3 drops of methyl red indicator solution to the vials and observed for the change in colour from methyl red to yellow. Change in colour was recorded as negative. VP test was carried out by adding 600 µl each of V-P reagents A and B to the second set of vials. The tubes were shaken gently for 30 s with the caps off to expose the media to oxygen and observed for the colour change after 15-30 min. The development of a crimson-to-ruby pink (red) colour indicated a positive reaction.

### 3.2.3.3.9 Citrate utilization

Simmon’s citrate agar (HiMedia) (pH 6.9) slants were prepared in 30 ml sterile glass vials, streak inoculated with the bacterial culture and incubated aerobically at 28±2°C for 2 days. Positive reaction was indicated by an increase in pH of agar, changing the colour of bromothymol blue indicator in the medium from green (pH 6.9 and below) to deep blue (pH 7.6 and higher).
3.2.3.3.10 Levan formation

Bacterial strains were streaked on specific medium (KH$_2$PO$_4$ 1 g; (NH$_4$)$_2$SO$_4$ 1 g; MgSO$_4$-7H$_2$O 1 g; yeast extract 2.5 g and sucrose 100 g dissolved in 1 L distilled water; Avigad, 1965) for levan formation and incubated for 24 h. Strains which exhibited slightly raised slimy white glistening appearance are chosen as positive for levan formation.

3.2.3.3.11 Utilization of carbon sources

The bacterial strains were also tested for the utilization of various carbon sources (Table ) using Hicarbohydrate™ kit as described by the manufacturer (HiMedia). The kit contains a basal media amended with specific carbohydrate and phenol red as an indicator. On fermentation of the carbohydrate, acid is liberated which lowers down the pH of medium and this change of colour is indicated by the pH indicator dye. To test the strains, cells were grown in nutrient broth to reach density of 0.5 O.D. at 600 nm. An aliquot of 50 µl of this suspension was inoculated to each well of Hicarbohydrate™ kit, incubated at 30°C for 24 h and the results were registered according to the instructions of the manufacturer i.e. positive test is indicated by a change of colour to yellow due to acid reaction. No change in colour or red/pink colour indicated negative reaction.

3.2.3.3.12 Selection of fluorescent pseudomonads for intrinsic antibiotic resistance

The strains were screened for intrinsic resistance to 19 antibiotics (Table 4.7) using antibiotic discs (Himedia, India) on nutrient agar medium. The diameters of inhibition zones were measured and correlated to the zone size interpretative chart supplied by the manufacturer.
3.2.3.4 Genotypic characterisation

3.2.3.4.1 Isolation of genomic DNA

Pure cultures of the bacterial strains were inoculated separately in 10 ml KB (King et al., 1954) broth and grown overnight at 28±2°C. Genomic DNA was isolated from the cultures following standard procedures (Graves and Swaminathan, 1993) as follows:

i. Pelleted 3 ml of the culture by centrifuging at 12,000 rpm for 5 min.
ii. The pellet was resuspended in 567 μl TE buffer by repeated pipetting and 30 μl of 10% SDS and 3 μl of 20 mg/ml proteinase K were added, mixed well and incubated for 1 h at 37°C.
iii. To the solution, 100 μl of 5 M NaCl was added and mixed thoroughly. Later, 80 μl of CTAB/NaCl solution was added, mixed well and incubated for 10 min at 65°C
iv. Equal volume of chloroform:isoamyl mix (24:1) was added and micro centrifuged at 4°C for 20 min at 13,000 rpm.
v. The supernatant was transferred to a fresh tube by first removing the protein debris with the help of a toothpick followed by adding equal amount of phenol:chloroform:isoamyl mix (25:24:1).
vi. The solution was mixed well and centrifuged at maximum speed for 20 min.
vii. Transferred the supernatant again to a fresh tube and extracted once again with chloroform:isoamyl mix. To the aqueous layer, 0.6 volume of isopropanol was added and mixed gently, kept for 15-30 min at room temperature and microcentrifuged for 15 min at 4°C.
viii. The pellet was washed twice with 70% ethanol and air-dried.
ix. Finally, the pellet was resuspended in 50 μl of nuclease free water.
x. The DNA was then treated with RNase (10 mg/ml) at 37°C for 1 h to avoid RNA contamination.
3.2.3.4.2 PCR amplification and sequencing of ITS region

To confirm the bacterial strains as fluorescent pseudomonads, either 16S-23S rRNA intervening sequence-specific primers ITS1F (5’-AAGTCGTAACAAGGGTAG-3’) and ITS2R (5’-GACCATAACCCCAAG-3’); or 16S rRNA primers, pA (5’-AGAGTTTGTGACTCAAAATTCACCHGACCHTATCC-3’; bases 8 to 27) and reverse primer PC5B (5’- TACCTTGTACGACGCTC-3’; bases 1507 to 1492) used to get an amplicon size of 560 bp (Kumar et al., 2002) and 1500 bp (Kuske et al., 1997), respectively.

For 16S-23S rRNA intervening sequence, amplifications were carried out in 50 µl reaction volume consisting of 10X buffer, 5.0 µl; 2 mM dNTPs, 5.0 µl; 3 U/µl Taq DNA polymerase, 0.33 µl; 100 ng/µl of each primer, 2 µl; 50-100 ng template DNA, 1 µl and H2O 34.67 µl in a Biorad (USA) thermal cycler using the PCR conditions 94°C for 4 min 94°C for 30 s 48°C for 30 s and 72°C for 30 s. The total number of cycles was 34, with the final extension of 72°C for 7 min.

For 16S rRNA, amplifications were carried out in 50 µl reaction volume consisting of 10X buffer, 5.0 µl (Genei, India); 2 mM dNTPs, 5.0 µl; 3 U/µl Taq DNA polymerase, 0.33 µl (Genei, India); 100 ng/µl primer, 2 µl; 35-45 ng template DNA, 1 µl and H2O, 34.67 µl in a Biorad (USA) thermal cycler using the PCR conditions 94°C for 4 min (initial denaturation), 94°C for 30 s (denaturation), 52°C for 1 min 30 s (annealing) and 72°C for 2 min (extension). The reaction control consisted of all components, except the genomic DNA. The total number of cycles was 35, with the final extension of 72°C for 7 min.

The 1500 bp PCR product was size separated, purified from contaminating products, cloned and sequenced as described earlier for 18S rRNA (762 bp) product.

3.2.3.4.3 Nucleotide sequencing and phylogenetic analysis

A sequence similarity search was done using GenBank BLASTN (Altschul et al., 1997). The nucleotide sequence of 16S rRNA was deposited in GenBank. The accession numbers of the 16S rRNA sequences of the strains are presented in Table 4.8. Phylogenetic analysis was performed using TREETOP software (http://www.genebee.msu.su/services/phtree_reduced.html).
3.2.4 Elucidation of functional traits of antagonistic fluorescent pseudomonads

The antagonistic strains were screened by plate assays for the production of the following functional traits.

3.2.4.1 Cellulases and pectinases

Cellulase and pectinase productions were determined as described (Cattelan et al., 1999). M9 medium agar (Na$_2$HPO$_4$ 6.0 g; KH$_2$PO$_4$ 3 g; NaCl 0.5 g; NH$_4$Cl 1.0; MgSO$_4$ 0.5 g; glucose 2 g; CaCl$_2$ 0.015 g; distilled water 1 L) (Miller, 1974) amended with 10 g of cellulose and 1.2 g of yeast extract per L of distilled water was used to test the cellulase activity. The strains were plated and incubated at 28°C for 8 days. Development of halos was considered as positive.

For determining the pectinase activity, 10 g pectin and 1.2 g yeast extract were amended in M9 medium agar and plated. After incubating at 28°C for 2 days, the cultures were flooded with 2 M HCl. Clear halos around the colonies were considered as positive for pectinase production.

3.2.4.2 P-solubilisation

Screening for phosphate solubilisation was done by a plate assay (Pikovskaya, 1948) using Pikovskaya’s agar medium (Hi-Media). The strains were plated and after incubation at 28±2°C for 5 days, the plates were observed for the zone of clearance.

3.2.4.3 Indole acetic acid (IAA)

The production of IAA was determined by using standard method (Bric et al., 1991). Single colony was streaked onto Luria-Bertani (LB) agar (tryptone 20 g, yeast extract 5 g, NaCl 5 g, agar 20 g dissolved in 1 L distilled water) amended with 5 mM L-tryptophan, 0.06% sodium dodecyl sulphate and 1% glycerol. Plates were overlaid with Whatman no. 1 filter paper (82 mm diameter) and bacterial strains were allowed to grow for a period of 3 days. After the incubation period, the paper was removed and treated with Salkowski’s reagent (Gordon and Weber, 1951) with the formulation of 2% 0.2 M ferric chloride in 35% perchloric acid.
Membranes were saturated in a Petri dish by soaking directly in the reagent and the production of IAA was identified by the formation of a characteristic red halo within the membrane immediately surrounding the colony.

### 3.2.4.4 Hydrogen cyanide (HCN)

Test for the production of HCN was carried out as described by Bakker and Schippers (1987). Fluorescent pseudomonads strains were grown at 28°C on a rotary shaker in tryptic soy broth. Filter paper (Whatman No. 1) was cut into uniform strips of 10 cm long and 0.5 cm wide saturated with alkaline picrate solution and placed inside the conical flasks in a hanging position. After incubation at 28°C for 48 h, the sodium picrate present in the filter paper was observed for a change in colour.

### 3.2.4.5 Siderophore

The production was assayed by a plate method using the ternary complex chromeazurol S (CAS)/Fe^{3+}/hexadecyltrimethylammonium bromide (HDTMA) (Schwyn and Neilands, 1987). CAS-blue agar (I L) was prepared using 60.5 mg CAS dissolved in 50 ml distilled water and mixed with 10 ml iron (III) solution (1 mM FeCl₃.6 H₂O in 10 mM HCl). Under stirring this solution was slowly added to 72.9 mg HDTMA dissolved in 40 ml water. The resultant dark blue liquid was amended into succinate medium (succinic acid 4 g; K₂HPO₄ 3 g, (NH₄)₂ SO₄ 7H₂O 0.2 g and agar 12.5 g dissolved in 1 L distilled water; pH 7.0). An inoculum (10 µl) of 48 h old fluorescent pseudomonad was placed onto the center of the succinate medium amended with the indicator and incubated at 28±2°C for 5 days. Production of siderophore was indicated by a bright zone with a yellowish fluorescence in the dark-blue medium.

### 3.2.4.6 Chitinase

The bacterial strains were evaluated for their chitinolytic ability on Chitinase Detection Agar (CHDA) (Wen et al., 2002) medium. Colloidal chitin was prepared from crab shell chitin (Sigma) as described (Berger and Reynolds, 1958). The crab shells were powdered and
digested overnight with concentrated HCl at 4°C. The digested chitin was washed repeatedly with distilled water to adjust the pH to 7.0. The chitin suspension in water was centrifuged and the pellet was collected, dried and used at 1% in the medium. A loopful of 48 h old cultures was streaked in triplicate on water agar medium incorporated with 0.4% colloidal chitin and incubated at 28±2°C. Development of clear halos around the colonies was recorded after 5 days of incubation.

3.2.5 ROLE OF CHITINASE IN ANTAGONISM OF FLUORESCENT PSEUDOMONADS USING SOIL REACTION AS AN INDICATOR

The effect of soil reaction on diversity and antifungal activity of rhizosphere populations of fluorescent pseudomonads associated with the host plants, tea, gladiolus, carnation and black gram grown in acidic soils under similar climatic conditions was assessed.

3.2.5.1 Site description and sample collection

Soil samples were collected from seven different locations (32°6 N, 76°3 E, 1300 m above msl) representing rhizospheres of mainly tea (Camellia sinensis Linn.), gladiolus (Gladiolus hortulanus L.H. Bailey), carnation (Dianthus caryophyllus Linn.) and black gram (Vigna mungo Linn.), around Palampur, the site of the institute. The soil texture was silty clay loam (30% clay, 53% silt and 17% sand) with an average pH of 5.2 and the climate is wet temperate with an average annual rainfall of 2491 mm. Rhizosphere soils from 15 healthy and mature plants were collected at regular intervals from each plot, to represent the entire area of cultivation, and then pooled before bacterial isolation.

3.2.5.2 Isolation and characterisation of fluorescent pseudomonads

Rhizosphere-colonising fluorescent pseudomonads were isolated from fresh intact root systems of all four-host plants and characterized as described in 3.2.3.
3.2.5.3 RAPD fingerprinting

The extent of molecular diversity of strains from each of the crop rhizospheres was analysed along with the reference strain. Fifty primers from the kits OPA, OPAA, OPB, OPG and OPH, each consisting of 20 random decamer primers were tested. The primers were supplied by Operon technologies, CA, USA, and the PCR reagents by Genei, India. Amplifications were carried out in 25 µl reaction volume consisting of 10 µl buffer with 1.5 mM MgCl$_2$, 2.5 µl; 2 mM dNTPs, 2.5 µl; 3 U/µl Taq DNA polymerase, 0.33 µl; 20 pM primer, 2 µl; 45 ng template DNA, in a Biorad (USA) thermalcycler using the PCR conditions 94°C for 3 min (initial denaturation), 94°C for 1 min (denaturation), 37°C for 1 min (annealing) and 72°C for 2 min (extension). The reaction control consisted of all components, except the genomic DNA. The total number of cycles was 40, with the final extension of 72°C for 10 min. The amplified products (25 µl) were size separated on 1.4% agarose gel containing 0.5 µg/ml ethidium bromide and photographed in a gel documentation system (Alpha Imager 2200). A 100 bp DNA ladder (GeneRuler plus, MBI, Fermentas) was used as molecular weight size markers. The analysis was repeated at least three times, fingerprints were compared and the bands which appeared consistently were evaluated. The pair-wise coefficient similarity based on presence and absence of bands and cluster analysis with unweighed pair group method arithmetic mean (UPGMA) were used to generate similarity matrix.

3.2.5.4 Restriction analysis of 16S rDNA

The PCR amplified 16S rDNA product (560 bp) was restriction digested for 3 h at 37°C in 25 µl reaction mixture containing 8 µl (300 ng) of PCR product, 2.5 µl of 10 µl PCR buffer, and 10 U of one of the following restriction enzymes, HindIII, BamHI, PstI, EcoRI, AluI, Rsal and MboI. Restriction digestion was then analysed by agarose gel electrophoresis (1.4%) containing 0.5 µg/ml ethidium bromide (Sambrook et al., 1989). A 100 bp ladder (MBI, Fermentas) was used as molecular size marker. The analysis was done at least twice with each enzyme.
3.2.5.5 Selection for acidic pH

Pure culture of each of the strains was plated in triplicate on KB (King et al., 1954) medium with the pH adjusted to 5.2, 5.8 and 6.4. The plates were incubated at 28±2°C for 2 days.

3.2.5.6 Test for in vitro antagonism

The fluorescent pseudomonads were tested for antagonism against FOD in dual culture assays on PDA at 5.2, 5.8 and 6.4 pH levels as described in 3.2.2.

3.2.5.7 HCN production, siderophore and chitinase assays

The assays were carried out as at three pH levels, 5.2, 5.8 and 6.4. Siderophore production was quantified as suggested (Reeves et al., 1983). The bacterial strains were grown in KB broth of 5.2, 5.8 and 6.4 pH and incubated at 28±2°C for 3 days and the culture supernatants were collected by centrifugation at 3000 g for 10 min and passed through bacterial proof filters 0.22 μm (Millipore, USA). The pH of the supernatant was adjusted to 2.0 with 1 N HCl and equal quantity of ethyl acetate was added in a separating funnel, mixed well and ethyl acetate fraction was collected. Five milliliters of ethyl acetate fraction was mixed with 5 ml of Hathway’s reagent (1.0 ml of 0.1 M FeCl3 in 0.1 N HCl to 100 ml distilled water+1.0 ml of 0.1 M potassium ferricyanide). The absorbance for dihydroxyl phenols was read at 700 nm in a Spectrophotometer. A standard curve was prepared with dihydroxy benzoic acid and the quantity of siderophore produced was expressed as μmol of benzoic acid/ml of culture filtrate.

Chitinase production was also quantitatively evaluated by colorimetric method (Boller and Mauch, 1988). The reaction mixture consisted of 10 μl of 1 M sodium acetate buffer (pH 4.0), 0.4 ml supernatant from culture filtrate of strains grown separately in KB broth of 5.2, 5.8 and 6.4 pH and 0.1 ml colloidal chitin (10 mg). After incubating for 1 h, the resultant chitin oligomers were treated with 2 ml dimethyl amino benzaldehyde (DMAB) for 20 min at 37°C and the absorbance was measured at 585 nm. N-acetylg glucosamine (GlcNAc) served as the standard. The enzyme activity was expressed as nmoles GlcNAc equivalents/min/ml.
3.2.6 ROLE OF EXTRACELLULAR CHITINASES IN ANTAGONISM OF FLUORESCENT PSEUDOMONADS USING CHITIN AMENDED MEDIA AS AN INDICATOR

3.2.6.1 Isolation and characterisation of extracellular chitinases

3.2.6.1.1 Preparation of fungal cell wall

Fungal cell wall preparation (Chet and Hutterman, 1980) was obtained from the mycelial mats grown on PDB for 8 days at room temperature (28±2°C). The mycelium was then collected by filtration through Whatman No.1 filter paper, washed with SDW and homogenized in chloroform and methanol (1:1 ratio). The resulting suspension was filtered and homogenized in acetone. The mycelium was washed with distilled water repeatedly to remove excess acetone and dried at 45°C. The dried cell wall material was used at 1% in the medium.

3.2.6.1.2 Bacterial growth on chitin-amended media

Selected strains of fluorescent pseduomonads were grown on medium containing colloidal chitin (1%), peptone (2%), glycerol (1%), Fusarium cell wall (1%) in different combinations, and incubated at 28±2°C for 48 h with continuous shaking. The bacterial cells were then collected by centrifuging the media (1 ml) at 6000 rpm for 10 min and resuspended in 1 ml of 0.1 M phosphate buffer. The population was assessed and calculated spectrophotometrically at 595 nm (Thompson, 1996).

3.2.6.1.3 Chitinase production on different media

Chitinase production on different media was quantitatively evaluated by colorimetric method as described in 3.2.5.7.
3.2.6.1.4 Chitinolytic ability of fluorescent pseudomonads

The bacterial strains were also evaluated for their chitinolytic ability as described in 3.2.4.6. Cell free culture (CFC) filtrates of the strains grown on chitin-amended medium were prepared by filtering the cultures by coarse filtration using Buckner flasks. This filtrate was again filtered through Millipore filters (pore size 450 nm) and 100 µl was placed in 8 mm diameter wells in chitin-water agar medium and incubated at room temperature (28±2°C). Observation on clearing zone around the treated wells was recorded 4 days later. Culture filtrate from each strain was treated similarly in three wells.

3.2.6.1.5 Protein separation by SDS-PAGE

The supernatant from the chitin medium was filtered through 0.22 µm bacterial proof filter (Millipore, USA) and collected in sterile conical flasks. Ammonium sulphate was added to the filtrate to 80% saturation (52.3 g/100 ml), incubated overnight at 4°C and centrifuged (15,000 x g for 15 min at 4°C). The protein pellet was dissolved in 0.1 mol/L sodium phosphate buffer, pH 7.0, and dialysed against distilled water overnight at 4°C. The dialysate was lyophilized using a lyophilizer (Ilshin, Korea) and used for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein contents of the samples were determined (Bradford, 1976) using bovine serum albumin as the standard. The proteins were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in polyacrylamide slab gels consisting of 4% stacking gel and 12% separating gel using the Hoefer mini-electrophoresis system (Amersham Biosciences, Sweden) (Laemmli, 1970). The samples were loaded at 50 µg per well with Rainbow molecular weight markers (Genei, India) to locate the position of the protein bands during semi-dry transfer. Electrophoresis was carried out at a constant voltage of 65 V and the gels were subjected to coomassie brilliant blue (200 mg coomassie brilliant blue, 40 ml methanol, 10 ml glacial acetic acid, 50 ml distilled water) or monochromatic silver staining as follows.

i.  After SDS-PAGE, the gel was soaked in 50% methanol for 1 h in a glass dish.
ii. The silver stain was prepared by adding solution A (0.8 g of silver nitrate in 4 ml de-ionised water) drop wise to solution B (21 ml of 0.36% NaOH mixed with 1.4 ml of freshly prepared 14.8 M ammonium hydroxide) with constant shaking and then the volume was made upto 100 ml with de-ionized water.

iii. The gel was stained in this solution for 15 min with gentle agitation on a platform shaker in dark.

iv. The gel was washed three times in de-ionized water for 10 min each.

v. Later, the gel was soaked in developing solution (1.25 ml of 1% citric acid mixed with 0.125 ml of 37% formaldehyde and made upto 500 ml with de-ionized water) until bands appeared (usually in less than 10 min).

vi. Finally, the gel was washed with de-ionized water and placed in 40:10:50 methanol: glacial acetic acid: water to arrest further stain development.

3.2.6.1.6 Western blotting

The electrophoretic transfer of proteins was carried out from gel to membrane in a Atto semi-dry blot transfer apparatus (140 mA, 30 min) (Atto Corporation, Japan) for 1 h.

i. After SDS-PAGE, the stacking gel was removed from the gel and the separating gel was incubated for 15 min in 200 ml of cathode buffer (0.3 M Tris, pH 10.4, 10% v/v methanol).

ii. Simultaneously two pieces of filter papers were incubated in anode buffer I (25 mM Tris, pH 10.4, 10% v/v methanol) for two min.

iii. Additionally one piece of filter paper was kept in anode buffer II (25 mM Tris, 40 mM glycine pH 9.4, 10% v/v methanol) for two min.

iv. Three pieces of filter paper were kept in cathode buffer for two min.

v. Membrane preparation: Before doing the electro transfer to PVDF membrane (Hybond-P PVDF, GE Biosciences), the membrane was wet in 100 % methanol for 15 s (membrane changes from opaque to semitransparent). After that, the membrane was soaked in Milli Q water for 2 min.

vi. For the equilibration of membrane was transferred to anode buffer II for 15 min
vii. Transfer: After doing the equilibration, the filter paper soaked in anode buffer I was kept on anode electrode plate in the center.

viii. Filter papers soaked in anode buffer II was placed on the top of the first two sheets.

ix. Membrane was placed on the top of the filter paper and then the gel was transferred onto the membrane taking care not to trap any air bubble in between by rolling with glass rods.

x. Immediately after placing the membrane on the gel, the filter papers soaked in cathode buffer was kept over it.

xi. The cathode electrode was kept over the filter papers and sufficient pressure was applied.

xii. After connecting the leads, the system was turned on for 2 h at 35 mA.

xiii. Membrane was washed twice for 10 min with 15 ml of 1X TBS buffer followed by 1 h incubation in blocking solution i.e.2% skimmed milk in TBS.

xiv. The membrane was again washed two times for 15 min each with 1X TBST.

xv. The membrane was then soaked in the primary antibody (tobacco chitinase antiserum) diluted to 1:5000 with diluent (Amersham Biosciences, Sweden) for overnight at room temperature in an orbital shaker.

xvi. Membrane was washed two times for 10 min with 20 ml of 1X TBST.

xvii. The membrane was then incubated in affinity purified goat anti-rabbit immunoglobulin (IgG), conjugated with alkaline phosphatase (1:1000) for 1 h at room temperature in an orbital shaker.

xviii. After each incubation, the membrane was washed thrice with Tris-buffered saline containing 0.05% Tween for 10 to 15 min each time to remove the unbound antibody.

xix. Immunological reaction was visualized by soaking (0.1 ml/cm²) the membrane in detection solutions A and B premixed at 1:1 ratio (ECL Advance Western Blotting Detection Kit). After incubating for 5 min, the blot was exposed to an X-ray film for 10 s. The film was then developed and fixed.
3.2.6.1.7 Chitinase detection after PAGE under non-denatured conditions

Chitinase activity was also detected under native condition on 7.5% separating gel copolymerized with 100 μM of the fluorogenic substrate (Sigma), 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (4-MU-(GlcNAc) for NAGase and 0.1% glycol chitosan (Sigma, USA) for chitosanase. In-gel detection of corresponding molecular weight of the protein was done using native protein markers (Genei). Chitinase was reactivated in the gels by removing SDS upon incubating the gel with 1% Triton-X in 100 mM Tris buffer, pH 8.0 (Hung et al., 2002) followed by incubation at 37°C for 30 min in 100 mM sodium acetate buffer pH 5.0. The enzyme activity appeared as fluorescent band under UV light because of enzymatic hydrolysis of fluorescent 4-methyl umbelliferone from the GlcNAc disaccharide.

3.2.6.1.8 In vitro antagonism of fluorescent pseudomonads and CFC filtrates

The fluorescent pseudomonads grown on chitin-amended media were tested for their antagonistic action against FOD by dual plate method as described in 3.2.2. CFC filtrates of selected fluorescent pseudomonads grown on chitin-amended media were prepared as described in 3.2.7.1.3 and assessed for their antifungal activity on the pathogen. Spore suspension of the fungus was prepared from 7 days old culture with SDW. Three ml of the suspension (1 x 10^8 spores/ml) was seeded in 20 ml of PDA. After 48 h, about 200 μl of cell free culture filtrate of each strain was pipetted out in three 8 mm wells made in PDA. The treated plates were incubated at 28±2°C. SDW was used in place of culture filtrates for control. The inhibition zone formation was measured at different time intervals up to 96 h.

3.2.7 CLONING AND CHARACTERISATION OF A GLYCOSYL HYDROLASE (GH5) WITH CHITINASE AND CHITOSANASE ACTIVITY

3.2.7.1 Isolation of genomic DNA

Isolation was carried out as described in 3.1.5.1 from 10 ml of overnight grown culture of a highly chitinolytic and antagonistic fluorescent pseudomonad strain P3(4) grown on colloidal chitin (1%) amended in peptone and glycerol.
3.2.7.2 PCR cloning and DNA sequencing

Amplifications were carried out in 50 µl reaction volume consisting of 10X buffer, 5.0 µl (Genei, India); 2 mM dNTPs, 5.0 µl; 3 U/µl Taq DNA polymerase, 0.33 µl (Genei, India); 100 nM of each primer (PChi34 5’-CACCCGATCAAGCCTACTTC-3’ and PChi35 5’-GTGCCAGTTCCGTAGTCCAT-3’), 2 µl; 50 ng template DNA, 1 µl and H2O, 34.67 µl in a Biorad (USA) thermal cycler using the PCR conditions 95°C for 3 min (initial denaturation), 95°C for 40 s (denaturation), 50°C for 40 s (annealing) and 72°C for 2 min (extension). The reaction control consisted of all components, except the genomic DNA. The total number of cycles was 30, with the final extension of 72°C for 10 min. A 947 bp amplicon was purified in 30 µl of nuclease free water with QIAquick PCR purification kit (Qiagen, USA), cloned and sequenced as described in 3.1.6.5 and 3.1.6.6. Translated nucleotide sequence was then analyzed for similarities by using BLASTN tool (www.ncbi.nlm.nih.gov:80/BLAST/) of National Centre for Biotechnology Information (NCBI).

3.2.7.3 Overexpression and purification of recombinant protein

3.2.7.3.1 Cloning

The PCR product was cloned into six-His-tagged protein expression pQE-30UA cloning vector (Qiagen, USA), which is designed for direct cloning. The reaction mix consisted of pQE-30 UA vector (50 ng/µl), 1 µl; PCR product (25 ng/µl), 3 µl; distilled water, 1 µl and 2X Ligation Master Mix, 5 µl. The mixture was gently mixed by pipetting and incubated at 16°C for 2 h.

3.2.7.3.2 Transformation of competent M15 cells

i. The ligation mix (10 µl) was transferred into a cold and sterile 1.5 ml microcentrifuge tube, and kept it on ice.

ii. An aliquot (100 µl) of frozen competent M15 [pREP4] cells prepared as detailed in section 3.1.5.4.2 was thawed on ice.
iii. The cells were gently resuspended and 100 μl of the cell suspension was transferred into the microcentrifuge tube with the ligation mix, mixed carefully, and kept it on ice for 20 min.

iv. The tube was transferred to a 42°C heating block for 90 s and immediately transferred to ice for at least 5 min.

v. About 500 μl of Psi broth was added to the cells and incubated for 90 min at 37°C on a rotary shaker (200 rpm).

vi. Aliquots of 50, 100 and 200 μl were plated on LB-agar plates containing 25 μg/ml kanamycin and 100 μg/ml ampicillin.

vii. The plates were incubated at 37°C overnight.

3.2.7.3.3 Screening

The transformants were screened for correct insertion and frame of the coding fragment by PCR analysis of the recombinant plasmid (isolated as described in 3.1.6.5.4) using different combinations of the forward vector and gene specific reverse primers as follows: promoter primers (5'-CCCGAAAAGTGCCACCTG -3' and PChi35 5'-GTGCCCCAGTTCGTAGTCCAT-3'); type III/IV primers 5'-CGGATAACAATTTCACACAG-3' and PChi35 5'-GTGCCCCAGTTCGTAGTCCAT-3' and gene specific primer pair (PChi34 5'-CACCCGATCAAGCCTACTTC-3' and PChi35 5'-GTGCCCCAGTTCGTAGTCCAT-3'). Amplifications were carried out in 50 μl reaction volume consisting of 10X buffer, 5.0 μl; 2 mM dNTPs, 5.0 μl; 3 U/μl Taq DNA polymerase, 0.33 μl; 100 ng/μl of each primer, 2 μl; 50-100 ng template DNA, 1 μl and H2O 34.67 μl in a Biorad (USA) thermalcycler using the PCR conditions 95°C for 5 min (initial denaturation), 94°C for 30 sec (denaturation), 56°C for 10 sec (annealing) and 72°C for 1 min (extension). The total number of cycles was 35, with the final extension of 72°C for 7 min.

The recombinant cells were plated on CHDA and chitosanase detection agar (0.5% colloidal chitosan, 0.2% K2HPO4, 0.1% KH2PO4, 0.07% MgSO4, 0.05% NaCl, 0.05% KCl, 0.01% CaCl2, and 0.05% yeast extract). Colloidal chitosan was prepared as described (Helisto et al. 2001). Five g of chitosan (Sigma, with 18% N-acetylation) was dissolved in 100 ml of 1 M HCl with continuous stirring while heating from room temperature to 50-60°C during 30-
40 min. The pH of solution was maintained at 6.5-7.5 by addition of 50% (w/v) NaOH. The precipitate was washed 8-10 times with distilled water, collected by centrifugation and used.

3.2.7.3.4 Nucleotide sequencing and deduced amino acid analysis

The nucleotide sequence of the 947 bp gene encoding GH5 was determined as detailed in 3.1.6.6 and was deposited in the nucleotide database (NCBI). The deduced amino acid sequence of the gene encoding 38 kDa GH5 protein was analysed for conserved domains using Conserved Domain Architecture Retrieval Tool (CDART) software of NCBI.

3.2.7.3.5 Purification

The purification of His₆ tagged protein from recombinant cells showing activity was done using His•Bind® Resin Chromatography following the manufacturer’s protocol (Novagen, USA). The QIAexpressionist (M/s Qiagen). Briefly, recombinant *E. coli* was grown in LB broth containing 100 µg/ml ampicillin and 25 µg/ml kanamycin to an OD₆₀₀ of 0.5, induced with 1 mM IPTG and further incubated for 8 h with shaking. The cells were harvested by centrifugation at 3000 × g for 15 min, and the pellet was lysed using a lysis buffer (6 M GuHCl, 0.1 M NaH₂PO₄, 0.01 M Tris Cl, pH 8.0) and centrifuged at 11,000 g for 15 min at room temperature to pellet the cellular debris. The supernatant was loaded onto Ni-NTA (nickel-nitrilotriacetate) affinity columns, centrifuged and the column was washed twice with wash buffer (6 M GuHCl, 0.1 M NaH₂PO₄, 0.01 M Tris Cl, pH 6.3). The bound recombinant protein was eluted with elution buffer (6 M GuHCl, 0.1 M NaH₂PO₄, 0.01 M Tris Cl, pH 4.5), dialyzed against Tris buffer (10 mM Tris Cl pH 8.0, 0.1% Triton X-100) overnight at 4°C to remove guanidine hydrochloride.

3.2.7.3.6 6xHis-protein tag staining

The recombinant protein was electrophoresed on a 10% sodium dodecyl sulphate polyacrylamide gel as described in 3.2.6.1.5 and visualized by staining with coomassie brilliant blue (Sigma). Pierce® 6xHis-protein tag staining (Thermo Scientific) was done as follows:
i. After electrophoresis, the gel was washed thrice by gently agitating with 100 ml of Milli-Q water for 20 min.

ii. The 6xHis protein tag stain (50 ml) was added to the gel and gently agitated for 5 min.

iii. The gel was washed twice with 100 ml of Milli-Q water for 15 min.

iv. The 6xHis protein tag developer (50 ml) was added to the gel and agitated for 15 min.

v. The gel was washed twice with 100 ml of Milli-Q water for 15 min.

vi. The gel was irradiated with ultraviolet light (~300 nm) using an UV transilluminator. 6xHis tagged proteins fluoresced as yellow bands when viewed under ultraviolet light.

3.2.7.3.7 Enzyme detection

3.2.7.3.7.1 Glycol chitin

Glycol chitin was obtained by acetylation of glycol chitosan as described by Trudel and Asselin (1989). Five gram of glycol chitosan (Sigma, USA) was dissolved in 100 ml of 10% acetic acid by grinding in a mortar. The viscous solution was allowed to stand overnight at 22°C. Methanol (450 ml) was slowly added and the solution was vacuum filtered through a Whatman No. 4 filter paper. The filtrate was transferred into a beaker and 7.5 ml of acetic anhydride was added with stirring. The resulting gel was allowed to stand for 30 min at room temperature and then cut into small pieces. The liquid extruding from the gel pieces was discarded. Gel pieces were transferred to a waring blender, covered with methanol and homogenized for 4 min at maximum speed. This suspension was centrifuged at 27,000 g for 15 min at 48°C. The gelatinous pellet was resuspended in about one volume of methanol, homogenized and centrifuged as in the preceding step. The pellet was resuspended in distilled water (500 ml) containing 0.02% (w/v) sodium azide and homogenized for 4 min. This was the final 1% (w/v) stock solution of glycol chitin.
3.2.7.3.7.2 Native PAGE analysis for hydrolytic activity

Chitinase and chitosanase were detected under native condition on 7.5% separating gel copolymerized with 100 µM of the fluorogenic substrate (Sigma), 4-methylumbelliferyl-\(\text{N-}\)acetyl-\(\beta\)-D-glucosaminide (4-MU-(GlcNAc) for NAGase and 0.1% glycol chitosan (Sigma, USA) for chitosanase. In-gel detection of corresponding molecular weight of the protein was done using native protein markers (Genei). Chitinase (NAGase) was reactivated in the gels by removing SDS upon incubating the gel with 1% Triton-X in 100 mM Tris buffer, pH 8.0 (Hung et al., 2002) followed by incubation at 37°C for 30 min in 100 mM sodium acetate buffer pH 5.0. The enzyme activity appeared as fluorescent band under UV light because of enzymatic hydrolysis of fluorescent 4-methyl umbelliferone from the GlcNAc disaccharide. For detection of chitosanase, the gels were incubated in 150 mM sodium acetate buffer at pH 5.0 for 5 min and then in 100 mM sodium acetate buffer at pH 5.0, containing 0.1% glycol chitosan (Sigma, USA) for 30 min at 37°C. The gels were finally transferred into a solution containing 0.01% (w/v) fluorescent brightener 28 (Sigma) in 500 mM Tris HCl (pH 8.9). After 5 min, the brightener solution was removed and the gels were rinsed with distilled water for more than 1 h. Lytic zones were visualized and photographed under UV light in alpha imager (Alpha InfoTech Corporation, UK).

3.2.7.4 Enzymatic assay

Chitinase assay was performed as described in 3.2.5.7. For chitosanase assay, the reagents A through F were prepared as follows:
A. 1000 mM acetic acid solution (Prepared 25 ml in deionized water using acetic acid)
B. 55 mM sodium acetate buffer, pH 5.5 at 37°C (Prepared 100 ml in deionized water using sodium acetate trihydrate and the pH was adjusted to 5.5 at 37°C using 1 N NaOH.)
C. 0.1% (w/v) chitosan substrate solution with 150 mM acetate buffer, pH 5.5 at 37°C (chitosan) (dissolved 50 mg of chitosan in 5 ml of reagent A. The solution was mixed for 1 to 3 h, until chitosan was completely dissolved. Once dissolved, the solution was diluted to 50 ml with reagent B and the pH of solution was adjusted to 5.5 at 37°C using 1 N NaOH.)
D. 500 mM sodium hydroxide solution (prepared 100 ml in deionized water using sodium hydroxide)

E. 16.4 mM p-hydroxy-benzoic hydrazide in 500 mM sodium hydroxide color reagent (PAHBAH) (Dissolved 250 mg of p-hydroxy-benzoic hydrazide with 100 ml of reagent D. The solution was stable for 2 h)

F. 1.5 mM D(+)glucosamine standard solution (prepared 100 ml in deionized water using D(+)glucosamine)

G. 50 mM sodium acetate buffer, pH 5.5 at 37°C (diluted 50 ml of reagent B to 55 ml with deionized water and the pH was adjusted to 37°C.)

H. Chitosanase enzyme solution (immediately before use, a solution containing 0.4 to 2.0 units/ml of chitosanase in cold reagent G was prepared)

The assay was carried out as follows:

i. A sample (0.975 ml) and blank solution (0.975 ml) for reagent C (chitosan) was pipetted into microfuge tubes. While the sample solution was equilibrated to 37°C, the blank solution was placed in an ice bath.

ii. Reagent H (enzyme soln) (0.025 ml) was added to the sample solution, mixed immediately by inversion and incubated at 37°C for exactly 10 min. The reaction was stopped by removing a 0.4 ml aliquot of test solution to a microfuge tube containing 0.8 ml of reagent E (PAHBAH).

iii. Similarly, reagent H (enzyme soln) (0.025 ml) was added to the blank solution in an ice bath. Immediately stopped the reaction by removing a 0.4 ml aliquot of test solution to a microfuge tube containing 0.8 ml of reagent E (PAHBAH).

iv. All the tubes were placed in a boiling water bath for exactly 5 min and then were placed in an ice bath to cool to room temperature.

v. The tubes were centrifuged in a microcentrifuge for 5 min, the supernatants were removed and A_{405nm} was recorded for the sample and blank.

One unit of chitosanase was defined as the amount of enzyme required to liberate 1.0 micromole of reducing sugars (D-Glucosamine equivalents) from chitosan per min at pH 5.5 at 37°C.
3.2.7.5 Characterisation of purified glycosyl hydrolase

The purified enzyme was characterized with respect to its optimum pH and temperature, stability at different temperatures and pH values and metal ion effect. To study the influence of temperature, chitinase and chitosanase activities were assayed in triplicate at different temperatures ranging from 10-70ºC at pH 5.0 of 50 mM sodium acetate buffer. To determine thermostability, the enzyme preparation in buffer was incubated at temperatures ranging from 10-70ºC for 1 h. The enzyme activities were assayed at 37ºC and pH 5.0. The enzyme activities were also assayed in triplicate at different pH values (pH 3.0 to 6.0) of 50 mM sodium acetate buffer and pH 6.5 to 9 of 50 mM Tris-HCl buffer. To determine pH stability, the enzyme preparation in buffer at different pH ranging from 3.0-9.0 was kept at room temperature for 2 h and the respective enzyme assays were performed.

The effect of metal ions such as MgSO₄, MnCl₂, CaCl₂, CuSO₄, HgCl₂, ZnCl₂, FeCl₃ and EDTA on enzyme activity was studied by incorporating the metal ions and EDTA at 5 mM concentration in pH 5.0 of 50 mM sodium acetate buffer. The enzyme preparation in buffer was incubated at room temperature for 1 h and the enzyme activities were determined under standard assay conditions.

3.2.7.6 Antifungal assay

The recombinant protein was also evaluated for its antifungal activity. To prepare pure cultures, the fungal spores of the respective pathogens were taken separately from agar slants with the help of a sterile loop, and resuspended in 1 ml SDW in a microfuge tube. The suspension was serially diluted to 10⁻⁴ dilution and isolated colonies of fungal pathogens were obtained by pour plating on PDA after incubating at 28±2ºC for 5 days. Spore suspensions of FOD and AS were prepared with SDW, filtered through a 44 µm (325 mesh) screen, quantified by counts with a haemocytometer and adjusted to the desired (3x10⁵ conidia/ml) concentration by dilution with SDW. Aliquots comprising the purified protein of 5, 10 and 20 µg in triplicate were mixed individually with 20 µl spore suspension to a total volume of 30 µl and added to 8 mm wells made on PDA plates. Spore suspension mixed with sodium phosphate buffer and sodium phosphate buffer alone served as controls. The plates were
incubated at room temperature (28±2°C) and the inhibition of mycelial growth was observed after 3 days of incubation. The experiment was repeated twice.

3.2.7.7 Statistical analyses

All the experiments were replicated thrice and were carried out in completely randomized design. Statistical analyses of the experiments were performed using the package, IRRISTAT version 92-1 developed by the International Rice Research Institute Biometrics Unit, The Philippines.