MATERIALS AND METHODS
2.1.1 Cultures

The details of the bacterial, fungal strains and plasmids used in the present study are listed in Table 2.1.

2.1.2 Media

2.1.2.1 Luria Bertani medium (LB medium)

To 900 ml of water, 10 g tryptone, 10 g NaCl, 5 g yeast extract and 15 g agar was added. The pH was adjusted to 7.2 and volume was made up to 1 litre

2.1.2.2 Chitinase detection agar

Colloidal chitin-10 g, Na$_2$HPO$_4$-0.065 g, KH$_2$PO$_4$ – 1.5 g, NaCl-0.25 g, NH$_4$Cl-0.5 g, MgSO$_4$-0.12 g, CaCl$_2$-0.005 g and agar 15 g. The pH was adjusted to 6.5 and the volume was made up to 1 litre

2.1.3 Chemicals

Agarose and other molecular biology grade chemicals were procured from Sigma-Aldrich (USA) unless otherwise stated. Antibiotics were purchased from Calbiochem. All other chemicals and routine laboratory media components for bacterial culture were of analytical grade and obtained from Merck, HiMedia laboratories (India) unless otherwise stated. Polymeric chitin and chitosan substrates were kindly provided by Dr. Dominique Gillete, Mahatani Chitosan Pvt., Ltd. (Veraval, India). Chitooligosaccharides with different degrees of polymerization (DP) were purchased from Seikagaku Corporation (Tokyo, Japan).
2.1.4 Kits and enzymes

GeneElute HP plasmid miniprep kit, Plasmid isolation kit, and Taq DNA polymerase were from Sigma- Aldrich (USA), and Gel extraction kit and DNeasy kit for genomic DNA isolation were procured from Qiagen (Germany). pGEM-T Easy was obtained from Promega (USA). *Pfu* DNA polymerase, T4 DNA ligase and all restriction enzymes were from MBI Fermentas (Germany).

2.1.5 Genomic DNA isolation from *Stm k279a*

Genomic DNA (gDNA) was isolated using DNeasy kit from *Stm k279a*. Broth cultures were harvested at the end of the exponential growth phase by centrifugation at 16000 g for 15 min. Pelleted cells were processed for gDNA isolation as per the manufacturer’s protocol.

2.2 SEM for *Stm k279a*

Overnight grown culture of *Stm k279a* cells in LB medium were centrifuged and the cell pellet was resuspended and fixed in a freshly prepared mixture of 2% glutaraldehyde (v/v) and 2% paraformaldehyde (v/v) in 0.1 M phosphate buffer (pH 7.2). After rinsing in phosphate buffer, samples were dehydrated in a graded ethanol series and reaction mixture was applied onto cover slips (10μL drops), and air dried to fix the sample. The cover slips containing the reaction mixtures were glued onto scanning electron microscopy (SEM) aluminum studs with carbon tape and sputter-coated (JEOL FC 1100) with gold-palladium. Scanning was performed in a Philips SEM at 20 kV.
2.3 Strain identification and confirmation using 16S rDNA

Molecular tools are now frequently used to analyze the diversity of specific genes in pure cultures and from microbial communities. To confirm the identity of the strain *Stenotrophomonas maltophilia*®434 (*Stm*) (Obtained from MTCC, IMTECH), gDNA (isolated as above) was used to amplify 16S rRNA conserved region using universal primers 27F and 1489R (Table 2.2) using thermocycler (Eppendorf Mastercycler Gradient, Germany) at 50°C annealing temperature. The PCR product was analyzed on 1% agarose gel and eluted for sequencing at Eurofins Pvt. Ltd., Bangalore, India. The partial sequences were matched with the nucleotide database available at GenBank, using BLAST tool in NCBI (National Centre for Biotechnology Information). A phylogenetic tree was constructed by using MEGA (Molecular Evolutionary Genetics Analysis) version 5 (Tamura *et al*., 2011). Simultaneously primers were designed for GH18 conserved domain using CODEHOP (Consensus Degenerate hybrid oligonucleotide primers) program. GH18 conserved region was amplified at gradient temperatures of 60±5°C using GH18 FP & RP primers (Table 2.2) and the amplicon was sequenced at Eurofins Pvt. Ltd., Bangalore, India.

2.4 Chitinolysis by *Stm* k279a on chitinase detection agar medium

Single colony of *Stm* k279a, grown on LB plate, was spotted on to the chitin agar plate containing minimal medium with colloidal chitin and incubated at 30°C for 4-5 days to observe the clearance zone around the bacterial colony.
2.5 Cloning and characterization of chitinases from *Stm* k279a

The genome sequence of *Stm* k279a was downloaded from NCBI FTP site ([ftp://ftp.ncbi.nih.gov/genomes/](ftp://ftp.ncbi.nih.gov/genomes/)) and searched for ORFs containing putative function for chitin modifying enzymes were selected for the study.

### 2.5.1 PCR amplification and cloning of *Stm* chitinases

Primers were designed (Table 2.2) for the amplification of chitin degrading/modifying genes from the g DNA of *Stm* k279a. Genes encoding *Stm* ChiA, *Stm* ChiB, *Stm* Nag, *Stm* CbpD1, *Stm* Pda1 and *Stm* Pda2 were amplified by gene specific primers using *Pfu* DNA polymerase. Amplicons (*Stm*ChiA, *Stm*ChiB and *Stm*Nag) were gel extracted using Qiagen gel clean up kit and cloned into bacterial expression vector pET22b (+) in *NcoI/XhoI, NcoI/HindIII* sites, respectively. Amplicon CbpD1 was cloned into *NcoI/XhoI* in pET22b (+), Pda1 and Pda2 were cloned into pRSET A expression vector in *BamHI/HindIII, BamHI/EcoRI* restriction sites. Vectors and amplicons were gel purified and ligated using T4 DNA ligase at 16°C for 16 h. The resultant plasmids were designated as pET22- ChiA, pET22- ChiB, pET22- Nag, pET22-CbpD1 pRSET- Pda1 and pRSET- Pda2. pET22- ChiA, pET22- ChiB, pET22- Nag were transformed into *E. coli* to express *Stm* ChiA, *Stm* ChiB and *Stm* Nag, respectively.

### 2.5.2 Expression and purification of *Stm* chitinases

#### 2.5.2.1 Expression

Highly efficient competent cells of *E. coli* rosetta-gami 2 (DE3) were transformed with the recombinant plasmids pET 22b*-Stm* ChiA, pET 22b -*Stm* ChiB and pET 22b -*Stm* Nag, and selected on ampicillin plates. The cells harboring positive plasmids were grown at 37 °C and
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1 mM IPTG was added to induce the cells at an OD$_{600}$ of 0.6 and continued for 3 h. The cells were harvested and further processed for purification of chitinolytic enzymes. The expressed recombinant proteins from plasmids pET 22 b-\textit{Stm} ChiA, pET 22 b -\textit{Stm} ChiB and pET 22 b -\textit{Stm} Nag were designated as \textit{Stm} ChiA, \textit{Stm} ChiB and \textit{Stm} Nag, respectively.

2.5.2.2 Protein purification

The expressed proteins of \textit{Stm} ChiA, \textit{Stm} ChiB and \textit{Stm} Nag were isolated from respective cell pellets by whole cell lysate using sonicator. The cell pellets of expressed \textit{Stm} ChiA, \textit{Stm} ChiB and \textit{Stm} Nag were resuspended in Ni-NTA equilibration buffer (50 mM NaH$_2$PO$_4$, 100 mM NaCl and 10 mM imidazole pH 8.0), and the cells were lysed by sonication at 20% amplitude with 30 x 15 s pulses (with 20 s delay between pulses) on ice, with a Vibra cell Ultrasonic Processor, converter model CV33, equipped with a 3 mm probe (Sonics, Newtown, CT, USA). The sonicated material was centrifuged at 15, 200 g for 10 min at 4°C in order to pellet the insoluble cell debris. At this stage, \textit{Stm} ChiA, \textit{Stm} ChiB, and \textit{Stm} Nag were found in the soluble fraction. The cleared lysate was applied to a Ni-NTA column (Sigma, USA) equilibrated with running buffer (50 mM NaH$_2$PO$_4$, 100 mM NaCl and 10 mM imidazole pH 8.0). \textit{Stm} chitinases were eluted by running four column volumes of elution buffer (50 mM NaH$_2$PO$_4$, 100 mM NaCl and 250 mM imidazole) through the column. The pure fraction containing chitinase was collected, concentrated and buffer-exchanged with 50 mM sodium acetate buffer pH 5.0 using Macrosep Centrifugal Devices (Pall Corporation, USA).
2.5.2.3 SDS-PAGE analysis

The protein samples were separated by SDS-PAGE on vertical slab gels according to Laemmli (1970). The stacking gel contained 4.5 % polyacrylamide in 0.125 M Tris-HCl, pH 6.8 and the resolving gel contained 12% polyacrylamide in 0.375 M Tris-HCl, pH 8.8. Electrode buffer contained 0.025 M Tris-HCl, 0.192 M glycine and 0.1% (w/v) SDS of pH 8.5. The samples were boiled at 100°C for 5 min in sample buffer [1% SDS (w/v) and 12% glycerol (v/v), in 0.063 M Tris-HCl, pH 6.8] and electrophoresis was carried out at 50V in stacking gel and at 100V in resolving gel. The gels were stained in a solution containing 0.5% (w/v) coomassie brilliant blue G-250, 30% (v/v) methanol and 10% (v/v) glacial acetic acid, and destained in a solution containing 30% (v/v) methanol and 10% (v/v) glacial acetic acid till the protein bands were visible against a clear background.

2.5.2.4 Zymogram analysis for Stm chitinases

Dot blot assay was used to detect the activity of purified recombinant chitinases. Polyacrylamide gel supplemented with 0.1% glycol chitin was prepared. Five µg of Stm chitinases were spotted on to the gel and incubated under humid conditions at 37°C for 3 h. The gel was stained with 0.01% calcofluor white M2R in 0.5M Tris-HCl pH 8.9 for 10 min at 4°C. Finally, the brightener solution was removed, and the gel was washed with distilled water for 10 min at 4°C. Zone of clearance was observed by placing the gel under UV transilluminator.
2.5.3 Characterization of *Stm* chitinases

2.5.3.1 Chitinase assay

Chitinase activity was determined by a modified Schales’ procedure (Reducing end assay) using chitin hexamer as the substrate (Imoto and Yagishita, 1971). The reaction mixture (40 µl) consisting of recombinant *Stm* chitinases (1.79 µM of *Stm* ChiA or *Stm* ChiB or *Stm* Nag) and chitin hexamer (300 µM) in 50 mM buffer (*Stm* ChiA: sodium acetate pH 5.0, *Stm* ChiB: sodium phosphate pH 7.0, *Stm* Nag: sodium acetate pH 5.0) incubated at 40°C for 1 h. After incubation, 300 µL of color reagent (0.5 M sodium carbonate and 0.05% potassium ferricyanide) was added and boiled for 15 min in dark. Reaction mixture was allowed to cool down, followed by estimation of reducing ends produced in triplicates. Supernatants were loaded onto 96 well microtitre plates. OD was measured at 420 nm using microtitre plate reader (Multiskan, Labsystems, Finland). One unit was defined as the amount of enzyme that liberated 1µmol of reducing amino sugar per minute. The reducing end assay was done as above unless stated otherwise.

2.5.3.2 Kinetic analysis

2.5.3.2.1 Kinetic analysis of *Stm* chitinases

Chitinase activity of *Stm* chitinases was measured by incubating the recombinant enzymes (5 µg of *Stm* ChiA or *Stm* ChiB or *Stm* Nag) with different concentrations of chitin hexamer (50- 600 µM) in 50 mM buffers (*Stm* ChiA: sodium acetate pH 5.0, *Stm* ChiB: sodium phosphate pH 7.0, *Stm* Nag: sodium acetate pH 5.0) with respective substrate controls in triplicates at 40°C for 1 h. The assay was done as described in 2.5.3.1. Enzyme activity was defined as the release of one micromole of GlcNAc per sec under standard experimental conditions.
conditions. Specific activity in nanokat/mg of protein was calculated and kinetic values were analyzed from three independent sets of data fitting to the Michaelis-Menten equation by non-linear regression function available in Graph Pad Prism version 5.0 (Graph Pad Software Inc., San Diego, CA).

2.5.3.2.2 Comparative analysis of kinetic parameters of chitinases on chitohexaose

Chitinases from *Stenotrophomonas maltophilia* (Stm), *Bacillus thuringiensis* (Bt), *B. licheniformis* (Bli) and *Serratia proteamaculans* (Sp) were compared to assess the kinetic properties. Chitinase activity of the aforesaid chitinases was measured by incubating the equimolar concentrations of the recombinant enzymes (1.79 µM of Stm ChiA or Stm ChiB or Stm Nag or Bt Chi or Bli Chi or Sp ChiA or Sp ChiB or Sp ChiC or Sp ChiD) with different concentrations of chitin hexamer (50-600 µM) in 50 mM sodium acetate pH 5.0 buffer. Respective substrate controls in triplicates were kept at 40°C for 1 h and chitinase assay was done as described in 2.5.3.1. Kinetic analysis was done as described in 2.5.3.2.1.

2.5.3.3 Optimum temperature

Optimum temperature for Stm chitinases was determined by incubating the enzymes (1.79 µM of Stm ChiA or Stm ChiB or Stm Nag) with chitin hexamer (300 µM) in 50 mM buffers (*Stm* ChiA: sodium acetate pH 5.0, *Stm* ChiB: sodium phosphate pH 7.0, *Stm* Nag: sodium acetate pH 5.0) for 1 h at 20, 40, 60, 80 or 100°C. Specific activity was determined under standard assay conditions as described in 2.5.3.1.
2.5.3.4 Stability at optimum temperature

*Stm* chitinases (1.79 µM of *Stm* ChiA or *Stm* ChiB or *Stm* Nag) were pre-incubated at 40°C for 1 h followed by reducing end assay with chitin hexamer (300 µM) in 50 mM buffers (*Stm* ChiA: sodium acetate pH 5.0, *Stm* ChiB: sodium phosphate pH 7.0, *Stm* Nag: sodium acetate pH 5.0) for 1 h. Specific activity was determined under standard assay conditions as described in 2.5.3.1.

2.5.3.5 Thermal stability

*Stm* chitinases (1.79 µM of *Stm* ChiA or *Stm* ChiB or *Stm* Nag) were pre-incubated at 30, 40, 50, 60, 70, 80, 90 or 100°C for 1 h, followed by chitinase assay with chitin hexamer (300 µM) in 50 mM buffers (*Stm* ChiA: sodium acetate pH 5.0, *Stm* ChiB: sodium phosphate pH 7.0, *Stm* Nag: sodium acetate pH 5.0) for 1 h. Specific activity was determined under standard assay conditions as described in 2.5.3.1.

2.5.3.6 Optimum pH

The optimum pH of *Stm* chitinases was determined by incubation of enzymes (1.79 µM of *Stm* ChiA or *Stm* ChiB or *Stm* Nag) in different pH buffers ranging from 2-12 for 1 h at 37°C under standard assay conditions using 300 µM of chitin hexamer as a substrate. The buffers used were 50 mM sodium citrate buffer (pH 2-5), 50 mM sodium acetate buffer (pH 5-6), 50 mM sodium phosphate buffer (pH 6-8), 50 mM glycine- NaOH buffer (pH 8-10) and 50 mM NaH2PO4-NaOH buffer (pH 10-12). Specific activities of the enzymes were determined under standard assay conditions as described in 2.5.3.1.
2.5.3.7 Substrate specificity of *Stm* chitinases

Reducing end assay (2.5.3.1) was performed using 1.79 µM of purified recombinant enzymes (*Stm* ChiA or *Stm* ChiB or *Stm* Nag) incubated with 50 µl of 0.1 % chitosan with different degrees of deacetylation (DDA 70 or 90) and water soluble chitosan (WSC) at 37 ºC for 1 h in 50 mM of sodium acetate buffer, pH 5.2.

2.5.3.8 Binding of recombinant *Stm* chitinases

2.5.3.8.1 Insoluble polymer substrate binding

Equimolar (1.79 µM) concentrations of recombinant proteins (*Stm* ChiA or *Stm* ChiB or *Stm* Nag) were incubated with 1 mg (dry weight) of α- chitin, β-chitin, Avicel (microcrystalline cellulose) and WSC in 1 ml of 50 mM sodium acetate pH 5.2 for 1 h, on ice. After incubation, reactions were stopped by centrifugation (16,100 g, 10 min and 4 ºC). Unbound protein was measured from the supernatant using BCA method. The relative percentage of bound protein was calculated as the total protein minus the unbound protein.

2.5.3.8.2 Soluble polymer substrate binding

The binding of the recombinant *Stm* ChiA, *Stm* ChiB and *Stm* Nag to soluble substrates was evaluated using affinity electrophoresis in 8 % polyacrylamide gels polymerized in the absence or presence of soluble polysaccharides (0.1 %) like laminarin, CM cellulose and glycol chitin. Electrophoresis was performed for 1.5 h at 4 ºC at a constant voltage of 80 V. The gels were stained with coomassie blue to detect the retardation in the mobility of the protein. Binding was assessed visually or, alternatively, the migration distances of the chitinases and reference proteins were measured directly on the resolving gels.
2.5.4 Analysis of hydrolysis products of Stm chitinases on chitooligosaccharides (CHOS) and chitin

2.5.4.1 Thin layer chromatography for colloidal chitin hydrolysis by Stm ChiA

Reaction mixture containing 1mg/ml of colloidal chitin as a substrate and 1.79 µM of Stm ChiA in sodium acetate buffer pH 5.0 was incubated at 40°C. Samples were withdrawn at intervals from 0 min -12 h and the reaction was stopped by the addition of equal volume of 0.1 N NaOH. The reaction products containing different CHOS were analyzed. Aliquots (20 µl) of the reaction mixtures were chromatographed on a silica gel plate (TLC silica gel 60, Merck Co., Germany) with a solvent system containing n-butanol, methanol, 25% ammonia solution-water [5:4:2:1(v:v:v:v)], and the products were detected by spraying the plate with aniline-diphenylamine reagent (400 µl aniline, 400 mg of diphenylamine, 20 ml of acetone and 3 ml of 85% phosphoric acid) and baking it at 180°C using hot air gun (Black &Decker, Germany) for 3 min.

2.5.4.2 High performance liquid chromatography (HPLC)

Analyses of the hydrolysis of chitin polymers and oligomers by Stm chitinases were conducted by incubating recombinant enzymes (1.79 µM of Stm ChiA or Stm ChiB or Stm Nag) in 50 mM buffer (Stm ChiA: sodium acetate pH 5.0, Stm ChiB: sodium phosphate pH 7.0, Stm Nag: sodium acetate pH 5.0). Reaction mixtures were incubated at 40°C in a thermomixer, and samples were collected at 0, 1, 2, 3, 5, 10, 15, 20, 30, 40, 60, 120, 180 and 720 min; 50 µL of the reaction mixture was transferred to an eppendorf tube containing 50 µL of 70% acetonitrile, to stop the reaction. For polymers, the reaction mixtures were centrifuged at 16,100 g for 10 min at 4°C to remove the undigested chitin polymers. The
supernatant was further concentrated (Eppendorf concentrator, Germany) till the complete evaporation of the solvent without heating. The residue was dissolved in 20 μL of 35% acetonitrile and reaction mixtures were stored at -20°C until analyzed by isocratic HPLC at 25°C using a Shimadzu10ATvp UV/VIS HPLC system (Shimadzu corporation, Tokyo, Japan) equipped with a Shodex Asahipack NH2P-50 4E column (4.6 ID x 250 mm) (Showa Denko K.K,USA). Twenty microliter of the reaction mixture was injected in to the HPLC using Hamilton syringe (HAMILTON Bonaduz, Switzerland). The liquid phase consisted of 70% acetonitrile: 30% MilliQ H₂O and flow rate was set to 0.70 ml/min, eluted CHOS were monitored by recording absorption at 210 nm. Based on the peak areas obtained from HPLC profiles, CHOS concentrations were calculated using authentic oligosaccharide solutions obtained from Seikagaku Corp., (Tokyo, Japan). CHOS HPLC mixture, which contains the equal weights of oligomer ranging from DP1- DP6 was used for standard graph preparation. Standard calibration curves of CHOS moieties were constructed separately for each oligosaccharide. These data points yielded a linear curve for each standard amino sugar with the R² values of 0.997-1.0, allowing molar concentrations of CHOS to be determined with a confidence.

2.5.4.2.1 Time-course analysis for α- and β- chitin

All the three recombinant enzymes (1.79 μM of Stm ChiA or Stm ChiB or Stm Nag) were incubated with 1 mg/ml chitin polymers (α- and β-) at 40°C for 12 h. Reaction mixtures were centrifuged at 16,100 g for 10 min at 4°C to remove the undigested chitin polymers. The supernatant was further concentrated (Eppendorf concentrator, Germany) till the complete evaporation of the solvent without heating. The residue was dissolved in 20 μL of 35%
acetonitrile and reaction mixtures were stored at -20°C until analyzed by isocratic HPLC at 25°C. Oligosaccharides produced were estimated against the standard slope.

2.5.4.2.2 Time-course analysis for α- and β- chitin up to 12 h

Polymers α- and β- chitin (1 mg/ml) were incubated with 1.79 μM of Stm ChiA at 40°C for 0, 2, 5, 10, 20, 30, 40, 60, 120, 180, and 720 min. Reaction products were quantified using HPLC as described in 2.5.4.2.

2.5.4.2.3 Scanning electron microscopy of α- chitin

α –chitin (1 mg/ml) suspension with and without Stm ChiA in 50 mM sodium acetate buffer, pH 5.0 was pre incubated for 16 h at 37 °C in 1.5 ml reaction tubes. Reaction mixture was applied onto cover slips (10 μL drops), and air dried to fix the sample. The cover slips containing the reaction mixtures were glued onto scanning electron microscopy (SEM) aluminum studs with carbon tape and sputter-coated (JEOL FC 1100) with gold-palladium. Scanning was performed in a Philips SEM at 20 kV.

2.5.4.2.4 Reaction time – course of DP6-DP2 substrates hydrolysis

Hydrolysis products generated by Stm ChiA, Stm ChiB and Stm Nag from DP2- DP6 substrates were analyzed on isocratic HPLC as described in 2.5.4.2. Reaction mixture containing 1.79 μM of Stm chitinases (Stm ChiA or Stm ChiB or Stm Nag) and 2.5 mM of each individual substrate ranging from DP2-DP6 incubated separately in a reaction tube containing 50 mM sodium acetate buffer pH 5.0 from 0 min- 24 h. Oligosaccharides produced were estimated against the standard slope of each substrate. Products were
quantified from respective peak areas by using standard calibration curves of CHOS ranging from DP1-DP6. Graphs were constructed using Origin 8.0 software.

2.5.5. Antifungal activity of Stm chitinases

*Stm* ChiA and *Stm* Nag were tested for antifungal activity against a range of phytopathogenic fungi on potato dextrose agar (PDA) plate. The zone of inhibition assay for antifungal activity was executed using 90x15mm petri plates containing 25 ml of PDA. After the mycelial colony had developed, sterile blank paper disks (0.5 cm in diameter) were placed around and at a distance of 1 cm away from the rim of the mycelial colony. An aliquot (20 µl containing 50 µg) of chitinase in 50 mM sodium acetate buffer (pH 5.0) was introduced to a disk. The plates were incubated at 24°C for 72 h until mycelial growth had enveloped peripheral disks buffer control and had produced crescents of inhibition around disks containing samples with antifungal activity. The fungi used in this test included *Fusarium solani, F. oxysporum, F. moniliforme, Macrophomina phaseolina, Colletotrichum dematium, Curvularia lunata* and *Rhizopus stolonifer*.

2.5.5.1 SEM for fungal spores

Spores of *F. oxysporum* were treated with 50 mM sodium acetate buffer pH 5.0 and 5 µg of *Stm* ChiA. These were incubated at 24°C for 12 h in 1.5 ml reaction tubes and observed under SEM as described earlier in 2.5.4.2.3.