Characterization of chitinases and a hexosaminidase from *Stenotrophomonas maltophilia* k279a

Thesis submitted for the degree of
DOCTOR OF PHILOSOPHY

by

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CERTIFICATE

This is to certify that Ms. KATTA SUMA has carried out the research work embodied in the present thesis under the supervision and guidance of Prof. Appa Rao Podile for a full period prescribed under the Ph.D. ordinances of this University. We recommend her thesis entitled **"Characterization of chitinases and a hexosaminidase from *Stenotrophomonas maltophilia* k279a"** for submission for the degree of Doctor of Philosophy of the University.

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DECLARATION

I hereby declare that the work embodied in this thesis entitled "**Characterization of chitinases and a hexosaminidase from *Stenotrophomonas maltophilia* k279a**" has been carried out by me under the supervision of Prof. Appa Rao Podile and this has not been submitted for any degree or diploma in this or any other University earlier.

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CONTENTS

ABBREVIATIONS

^{o}C	degree centigrade/degree Celsius
C-terminal	carboxy terminal
CBD	carbohydrate binding domain
ChBD	chitin binding domain
CeBD	cellulose binding domain
dNTPs	deoxy nucleotide triphosphates
DNA	deoxy ribonucleic acid
DDA	degree of deacetylation
EDTA	ethylene diamine tetra acetic acid
g	gram
GlcNAc	N-acetyl glucosamine
h	hour (s)
IPTG	isopropyl β-D-thiogalactoside
Km	Michaelis Menton constant
kb	kilobase pair
kDa	kilodalton
LB	Luria-Bertani
L	litre
M	molar
mg	milligram
min	minute
ml	milliliter
mM	millimolar
mm	millimeter
ng	nanogram
Ni-NTA	nickel-nitroacetic acid agarose
nm	nanometer

nM	nanomolar
N-terminal	amino terminal
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
SDS	sodium dodecyl sulphate
Tris	tris-(Hydroxymethyl) aminoethane
U	Unit
μg	microgram
μL	microlitre
μmol	micromole
CHOS	chitooligosaccharides

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INTRODUCTION

1.1 Chitin is a renewable amino sugar polymer

Chitin is an abundant extracellular amino-sugar polymer that in association with other biomacromolecules (carbohydrates, proteins) forms an essential structural component. The polysaccharide, sometime referred to as "animal cellulose", is a major cell wall component of many agronomically important pests, including cell walls of fungi as well as exoskeletons in arthropods such as crustaceans (e.g., crabs, lobsters and shrimps) and insects. It is also an integral component in the radulas of mollusks, and the beaks of cephalopods including squid and octopuses. The worldwide annual crustacean shells production was estimated to be ~ 3 million tons.

Structurally chitin may be compared to the polysaccharide cellulose and functionally to the protein keratin. It is a β-(1→4)-linked homopolymer of N-acetyl-D-glucosamine (GlcNAc), differs with cellulose in having an acetamide group (NH.CO.CH3) in place of hydroxyl (–OH) group at the C2 position of glucose (Fig 1.1A). Chitin is one of the largest forms of renewable biomass on earth, second only to cellulose.

An enormous amount of chitin is synthesized in the biosphere. About 10^{11} metric tons is produced annually in the aquatic biosphere alone. However, there is no substantial accumulation of chitin in ocean sediments due to a wide distribution of marine bacteria that degrade and catabolize chitinous particles, allowing carbon and nitrogen to return to the ecosystem. Chitin has become a subject of great interest not only as an under-utilized resource but also as a new functional biomaterial of high potential in various fields.

1.2 Chitin occurs in three different forms

Three different crystalline polymorphic forms of chitin exist in natural conditions. These three forms differ in the packing and polarity of GlcNAc chains. The most abundant α-chitin, where sheets of GlcNAc are antiparallel, is present in arthropods and fungi. β- chitin made of parallel chains of GlcNAc has been identified in the pen of squid *Loligo,* and the spines of several diatoms. The third form, γ- chitin, is rarest among the three in which two GlcNAc chains run in one direction, and another in opposite direction. This form has been reported in cocoon fibres of the beetle *Ptinus.* Analysis of the tensile mechanical properties of the three forms of chitins revealed that all of them behaved as viscoelastic polymers, but varied in stiffness, strength and extensibility by several orders of magnitude. α- chitin being the stiffest of all, with higher intermolecular hydrogen bonding, exhibits highest maximum tensile strength.

1.3 Chitosan is deacetylated form of chitin

N- Deacetylation of chitin with enzyme or alkaline solution produces chitosan, which is a cationic biopolymer. Chitin can be partially or fully de-acetylated to yield chitosans. Chitosan is the only known natural polycationic polymer with numerous unique and biotechnologically interesting properties. Chitosan has been used to protect crop plants from disease. Chitosan has antimicrobial properties which can be used in tissue and paper coating. Chitosan can bind heavy metals and proteins and is, therefore, used in drinking water purification and in waste water treatment. Chitosan can also be used in wound dressings to promote scar-free wound healing (Rinaudo, 2006).

1.4 Chitin can be modified either chemically or enzymatically

Chitin is an intractable polymer, as it is insoluble in water and many organic solvents. Current methods to modify chitin and transform it to useful carbohydrate products require harsh chemical treatments that incur problems of undesirable by-products, hydrolysis and chemical modification. Acid hydrolysis produces lower yields of oligosaccharides and a large amount of monomeric GlcNAc (Wang *et al*., 2006). Industry-level preparation of water-soluble chitin oligosaccharides has been obtained largely by hydrolysis of chitin with chemical reagent e.g. hydrochloric acid or using chitinolytic enzymes (Einbu *et al*., 2007; Suginta *et al*., 2005; Wang *et al*., 2006). Oligosaccharides produced by acid hydrolysis might be toxic because of chemical changes during conversion. Production of specific oligosaccharides by chemical methods is challenging. It requires selective protection and manipulation of chemically similar saccharide donors and acceptors, and also not easily repeatable. Using chemical reagents, however, is less favorable owing to environmental pollution and lower yields for specific size of chitin-oligosaccharides. The salts of chitooligosaccharides are not suitable for oral administration owing to bitter taste. Although GlcNAc is produced by acid hydrolysis of chitin, this procedure still has problems such as high cost, and low yield (Sakai, 1995), and acidic wastes are generated. N-acetylation of glucosamine is also possible to produce GlcNAc. This product, however, is not approved as a natural material since it involves chemical process. Enzymatically modified and synthesized chitinous polymers and oligosaccharides possess versatile functional properties (Rinaudo, 2006) useful for targeted biological functions.

1.5 A repertoire of enzymes are involved in chitin hydrolysis

The degradation of abundant insoluble carbohydrate polymers such as cellulose and chitin is achieved in nature with the help of an array of glycosyl hydrolases with different substrate preferences and product specificities. Enzymatic hydrolysis of chitin is complex, multi-faceted, interconnected and highly harmonized. It involves intracellular and extracellular cascades of biochemical and biophysical transformations. In chitin producing organisms, chitinolytic enzymes are essential for maintaining normal life functions such as morphogenesis in arthropods or cell division and sporulation of yeast and other fungi. The complete hydrolysis of chitin requires two hydrolytic enzymes namely chitinases and hexosaminidases. Bacterial chitinases are grouped into glycosyl hydrolase family 18 and 19. Hexosaminidases belong to glycosyl hydrolase family 3, 20 and 84 based on the conserved amino acid sequence similarity in the catalytically active site. All hexosaminidases have a common evolutionary origin and no relationship to chitinases (Somerville and Colwell, 1993). Exochitinases, act either at reducing end or at the non-reducing end of the chitin chain. Endochitinases hydrolyze internal β-1, 4- glycosidic linkages. Many plant endochitinases, especially those with a high isoelectric point, exhibit an additional lysozyme or lysozyme-like activity.

1.6 Chitinases often recruit accessory domains

Multi-domain organization is considered to be an important mechanism of adaptation and evolution of new functions. Chitinase encoding genes have few accessory domains like chitin binding domain (ChBD), Fibronectin type III domain (FN3) and polycystic kidney disease domain (PKD) other than the catalytically active glycosyl hydrolase domain (GH). van

Aalten *et al*. (2000) provided insight into the catalytic activity of ChiB enzyme and explained the role of ChBD in substrate binding. Recombinant chitinases from *B. licheniformis* were constructed by deleting the C-terminal chitin-binding domain and replacing it with cellulose-binding domain resulting in improved activity and stability of the hybrid chitinase (Neeraja *et al*., 2010a) suggesting the substrate binding role of ChBD in assisting the catalytic activity.

The chitin- binding protein (Cbp21) produced by *Serratia marcescens* potentiates chitinase action by disrupting the structure of the β-chitin substrate (Kolstad *et al*., 2010) strongly suggesting the synergistic effects. One enzyme increasing substrate accessibility for other enzyme by a hitherto unknown disruptive mechanism involving the CBMs. Deletion of the two FnIII domains of ChiA1 from *Bacillus circulans* did not affect chitin-binding, but strongly reduced chitin hydrolyzing activity (Watanabe *et al*., 1994). PKD domain of ChiA participates in effective hydrolysis of powdered chitin through the interaction between two aromatic residues (W30 and W67) and chitin molecule (Orikoshi *et al*., 2005). Wang *et al*. (2010) described the role of PKD domain in collagenolytic serine protease and showed that binding of PKD domain helps in the swelling of the collagen further improving the catalytic efficiency.

1.7 Chitinases exhibit different modes of action

Efficient chitin degradation often requires the combined action of multiple chitinolytic enzymes including chitinases and hexosaminidases. Synergistic effect of multiple enzymes on degradation of chitin (or analogous cellulose) was observed in the simultaneous action of different types of hydrolases (Boisset *et al*., 2000; Gaudin *et al*., 2000). Chitin is degraded to GlcNAc by the cooperative action of two enzymes. Chitinase (EC 3.2.1.14) first hydrolyzes

chitin to chitooligosaccharides (CHOS), and β-N- acetyl hexosaminidase (EC 3.2.1.52) catalyzes hydrolysis of higher chain length chitooligosaccharides into GlcNAc (Merzendorfer and Zimoch, 2003).

Chitinases are classified into families 18 and 19 of glycoside hydrolases based on their amino acid sequence similarity (Henrissat, 1991; Henrissat and Bairoch, 1993; http://www.afmb.cnrs-mrs.fr/CAZY/) at the catalytically active domain. The catalytic domains of family 18 chitinases are characterized as having $(\beta / \alpha)_8$ barrel folds, whereas those of family 19 chitinases have a high α-helical content and a structure similar to those found in chitosanases and lysozymes.

The family 18 chitinases, which include most chitinases from bacteria and some fungi, catalyze the hydrolysis of chitin and related substrates *via* a "substrate-assisted" mechanism involving the formation of an oxazolinium ion intermediate (Fig 1.1B). Family 18 chitinases show variation among themselves with respect to the presence of accessory domains, the architecture of the substrate binding cleft and mode of interaction with the polymeric substrate (exo- or endo- activity) (Watanabe *et al*., 1994; van Aalten *et al*., 2000; Suzuki *et al*., 2002). Several family 18 chitinases contain one or more accessory domains involved in interaction with the substrate. The active site cleft of family 18 chitinases may be shallow and open, as in the case of endo chitinase hevamine (van Scheltinga *et al*., 1996) or deep and almost closed tunnel shape (Davies and Henrissat, 1995) as in exochitinases such as ChiA and Chi B from *Serratia marcescens* (Perrakis *et al*., 1994; van Aalten *et al*., 2000).

Family 19 enzymes employ an inverting mechanism, as determined by NMR (Fukamizo *et al*., 1995) and HPLC (Iseli *et al*., 1996). Structural characteristics and available biochemical

data (Heggset *et al*., 2009) suggest that GH19 chitinases are non-processive endo-acting enzymes. Hydrolysis of a glycoside with net inversion of anomeric configuration is generally achieved *via* one step, single-displacement mechanism involving oxocarbenium ion-like transition states (Fig 1.2A). The reaction occurs with acid/base assistance from two amino acid side chains, normally glutamic or aspartic acids that are typically located 6-11 A^{o} apart.

β-N-acetyl hexosaminidases (EC 3.2.1.52) belong to CAZy glycosyl hydrolase families 3, 20 and 84. They behave like typical exo-enzymes, catalyzing the cleavage of terminal β-D-GlcNAc and β-D-GalNAc residues in N-acetyl-β-D-hexosaminides. The three families of functionally- related enzymes, although possessing no sequence homology, share some basic features of both their structure and catalytic mechanism. The most abundant GH family 20 utilizes classical hexosaminidase substrate-assisted catalysis. The oxygen of the substrate's C-2 acetamido group acts as the catalytic nucleophile, forming an oxazoline intermediate (Fig 1.1B).

Bacterial β-N-acetylhexosaminidases or β-N-acetylglucosaminidases have been intensively studied due to their important physiological role in cell wall recycling. In marine chitinolytic bacteria such as *Vibrio furnissii* or *Alteromonas* sp., β-N-acetylhexosaminidases participate in chitin degradation and formation of chitinase inducer (Keyhani and Roseman, 1996; Tsujibo *et al*., 1999). The crystal structure of a bacterial β-N-acetyl hexosaminidase (Tews *et al*., 1996) revealed that the protein folding is highly conserved among GH family 20 β-N-acetyl hexosaminidases. The common feature of all known structures is the (β/α)8-barrel (TIM-barrel) architecture of the catalytic domain that houses the active site within loops extending from the C- termini of the strands that constitute the β-barrel. Properties of bacterial β-N-acetyl hexosaminidases were elucidated for *Vibrio harveyi* 650 (Suginta *et al*.,

2010), *Pseudomonas fluorescens* JK-0412 (Park *et al*., 2010a) and *Alteromonas* sp. strain O-7 (Tsujibo *et al*., 2002). The bacterial enzymes differ from the human hexosaminidases mainly by subunit constitution. Bacterial hexosaminidases are monomeric, while the human hexosaminidases form active dimers with two catalytic sites. Moreover, the human hexosaminidase α- and β-subunits are post-translationally cleaved, to yield the mature enzyme composed of two and three polypeptide chains, respectively (Slamova *et al*., 2010).

1.8 Transglycosylation of chitinous substrates is not uncommon

In addition to hydrolytic activity, some chitinases and hexosaminidases possess certain level of transglycosylation activity, that is, the ability to transfer the released oligosaccharide moiety to a suitable acceptor to form a new glycosidic bond (Fig 1.2B) (Bardales and Bhavanandan, 1989; Chipman *et al*., 1968; Eneyskaya *et al*., 1997; Umekawa *et al*., 2008). The transglycosylation activity of family 18 chitinases is of special interest because there are potential applications for CHOS with specific degree of polymerization (DP). Family 18 chitinases are retaining glycoside hydrolases. In retaining glycoside hydrolases, the transglycosylation reaction occurs through a double-displacement mechanism. In the first step the glycosidic oxygen is protonated by a catalytic acid, and the anomeric carbon is a target for a nucleophilic attack from the catalytic base, leading to cleavage of the glycosidic bond and to formation of a glycosyl enzyme intermediate. In the second step, the intermediate decomposes with different possible outcomes. Hydrolysis is attained by attack of a water molecule on the glycosyl enzyme intermediate that is assisted by the conjugated base of the catalytic acid residue and that leads to release of a hydrolyzed sugar. Alternatively, transglycosylation may occur if the water molecule is outcompeted by another acceptor such as a carbohydrate or an alcohol.

Since transglycosylation is a kinetically controlled reaction, efficient transglycoslation requires an enzyme with an active site architecture that disfavors correct positioning of the hydrolytic water molecule and/or favors binding of incoming carbohydrate molecules, through strong interactions in the aglycon subsites (Zakariassen *et al*., 2011). The transglycosylation activity of these chitinases implicates a great potential for the synthesis of oligosaccharides and polysaccharides of chitin origin. Kobayashi *et al*. (1996) have successfully applied the chitinases from *Bacillus* sp. for the chemoenzymatic synthesis of artificial chitin and related polysaccharide derivatives, using N, N^0-diacetylchitobiose oxazoline and its modified forms as highly activated monomers for the chitinase-catalyzed polymerization.

1.9 Bacteria are a rich source of chitinolytic enzymes

All chitin containing organisms produce chitinases for different biological functions. Several bacteria utilize chitin to obtain C and N from the environment. Given the great diversity of possible chitin structures, perhaps it is not surprising that bacteria typically produce more than one type of chitin degrading/modifying enzymes. Presumably, a bacterium produces different chitinases to efficiently hydrolyze the different forms of chitin found in nature. It is known that one single chitinase is not equally efficient in hydrolyzing α and β forms of chitin.

Species of *Serratia*, *Bacillus,* and *Vibrio* have been reported to secrete several chitinolytic enzymes and chitin-binding proteins into the extracellular environment, which are thought to degrade chitin synergistically. Various chitinases were isolated from aerobic microorganisms such as *Bacillus circulans* (Alam *et al*., 1996, Watanabe *et al*., 1990), *Serratia marcescens*

(Brurberg *et al*., 1996; Harpster and Dunsmuir, 1989; Hines *et al*., 1988; Jones *et al*., 1986), *Serratia proteamaculans* (Purushotham *et al*., 2012) an *Aeromonas* sp. (Shiro *et al*., 1996; Ueda *et al*., 1996), an *Alteromonas* sp. (Tsujibo *et al*., 2002), *Streptomyces plicatus* (Robbins *et al*., 1992), *Streptomyces olivaceoviridis* (Blaak *et al*., 1993; Radwan *et al*., 1994; Romaguera *et al*., 1992), and *Janthiobacterium lividum* (Gleave *et al*., 1995). Several genes encoding chitinases were cloned in *Escherichia coli* and characterized in detail along with their hydrolysed products (Gleave *et al*., 1995; Jones *et al*., 1986; Shiro *et al*., 1996; Sitrit *et al*., 1995).

Stenotrophomonas maltophilia occurs ubiquitously in the environment, soil and plants being main environmental reservoirs. *S. maltophilia* is a typical, often dominant member of the microbial communities and is distributed worldwide (Denton and Kerr, 1998). The genus *Stenotrophomonas* is phylogenetically placed in the gammaproteobacteria and belongs to the family *Xanthomonadaceae*. *S. maltophilia* (referred to as *Stm*) is gram-negative, rod- shaped and aerobic. *Stm* has an extra range of activities that include beneficial effects for plant growth and health and degradation of xenobiotic compounds (Lee *et al*., 2002), possessing a potential for soil decontamination (bioremediation) besides being chitinolytic. The degradation of linear polysaccharide chitin by *Stm* involves a cascade of chitin degrading and modifying enzymes, which include two chitinases, two chitin binding proteins, two polysaccharide deacetylases and a β- N- acetyl hexosaminidase.

1.10 Analysis of end products of chitin

The non-toxic, biodegradable and biocompatible properties of chitin and chitosan provide much potential for many food, pharmaceutical and biotechnology applications. Chitin and

chitosans can also be partially or fully depolymerised to yield GlcNAc and glucosamine oligomers or monomers, respectively. Monomers are used successfully to treat arthritis (Salvatore *et al*., 2000). The existing chemical methods for the breakdown of these polymers are highly expensive. Chitin and chitosan oligomers with different DP and different degrees of acetylation (DA) have been described to have more specific biological activities including wound healing, anti-tumour, elicitation of plant defense responses etc. (Aziz *et al*., 2006). However, the enzymology of chitin-degradation and chitin-deacetylation is not completely worked out and the diversity of the genes encoding these enzymes has not been fully exploited to precisely hydrolyze or modify the chitin and chitosan to produce defined products.

Hydrolytic and transglycosylated end products of chitinases and hexosaminidases can be qualitatively and quantitatively assessed by techniques such as TLC, HPLC, MALDI-TOF, MS and NMR. NH_2 and amide 80 columns are used to detect enzymatically produced oligosaccharides of different chain length and anomeric configuration (Fukamizo and Koga, 1995), respectively.

1.11 Chitinases as biocontrol agents

Chitin degrading and modifying enzymes can be exploited as powerful biocontrol agents exhibiting broad range of activity (Neeraja *et al*., 2010b). Roberts and Selitrennikoff (1988) reported that plant and bacterial chitinases differ markedly in antifungal activity. This difference in biological activity correlates with differences in their substrate specificities. Bacterial enzymes like glucanases, chitinases and chitosanases target and degrade the cell walls of phytopathogenic fungi. Biological control of plant pathogens provide an attractive

alternative means for the management of plant diseases without the negative impact of chemical fungicides, which are usually costly, can cause environmental pollution, and may induce pathogen resistance (Kishore and Pande, 2007; Reyes-Ramirez *et al*., 2004). Purified preparations of chitinase from *Bacillus* or *Serratia* sp. showed significant reduction in the severity of citrus fruit rot, rust and late leaf spot of groundnut rust (Manjula *et al*., 2004; Kishore *et al*., 2005). *Serratia marcescens* has been reported to control *Rhizoctonia solani, Fusarium oxysporum* and *Botrytis cinerea* (Someya *et al*., 2000). *Stm* is also known for its biocontrol ability. Kobayashi *et al*. (2002) reported that chitinase from *Stm* 34S1, had the ability to suppress summer patch disease on Kentucky bluegrass, supporting a role for the enzyme in the biocontrol activity. Chang *et al*. (2010) reported antifungal activity of *Bacillus subtilis* NPU 001 chitinase against a fungal pathogen *Fusarium oxysporum.* Chitinase from *S. proteamaculans* inhibited fungal spore germination and hyphal growth of pathogenic *F. oxysporum* and *Aspergillus niger* (Mehmood *et al*., 2009). Chitinase A produced by *B. thuringiensis* subsp. *colmeri* inhibits spore germination of two fungal pathogens (Liu *et al*., 2010). Control of late blight (*Phytophthora capsici*) in pepper plant with a compost containing multitude of chitinase-producing bacteria has been reported by Chae *et al*. (2006). Inhibition of fungal growth was more effective when β-1,3-glucanase and chitinase acted synergistically (Lim *et al*., 1991; Lorito *et al*., 1993). Neeraja *et al*. (2010b) discussed in detail about the possible biotechnological approaches to develop bacterial chitinases as a bioshield against fungal diseases of plants.

1.12 Significance of end products in biological systems

During the last decade, chitinases have received increased attention because of their wide range of applications. Chitin research carries practical aspect as the physiologically crucial

synthesis and hydrolysis of the polymer have been regarded as suitable, attractive and largely selective targets for interference and for producing effective pharmaceuticals and safe biopesticides. Production of GlcNAc and CHOS is of interest to the food, agriculture and biotechnology sectors. Their utilization is a way to improve biofertilizers, which can be comprised of microorganisms and elicitors of both plant pathogen defense responses and enhanced growth. This constitutes an interesting possibility for both plant growth promotion and biocontrol of plant pathogens. The potential to produce large amounts of these compounds makes them particularly promising for use as elicitors for disease control and growth promotion in sustainable agricultural systems.

Chitin and chitosan degradation products such as CHOS and GlcNAc have very crucial applications. The GlcNAc has been used as a nutritional substrate for pediatric chronic inflammatory bowel disease and pharmaceutical therapy of osteoarthritis (Shikhman *et al*., 2005). Hexa- N-acetyl- chitohexaose, $(GlcNAc)_{6,}$ has immunopotentiating and antitumor functions (Tokoro *et al*., 1989). Chitosan and CHOS have attracted considerable interest due to their biological activities, namely, antimicrobial (Zhao and Xia, 2006), hypocholesterolemic (Kim and Rajapakse, 2005; Liao *et al*., 2007; Zhou *et al*., 2006), immunity-enhancing and antitumor effects (Xia, 2003), in drug delivery (Agnihotri *et al*., 2004; Bravo-Osuna *et al*., 2007; Liao *et al*., 2007; Park *et al.,* 2010b; Sinswat and Tengamnuay, 2003) and accelerating calcium and ferrum absorption (Bravo-Osuna *et al*., 2007; Deuchi *et al*., 1995; Jeon *et al*., 2000; Jung *et al*., 2006; Liao *et al*., 2007).

Studies on the biological activities of chitosan and its oligomers have been increasing, as no single type of chitosan or its oligomer exerts all of the above bioactivities. Moreover, different chitosan derivatives and enzymatic products have different structures and

physicochemical properties, which may result in novel bioactivities or novel findings in known bioactive compounds (Xia *et al*., 2011).

1.13 Significance of the present study

The CAZy database shows nine genes from the genome sequence of *S. maltophilia* k279a potentially involved in chitin turnover, coding for the following: Two family 18 chitinase (*Stm* ChiA and *Stm* ChiB); two family 19 chitinases, two family 33 CBPs (*Stm* Cbp D1 and *Stm* Cbp D2); and a family 20 N- acetylhexosaminidase (*Stm* Nag); two carbohydrate esterase family 4 proteins (*Stm* Pda1 and *Stm* Pda2). The presence of wide variety of genes involved in chitin turnover by *S. maltophilia* k279a, the potential of this organism to produce useful products from chitin hydrolysis and antifungal activity of these enzymes on agronomically important fungal pathogens were promising. The objective of this study, therefore, was to characterize the hydrolytic and transglycosylation properties of the heterologously expressed chitinases and a hexosaminidase of *Stm* k279a.

1.14 Detailed objective

Cloning, heterologous expression and characterization of chitinases and a hexosaminidase from *Stm* k279a, was carried out with the following objectives:

- Cloning, expression and purification of chitin degrading/modifying genes
- Characterization of chitinases and a hexosaminidase
  - Comparative account of kinetic parameters of a few bacterial chitinases on chitohexaose
  - Temp. optima, stability at optimum temperature, thermal stability, pH optima
  - Substrate binding analysis
- End product analysis for ChiA, ChiB and Nag using HPLC
- Antifungal activity of ChiA, ChiB and Nag

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_2HPO_4-0.065 g, KH_2PO_4 – 1.5 g, NaCl-0.25 g, NH_4Cl-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^{o}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^{o}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/) 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 CbpD1was 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^{o}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 $^{\circ}$C and

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-*Stm* ChiA, pET 22 b -*Stm* ChiB and pET 22 b -*Stm* Nag were designated as *Stm* ChiA, *Stm* ChiB and *Stm* Nag, respectively.

2.5.2.2 Protein purification

The expressed proteins of *Stm* ChiA, *Stm* ChiB and *Stm* Nag were isolated from respective cell pellets by whole cell lysate using sonicator. The cell pellets of expressed *Stm* ChiA, *Stm* ChiB and *Stm* Nag were resuspended in Ni-NTA equilibration buffer (50 mM NaH_2PO_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, *Stm* ChiA, *Stm* ChiB, and *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 NaH2PO4, 100 mM NaCl and 10 mM imidazole pH 8.0). *Stm* chitinases were eluted by running four column volumes of elution buffer (50 mM NaH_2PO_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. Poly acrylamide 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^{o}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^{o}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^{o}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^{o}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. 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^{o}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^{o}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^{o}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^{o}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^{o}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 NaH_2PO_4-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°C). 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°C 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 chitoloigosaccharides (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^{o}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^{o}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 H_2O 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 R^2 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^{o}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^{o}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^{o}C for 12 h in 1.5 ml reaction tubes and observed under SEM as described earlier in 2.5.4.2.3.

RESULTS

3.1 Selection of organism for the present study

Stenotrophomonas maltophilia (MTCC*434) was obtained to study the chitin degrading/ modifying enzymes produced by this organism. The identity and chitinolytic ability of the organism was confirmed before cloning chitinases.

3.1.1 SEM for *S. maltophilia* MTCC*434

Overnight grown cells of *S. maltophilia* *434 in LB medium were observed under SEM (Fig 3.1). The cells of *S. maltophilia* appear straight or slightly curved nonsporulating gram-negative bacilli, occur singly or in pairs. The approximate length and width of individual cell is 2.12 µ and 0.6µ, respectively.

3.1.2 Confirmation and phylogenetic analysis of *Stm* k279a

Genomic DNA of *S. maltophilia* (MTCC*434) was isolated using DNeasy kit (Qiagen, Germany). The 16S rRNA gene sequence of *S. maltophilia* MTCC*434 was amplified with 27F and 1489R primers using *Taq* DNA polymerase (Fig. 3.2A). 1.5 kb of 16S rDNA was sequenced and submitted for BLAST search at NCBI. The amplified sequence showed 100% homology to *S. maltophilia* k279a. The complete 16S rRNA gene sequence was determined and a phylogenic tree was constructed (Fig. 3.3). A BLAST search of the GenBank database identified *Stenotrophomonas maltophilia* IAM 12423 (GI: 343200890) as the closest neighbour with 100% homology.

Similarly the GH18 domain was amplified using degenerate primers GH18 FP and GH18 RP at gradient temperatures with *Taq* DNA polymerase (Fig 3.2B) and cloned into pGEMT vector. 0.7kb of amplified GH18 domain was released from pGEMT vector by restriction digestion with *EcoR* I enzyme (Fig 3.2C). Sequence of the amplified conserved domain of

GH18 blasted against nucleotide database at NCBI further confirmed that the organism belonged to the type strain k279a of *S. maltophilia* and therefore referred to as *Stm* k279a.

3.2.2 Screening for chitinolytic ability

Active culture of *Stm* k279a was spotted on agar containing colloidal chitin as a sole source of carbon and nitrogen. After 4 days of incubation at 28^{o}C, wide zone of clearance was observed around the *Stm* k279a colony, indicating the chitinolytic potential of *Stm* k279a (Fig 3.4).

3.3 Cloning and characterization of chitinases

3.3.1 Amplification and cloning of degrading/modifying chitinases

Broth culture of *Stm* k279a was harvested at the end of the exponential growth phase by centrifugation at 16,100 g for 15 min. The cell pellet was processed for gDNA isolation using DNeasy kit (Qiagen, Germany). Six genes involved in chitin degradation/modification were identified (Table 3.1) from genomic resources of *Stm* k279a and were cloned using PCR-based approaches. Six chitinases of *Stm* k279a (referred to as *Stm* chitinases or with prefix *Stm*) were amplified using gene specific primers using gDNA as template. The amplicons, 2kb of *Stm* ChiA, 1.1kb of *Stm* ChiB were cloned into *NcoI/XhoI* sites of pET22b (+), 2.27kb of *Stm* Nag was cloned into *NcoI/HindIII* sites of pET22b (+) (Fig 3.5). A 1.1kb of *Stm*CbpD1 was also cloned into *NcoI/XhoI* sites of pET22b (+), while 0.91kb of *Stm* Pda1 and 0.78kb of *Stm* Pda2 were cloned into *BamHI/HindIII*, *BamHI/EcoRI* sites of pRSET-A, respectively (Fig 3.6). Double-digestion of pET22-ChiA, pET22-ChiB, pET22-CbpD1 with *NcoI/XhoI*, pET22-Nag with *NcoI/HindIII*, pRSET-Pda1 with *BamHI/HindIII* and pRSET-

Pda2 with *BamHI/EcoRI* released the respective inserts and were confirmed by automated DNA sequencing (Europhins, India).

3.3.2 Expression and purification of *Stm* chitinases

Stm ChiA, *Stm* ChiB and *Stm* Nag genes were over expressed in *E.coli*, with C-terminal His-tag. The expressed proteins (*Stm* ChiA, *Stm* ChiB and *Stm* Nag) were extracted from whole cell lysate and isolated in soluble form. The extracted proteins were purified using Ni-NTA agarose chromatography. SDS-PAGE analysis of the purified *Stm* chitinases revealed a molecular mass of 70.5kDa, 41.6kDa and 83.6kDa which correspond to the expected molecular mass (Table 2.1) of *Stm* ChiA, *Stm* ChiB and *Stm* Nag, respectively (Fig 3.5).

3.3.3 Dot blot activity of *Stm* chitinases

The purified recombinant *Stm* chitinases were spotted on glycol chitin substrate containing gel to detect the activity. Only *Stm* ChiA showed activity zone on glycol chitin (Fig 3.5).

3.3.4 Characterization

3.3.4.1 Michaelis –Menten kinetic analysis for three *Stm* chitinases

The kinetic values of the three *Stm* chitinases were determined with chitohexaose as a substrate using the reducing end assay. Specific activity (nanokat/mg of protein) and substrate concentration (µmoles) data were directly fitted to the Michaelis-Menten equation by non-linear regression function of Graph Pad Prism version 5.0 software. The curve fitting for the enzymes showed highest V_{max} value for *Stm* ChiB (10.91) followed by *Stm* Nag (9.953) and *Stm* ChiA (7.835). The K_m for *Stm* ChiB (691) was lower than those of *Stm* ChiA (1281) and *Stm* Nag (1454) indicating the substrate- binding affinity of *Stm* ChiB was highest

compared to the other two enzymes (Fig 3.7). Among the *Stm* chitinases, catalytic activity (k_{cat}) of *Stm* ChiB was highest (6163) followed by *Stm* Nag (5563) and *Stm* ChiA (4401). *Stm* ChiB (8.9) displayed the highest overall catalytic efficiency (k_{cat}/K_m) when compared to *Stm* ChiA (3.4) and *Stm* Nag (3.8) (Table 3.2).

3.3.4.2 Comparative kinetic analysis of a few bacterial chitinases

Kinetic analysis for chitinases from a few bacterial sources (*Stenotrophomonas maltophilia, Bacillus thuringiensis*, *B. licheniformis* and *Serratia proteamaculans*) was performed with chitohexaose as a substrate using reducing end assay. Specific activity (nanokat/mg of protein) and substrate concentration (µmoles) data were directly fitted to the Michaelis-Menten equation by non-linear regression function of Graph Pad Prism version 5.0 software (Fig 3.8). The curve fitting for the enzymes exhibited highest substrate affinity (K_m) in the order of preference *Sp* ChiD (82.6) > *Sp* ChiA (225.2) > *Sp* ChiB (252.3) > *Bt* Chi (317.8) > *Stm* ChiB (691) > *Sp* ChiC (882) > *Stm* ChiA (1281) > *Stm* Nag (1454) > *Bli* Chi(1496) and overall catalytic efficiency (k_{cat}/K_m) in the following order *Sp* ChiD (31) > *Sp* ChiB (9.5) > *Stm* ChiB (8.9) > *Sp* ChiA (8.2) > *Sp* ChiC (6.1) > *Bt* Chi (5.3) > *Stm* Nag = *Bli* Chi(3.8) > *Stm* ChiA (3.4). Among the bacterial chitinases analyzed here, *Sp* ChiD exhibited highest substrate affinity and overall catalytic efficiency compared to other chitinases on hexamer substrate (Table 3.3).

3.3.4.3 Optimum temperature

The *Stm* chitinases were incubated with chitohexaose, at different temperatures (20-100^{o} C) to determine the optimum temperature for the three chitinases. The three *Stm* chitinases had

temperature optimum at 40^{o}C. The *Stm* ChiB had highest specific activity at 40^{o}C compared to *Stm* ChiA and *Stm* Nag (Fig 3.9A).

3.3.4.4 Stability at optimum temperature

Pre-incubation of recombinant *Stm* chitinases at optimum temperature (40^{o}C) followed by a reducing end assay using chitohexaose as a substrate revealed that the activity of enzyme was unaffected, at least up to 4 h, for all the three *Stm* chitinases (Fig 3.9B).

3.3.4.5 Thermal stability

Recombinant *Stm* chitinases were pre-incubated at 30-100^{o}C followed by reducing end assay. The results indicated that *Stm* ChiA is thermally stable as it retained more than 90% of its initial activity between 30-50^{o}C (up to 1h), followed by *Stm* ChiB and *Stm* Nag are thermally stable up to 40^{o}C (Fig 3.9C).

3.3.4.6 Optimum pH

The effect of pH on the hydrolytic activity of *Stm* ChiA, *Stm* ChiB and *Stm* Nag using chitohexaose as the assay substrate was determined. Significant activity of *Stm* ChiA was observed with sodium citrate buffer pH 3.0 and sodium acetate pH 5.0, whereas maximum activity of *Stm* ChiB was seen at pH 4.0 of sodium citrate buffer and 7.0 when sodium phosphate buffer was used. *Stm* Nag exhibited highest specific activity at 5.0 in sodium acetate buffer. Enzyme assay with different pH range buffers revealed that *Stm* ChiA and *Stm* Nag exhibited highest specific activity in sodium acetate buffer pH 5.0 and *Stm* ChiB exhibited highest specific activity in sodium phosphate buffer pH 7.0 (Fig 3.10).

3.3.4.7 Substrate specificity of *Stm* chitinases

The *Stm* chitinases, *Stm* ChiA or *Stm* ChiB or *Stm* Nag were incubated with chitosan having different degree of deacetylation (DDA 70, 90 and water soluble) at 37ºC and pH 5.0 followed by reducing end assay to study their substrate specificity. The results suggested that *Stm* ChiA and *Stm* Nag hydrolyze de-acetylated chitosan. Both the enzymes produced maximum reducing ends with water soluble chitosan. Among the three, only *Stm* ChiA exhibited activity towards 70% and 90% DDA chitosans. *Stm* ChiB behaved like an exclusive chitinase as it did not show any activity towards different DDA chitosans (Fig 3.11).

3.3.4.8 Binding of *Stm* chitinases towards insoluble and soluble polymeric substrates

3.3.4.8.1 Insoluble substrate binding

The binding ability of *Stm* chitinases to insoluble substrates was investigated on ice, to minimize enzyme-mediated hydrolysis of the bound substrate. Among all *Stm* chitinases, *Stm* Nag displayed binding towards all the four insoluble polymers and exhibited 100% binding towards chitosan substrate. *Stm* ChiB exhibited 45 % binding towards β-chitin and 11% towards Avicel. *Stm* ChiA showed highest binding preference (96%) to chitosan, followed by α- and β- chitin (42%) and no binding was observed with Avicel (Fig 3.12).

3.3.4.8.2 Soluble substrate binding

Binding preference of *Stm* chitinases towards soluble polysaccharide substrates was investigated by affinity electrophoresis in native PAGE with and without polysaccharides. Binding is attributed to the retardation in the mobility of the proteins. Comparison of

electrophoretic pattern of proteins in presence or absence of the substrates revealed that *Stm* chitinases were not affected by the presence of CM-cellulose, laminarin or without substrate. The mobility in retardation was observed only with *Stm* ChiA in presence of glycol chitin. *Stm* ChiB and *Stm* Nag did not bind to glycol chitin (Fig 3.13).

3.3.5 Analysis of hydrolysis products of *Stm* chitinases on chitooligosaccharides (CHOS) and chitin

3.3.5.1 Thin layer chromatography for colloidal chitin hydrolysis by *Stm* ChiA

Efficiency of *Stm* ChiA was assessed by the hydrolytic ability of the recombinant enzyme on chitinous polymers and oligomers. Reaction end products were separated and analyzed on TLC. Only *Stm* ChiA displayed hydrolytic activity on colloidal chitin substrate. Hydrolysis products were observed from the 5^{th} min onwards. Di-acetyl chitobiose (DP2) was the major end product released by *Stm* ChiA up to 720 min on colloidal chitin (Fig 3.14).

3.3.5.2 HPLC product analysis on chitin polymers

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

All the three recombinant *Stm* chitinases were tested for their activity on chitin polymers (α- and β- chitin). Hydrolysis products were quantified after 12 h using HPLC. Among all, only *Stm* ChiA exhibited activity on chitin polymers. *Stm* ChiB and *Stm* Nag did not show activity towards chitin polymers. *Stm* ChiA preferred β- chitin than α-chitin for its activity. The oligomers produced from the β- chitin were five-fold higher than from α-chitin (Fig 3.15).

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

Hydrolysis products were quantified from 0 min -12 h by the action of *Stm* ChiA on chitin polymers α- and β- chitin. Hydrolysis started from 0 min in β- chitin and 2 min in α – chitin. Major end product released by the action of *Stm* ChiA on chitin polymers was di-acetyl chitobiose (DP2) (Fig 3.16).

3.3.5.2.3 Scanning electron microscopy of α- chitin

Morphological changes were observed when α- chitin powder was treated with *Stm* ChiA. Significant changes in surface properties were visible. The surface of buffer-treated chitin powder appeared smoother and retained the original crystalline morphology, where as *Stm* ChiA- treated chitin displayed deformed crystalline morphology (Fig 3.17).

3.3.5.3 Product profile of *Stm* chitinases on chitin oligosaccharides

Hydrolysis products generated by *Stm* ChiA, *Stm* ChiB and *Stm* Nag from DP2- DP6 substrates were analyzed on isocratic HPLC. Oligosaccharides produced were estimated against the standard slope of each substrate and graph was constructed using Origin 8.0 software

3.3.5.3.1 Dimer (DP2) as substrate

Stm ChiA released monomer upon prolonged incubation (24 h) of dimer as a substrate. *Stm* ChiB hydrolyzed dimer slowly. Hydrolysis products were detectable from 30^{th} min and complete hydrolysis of dimer to monomer occurred by the end of 12 h. *Stm* Nag behaved like a true chitobiase with dimer substrate. Hydrolysis started immediately after the addition of

enzyme i.e, at 0 min and within two minutes it cleaved the entire dimer to monomer (Fig 3.18).

3.3.5.3.2 Trimer (DP3) as substrate

Stm ChiA was not readily accepting trimer for hydrolysis. However, upon prolonged incubation (1 h) *Stm* ChiA hydrolysed trimer and released very low concentration of the monomer and dimer. *Stm* ChiB hydrolyzed the trimer from 2nd min onwards. After 60 min, entire trimer got converted in to mono and dimer. Hydrolysis of trimer by *Stm* Nag started at 0 min, and cleaved trimer readily to give monomer as a major product within 5 min (Fig 3.19).

3.3.5.3.3 Tetramer (DP4) as substrate

Stm ChiA was active at initial time points on the tetramer substrate and exhibited transglycosylation activity at the 60th min. There was a clear decrease in the concentration of dimer with a concomitant increase in the concentration of tetramer by 1 h, both in the chromatogram and in the quantification profile (Fig 3.20). Dimer was obtained as a major end product by tetramer hydrolysis with *Stm* ChiA. *Stm* ChiB cleaved tetramer to give monomer as the major end product after 3 h. The product formation was biphasic for both *Stm* ChiA and *Stm* ChiB. In the first phase (by 20 min) 50% of substrate was hydrolyzed. The second phase was much longer (160 min) for the maximum conversion of substrate. *Stm* Nag cleaved tetramer completely at 30th min giving monomer as the end product (Fig 3.20).The cleavage of the substrate was rapid in the first 10 min (> 90% of the activity was complete), and in the later part of 20 min the remaining substrate was cleaved.

3.3.5.3.4 Pentamer (DP5) as substrate

On pentamer substrate, the three *Stm* chitinases exhibited different product profile. *Stm* ChiA cleaved the pentamer to trimer and dimer in the initial time points (within 2 min) showing a rapid hydrolysis, on prolonged incubation even up to 3 h only a minute decrease in the concentration of trimer and increased concentration of monomer was noticed. *Stm* ChiB gradually cleaved pentamer to give monomer as the end product. *Stm* Nag hydrolyzed most of pentamer at the end of 30th min to give monomer as the major end product (Fig 3.21).

3.3.5.3.5 Hexamer (DP6) as substrate

When hexamer was incubated with *Stm* ChiA, hydrolysis started from the 2nd min. At the end of 10th min, hexamer was completely hydrolysed. Again by the 15th min there was a decrease in the concentration of dimer, trimer and tetramer, and simultaneous formation of the hexamer was observed. This clearly indicated the involvement of transglycosylation of *Stm* ChiA acting on hexamer. When the substrate is limiting for the enzyme to show hydrolytic activity, *Stm* ChiA amalgamated the available oligomers to produce hexamer. This transient transglycosylation lasted at least up to 30 min. At the end of 12 h, *Stm* ChiA produced only dimer as the end product. A gradual cleavage pattern was observed, when *Stm* ChiB was incubated with hexamer. At the end of 60th min, most of the hexamer got converted into monomer. *Stm* Nag showed rapid hydrolysis of hexamer. At the end of 10th min, most of the hexamer got converted to monomer (Fig 3.22).

The end products released by *Stm* chitinases on CHOS are summarized in Table 3.4. *Stm* ChiA released DP2 as the major product, whereas *Stm* ChiB and *Stm* Nag produced DP1 as the predominant end product.

3.3.5.4 Antifungal activity of *Stm* chitinases

Stm chitinases (*Stm* ChiA and *Stm* Nag) were tested for antifungal activity in terms of inhibition of hyphal extension on seven fungi *viz. F. oxysporum, F. solani, M. phaseolina, F. moniliforme, C. dematium, C. lunata* and *R. stolonifer*. *Stm* ChiA and *Stm* Nag showed strong antifungal activity against *F. oxysporum*. Antifungal activity towards *F. solani, M. phaseolina, F. moniliforme* and *C. dematium* was discernible. However, *Stm* ChiA and *Stm* Nag had no antifungal effect on *C. lunata* and *R. stolonifer* (Fig 3.23; Table 3.5). When high concentration (0.5mg) of *Stm* ChiA alone incubated with *F. oxysporum* exhibited good antifungal activity on PDA plate by restricting the sporulation (Fig 3.24).

3.3.5.4.1 Scanning electron microscopy for fungal spores

To observe the effect of *Stm* ChiA on inhibition of sporulation, *Stm* ChiA-treated *F. oxysporum* spores and buffer-treated spores were observed under SEM to observe the changes in surface morphology. *Stm* ChiA- treated spores exhibited deformed, perforated and shrunken morphology (Fig 3.25).

DISCUSSION

4.1 *Stenotrophomonas maltophilia* is a potential chitinolytic bacterium

Chitin is a homo polymer of *N*-acetyl glucosamine linked by β- (1-4) glycosidic bonds. Chitin is the most abundant polysaccharide next to only cellulose, it is complex, crystalline, high molecular weight, extracellular amino-sugar polymer, which is non-toxic and biodegradable. Utilization of the chitin is difficult because of its insolubility in water (intermolecular hydrogen bonds), compact crystalline nature. To recycle such an abundant biomass, nature evolved several mechanisms involving microorganisms. Bacteria are the largest group explored for chitinolytic potential. Bacteria play a major role in turnover of chitin biomass and metabolize crystalline chitin as source of C and N. From ecological point of view, there is a great interest in the enumeration of chitinoclastic microorganisms from natural sources.

In the present study, we have isolated the chitinolytic genes and characterized the chitin degrading enzymes from *S. maltophilia* k279a which belongs to family Xanthomonadaceae. *Stm* represents a rhizosphere bacterial species of potential agronomic importance. The organism is aerobic, non- fermentative, Gram-negative, rod-shaped and its full genome sequence is available since 2008. Traits of *Stm* associated with biocontrol mechanisms include antibiotic production (Jakobi *et al*., 1996; O'brien and Davis, 1982), extracellular enzyme activities such as protease and chitinase (Dunne *et al*., 1997; Kobayashi *et al*., 1995; O'brien and Davis, 1982; Zhang and Yuen, 2000), and rhizosphere colonization (Dunne *et al*., 1997; Elliott *et al*., 1987; Kobayashi *et al*., 1995; Lambert *et al*., 1987; Lambert *et al*., 1990).

Complete genome sequence search of *Stm* k279a revealed 2 chitinases, 1 hexosaminidase, 2 chitin binding proteins (CBPs), two polysaccharide deacetylases (Pdas), and one unannotated GH19 sequence, which are capable of converting the chitin biomass to GlcNAc. Due to the presence of a rich diversity of genes for chitin degradation and /or modification, *Stm* was selected for the study. The domain architecture of these chitinases deduced from their amino acid sequences is depicted in Table 4.1.

The complete hydrolysis of chitin requires cooperative and synergistic action of chitinases and hexosaminidases. Zechmeister and Toth (1939) reported the key observation, the insoluble chitin polymer was converted to its monomer, GlcNAc, by two enzymes, a "polysaccharidase" (chitinase), and a "disaccharidase," (β-*N*-acetyl glucosaminidase or chitobiase). Suzuki *et al*. (2002) reported the synergistic action of chitinases ChiA, ChiB, and ChiC1 of *Serratia marcescens* 2170 on chitin degradation. The hydrolytic activity of chitinases is either by single or multiple forms of the enzyme. The multiple forms may be due to expression from different genes or due to proteolytic processing, as in case of *Bacillus circulans* and *Serratia marcescens* (Alam *et al*., 1996; Gal *et al*., 1998).

Several methods were reported for the detection of chitinase activity using different chitinous substrates and chemicals. A simple and inexpensive method to identify chitinolytic microorganisms on agar containing chitinous polymer was developed (Howard *et al*., 2003). Since chitinases diffuse through agar, identification of chitinolytic bacteria or genomic clones encoding chitin-degrading enzymes was performed by monitoring the degradation of polymeric chitin incorporated into an agar medium. The larger and clearer zone of chitin hydrolysis around the colony was attributed to the chitinolytic ability of the organism. *Stm* was tested on chitinase detection agar medium, and a clear zone around the colony was

formed indicating the strong chitinolytic activity. A simple, rapid and less expensive method to detect chitinase activity on agarose plates using a fluorescent brightener Ranipal (Anil *et al*., 2007). Chitinase activity was detected after protein separation by SDS-PAGE and glycol chitin containing agar (Trudel and Asselin, 1989). Determination of chitinolytic activity of chitinase, glucosaminidase and chitobiosidase in solution can be achieved by rapid method as described by Tronsmo and Harman (1993). In the present study, we have estimated the chitinase activity calorimetrically by estimating the number of reducing amino sugars produced by using modified Schale's procedure (Imoto and Yagashita, 1971). This method has the advantage of estimating the number reducing amino sugars in solution released due to chitinase activity compared to other known procedures.

We have amplified and sequenced the 16S rDNA gene of *Stm* to confirm the taxonomic affiliation of the isolate. The BLAST search of the 16S rDNA sequence showed 100% homology to *S. maltophilia* k279a as well as *S. maltophilia* IAM 12423 (GI: 343200890). Phylogenetic tree was constructed along with closely related 16S rDNA sequences of different bacterial species (Fig. 3.1). The strain used in the study was, therefore, considered as *S. maltophilia* k279a. Similarly, Tyler *et al*., (1995) distinguished between *Stm* and seven species of *Pseudomonas* by identifying differences in the sequences of 16S to 23S rDNA internal transcribed spacers.

4.2 Cloning and characterization of chitin degrading/modifying enzymes from *Stm* k279a

4.2.1 Search for homologs of *Stm* k279a chitin modifying enzymes at NCBI using BLAST

4.2.1.1 *Stm* ChiA

The amino acid sequence of *Stm* ChiA was BLASTed at NCBI database to search for protein homologues. The results displayed 75% sequence similarity to the sequence of ChiA from *Lysobacter enzymogenes* (AAT 77164.1), 65% to GH18 domain of *Xanthomonas albilineans* (YP003376658.1), 55% to chitinase of *Burkholderia ambifaria* (ZP02909207.1), and 47% to GH18 of *Streptomyces coelicolor* A3(2) (NP 629515.1).

The SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) program predicted the presence of a putative signal peptide, typical of Gram-negative and Gram-positive bacteria, with a cleavage site located between amino acid residues Ala-28 and Ala-29. The sequences were tested for functional domains or motifs using SMART (a Simple Modular Architecture Research Tool) data base. *Stm* ChiA contained an N-terminal chitin binding domain 3 (ChtBD3), polycystic kidney disease domain (PKD), fibronectin type 3 domain (FN3) and a family 18 catalytic domain (GH18).

4.2.1.2 *Stm* ChiB

The BLAST search for *Stm* ChiB displayed 96% identity to chitinase A1 of *Stenotrophomonas* sp. SKA14 (ZP 05136728.1), 41% to putative chitinase A1 from *Paenibacillus sp.* HGF 7 (ZP08512913.1), 40% to catalytic domain of ChiA1 of *Bacillus circulans* WL 12 (1ITX_A) and 38% to family 18 chitinase of *Chitiniphilus shinanonensis* (BAF 02588.1). *Stm* ChiB contained a single catalytic domain GH18 similar to that of *Serratia proteamaculans* ChiD (Purushotham, 2011).

4.2.1.3 *Stm* Nag

Stm Nag exhibited 62% identity to NAGase of *Pseudoxanthomonas suwonensis* (YP 004145298), 48% to NAGase from *Xanthomonas perforans* 91-118 (ZP06729723.1), 41% to Nag C from *Streptomyces thermoviolaceus* (BAC 76622), 36% to Nag 2 from *Vibrio harveyii* 650, 34% to GlcNAcase A from *Pseudoalteromonas piscicida* (BAB17855), and 26% to chitobiase from *Serratia marcescens*. *Stm* Nag contained family 20 catalytic domain, similar domain architecture was observed in *S. marcescens* chitobiase (Chb) (Tews *et al*., 1996).

4.2.1.4 *Stm* CbpD1

Stm CbpD1 had 53% homology with chitin binding protein D from *Pseudomonas aeruginosa* PA7 (YP 001350011.1), 48% to chitin binding protein from *Myxococcus xanthus* (YP 631148.1) and 35% to chitin binding domain 3 protein B from *Streptomyces* sp.4 (ZP 09182986.1) having a ChtBD3 as a conserved domain.

4.2.1.5 *Stm* Pda1

Stm Pda1 had 64% homology with polysaccharide deacetylase from *Xylella fastidiosa* Ann1(ZP 00680562.1), 61% to putative chitin deacetylase from *Xanthomonas vesicatoria* (ZP 08178658.1) and 43% to chitooligosacchride deacetylase from *Ralstonia eutroph*a H16 (YP 84181.1).

4.2.1.6 *Stm* Pda2

Stm Pda2 had 49% homology with polysaccharide deacetylase from *Rhodanobacter* spp. 2APBS1 (ZP 08953748.1), 28% to putative xylan/chitin deacetylase from *Rheinheimera* spp.

A13L (ZP 08570187.1) and 26% to chitooligosacchride deacetylase (Nodulation protein B) from *Paenibacillus polymyxa* E681 (YP 003870877.1).

The BLAST search of protein homologues for different *Stm* chitinases revealed the evolutionary divergence of *S. maltophilia* from different genera. However, it has close affinity with *Stenotrophomonas*. spp. SKA14 and *Lysobacter enzymogenes*.

4.2.2 Catalytic activity

The presence or absence of the accessory domains in the protein sequence of the chitinases and chitin modifying enzymes influence the catalytic activity. *Stm* chitinases differ amongst each other with different domain organization. The role of accessory domains in the specific activity of enzymes was assessed by performing kinetic studies for *Stm* chitinases.

Colloidal chitin or chitin derivatives are mostly used for activity measurements in enzyme assays. However, these substrates are not suitable for kinetic analysis as they are of heterogeneous nature (Bokma *et al*., 2000). Hence, we have used a soluble substrate, chitin hexamer. Michaelis-Menten kinetic parameters were determined using different concentrations of chitin hexamer as a substrate.

Kinetic analysis of *Stm* chitinases revealed that *Stm* ChiB had highest catalytic efficiency (*k*cat/*K*m) and substrate affinity (*K*m) than *Stm* ChiA and *Stm* Nag. *Stm* ChiB displayed two-fold high substrate affinity and overall catalytic efficiency compared to *Stm* ChiA and *Stm* Nag (Fig 3.6). From this analysis it can be assumed that GH18 domain has preference towards hexameric substrate than GH20 domain. The kinetic analysis for the *Stm* Nag showed higher substrate affinity and overall catalytic efficiency over Nag 2 from *Vibrio*

harveyii 650 [k_{cat}/K_m 166 s^{-1} M^{-1} , K_m 421 µM] (Suginta *et al*., 2010). NagC from *Streptomyces thermoviolaceus* (Kubota *et al*., 2004) and chitobiose from *Vibrio parahemolyticus* (Zhu *et al*., 1992) showed similar kinetic parameters like *Stm* Nag.

Among *Bacillus* chitinases, *B. thuringiensis* chitinase displayed five-fold greater affinity and overall catalytic efficiency when compared to *B. licheniformis* chitinase. This was observed with both polymer and oligosaccharide substrates. In *S. proteamaculans* chitinases, *Sp* ChiD exhibited 3-fold greater affinity towards hexamer substrate when compared to ChiA and ChiB, and 10-fold greater affinity than ChiC. Comparative kinetic analysis of a few bacterial chitinases revealed that among all, *Sp* ChiD is catalytically active towards hexamer, and *Stm* ChiB exhibited high activity in terms of k_{cat} and k_{cat}/K_m among *Stm* chitinases. Unique feature among these two enzymes was to have a single GH18 domain. We can infer that single catalytic domain favoured efficient hydrolysis of soluble CHOS. Our results in the present study ascertained this fact by the behavior of these two enzymes on polymer substrates. The enzymes *Sp* ChiD and *Stm* ChiB did not show significant activity towards chitinous polymers as both these enzymes do not contain accessory domains. Enzymes without accessory domains are not active on polymer substrates as these domains are helping in binding to the polymeric substrates making them accessible for enzymatic hydrolysis (Kawase *et al*., 2006).

The PKD domain was found in N-terminal region of both the chitinases that were effective in hydrolysis of crystalline α-chitin (Orikoshi *et al*., 2005), So, we can assume that the enzymes containing accessory domain cleave the polymer to shorter chain length oligomers making them better substrates for *Sp* ChiD and *Stm* ChiB, to release products like GlcNAc, the energy source for the bacterium. Kinetic analyses for Sp chitinases towards colloidal chitin

are in contrast to hexamer substrate. *Sp* ChiA exhibited highest substrate affinity and overall catalytic efficiency on colloidal chitin and *Sp* ChiD showed very poor K_m & k_{cat}/ K_m values (Purushotham *et al.*, 2012). Our results are in conformity with the earlier findings on *Sp* chitinases. The results revealed that, among different bacterial chitinases used in this study, *Sp* ChiD exhibited highest K_m & k_{cat}/ K_m and *Bli* Chi showed low K_m & k_{cat}/ K_m values (Table 3.3).

The difference in the kinetic parameters among bacterial chitinases also could be due to difference in the amino acid sequences of catalytic domains in conjunction with the presence (*Stm* ChiA, *Sp* ChiA- *Sp* ChiC, *Bt* Chi and *Bli* Chi) or lack of (*Stm* ChiB, *Stm* Nag and *Sp* ChiD) of different accessory domains (Table 4.1). It is evident that the primary role of these accessory domains (CBMs, PKD and FN3) is to potentiate catalytic activity by disrupting the substrate, rather than simply to promote enzyme-substrate binding. Watanabe *et al*. (1994) showed that deletion of the two FN3 domains of ChiA1 from *Bacillus circulans* did not affect chitin-binding, but strongly reduced chitin hydrolyzing activity.

The difference in the kinetic parameters of *Stm* chitinases could be attributed to the difference in the amino acid sequence. More specifically, the variation in the affinity of the chitinases could be due to amino acid sequence variation that imparts a strong or weak binding affinity towards the polymeric substrate or a more or less efficient catalytic process.

4.2.3 Effect of temperature on chitinolytic activity

All the three *Stm* chitinases exhibited highest specific activity at an optimum temp of 40^{o}C (Fig 4A), similar to *S. proteamaculans* chitinases (Purushotham *et al*., 2012) and Chi L of *B. pumilis* SG2 (Ghasemi *et al*., 2010) and CHIT100 from *Serratia plymuthica* HRO- C48 (Frankowski *et al*., 2001), while chitinases from *B. thuringiensis* and *B. licheniformis*

exhibited optimum temperature at 60^{o}C (Neeraja *et al*., 2010c) higher than *Stm* chitinases. *Stm* Nag was active at 40^{o}C (Fig.3.8 A), similar to *β-N*-acetylglucosaminidase from *Alteromonas* spp. strain 0-7 (Tsujibo *et al*., 1995), *β-N*-acetylglucosaminidase from *V. furnissii* (Keyhani and Roseman, 1996). The three *Stm* chitinases retained their specific activity with a marginal loss at 40^{o}C after pre-incubation of 4 h. *Stm* ChiA showed higher thermal tolerance than *Stm* ChiB and *Stm* Nag. *Stm* ChiA was thermally stable as it retained more than 90% of its initial activity between 30-50 ^{o}C up to 1 h. *Stm* ChiB and *Stm* Nag were thermally stable up to only 40^{o}C, while *Stm* ChiB lost 80% of the initial activity at 50^{o}C. On the other hand 50% of the initial activity was retained at 50^{o}C for *Stm* Nag.

4.2.4 Effect of pH

The three *Stm* chitinases were optimally active in acidic to neutral pH (3.0-7.0) (Fig 3.9). *Stm* ChiA displayed activity over a broad range of pH (7.0-12.0) similar (but not identical) to *Pt* ChiA from *Paecilomyces thermophila* (Kopparapu *et al*., 2011; Kudan and Pichyangkura, 2009). *Stm* ChiA exhibited highest specific activity at sodium citrate buffer of pH 3.0 and sodium acetate buffer pH 5.0. The *Stm* ChiB was optimally active at pH 7.0 similar to that of CHIT 100 of *Serratia plymuthica* HRO- C48 (Frankowski *et al*., 2001). The *Stm* Nag exhibited activity over a broad pH range similar to *Bacillus cereus* TKU 006 (Wang *et al*., 2009) and *B. atrophaeus* SC081(Cho *et al*., 2011) with an optimum activity at pH 5.0. The chitinases showing optimum activity in both alkaline and acidic pH is not uncommon (Kawase *et al*., 2006) as observed for *Stm* ChiA and *Stm* Nag in this study. Although not clear one possible explanation could be such chitinases have a deep catalytic cleft formed by the two insertion domains. These domains make the top of the catalytic cleft narrow and might interfere with the entrance of long-chain soluble substrate into the catalytic cleft in the

neutral pH range. At extremely high or low pH, conformational change occurs that makes the top of the catalytic cleft wider or the cleft structure more flexible. This results in long-chain soluble substrate easily entering into the catalytic cleft and the chance of hydrolysis increases.

4.2.5 Activity on chitosans of different Degree of De Acetylation (DDA)

Chitinases are reported to be chitosanolytic as well. Chitosans are very valuable substrates for in depth studies of processivity in family 18 chitinases (Sikorski *et al*., 2006). *Stm* ChiA was able to hydrolyze chitosans of different DDA, but preferably water soluble chitosan. *Stm* Nag has very feeble activity towards water soluble chitosan, and *Stm* ChiB did not hydrolyze any of the chitosan.

4.2.6 Substrate binding properties of *Stm* chitinases

4.2.6.1 Insoluble substrates

To establish the relationship between specific activity and the substrate binding capability of *Stm* chitinases on different insoluble polymers was assessed and compared with different substrates (Fig. 3.11). *Stm* ChiA did not show binding towards Avicel. *Stm* ChiB showed binding preference to β-chitin, but did not show binding to α-chitin and chitosan. However, binding towards Avicel was negligible. The importance of the accessory domains, most importantly ChBD (chitin-binding domain) in binding to insoluble substrates was known. The C-terminal binding region was important for strong affinity of the enzyme to insoluble chitin (Watanabe *et al*., 1990). Morimoto *et al*. (1997) explained the role of highly conserved aromatic amino acids tryptophan (W) and tyrosine (Y) in chitin binding domain (ChBD). These amino acids may play a crucial role during binding to the pyranosyl rings of

N-acetyl glucosamine residues in chitin. Deletion of the CBD from the *B. circulans* chitinase A1 reduced the rate of hydrolysis of colloidal chitin by approximately 50% (Svitel and Kirchman, 1998). *Stm* Nag exhibited binding towards all the polymers.

4.2.6.2 Soluble substrates

Binding of three *Stm* chitinases to soluble polysaccharides was investigated by native PAGE with and without polysaccharides. Electrophoretic mobility of the *Stm* chitinases was only affected by the presence of glycol chitin (Fig. 3.12). Among *Stm* chitinases only *Stm* ChiA exhibited strong affinity towards the glycol chitin substrate indicating that accessory domains are crucial for the hydrolysis of chitin polymer which is in accordance with the previous results. The binding ability of *Stm* ChiA is similar to that of BliGH, BliGh-CeBD towards glycol chitin substrate (Neeraja *et al*., 2010a).

4.2.7 Hydrolytic activities on chitinous substrates

4.2.7.1 TLC analysis of products

Only *Stm* ChiA was active on polymers probably due to the presence of accessory domains. To check the hydrolytic activity of *Stm* ChiA on colloidal chitin was assessed by TLC was performed and followed the products formation over a period of time (Fig 3.13). We could see the concentration of dimer enhanced in addition to very faint concentrations of higher chain oligosaccharides observed during extended incubation. Colloidal chitin hydrolysis by chitinase A of *Vibrio carchariae* and *B. licheniformis* (Suginta *et al*., 2005; Songsiriritthigul *et al*., 2010 also had similar pattern. Our results reveal the endo-mode of action of chitinase A on chitin polymers.

4.2.7.2 HPLC analysis of products

4.2.7.2.1 Polymeric substrates

Activity profile of *Stm* ChiA on α- chitin and β- chitin was observed for 12 h, to see the activity profile from 0 min to 12 h (Fig 3.15). The results indicated that *Stm* ChiA preferentially hydrolyzed β- chitin. Similarly, chitinase A from *S. marcescens* preferentially hydrolyzed β- chitin than α- chitin and predominantly released dimers (Horn *et al*., 2005).

4.2.7.2.2 Oligomeric substrates (DP2-DP6)

Reaction time course of CHOS hydrolysis was analyzed by HPLC (Fig. 317-3.21). Hydrolysis of DP2 with *Stm* ChiA yielded very low amount of DP1 after 24 h indicating that DP2 is not preferred substrate for the *Stm* ChiA hydrolysis. Similarly, chitinase A from *V. carchariae* and *B. licheniformis* did not hydrolyze DP2 substrate after 57 h and 18 h, respectively (Suginta *et al*., 2005; Songsiriritthigul *et al*., 2010). Sakai *et al*. (1998) showed that three chitinases (Chi L, Chi M and Chi S) from *Bacillus* strain MH-1 did not hydrolyze DP2 substrate at all. On DP3 substrate, *Stm* ChiA produced low concentration of monomer and dimer after 1 h. In contrast *V. carchariae* chitinase required 57 h to completely hydrolyze trimer (Suginta *et al*., 2005). *Stm* ChiA hydrolyzed DP4 readily from the 0 min, and it cleaved tetramer mainly in the middle, releasing dimers. Trimer was also produced but in lower quantity (< 20% of the dimers at 60 min of reaction). *Stm* ChiA exhibited transglycosylation activity at the 60^{th} min. Based on these results we can infer that *Stm* ChiA prefers higher chain length oligosaccharides for the hydrolysis. As soon as the substrate was a limiting factor, the enzyme transglycosylated the available oligomers (Fig 1.4). DP5 substrate got readily cleaved by *Stm* ChiA at the initial time points. On DP6 substrate also

Stm ChiA exhibited transglycosylation activity. The increase in the concentration of the hexamer at 15th min could be due to the transient transglycosylation activity of the enzyme where DP2, DP3, and very low concentrations of DP4 are acting as substrates for transglycosylation. *Stm* ChiA is exhibiting transglycosylation on even chain length oligosaccharides starting from DP4. Hydrolysis pattern of *Stm* ChiA was similar to chitinase A from *B. licheniformis* (Songsiriritthigul *et al*., 2010). *Stm* ChiB and *Stm* Nag hydrolyzed DP2-DP6 to give DP1 as the major end product. The formation of other intermediate oligomers indicated that these enzymes are endo–acting enzymes on oligomeric substrates. *Stm* Nag was truly a chitobiase with dimer. Unlike *Stm* Nag, Nag2 from *Vibrio harveyii* 650 required 25 h for complete hydrolysis of the DP6, *β-N*-acetylglucosaminidase from *Pseudomonas fluorescens* JK-0412 (Park *et al*., 2010a) and *Aeromonas hydrophila* strain SUWA-9 (Lan *et al*., 2004) attained complete DP5 hydrolysis by 24 and 7 h, respectively. NagC from *Streptomyces thermoviolaceus* exhibited activity only on DP2-DP5 substrates and no activity on DP6 substrate (Kubota *et al*., 2004). The activity of *Stm* Nag on DP2, in the present study, started at 0 min and completely hydrolyzed within two minutes indicating the rapid rate of hydrolysis unlike other known hexosaminidases. Our results establish the fact that *Stm* Nag is an exclusive chitobiase.

4.2.8 Antifungal activity

Chitinolytic bacteria have received much attention as potential biocontrol agents due to their ability to lyse hyphae of fungal pathogens. Hyphal tips and germinating spores appear to be susceptible to the lytic activities of chitinolytic bacteria. Purified chitinase preparations of potent biocontrol strains can cause deformation of fungal hyphae (Ordentlich *et al*., 1988; Zhang and Yuen, 2000; Manjula *et al*., 2004).

S. maltophilia k279a is well known for its biocontrol ability due to its ability to produce diverse group of enzymes. Kobayashi *et al.* (2002) reported that chitinase from the strain *Stm* 34S1 had the ability to suppress summer patch disease on Kentucky bluegrass, supporting a role for the enzyme in the biocontrol activity. But, very few biocontrol traits have been characterized in detail. The present study reports the involvement of *Stm* chitinase A in alterations in the structure of *F. oxysporum* fungal mycelium and spores. It was confirmed by scanning electron microscopy (Fig 3.23.2). *Bacillus cereus* chitinase showed antifungal activity towards *F. oxysporum* and *R. solani* (Pleban *et al*., 1997).

Genome sequence of *Stm* k279a consists of putative un annotated GH19 gene sequence. This gives us a clue that the role of *Stm* k279a in biocontrol ability could also be due to the presence of a variety of chitinase gene sequences in the genome. Most probably, family 19 chitinases were transferred from plants to bacteria by horizontal gene transfer. In plants, family 19 chitinases are thought to form part of a defense mechanism against chitin-containing fungal pathogens. The family 19 chitinases may attack the hyphal tips, which are believed to consist of newly synthesized chitin that is not firmly crystallized (Kawase *et al*., 2006). Only a few chitinolytic bacteria possess family 19 chitinases, and these also display antifungal activity (Watanabe *et al*., 1999; Kawase *et al*., 2006). Role of chitinases in antifungal activity and presence of different glycosyl hydrolase domains contribute to over all biocontrol ability of *S. maltophilia* k279a towards fungal pathogens. Presence of C-terminal ChBD in *Streptomyces griseus* HUT 6037 increased the antifungal ability of the chitinase (Itoh *et al*., 2002).

Our present work focused on cloning and characterization of chitin degrading/modifying enzymes from *S. maltophilia* k279a and the production of CHOS by the action of these

enzymes. A search for effective biological plant protection methods currently is of high interest in view of the growing area of ecological crops. *Stm* chitinases can be used in industries for conversion of chitinous waste into biologically active CHOS. Results presented in this study will be useful to design appropriate strategies for enzymatic synthesis of CHOS.

SUMMARY AND CONCLUSIONS

Background and Objective

Chitin is an amino sugar polymer consisting of N-acetylglucosamine units linked with β-1,4-glycosidic bonds. Chitin is present in fungal cell walls as well as insect exoskeleton and it is also a principal structural component in different phyla. Chitin biomass turnover is achieved by micro-organisms by utilizing a complex chitinolytic system to degrade and generate products that are useful for the welfare of human beings. Chitinases are the hydrolyzing enzymes that degrade chitin. Multiple chitinases are characteristically produced to convert chitin into useful oligomers. The chitinolytic system of bacteria also includes chitin binding proteins which are helping chitinases in a synergistic manner for efficient hydrolysis of chitin. Many bacterial genera have evolved chitinases with different structure and function.

The present study aimed at cloning the chitinase encoding genes from *S. maltophilia* k279a followed by their characterization to understand the role of bacterial chitinases in chitinous biomass turnover, oligomer production and antifungal ability. *S. maltophilia* k279a is one of the chitinolytic strains with several genes coding for chitinolytic machinery. Complete genome sequence of *S. maltophilia* k279a revealed 2 chitinases, 1 hexosaminidase and 1 chitin binding protein (Cbp) and two polysaccharide deacetylases (Pdas) which are capable of converting the chitin biomass to GlcNAc. The properties of 2 chitinases and 1 hexosaminidase (*Stm* ChiA, *Stm* ChiB and *Stm* Nag) and cloning of 1 Cbp (*Stm* CbpD1), 2 Pdas were investigated in this study.

5.1 Cloning and characterization of *S. maltophilia* k279a chitinases and a hexosaminidase

S. maltophilia k279a strain was confirmed using 16S rDNA analysis. *S. maltophilia* k279a was screened for its chitinolytic ability on chitinase detection agar medium. After 5 days of incubation a clear chitinolytic zone was observed around the colony.

Two chitinases and a hexosaminidase (*Stm* ChiA, *Stm* ChiB and *Stm* Nag) were amplified without signal peptide, cloned in to pET 22b vector, expressed in *E. coli* and purified using Ni-NTA agarose column chromatography. Purified enzymes were characterized in terms of kinetics, optimum pH and temperature, substrate preference, binding preferences towards the insoluble and soluble substrates. Analysis of products like CHOS was performed using TLC and HPLC. Purified proteins were tested for their antifungal ability against different fungal pathogens.

Kinetic analysis of *Stm* chitinases showed that *Stm* ChiB had highest catalytic efficiency and substrate affinity than *Stm* ChiA and *Stm* Nag. *Stm* ChiA and *Stm* Nag also displayed similar values for overall catalytic efficiency and substrate affinity. Comparative kinetic analysis of bacterial chitinases (*Stenotrophomonas maltophilia*, *Bacillus thuringiensis*, *Bacillus licheniformis* and *Serratia proteamaculans)* revealed that among all the enzymes analyzed, *Sp* ChiD followed by *Stm* ChiB exhibited highest substrate affinity and overall catalytic efficiency on hexamer substrate. *Stm* chitinases displayed optimum activity between pH 3.0-7.0. Compared to the other *Stm* chitinases, *Stm* Nag was active over a broad range of pH. All three *Stm* chitinases were optimally active at 40^{o}C and exhibited stability up to 4 h at optimum temperature. Among the *Stm* chitinases, *Stm* ChiA had highest hydrolytic activity

on water soluble chitosan. Only *Stm* ChiA exhibited binding towards soluble chitin (glycol chitin) from among the tested soluble polysaccharides.

All *Stm* chitinases were tested on CHOS (DP2-DP6) substrates. *Stm* ChiA released dimer (DP2) as major end product from DP4-DP6 substrates and did not prefer DP3 and DP2 oligomers for effective hydrolysis. *Stm* ChiA exhibited transglycosylation s on even chain length oligosaccharides such as DP4 and DP6. *Stm* ChiA was active on polymers α- and β-chitin, released dimer (DP2) as the major end product. Both on polymers and oligosaccharides, *Stm* ChiA exhibited endo-mode of action. *Stm* ChiB and *Stm* Nag formed monomer (DP1) as major end product from oligosaccharides (DP2-DP6). *Stm* Nag behaved like a true chitobiase on dimer (DP2) and produced monomer immediately after the addition of enzyme. Only *Stm* ChiA released DP1-DP3 products from polymeric substrates. *Stm* ChiB and *Stm* Nag exhibited both exo/endo-mode of action on oligomeric substrates.

Stm chitinases (*Stm* ChiA and *Stm* Nag) were tested for antifungal activity by their ability to inhibit hyphal extension on different fungal species. *Stm* ChiA showed strong antifungal activity towards *F. oxysporum*.

5.2 Major findings in the present work

An overview of the cloning, expression and characterization of chitinases of *S. maltophilia* k279a was given in Table 4.2.

* Three chitinases (*Stm* ChiA, *Stm* ChiB and *Stm* Nag) and three chitin modifying enzymes (*Stm* CbpD1, *Stm* Pda1 and *Stm* Pda2) were cloned, heterologously expressed and purified.
* *Stm* ChiA, *Stm* ChiB and *Stm* Nag were cloned, expressed and purified in their active form.

* All three enzymes had 40^{o}C as optimum temp and optimum pH was in acidic - neutral range.
* *Stm* ChiA had greater affinity towards chitosan and soluble polysaccharide glycol chitin, while *Stm* ChiB showed affinity towards β-chitin, and *Stm* Nag showed affinity towards chitosan.
* *Stm* ChiB displayed two-fold high substrate affinity and overall catalytic efficiency compared to *Stm* ChiA and *Stm* Nag.
* Comparative analysis of a few bacterial chitinases revealed that *Sp* ChiD exhibited lower K_m and higher k_{cat}/K_m towards the hexameric substrate.
* Predominantly chitobiose was released by *Stm* ChiA on α and β forms of chitin. *Stm* ChiB and *Stm* Nag were not active on polymeric substrates
* *Stm* ChiA is an endo- acting enzyme, releasing dimers as major end product from CHOS and exhibited transglycosylation on even chain length oligomers.
* GlcNAc was the end product released by *Stm* ChiB and *Stm* Nag on oligomers exhibiting both exo/endo mode of action for *Stm* ChiB and *Stm* Nag.
* *Fusarium oxysporum* spores were sensitive to *Stm* ChiA.

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