Auxiliary role of bacterial chitin binding proteins in chitin digestion

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

by

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CERTIFICATE

This is to certify that Ms. Manjeet Kaur 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 under the Ph.D. ordinances of this University. We recommend her thesis entitled **"Auxiliary role of bacterial chitin binding proteins in chitin digestion"** for submission for the degree of Doctor of Philosophy of the University.

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DECLARATION

This is to declare that the research work embodied in this thesis entitled **"Auxiliary role of bacterial chitin binding proteins in chitin digestion"** has been carried out by me under the supervision of Prof. Appa Rao Podile and is free from plagiarism. This has not been submitted for any degree or diploma in any other university earlier.

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Prof. Appa Rao Podile
(Research Supervisor)

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Manjeet Kaur

Dedicated to my beloved husband

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ABBREVIATIONS

AA	:	Auxiliary activity
A_{595}	:	Absorbance at 595 nm
BLAST	:	Basic Local Alignment Search Tool
bp	:	Base Pair
CAZy	:	Carbohydrate Active Enzyme
CBM	:	Carbohydrate Binding Module
CBP	:	Chitin Binding Protein
CD	:	Circular Dichroism
CDA/CDB	:	Czapek Dox Agar/ Czapek Dox Broth
CF	:	Cytosolic Fraction
CHOS	:	Chito-oligosaccharides
C-terminal	:	Carboxy terminal
°C	:	degree Celsius
DP	:	Degree of Polymerisation
ESI	:	Electron Spray Ionisation
FnIII	:	Fibronectin type III
g	:	Gram
xg	:	times gravity
gDNA	:	Genomic Deoxy Ribo Nucleic Acid
$\Delta G°$	:	Gibbs free energy
GH18	:	Glycosyl Hydrolase 18
GlcNAc	:	N-acetyl Glucosamine
h	:	Hour(s)
HPTLC	:	High Performance Thin Layer Chromatography

IPTG	:	Isopropyl β-D-thiogalactoside
K_B	:	Binding constant
Kb	:	Kilo Base
K_D	:	Dissociation constant
kDa	:	Kilo Dalton
L	:	Litre
LBA/LBB	:	Luria-Bertani Agar/ Luria-Bertani Broth
LPMO	:	Lytic Polysaccharide Mono-oxygenase
M	:	Molar
MALD-TOF	:	Matrix Assisted Laser Desorption –Time of Flight
mg	:	Milli Gram
min	:	Minute
mL	:	Milli Litre
mM	:	Milli Molar
MS	:	Mass Spectrometry
MW	:	Molecular Weight
NAG	:	*N*-acetyl Glucosamine
NCBI	:	National Centre for Biotechnology Information
N-terminal	:	Amino terminal
nm	:	Nano-meter
PAGE	:	Poly Acrylamide Gel Electrophoresis
P_{bound}	:	Bound Protein
PCR	:	Polymerase Chain Reaction
PDB	:	Protein Data Bank

PDA/PDB	:	Potato Dextrose Agar/Potato Dextrose Broth
PF	:	Periplasmic Fraction
P_{Free}	:	Free Protein
rpm	:	Revolutions Per Minute
SDS	:	Sodium Dodecyl Sulphate
sec	:	Seconds
TIM	:	Triose-phosphate Isomerase
TLC	:	Thin Layer Chromatography
µg	:	Micro Gram
µM	:	Micro Molar
w/v	:	Weight/Volume

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1.1 Chitin is high molecular weight, un-branched, obstinate, helical homo-polymer which is insoluble in most solvents

Taking the global biomass of arthropods into consideration, chitin polymerization is a major synthetic event, next to cellulose. Chitin is predominantly found in invertebrates; notably arthropods (insects, crustaceans), and to a minor extent in molluscs, annelids and nematodes (eggs). Chitin is also an integral component of fungal cell walls (except Oomycetes). But, it is absent in plants and vertebrates. Chitin is a polysaccharide of *N*-acetyl glucosamine (NAG) residues which are linked by β-1, 4 linkages (Figure 1.1). It is insoluble in water and also in most of the organic solvents. It is a helical homo-polymer where the helix is stabilized by intra-molecular hydrogen bonds. These polysaccharide chains are assembled into micro-fibrils. The micro-fibrils constitute the fibrous component of insect exoskeleton.

X-ray diffraction studies of various chitin crystallites revealed that an anti-parallel arrangement of polymers (α-chitin) is the most abundant form. This anti-parallel arrangement favors the formation of numerous inter- and intra- hydrogen bonds which makes α-chitin more stable. In β-chitin, parallel arrangement establishes intermolecular hydrogen bonds resulting in less densely packed structure. Therefore, in contrast to α-chitin, β-chitin has weak intermolecular force, and exhibit higher reactivity and affinity for solvents than α-chitin. The third least studied and the rarest form is γ-chitin which occurs as a combination of the above mentioned two arrangements. Here, two chains run in one direction and another chain runs in opposite direction. It is also suggested that γ-chitin is formed as a result of distortion in the α- and β-chitin. Depending on the organism and on its role, chitin adopts any of the three different polymorphic structures. The α-chitin is found in rigid and resistant structures such as cuticle of arthropods, fungal and yeast cell walls, in krill, in lobster and crab tendons and shells, and in shrimp's shells. The β- and γ-chitin forms occur in resistant but flexible structures.

Crustaceans' shells originating from industrial processing of sea-food are the main raw materials for industrial production of chitin. Chemical synthesis of chitin is a difficult and costly task. Japan, the U.S. and China are the largest producers of chitin, while to a lesser extent it is also produced in India, Norway, Canada, Italy, Poland, Chile and Brazil. Annual production of chitin in aquatic biosphere alone is estimated to be more than 10^{11} metric tons. Its degradation is thus a key step in recycling of nutrients into the environment. However, the major problem is that chitin polymers form extremely dense and resilient bonds. Indeed, as already mentioned, the main biological function of chitin is to make organism hard and durable. This makes it extremely difficult to hydrolyze chitin, as hydrolytic enzymes (chitinases) decompose crystalline chitin substrates very slowly.

1.2 For efficient chitin degradation, chitinolytic bacteria produce chitinases and chitin binding proteins/oxido-hydrolases

1.2.1.1 Chitinases hydrolyze chitin into monomers, dimers and chito-oligomers

During past several years, bacteria have been exploited for production of chitinases which have wide range of applications. Chitinases are hydrolytic enzymes which convert chitin to *N*-acetyl glucosamine, chitobiose or chito-oligosaccharides (CHOS). Chitinases are found in a wide range of organisms like bacteria, fungi, plants and animals. Based on usage and physiology, different organisms produce chitinases for different reasons. Plant chitinases serve a role in defense against bacterial, fungal and nematode pathogens. In insects, chitinases are involved in cuticle turnover and also in digesting the nutrients. In fungi, chitinases help in degradation and mobilization of organic substrate. Yeasts produce chitinases for efficient cell separation.

Chitinases production in bacteria is to degrade polymers with NAG residues; after digestion and subsequent processing NAG forms a nutrient source for bacteria. Also, chitin degradation for recycling of nutrients into the environment is mainly carried out by bacterial chitinases. Thus, the chitinases serve an important role to maintain balance in ecosystem. Depending on the bacterial source, chitinases are active in a wide range of pH and temperature conditions.

Chitin degradation by chitinases is not an easy task. Chitinases should be able to associate with crystalline chitin substrate and guide a single polymeric chain into the catalytic centre. To fasten this process, bacterial chitinolytic machinary produces an array of chitinases *viz*., exo-chitinases, endo-chitinases and *N*-acetyl hexosaminidases. Endo-chitinases cleave internal β-1, 4 linkages which join NAG residues. Exo-chitinases cleave the residues from either reducing or non-reducing end of chitin. Cleavage of chitin by exo- or endo-chitinases is random; this results in production of monomers, dimers or CHOS with shorter CHOS being the dominant one. These CHOS become substrate for *N*-acetyl hexosaminidases. Bacteria finally convert NAG to acetate, ammonia and fructose-6-phosphate, to use in various cell processes. In fact, conversion of insoluble chitin, which mainly consists of insoluble carbon and nitrogen, is primarily mediated by chitinolytic bacteria.

In sea, where most of the chitin is produced annually, most of the marine bacteria are responsible for conversion of chitin into its soluble form. One such well-studied marine bacterium is *Vibrio* sp. Marine bacterium carries out chitin degradation in four steps, first they sense chitin, which is carried out either by chemotaxis or by random collisions (Figure 1.2). Once they sense the substrate, they get attached to it. After attachment, they produce an array of enzymes (specifically chitinases) and other proteins to hydrolyze chitin polymer and finally the hydrolyzed products are taken inside which are then catabolized inside by the bacterium.

Various genes involved in chitinolytic machinery of marine and soil bacteria have been cloned and sequenced. A plethora of bacterial species like *Serratia marcescens*, *S. proteamaculans*, *Bacillus thuringiensis*, *B. licheniformis*, *B. amyloliquefaciens*, *Stenotrophomonas maltophilia*, *Enterococcus faecalis*, *Streptomycces griseus* etc., are known to produce chitinolytic enzymes. Various species of *Enterobacter*, *Vibrio*, *Aeromonas* etc., also produce chitinolytic enzymes in high intensity.

1.2.1.2 Mode of action of chitinases

Glycosidic bonds are hydrolyzed by enzymes by acid catalysis which requires a proton donor and a nucleophile. Hydrolysis usually gives rise to overall retention or inversion of anomeric forms. In both the conditions, the proton donor is within the hydrogen bonding distance of glycosidic oxygen. In inverting enzymes, a water molecule must be lodged in between the base and sugar such that the anomeric carbon of sugar and nucleophilic catalytic base are far apart. Whereas, in retaining enzymes both are in close proximity as shown in Figure 1.3. On the basis of sequence identity, chitinases are divided into two different families *viz.*, Family 18 and 19 (Henrissat and Davies, 1997). Family 19 chitinases are mainly found in plants and are involved in defense, where they hydrolyze chitin present in pathogens (eg. fungi) or insects. Hydrolysis by family 19 chitinases occurs *via* single displacement mechanism (Brameld and Goddard, 1998) where inversion of anomeric forms takes place. The characteristic feature of family 19 chitinases is that the catalytic residues are far apart. The general mechanism followed by family 19 chitinases is shown in Figure 1.4 A. Glutamic acid serves as a proton donor and protonates β-1, 4- glycosidic oxygen atom. This forms an oxo-carbonium ion intermediate. To complete this reaction, a water molecule attacks the carbon atom (C1) of oxo-carbonium ion. As mentioned above, in inverting enzymes separating location of catalytic residues allows water molecule to dwell in between C1 atom and carboxyl oxygen. This accommodation of water

molecule results in anomeric inversion products. High percentage of α-helices with two lobes in the catalytic domain is characteristic feature of family 19 chitinases. However, family 18 chitinases have a catalytic $(\alpha/\beta)_8$- barrel domain with DxDxE motif on fourth β-strand (Vaaje-Kolstad et al., 2004).

Most of the living organisms possess family 18 chitinases, which have a different mechanism of catalysis. Here, catalysis occurs *via* substrate-assisted mechanism (Scheltinga et al., 1996), unlike family 19 chitinases which follow single displacement mechanism. Figure 1.4 B shows the mechanism followed by family 18 chitinases. In family 18 chitinases, the transition state is stabilized when catalytic carboxylate donates a proton to the leaving group.

1.2.1.3 Domain architecture of family 18 chitinases

Most of the family 18 chitinases possess multi-modular structure. Glycosyl hydrolase 18 (GH18) domain is primarily responsible for hydrolysis of chitin substrates. In addition to these, GH18 chitinases may possess one or several carbohydrate binding modules (CBM) which are most often tethered to GH18 *via* one or more fibronectin III domains (FnIII).

GH18 domain consists of triose phosphate isomerase (TIM) barrel-fold which is made up of 8 alternating β- strands and α-helices, and is represented as $(\alpha/\beta)_8$-barrel. The eight alternating β- strands and α-helices curve around and close to form a doughnut-shaped structure. Outer wall of this doughnut is occupied by α-helices and β- sheets form the inner core (Figure 1.5). Some GH18 domains are known to have one (α+β) fold/insertion, usually between β7 strand and α7 helix (Zees et al., 2009).

On the basis of sequence identity and structure similarity, family 18 chitinases have been divided into three subfamilies A, B and C (Watanabe et al., 1993). Chitinases which possess (α+β) insertion are categorized in subfamily A and those which lack this fold are grouped in subfamily B. *Sm*ChiA and *Sm*ChiB from

Serratia marcescens and *Sp*ChiA and *Sp*ChiB from *S. proteamaculans* possess (α+β) insertion and belong to subfamily A of GH18, whereas *Sm*ChiC and *Sp*ChiC, belong to subfamily B as both these lack (α+β) insertion. The (α+β) insertion is responsible for deepening the substrate binding groove in many family 18 chitinases (Suzuki et al., 2002). Deepening of substrate binding groove forms a tunnel, where polymer chain tends to remain closely associated with catalytic centre of enzyme. This results in improved hydrolytic efficiency, as enzyme sticks to the substrate and degrades it in a processive manner. Deep substrate binding groove is a characteristic feature of exo-enzymes.

Absence of (α+β) insertion from catalytic cleft makes it shallow. One such example of enzyme lacking (α+β) insertion is a plant chitinase, hevamine (Scheltinga et al., 1994). Shallow catalytic clefts make the enzyme endo-acting and non-processive.

FnIII domains are the most common evolutionarily conserved protein folds, which are mostly found in modular proteins. These domains show sequence similarity to FnIII domains present in animals and hence are named as FnIII domains. Fibronectin is an extracellular matrix protein which is found throughout eukaryotes. Fibronectin is multimodular and consists of FnI, FnII and FnIII modules. Out of the three, FnIII is an evolutionarily conserved protein domain. FnIII domains were exclusively found in GHs and due to their high similarity to animal FnIIIs, it is believed that prokaryotes have acquired these domains from animals *via* horizontal gene transfer. Role of FnIII domains in prokaryotes is less known. However, it is believed that FnIII domains work as linkers that connect two same or different domains together. At the same time, presence, selection and conservation of FnIII domains during evolution also highlights that FnIIIs may have an important role in protein function. Fisher et al. (1999) predicted that FnIII domain might unfold or refold while protein executes mechanical functions.

Therefore, proteins with FnIII domains might gain more access into the substrates.

Carbohydrate binding modules (CBMs) are often found connected to GH18 domains sometimes *via* one or more FnIII domains. These CBMs are non-catalytic and play a role in efficient polysaccharide recognition. CBMs were previously defined as cellulose binding domains (CBDs), as the first known binding modules were known to bind primarily to cellulose. Further, as these binding domains were found to bind to a wide range of carbohydrates, they are referred to as CBMs. CBMs may bind to (or recognize) crystalline cellulose, non-crystalline cellulose, chitin, β-1,3-glucans, β-(1,3)/(1,4)- mixed linkage glucans, xylans, mannans, galactan and starch (Boraston et al., 2004).

Most of the CBMs consist of β-sheets as their most important secondary structure element. On the basis of topology of ligand binding sites, CBMs have been classified into three different types (Boraston et al., 2004). Type A CBMs are characterized by a flat, platform like hydrophobic surface which is composed of aromatic residues. These planar surfaces interact with crystalline chitin or cellulose like substrates. In type B CBMs, aromatic residues in ligand binding site interact with single chain of polysaccharide and display a cleft arrangement. Type B CBMs recognize substrates like β-1,3-glucans, mixed β-(1,3)/(1,4) - glucans, β-1,4-mannans, galactomannan and glucomannan. Type C CBMs bind only to mono-, di- or trisaccharides. This is due to steric interaction in ligand binding sites.

Based on amino acid sequence similarity, these CBMs have been classified into 69 families of CAZy database (previously known CBM33 family- now been placed under family AA10 of LPMOs in CAZy database). Mostly, these CBMs are found tethered to GH domains, where they help GH to degrade insoluble substrates. Some CBMs target plant cell walls where they bind to insoluble starch which is storage polysaccharide in plants. CBMs may play divergent roles, like

they may have a proximity effect and a targeting function and a disruptive function. The CBMs also play a role in root colonization, pathogen defence, plant development and polysaccharide biosynthesis (Guillen et al., 2010).

Due to efficient substrate recognition and binding ability, CBMs bring the enzyme (especially hydrolytic domain to which CBM is attached) in close proximity of substrate. This results in fast and efficient degradation of insoluble polysaccharides. CBMs thus help hydrolytic domain of the enzyme to come closer to the substrate. Usually, CBMs bind to the polysaccharides which are substrates for their associated hydrolytic domain. N-terminal CBM2a of Cel6A from *Cellulomonas fimi* was reported to carry out non-catalytic disruption of crystalline cellulose (Din et al., 1994). When CBMs occur independently they are referred as binding proteins. CBMs which were previously placed in family 33 of CAZy database bind to chitin and thus referred to as chitin binding proteins (CBPs). These CBPs are now known to oxido-hydrolyze crystalline substrates.

1.2.2.1 CBPs increase substrate accessibility and efficiency of chitinases

CBPs were earlier known to be non-catalytic proteins that aid chitinases in hydrolyzing crystalline chitin. Efficient chitin degradation in *S. marcescens* was shown to be dependent on the action of CBP21 (Vaaje-Kolstad et al., 2005). CBP21 accelerates hydrolysis of β-chitin by chitinase A and C and was essential for complete degradation of chitinase B. CBPs from other organisms were also found to show synergism with chitinases (Table 1.1). Figure 1.6 shows degradation of chitin by chitinases in absence or presence of chitin (Eijsink et al., 2008). It clearly illustrates that in presence of CBPs, accessibility of substrate is increased, which increases chitinase efficiency. Hence, chitin degradation is faster in presence of CBPs as compared to when only chitinases were present. It means that chitinases and CBPs work in synergy with each other for efficient chitin degradation.

CBPs from different organisms showed differential binding preferences (Table 1.1). Some CBPs bind specifically α-chitin, some to β-chitin and some CBPs bind to both α- and β-chitin. *Sp*CBP28 from *S. proteamaculans* 568 does not bind to α-chitin, β-chitin, colloidal chitin or avicel. Also there are some chitinases which are self efficient and do not need support of CBPs for chitin degradation. For example, *Bli*Chi from *Bacillus licheniformis* did not show increased product concentration when incubated with chitin substrates in presence of *Bli*CBP/*Bt*CBP/*Sp*CBP21 (Manjeet et al., 2013). As some CBPs showed binding and synergistic action with chitinases towards crystalline chitin and some do not, the role of these CBPs could be different under different conditions.

1.2.2.2 Antifungal activity of CBPs

Chitinolytic bacteria *viz.*, *Enterobacter agglomerans* (Chernin et al., 1995), *S. marcescens* and *Xanthomonas maltophilia* (Kobayashi et al., 1995), *Stenotrophomonas maltophilia* (Zhang and Yuen, 2000) and *B. thuringiensis* serovar *konkukian* (Mehmood et al., 2011) are known for their antagonistic effect on fungal pathogens. The synergistic effect of enzymes and antifungal compounds has been reported for *S. marcescens* (Someya et al., 2001). Since the fungal cell wall is chiefly made up of chitin, in addition to glucans and mannans, CBPs from plants and bacteria have been explored for their antifungal activity (Huang et al., 2000; Van Parijis et al., 1991). CBP from *Streptomyces tendae* Tu901 interferes with growth polarity in fungi (Bormann et al., 1999), while Cbp50 from *B. thuringiensis* serovar *konkukian* was antifungal (Mehmood et al., 2011).

1.2.2.3 CBPs are oxido-hydrolytic

Vaaje-Kolstad et al., (2010) described for the first time an enzyme (*Sm*CBP21: previously CBM33 now belongs to AA10 type) that generated oxidized CHOS due to its oxido-hydrolytic activity. Due to oxido-hydrolytic activity, CBM33s have been reclassified under auxiliary activity (AA) family of LPMOs. LPMOs

are divided into three families and named as AA9, AA10 and AA11. LPMOs belonging to family AA9 (previously GH61) are mainly found in fungi, AA10 (previously CBM33) occur in bacteria and viruses and those which have a mixed character of AA9 and AA10 are grouped under AA11. *Ef*CBM33A from *Enterococcus feacalis* (Vaaje-Kolstad et al., 2011), *Bl*AA10A from *B. licheniformis*, *Tf*AA10B from *Thermobifida fusca*, *Ba*AA10A from *B. amyloliquefaciens* (Hemsworth et al., 2013), *Hj*AA9B from *Hypocrea jecorina* (Karkehabadi et al., 2008), *Ta*AA9A from *Thermoascus auranticus* (Quinlan et al., 2011), *Nc*LPMO9C from *Neurospora crassa* (Isaksen et al., 2014) were oxido-hydrolytic.

The three dimensional structure of AA10 type LPMOs from *S. marcescens* (*Sm*CBP21; AAU88202.1), *E. faecalis* (*Ef*CBM33A; AAO80225.1), *B. amyloliquefaciens* DSM7 (*Ba*CBM33 DSM7; CBI42985.1), *Burkholderia pseudomallei* 1710b (*Bp*AA10A; ABA49030.1), *Vibrio cholerae* 01 biovar E1 Tor str. N16961 (GbpA; AAF96709.1) and *Thermobifida fusca* (E7; AAZ55306.1) are available.

1.2.2.4 Mode of action of CBPs/AA10 LPMOs

The mode of action of CBP21 from *S. marcescens* was described by Vaaje-Kolstad et al., (2010). Disruption of chitin by CBP21 was shown to occur in two steps: hydrolysis and oxidation. CBP21 introduces chain breaks in the most inaccessible regions of crystalline chitin substrates. These chain breaks resulted in oxidation of chitin chain ends. Oxidation of chitin and introduction of charge disrupt the normal chair conformation of NAG residues (Figure 1.7). CBP21 thus generated oxidized CHOS. Using NMR and isothermal calorimetry it was shown that CBP21 is an overall rigid molecule. However, there is a metal binding site which shows high local flexibility. Binding of CBP21 to crystalline chitin was metal-dependent. Metal binding studies further confirmed that ions, preferably

Cu^{1+} followed by Cu^{2+}, bind to a site which is present in between His28 and His114 (Aachmann et al., 2012).

1.2.2.5 Amino acid residues that are important for efficient substrate interaction

The amino acid residues involved in interaction and binding of CBPs to insoluble chitin substrates have been identified using structural data, site-directed mutagenesis and NMR. Importance of tryptophan residues in CHB1 of *Streptomyces olivaceoviridis* was reported by Zeltins and Schrempf (1997). CHB1 interacts highly specifically to α-chitin; all the mutants showed reduced binding and mutation of Trp57 to Leu or Tyr resulted in 90% reduction in binding capacity of CHB1 towards α-chitin.

Using site-directed mutagenesis, Vaaje-Kolstad et al., (2005) investigated importance of six conserved, surface located amino acid residues (Tyr-54, Glu-55, Glu-60, His-114, Asp-182 and Asn-185) in binding of CBP21 to β-chitin. Multiple sequence alignment revealed that these residues are highly conserved in most of the CBPs. Out of the six, Tyr54 was only conserved surface exposed aromatic amino acid in CBP21. Determination of dissociation constant showed that all mutants had reduced affinity (increased K_d) towards chitin. Y54A and E60A had highest values of K_d. E55A and H114A showed significant reduction in binding and lost affinity for parts of chitin surface.

This was further confirmed when spectral shifts of these amino acids were observed in NMR. These amino acid residues line up along the substrate binding surface, and this exactly matches the width of single polymeric chain. However, CBP21 binds to crystalline chitin instead of single polymeric chain, suggesting that this narrow stretch of amino acid residues binds perpendicularly to crystalline chitin (Aachmann et al., 2012). Metal binding studies revealed that an ion interaction site is present in between His28 and His114 residues of CBP21. These

residues in the catalytic centre bind to divalent metal ions. This metal binding site is flexible as it can accommodate a variety of metal ions.

1.2.2.6 Domain architecture of CBPs

Most of the CBPs possess only CBM33 domain (renamed as AA10). However, there are reports where in addition to CBM33, several accessory domains are present (Figure 1.8). E8/*Tf*AA10B from *Thermobifida fusca* possesses two CBMs (CBM33 and CBM2) which are interconnected by one FnIII domain (Moser et al., 2008). *Bt*CBP from *B. thuringiensis* sv. *kurstaki* and cbp50 from *B. thuringiensis* sv. *konkukian* possess CBM33 and CBM5, interconnected by two FnIIIs (Manjeet et al, 2013; Mehmood et al., 2011). *Bli*CBP from *B. licheniformis*, *Sp*CBP21, *Sp*CBP50 and *Sp*CBP21 from *S. proteamaculans*, CBP21 from *S. marcescens*, CHB1, CHB2 and CHB3 from *Streptomyces* and *Ef*CBM33A from *Enterococcus faecalis* possess only CBM33/AA10 domain. Most of the CBPs are known to have N-terminal signal peptide. When amino acid sequence was analyzed in Smart database, some CBPs also possess a trans-membrane domain like *Bli*CBP from *B. licheniformis* DSM13, *Ef*CBM33A from *E. faecalis*, E8/*Tf*AA10B from *T. fusca* and CelS2 from *Str. coelicolor*. These domains mostly consist of hydrophobic residues. Trans-membrane domains in proteins play a role in receptor complex assembly and in trans-membrane signaling. However, the role of these trans-membrane domains in CBPs is still not known.

1.3 CBP-CHOS interaction can be studied using fluorescence spectrometry or circular dichroism

1.3.1 Fluorescence spectrometry

Proteins have intrinsic fluorescence because of presence of aromatic amino acid residues, mainly due to tryptophan, and with some emissions due to tyrosine and phenylalanine as well. Some of the disulfide bonds also contribute to absorption. Tryptophan is a relatively rare amino acid as many proteins contain only one or a

few residues. Thus, fluorescence by tryptophan residues is a sensitive measurement for analysis of conformational states of a protein.

We can study ligand protein interaction by either recording the emission spectra (holding the excitation wavelength constant, ε_λ) or by recording excitation spectra (keeping emission wavelength constant, λ_{em}). When there is an interaction between a protein and a ligand, there can be a change in three dimensional structure of protein. Due to this structural change there can be a change in intrinsic fluorescence of a protein. This change in intrinsic fluorescence can be recorded by fluorescence spectrometer either by recording λ_{em} or ε_λ. If there is a linear relationship in fluorescence changes, these values can be used to calculate binding constant (K_B), Gibbs free energy (ΔG^o) and number of binding sites (n) in a protein. Different equations can be used to for calculation. One such equation is Stern-Volmer equation.

Modified Stern-Volmer equation can be used to analyze fluorescence quenching mechanism,

$$\text{Log } [(F_0\text{-}F/F)] = \log K_S + n * \log (Q)$$

where, n is the slope and corresponds to the number of binding sites, K_S is the binding constant and Q is the quencher concentration.

The free energy could be calculated from the following equation:

$$\Delta G^o = -RT \ln K$$

where, ΔG^o is the free energy change, R is the gas constant at room temperature and K is the binding constant which is obtained from the fluorescence data.

Whenever there is complex formation after interaction of protein and ligand, it results in quenching of fluorescence spectra. Quenching refers to the process which decreases the fluorescence intensity of a given substance. Thus, fluorescence spectrometry has been considered as a best method to study protein-ligand interaction, like interaction of human serum albumin and α-1-glycoprotein (Yeggoni et al., 2014). Zeltins and Schrempf (1997) generated site-directed

mutants of CHB1, a CBP from *Str. olivaceoviridis*, to know the role of individual tryptophan residues. They used fluorescence spectrometry to analyze changes in spectrum of CHB1 and its site-directed mutants in presence or absence of chitotriose or chitopentaose. These studies indicated that presence of chitotriose did not interfere with CHB1 binding whereas the λ_{max} in fluorescence spectrum was reduced in presence of chitopentaose.

1.3.2 Circular dichroism

Circular dichroism (CD) has been a widely recognized technique for analyzing structure of proteins. Different structural elements (α-helix, β-sheets and random coils) in a protein give rise to a characteristic CD-signal (Figure 1.9). Using CD, we can characterize a protein on the basis of its secondary and tertiary structure (Kelly et al., 2005). Secondary structure of protein can be determined in far-UV region (190-240 nm). Absorption in this region arises due to peptide bond. Different algorithms have been designed that can estimate the secondary structure composition of proteins using far-UV CD spectra. Algorithms which are widely used for CD spectra analysis are CDNN (Böhm et al., 1992), SELCON (Sreerama and Woody, 1993), VARSLC (Manavalan and Johnson, 1987), CDSSTR (Johnson, 1999), K2D2 (Andrade et al., 1993), CONTIN (Provencher et al., 1981) and DICHROWEB (Lobley et al., 2002; Whitmore and Wallace, 2004). Tertiary structure of protein can be determined in near-UV region (260-320 nm). In near-UV region, aromatic amino acids give a characteristic wavelength profile. Trp shows a peak in between 290-305 nm, Tyr and Phe show peak in between 275-282 nm and 255-270 nm, respectively (Figure 1.10). Near-UV spectrum of a protein depends on the number of aromatic amino acid residues, their spatial arrangement and also on the type of aromatic amino acid residue.

Protein-ligand interaction can be detected by analyzing the structural changes in protein. CD signals can arise from ligands which do not have any intrinsic chirality, but they acquire it when they interact with protein. CD can be used to

detect the optimum concentration of ligand which is required to induce conformational changes in protein. Due to protein-ligand interaction, there can be a shift in tertiary structure fingerprint of a protein, and also there can be changes in secondary structure elements of protein. For example, α-helix may switch to β-sheets or random coils or *vice-versa* (Hope et al., 1996).

CD can also be used to study thermal stability of a protein. With increase in temperature protein its changes conformation, and hence CD spectrum changes. Entire spectrum in far- or near-UV region can be studied at different temperatures. Or else, by choosing a single wavelength, temperature conditions can be changed, this will help in analyzing some specific feature of protein structure. Similarly, effect of pH, salts, drugs etc. on protein structure can also be studied using CD.

1.4 Oxidized CHOS generated by CBPs and crystalline chitin interaction can be analyzed using mass spectrometry

Mass spectrometry (MS) generates spectra of samples based on mass. It determines the elemental and isotopic signature of a sample. MS works by first ionizing the chemical compounds, and then measuring mass to charge (m/z) ratio of these ions. Based on the ionization sources, ESI (electron spray ionization) and MALDI (matrix-assisted laser desorption ionization) are the most common and sensitive ionization mechanisms. ESI and MALDI have also been used widely to detect CHOS. Identification and characterization of CHOS can also be done using ion exchange chromatography (Haebel et al., 2007) and gel filtration chromatographic (Sorbotten et al., 2005) techniques. MALDI-TOF MS assay for oxidized CHOS was described by Vaaje-Kolstad et al., (2010). They described *Sm*CBP21 (previously CBM33, now belongs to AA10 type) that generated oxidized CHOS due to oxido-hydrolytic activity. The products were detected using mass spectrometry. They further detected that these products are CHOS with a normal sugar at the non-reducing end and an oxidized sugar at the other.

Addition of reductants like ascorbic acid increased production of these oxidized CHOS.

1.5 Applications of CBPs in production of biofuels

Crystalline cellulose and chitin have emerged as efficient sources for biofuel production (Himmel et al., 2007). However, it is difficult to convert them into soluble sugars due to their crystalline nature. Conversion of chitin and cellulose to soluble sugars is primarily mediated by enzymatic hydrolysis. Reports on conversion of crystalline chitin to soluble sugar molecules, using CBPs (AA10-LPMOs), have opened new avenues for production of biofuels. CBPs/oxido-hydrolases disrupt the normal chair conformation of *N*-acetyl glucosamine and this finally leads to disruption of crystalline chitin. This oxido-hydrolytic activity of CBPs/LPMOs can be utilized on a large scale in various industries as a start up process to hydrolyze crystalline chitin substrate. Hydrolysis of chitin substrate generates CHOS which are used by various industries like in pharmaceuticals, medicine, agriculture and cosmetics. However, usefulness of oxidized CHOS is yet to be identified.

1.6 Our attempt to answer some questions about CBPs in chitin digestion

We have used CBPs and chitinases from three different organisms: *Bacillus thuringiensis* (*Bt*), *B. licheniformis* (*Bli*) and *S. proteamaculans* (*Sp*). CBPs and chitinases (Chi) from these organisms are designated by using abbreviation of organism's genus and species name (first letter) as prefix, like *Bt*Chi and *Bt*CBP are chitinase and CBP from *B. thuringiensis*. Similarly, we used *Bli*Chi and *Bli*CBP from *B. licheniformis* and *Sp*ChiB, *Sp*CBP21 and *Sp*CBP28 from *S. proteamaculans*. Using these three different chitinases and four different CBPs we made an attempt to answer following questions:

- What are the optimum pH and temperature conditions for binding of *Bt*CBP, *Bli*CBP and *Sp*CBP21 to crystalline chitin?
- Do all chitinases work in combination with CBPs?
- Is there any specificity between chitinases and CBPs?
- Do all bacterial CBPs increase antifungal activity of chitinases?
- What is the role of accessory domains (FnIII and CBM5) in *Bt*CBP?
- Whether *Sp*CBP28 has lost its binding/activity due to mutation in any or all of the essential amino acid residues?

2.1.1 Bacterial cultures and plasmids

Bacillus thuringiensis serovar *kurstaki* (ATC33679), *Bacillus licheniformis* (DSM13), *Serratia proteamaculans* 568 (ATCC19323), *Escherichia coli* DH5α and BL21 (DE3) were used from the collection of strains in our laboratory.

Full length clones of chitinases and CBPs from *Bacillus thuringiensis* serovar *kurstaki* (*Bt*Chi and *Bt*CBP), *Bacillus licheniformis* (*Bli*Chi and *Bli*CBP), deletion mutant of *Bli*Chi designated as *Bli*GH, *Serratia proteamaculans* 568 (*Sp*ChiB, *Sp*CBP21 and *Sp*CBP28) were obtained from Neeraja (2010a) and Purushotham (2012).

Fungal isolates *viz*., *Alternaria alternata*, *Fusarium oxysporum*, *Colletrotrichum falcatum*, *Rhizopus* sp., *Aspergillus* and *Penicillium* sp. were used from the collection of strains in our laboratory.

2.1.2 Media used

2.1.2.1 Luria Bertani Broth/ Agar (LBB/LBA)

One litre of LBB consists of 10 g tryptone, 10 g NaCl and 5g yeast extract. The pH was adjusted to 7.5 and 1.5% agar was added wherever required to prepare LBA.

2.1.2.2 Potato dextrose broth/Agar (PDB/PDA)

Potato dextrose broth was procured from Himedia Laboratories (Mumbai, India).

2.1.2.3 Czapek dox broth/Agar (CDB/CDA)

One litre of CDB consists of 30 g sucrose, 3 g sodium nitrate, 1 g di-potassium phosphate, 0.5 g magnesium sulfate, 0.5 g potassium chloride and 0.01 g ferrous sulfate. Final pH was adjusted to 7.3. Agar (1.5%) was added wherever required.

2.1.3 Chemicals

The chemicals used in present study were of analytical grade and procured from Sigma-Aldrich (MO, USA), GE Health care (Uppsala, Sweden), Fermentas (Ontario, Canada), Himedia labs (Mumbai, India), Thermo Scientific (USA) and Qualigens fine chemicals (Mumbai, India). Polymeric substrates, α- chitin (extracted from shrimp shells, 60 mesh powder) and β- chitin (extracted from squid pen, 200 µm), were provided by Dr. Dominique Gillete, Mahatani Chitosan (Veraval, India). CHOS with different degree of polymerization (DP) were purchased either from Seikagaku Corporation (Tokyo, Japan) through Cape Cod (USA) or from Megazyme (Co. Wicklow, Ireland).

2.1.4 Kits

Plasmid isolation and Gel extraction kits were purchased from Sigma Aldrich (USA).

2.1.5 Enzymes

Restriction enzymes (*Nco*I and *Xho*I) and T4 DNA ligase were purchased from MBI Fermentas (Ontario, Canada), *Pfu* and Phusion DNA polymerase were from New England Biolabs (Massachusetts, England) and Taq DNA Polymerase was from Sigma Aldrich.

2.2 Plasmids

Brief details of plasmids used and the designation of constructs used in this study are given in Tables 2.1 & 2.2.

2.3 Primers used for PCR

List of primers used for generation of different deletion and site-directed mutants is given in Tables 2.1 & 2.2. All primers used were purchased from MWG Biotech (Ebersberg, Germany).

2.4 Generation of deletion mutants of *Bt*CBP

Details of deletion mutants of *Bt*CBP generated using overlap extension PCR are shown in Figures 2.1 and 2.2.

2.4.1 PCR amplification and cloning of *Bt*CBPΔCIII, *Bt*CBPΔNIII and *Bt*CBPΔCΔNIII

To generate *Bt*CBPΔCIII deletion mutant, where C-terminal FnIII was deleted, N-terminal CBM33/AA10 and FnIII encoding gene with C-terminal overhang was amplified using *Btcbp* plasmid as template. Gene was amplified using 56°C of annealing temperature and gene specific forward primer (*Bt*CBPFp) and reverse primer with overhang (*Bt*CBPΔCIIIRpO). C-terminal CBM5 was amplified with 56°C annealing temperature using internal forward primer (*Bt*CBM5IFp) and reverse primer (*Bt*CBPRp). Both N- and C- terminal amplified products were loaded on 1% agarose gel and purified using gel extraction kit. Purified gel extraction products were added in ratio of 1:1 and used as template for fusion PCR. Fusion PCR was carried out at an annealing temperature of 56°C. *Pfu* DNA polymerase was used for PCR amplification and extension time was followed according to manufacturer's protocol. Fusion PCR product was loaded on 1% agarose gel, purified using gel extraction kit and double-digested using restriction enzymes *Nco*I and *Xho*I. Double-digested purified fusion products were ligated to double- digested expression vector pET22b (+). Ligation was kept at 16°C for 16 h. The resultant plasmid was designated as pET22b- *Bt*CBPΔCIII to express *Bt*CBPΔCIII.

*Bt*CBPΔNIII deletion mutant was generated by amplifying N-terminal CBM33/AA10 with overhang and C-terminal FnIII and CBM5 using *Btcbp* plasmid as template. Amplification was done using forward primer (*Bt*CBPFp) and reverse primer with overhang (*Bt*CBM33/AA10RpO2) for N-terminal CBM33/AA10 and forward primer (*Bt*CBPΔNIII-IFp) and (*Bt*CBM5Rp) for C-terminal FnIII and CBM5. Fusion PCR was kept as mentioned above for *Bt*CBPΔNIII. Annealing temperature was 56°C for amplification of individual N- and C-terminal regions and also for fusion. Extension time was used as mentioned in manufacturer's protocol of *Pfu* DNA polymerase. Fusion product and expression vector pET22b (+) were double-digested and ligated using T4 DNA ligase at a temperature of 16°C for 16 h. The resultant plasmid was designated as pET22b- *Bt*CBPΔNIII to express *Bt*CBPΔNIII.

*Bt*CBPΔCΔNIII deletion mutant, where both FnIIIs were deleted, was generated by amplifying N-terminal CBM33/AA10 (using *Bt*CBPFp and *Bt*CBM33RpO3) and C-terminal CBM5 (using *Bt*CBM5IFp and *Bt*CBM5Rp). Fusion PCR was kept as mentioned above for *Bt*CBPΔNIII. Annealing temperature was kept 56°C for amplification of individual N- and C-terminal regions and also for fusion. Extension time was used as mentioned in manufacturer's protocol of *Pfu* DNA polymerase. Fusion product and expression vector pET22b (+) were double-digested and ligated using T4 DNA ligase at a temperature of 16°C for 16 h. The resultant plasmid was designated as pET22b- *Bt*CBPΔCΔNIII to express *Bt*CBPΔCΔNIII.

2.4.2 PCR amplification and cloning of *Bt*CBPFnIII(N+C)

Both N- and C- terminal FnIII domains were PCR amplified together using gene specific forward (*Bt*CBPFnIIIFp) and reverse (*Bt*CBPFnIIIRp) primers (listed in Table 1) at an annealing temperature of 56°C. The amplified products were loaded on 1% agarose gel, gel purified and digested using *Nco*I and *Xho*I restriction enzymes. Double digested amplicon was ligated to double-digested

pET22b (+) expression vector. Ligation conditions were same as mentioned above. The resultant plasmid was designated as pET22b- *Bt*CBPFnIII(N+C) and expressed as *Bt*CBPFnIII(N+C).

2.4.3 PCR amplification and cloning of *Bt*CBM33/AA10 and *Bt*CBM5

Genes encoding *Bt*CBM33/AA10 and *Bt*CBM5 in *Bt*CBP were amplified using *Btcbp* plasmid at annealing temperature of 56°C with gene specific forward (*Bt*CBPFp and *Bt*CBM5Fp, respectively) and reverse (*Bt*CBM33/AA10Rp and *Bt*CBPRp, respectively) primers. Expression vector and amplicons were double-digested with *Nco*I and *Xho*I, gel purified and ligated using T4 DNA ligase at 16°C for 16 h.

2.5 Generation of site-directed mutants of *Sp*CBP28

Single mutants of *Sp*CBP28 with I79E, L84E and G263N mutations were generated as described by Song-Hua and Madison (1997) with pET-22b-*Sp*CBP28 (WT) as template. Mutagenic primers (Mp) were designed to generate single mutants as listed in Table 2.2. Amplification was carried out in two different steps using Phusion DNA polymerase. In the first step of PCR, mutagenic primer and either reverse or forward primer was used to generate mega-primer at an annealing temperature of 52°C and polymerization time of 90 sec. The product from the 1st step of PCR was used as megaprimer for the second step of PCR with annealing temperature of 60°C and polymerization time of 80 sec. Expression vector, pET-22b (+) and amplicons were double digested with *Nco*I and *Xho*I at 37°C for 4 h, followed by PCR clean up. Double-digested amplicons were ligated to *Nco*I and *Xho*I sites of the expression vector using T4 DNA ligase at 16°C for 16 h. The mutants were designated as pET22b-*Sp*CBP28-I79E, pET22b-*Sp*CBP28-L84E and pET22b-*Sp*CBP28-G263N for *Sp*CBP28-I79E, *Sp*CBP28-L84E and *Sp*CBP28-G263N.

Double and triple mutants of *Sp*CBP28 with I79E+L84E, I79E+G263N, L84E+G263N and I79E+L84E+G263N mutations were generated using *Dpn*I-mediated site-directed mutagenesis method based on quick change site-directed mutagenesis protocol (Papworth et al., 1996). PCR amplification was done using respective phosphorylated primers (as listed in Table 2.2) for each mutant at an annealing temperature of 60°C. Single mutants were used as template for generating double mutants and double mutants were used as template for generating triple mutant. PCR products were purified using gel extraction-purification kit and 200 ng of each product was used for self ligation. Ligation was carried out at 22°C for 30 min using T4 DNA fast ligase, followed by 10 min incubation at 65°C to inactivate T4 DNA fast ligase. Further, ligation product was digested using *Dpn*I fast digest restriction enzyme to remove any parent plasmid template. Digestion was carried out at 37°C for 1 h, followed by *Dpn*I inactivation at 80°C for 5 min. Digested plasmids were transformed into NEB 5α competent cells. Desired mutation was confirmed after sequencing. The mutant plasmids were designated as pET22b-*Sp*CBP28-I79E+L84E, pET22b-*Sp*CBP28-I79E+G263N, pET22b-*Sp*CBP28-L84E+G263N and pET22b-*Sp*CBP28-I79E+L84E+G263N for *Sp*CBP28-I79E+L84E, *Sp*CBP28-I79E+G263N, *Sp*CBP28-L84E+G263N and *Sp*CBP28-I79E+L84E+G263N.

2.6 Protein expression and purification of CBPs, deletion and site-directed mutants, and chitinases

Plasmid of desired clone (pET22b-*Bt*CBP, pET22b-*Bli*CBP, pET22b-*Sp*CBP21, pET22b-*Bt*Chi, pET22b-*BliGH*, pET22b-*Bli*Chi, pET22b-*Sp*ChiB, pET22b-*Bt*CBPΔCIII, pET22b-*Bt*CBPΔNIII, pET22b-*Bt*CBPΔCΔNIII, pET22b-*Bt*CBPFnIII(N+T) pET22b-*Bt*CBM33/AA10, pET22b-*Bt*CBM5, pET22b-*Sp*CBP28, pET22b-*Sp*CBP28-I79E, pET22b-*Sp*CBP28-L84E, pET22b-*Sp*CBP28-G263N, pET22b-*Sp*CBP28-I79E+L84E, pET22b-*Sp*CBP28-I79E+G263N, pET22b-*Sp*CBP28-L84E+G263N and pET22b-*Sp*CBP28-I79E+L84E+G263N)

were transformed into *E. coli* BL21 (DE3) competent cells using ampicillin resistance with working concentration as 100 µg/mL. Single colony was inoculated in LBB with ampicillin and incubated at 37°C for 12 h with constant shaking. Protein expression was done using auto-induction media as described by Studier (2005) when 1% of starter culture was used as inoculum. The expressed proteins were designated as *Bt*CBP, *Bli*CBP, *Sp*CBP21, *Bt*Chi, *BliGH*, *Bli*Chi, *Sp*ChiB, *Bt*CBPΔCIII, *Bt*CBPΔNIII, *Bt*CBPΔCΔNIII, *Bt*CBPFnIII(N+T), *Bt*CBM33/AA10, *Bt*CBM5, *Sp*CBP28, *Sp*CBP28-I79E, *Sp*CBP28-L84E, *Sp*CBP28-G263N, *Sp*CBP28-I79E+L84E, *Sp*CBP28-I79E+G263N, *Sp*CBP28-L84E+G263N and *Sp*CBP28-I79E+L84E+G263N.

After expression, culture was harvested by centrifugation at 4200xg for 10 min. Pellet obtained from centrifugation was resuspended in lysis buffer (50 mM NaH_2PO_4; 300 mM NaCl; 10 mM imidazole, pH 8.0) and sonication was performed (10 sec on; 20 sec off pulse and 32% amplitude) on ice with a Vibra cell ultrasonic processor, converter model CV33, equipped with 3 mm probe (Sonics, Newton, CT, USA). Sonication was followed by centrifugation at 40, 000 g for 40 min at 4°C. Periplasmic fraction (PF) isolation was done wherever required using pET manual protocol (Novagen). Cell pellet was resuspended in PF1 buffer (30 mM Tris-Cl, 20% sucrose, 1mM EDTA, pH 8.0) followed by gentle stirring for 10 min, and centrifugation at 4200 g for 10 min. Pellet obtained after centrifugation was further resuspended in PF2 (5 mM $MgSO_4$) followed by gentle stirring for 10 min at 4°C and centrifugation at 4200 g for 10 min at 4°C. Supernatant from PF isolation or sonication was collected and purified using Ni-NTA His-tag chromatography as mentioned in manufacturer's protocol (Sigma Aldrich, USA). Pure protein eluted in 250 mM imidazole elution buffer (NaH_2PO_4; 300 mM NaCl; 250 mM imidazole, pH 8.0). Purified fractions were pooled and concentrated using amicon filters (10 or 2 kDa MWCO; Millipore). Concentrated protein was buffer exchanged and stored at 4°C till use.

2.7 Visualization of pure protein using SDS-PAGE analysis

Purified *Bt*CBP, *Bli*CBP, *Sp*CBP21, *Sp*CBP28, *Bt*Chi, *BliGH*, *Bli*Chi, *Sp*ChiB, *Bt*CBPΔCIII, *Bt*CBPΔNIII, *Bt*CBPΔCΔNIII, *Bt*CBPFnIII(N+T), *Bt*CBM33/AA10, *Bt*CBM5, *Sp*CBP28-I79E, *Sp*CBP28-L84E, *Sp*CBP28-G263N, *Sp*CBP28-I79E+L84E, *Sp*CBP28-I79E+G263N, *Sp*CBP28-L84E+G263N and *Sp*CBP28-I79E+L84E+G263N were resolved and visualized using SDS-PAGE analysis on vertical slab gel units. Lower resolving gel (12 or 15%) and upper stacking gel (4%) were prepared in 0.375 M (pH 8.8) and 0.125 M (pH 6.8) Tris-Cl buffer, respectively. Electrode buffer was prepared using 0.025 M Tris-Cl, 0.192 M glycine and 0.1% (w/v) SDS; pH 8.5. Sample buffer (4X) contained 40 % glycerol, 240 mM Tris-Cl (pH 6.8), 8% SDS, 0.04% bromophenol blue and 5% beta- mercaptoethanol. Sample buffer was diluted to 1X final concentration. Electrophoresis was carried out at recommended voltage as per length of gel plates. For 15% SDS-PAGE electrophoresis was performed at low voltages. Gels were stained using colloidal Coomassie staining solution (20 % v/v ethanol, 8 % w/v ammonium sulphate, 0.08 % w/v Coomassie brilliant blue G-250, 0.35 M *o*-phosphoric acid). Destaining of gel was done using mQ/double distilled water till clear protein bands were visible.

2.8 Binding assay to test binding of CBPs towards α- and β- chitin

Substrate binding assay for *Bt*CBP, *Bli*CBP, *Sp*CBP21, *Sp*CBP28, *Bt*Chi, *BliGH*, *Bli*Chi, *Sp*ChiB, *Bt*CBPΔCIII, *Bt*CBPΔNIII, *Bt*CBPΔCΔNIII, *Bt*CBPFnIII(N+T), *Bt*CBM33/AA10, *Bt*CBM5, *Sp*CBP28-I79E, *Sp*CBP28-L84E, *Sp*CBP28-G263N, *Sp*CBP28-I79E+L84E, *Sp*CBP28-I79E+G263N, *Sp*CBP28-L84E+G263N and *Sp*CBP28-I79E+L84E+G263N was done as described by Manjeet et al., (2013) with slight modifications. The reaction mixture (100 µL) containing 100 µg of *Bt*CBP, *Bli*CBP, *Sp*CBP21, *Sp*CBP28, *Bt*Chi, *BliGH*, *Bli*Chi, *Sp*ChiB, *Bt*CBPΔCIII, *Bt*CBPΔNIII, *Bt*CBPΔCΔNIII, *Bt*CBPFnIII(N+T), *Bt*CBM33/AA10, *Bt*CBM5, *Sp*CBP28-I79E, *Sp*CBP28-L84E, *Sp*CBP28-G263N,

*Sp*CBP28-I79E+L84E, *Sp*CBP28-I79E+G263N, *Sp*CBP28-L84E+G263N or *Sp*CBP28-I79E+L84E+G263N was incubated with 0.5 µg of α- or β- chitin for 24 h at required temperature (ranging from 10- 100°C) and pH (ranging from 3-12), with constant shaking (650 rpm) in thermomixer (Thermomixer comfort; Eppendorf, Germany). For characterization at different pH, the buffers used were 50 mM sodium citrate buffer (pH 3-5), 50 mM sodium acetate buffer (pH 4-5), 50 mM sodium phosphate buffer (pH 6-8), 50 mM tris-Cl buffer (pH 7-9), 50 mM glycine-NaOH buffer (pH 8-9) and 50 mM NaH_2PO_4-NaOH (pH 10-12). After incubation, reaction mixture was centrifuged at 16,000g for 30 min. Protein concentration in supernatant was measured as described above. Amount of bound protein (P_{bound}) was calculated by subtracting amount of free protein present in supernatant (P_{free} or S) from total protein added initially (P_{total}, also mentioned as control: C). Percent bound protein was calculated using formula: $\%P_{bound}=[P_{bound}/P_{total}]*100$ and represented as bar graphs. Wherever required, pellet obtained after centrifugation was washed twice in same buffer as used for reaction mixture. Fractions obtained in these two steps were named as W1 and W2, respectively. Bound protein was eluted by boiling the washed pellet in 100 µL of 1% SDS in two steps and fractions were called E1 and E2, respectively. Fractions obtained above S, C, W1, W2, E1 and E2 were resolved on SDS-PAGE. All experiments were done in triplicates.

2.9 Binding assay to test binding of CBPs towards β- chitin at different temperature

Substrate binding assay for *Bt*CBP, *Bli*CBP and *Sp*CBP21 as mentioned above. The reaction mixture (100 µL) containing 100 µg of *Bt*CBP, *Bli*CBP or *Sp*CBP21 was incubated with 0.5 µg of β- chitin for 1 h at required temperature (ranging from 10- 100°C) in Tris-Cl buffer pH 7.0, in thermomixer (Thermomixer comfort; Eppendorf, Germany). After incubation, reaction mixture was centrifuged at 16,000g for 30 min. Protein concentration in the supernatant was measured as

described above. Amount of bound protein (P_{bound}) was calculated as mentioned above and represented as bar graphs. All experiments were done in triplicates.

2.10 Conformational changes in CBPs at different temperatures using CD

To assess conformational changes in *Bt*CBP, *Bli*CBP, *Sp*CBP21, *Bt*CBM33/AA10 and *Bt*CBM5 at different temperatures, CD spectra were measured in far UV region: 190-260 nm on Jasco J720 spectro polarimeter. Temperature control was provided by Peltier thermostat. Purified *Bt*CBP, *Bli*CBP, *Sp*CBP21, *Bt*CBM33/AA10 or *Bt*CBM5 was mixed with 800 µL of 10 mM tris-Cl buffer (final protein concentration: 0.2 mg/mL). Each spectrum was recorded 5 times at 20 nm/min scan rate, and 10.0 mdeg sensitivity.

2.11 Binding assay to test binding of CBPs towards β- chitin at different pH conditions

Substrate binding assay for *Bt*CBP, *Bli*CBP and *Sp*CBP21 as mentioned above. The reaction mixture (100 µL) containing 100 µg of *Bt*CBP, *Bli*CBP or *Sp*CBP21 was incubated with 0.5 µg of β- chitin for 1 h at 37°C in and pH (ranging from 3-12), in thermomixer (Thermomixer comfort; Eppendorf, Germany). For characterization at different pH, the buffers used were 50 mM sodium citrate buffer (pH 3-5), 50 mM sodium acetate buffer (pH 4-5), 50 mM sodium phosphate buffer (pH 6-8), 50 mM Tris-Cl buffer (pH 7-9), 50 mM glycine-NaOH buffer (pH 8-9) and 50 mM NaH_2PO_4-NaOH (pH 10-12). After incubation, reaction mixture was centrifuged at 16,000g for 30 min. Protein concentration in the supernatant was measured as described above. Amount of bound protein (P_{bound}) was calculated as mentioned above and represented as bar graphs. All experiments were done in triplicates.

2.12 Time course binding of *Bt*CBP and *Bli*CBP towards α and β-chitin

Substrate binding assay of *Bt*CBP and *Bli*CBP towards α- and β-chitin was done as mentioned above (section 2.8). Percent binding was assessed at different time points up to 24 h. All experiments were done in triplicates.

2.13 Binding kinetics of CBPs: Determining K_d and B_{max} of *Bt*CBP and *Bli*CBP

Dissociation constants (K_d and B_{max}) of *Bt*CBP and *Bli*CBP were calculated as described by Vaaje-Kolstad et al (2005) with slight modifications. Reaction mixture with varied concentration of *Bt*CBP and *Bli*CBP (10, 20, 50, 100, 150, 200, 300 and 400 µg) was incubated for 15 h for α-chitin and 9 h for β-chitin for both *Bt*CBP and *Bli*CBP. After incubation, reaction mixture was centrifuged and P_{free} and P_{bound} were calculated as mentioned above. P_{free} and P_{bound} values were plotted to fit into GraphPad Prism software version 5.0. All data sets were fitted to the equation for one-site binding by non-linear regression function to calculate K_d and B_{max} values.

2.14 Dot blot assay for testing activity of *Bt*Chi, *BliGH* and *Bli*Chi

Dot blot assay was carried out to test the activity of *Bt*Chi, *BliGH* and *Bli*Chi on glycol chitin. A glycol chitin gel was prepared which contains 0.1% of glycol chitin. Five µg of each *Bt*Chi, *BliGH* or *Bli*Chi was placed on glycol chitin gel. Gel was incubated in humid chamber at 37°C for 12 h. After incubation, gel was stained using calcofluor white M2R stain (0.01%). Gel was washed thoroughly with distilled water and visualized under UV-transilluminator.

2.15 Standardization of different conditions for chitinase assay

2.15.1 Standardization of chitinase concentration

The concentration of chitinase was standardized by performing chitinase assay as mentioned by Neeraja et al., (2010a) using different concentrations of *Bt*Chi till concentration of products reached saturation. The reaction mixture with colloidal chitin (0.5 mg/mL) and different concentrations of *Bt*Chi in 50 mM sodium phosphate buffer pH 8.0 was incubated at 37°C for 1 h. After incubation, reaction mixture was centrifuged at 16,100 g for 20 min and 40 µL of supernatant was used to estimate the release of reducing groups. The concentration of *Bt*Chi, just before the saturation level was used for further experiments.

2.15.2 Stability of chitinases at 37°C

To test the stability of chitinases at 37°C, chitinase and dot blot assay for *Bt*Chi/*Bli*Chi/*Sp*ChiB were performed as mentioned in sections 2.14; 2.15.1. For chitinase assay, reaction mixtures were incubated at 37°C for different time points, up to 24 h. Product concentration was measured as mentioned above.

2.16 Synergistic effect of CBPs and chitinases

2.16.1 Synergistic effect of CBPs and chitinases on chitin substrates

2.16.1.1 Chitinase assay

Chitinase assay to test hydrolytic activity of *Bt*Chi, *Bli*Chi and *Sp*ChiB on α- and β-chitin was determined as mentioned by Neeraja et al., (2010a) and briefly described in section 2.15.1 with slight modifications. Reaction mixture containing 0.2 nmoles of *Bt*Chi, *Bli*Chi or *Sp*ChiB was incubated with 0.5 mg/mL of α- or β-chitin in 50 mM sodium phosphate buffer, pH 8.0 at 37°C for 24 h with constant shaking (450 rpm) in thermomixer (Thermomixer comfort; Eppendorf, Germany). After incubation, reaction mixture was centrifuged at 16,100 g for 20 min.

Supernatant (40 µL) was used to measure the release of reducing groups. To study the synergistic effect of chitinases with CBPs, 0.28 nmoles of *Bt*CBP, *Bli*CBP or *Sp*CBP21 was also added to the reaction mixture along with chitinase.

2.16.1.2 High performance thin layer chromatography (HP-TLC)

Reaction mixtures containing 0.2 nmoles of purified chitinases and 0.28 nmoles of purified CBPs were incubated with 0.5 mg/ml of chitin substrates (α- chitin, β-chitin and colloidal chitin) at 37˚C for 24 h. After incubation, the reaction mixture was centrifuged at 13,000 g for 20 min. Fifteen µl of the supernatant containing the hydrolyzed products was loaded onto the silica gel coated TLC plate using HP-TLC. The plates were dried carefully and run against the solvent (n-butanol, methanol, 25% ammonia and water in ratio of 5:4:2:1 v/v/v/v) in a chromatography chamber till the solvent reaches 3/4th of the TLC plate. The plate was dried and sprayed with spraying solution (aniline-100 µl, diphenylamine-100 mg, acetone-5 ml and *o*-phosphoric acid-750 µl) followed by heating at 180˚C to develop the spots on the TLC plate.

2.16.2 Synergistic effect of CBPs and chitinase on fungi

2.16.2.1 PDA plate assay

To visualize antifungal activity of chitinases on *Alternaria alternata*, *Fusarium oxysporum*, *Colletrotrichum falcatum*, *Rhizopus* sp., *Aspergillus* sp. and *Penicillium* sp., diffusion assay was performed in PDA. Small amount of fungus was inoculated at the centre of the PDA plate and wells were made onto the PDA with the help of sterile borer. The fungus was allowed to grow at 27°C in dark, till it covers half of the PDA plate before reaching wells. Afterwards, test sample (different concentrations of chitinase and/or CBP) was inoculated into the wells and plate was again incubated at 27°C in dark. Nystatin and buffer were used as positive and negative control, respectively.

2.16.2.2 Isolation, counting and staining of spores

PDA plate with full grown *Alternaria alternata* in its sporulating stage was used for spore isolation. Ten mL of sterile mQ water was added onto the growing fungus and scrapped gently with sterile glass slide. Spores containing mQ water was then filtered through muslin cloth twice to avoid any contamination with hyphae. Spores were then observed under microscope (Leica, Switzerland) to test purity. Spore count was done using haemocytometer. Spores were stained using calcofluor white M2R stain (0.01%) for 2 min and washed thoroughly with distilled water. These spores were then analyzed using confocal microscope.

2.16.2.3 Evaluation of *in vitro* fungal growth in microtiter plates

The growth of *Alternaria alternata*, in the absence or presence of chitinases and/or CBPs, was assessed using microtiter plate assay with slight modifications (Podile and Prakash 1996). The assay was carried out in Corning, flat, transparent, sterile 96- well plate. To 50 µL of spores suspended in PDB/CDB, CBP (20 mM) and/or chitinase (200 µg) were added into the appropriate well of microtiter plate. PDB/CDB was added to make a final volume of 150 µL. Spores resuspended in PDB/CDB served as control. Six replicates were used for each treatment. Plates were then shaken for 1 min to mix spores and test solution. Before reading absorbance spores were sediment by keeping plates undisturbed for 30 min at 27°C and absorbance at 595 nm (A_{595}) was measured for time (t)=0. Plates were again incubated in dark at 27°C and absorbance was measured for up to 34 h. Statistical analysis was done using one-way analysis of variance (ANOVA), followed by Tukey's HSD post hoc test (SPSS Inc., Chicago, IL, USA). Significance was determined at 0.05 level.

2.17 CBP-CHOS interactions

2.17.1 Circular dichroism

Ligand binding studies were done in near UV region (250-320 nm) at 25±0.2°C. Purified *Bt*CBP, *Bli*CBP, *Sp*CBP21, *Bt*CBM33/AA10 or *Bt*CBM5 were dissolved in 200 µL of 10 mM tris-Cl buffer (protein concentration: 1 mg/mL). Stock solution (20µg/200µL) of water soluble fully acetylated CHOS with degree of polymerization (DP) 6 was prepared in 10 mM tris-Cl buffer. *Bt*CBP, *Bli*CBP, *Sp*CBP21, *Bt*CBM33/AA10 or *Bt*CBM5 and CHOS with DP6 were mixed and incubated for 5 min prior to recording of CD-spectrum. CD curves of CHOS-protein mixtures were corrected by subtracting controls (CHOS alone) and blanks (buffer). Data was analyzed using OriginPro8.

2.17.2 Fluorescence spectrometry

A fixed concentration of *Bt*CBP, *Bli*CBP, *Sp*CBP21, *Bt*CBM33/AA10 or *Bt*CBM5 (0.001 mM) was used to study their interactions with CHOS (with DP3/6; concentration 0.005mM) using Perkin Elmer LS55 fluorescence spectrometer. To study the binding kinetics, different concentrations of CHOS ranging from 0.005 to 0.05mM in 10 mM tris-Cl, pH 7.0 were used. The data was analyzed and graphs were plotted using OriginPro8 software. *Bt*CBP, *Bli*CBP, *Sp*CBP21, *Bt*CBM33AA10 or *Bt*CBM5 were excited at 285nm and emission spectra were collected in the wavelength range of 300-500nm at room temperature. The slit width (band width) was fixed to 10 nm for emission and excitation. The binding constant (K_B) and Gibbs free energy (ΔG°) were calculated using Stern-Volmer equation (Yeggoni et al., 2014).

2.18 Analysis of oxidized CHOS using MS

2.18.1 Reactions with *Bt*CBP, *Bt*CBM33/AA10 and *Bt*CBM5

Oxido-hydrolytic activity of *Bt*CBP, *Bt*CBM33/AA10 and *Bt*CBM5 was studied as described by Vaaje-Kolstad et al., (2010) with slight modifications. Stock solution (10 mg/mL) of β- chitin was prepared in 10 mM tris-Cl buffer, pH 7.0. Fifty µL of this stock solution (with continuous stirring) was added to the reaction mixture containing 1 µM of *Bt*CBP, *Bt*CBM33/AA10 or *Bt*CBM5. Ascorbic acid (1.0 mM) was used as a reducing agent to boost oxidative hydrolysis. Reactions were incubated at 37°C, 1000 rpm for 4 days on thermomixer (Thermomixer comfort; Eppendorf, Germany). After incubation, reaction mixture was centrifuged at 16,000 g for 30 min. Supernatant was collected and concentrated to 20 µL and then purified using Thermo-Scientific Hypersep^{TM} tip. Oxidized products were analyzed using MALDI-TOF or Nano-ESI mass spectrometry.

2.18.2 Matrix-assisted laser desorption – time of flight (MALDI-TOF) MS

One µL of 10 mg/mL mixture of 2, 5- dihydroxybenzioc acid (DHB) in 50% acetonitrile was applied to a MTP 384 target plate ground steel TF (Bruker Daltonics). One µL sample (*Bt*CBP, *Bt*CBM33/AA10 or *Bt*CBM5) was then mixed with 1µL droplet of DHB on plate and dried under stream of warm air. The samples were analyzed with an Ultraflex MALDI-TOF/TOF instrument (Bruker Daltonics GmbH, Bremen, Germany) with a Nitrogen 355 nm laser beam. The instrument was operated in positive ion mode. Signals were acquired over a range of *m/z* 0-4000. Peak lists were generated using mmass software (Strohalm et al., 2010).

2.18.3 Nano-Electron spray ionization (Nano-ESI) MS

*Sp*CBP28 or *Sp*CBP28-I79E+L84E+G263N was incubated with β- chitin (580# or 100-170 # mesh size) for 4 days at 37°C, 1000 rpm in tris-Cl buffer 20mM, pH

8.0. *Bt*CBP was used as positive control. After incubation, reaction mixture was centrifuged and supernatant was concentrated. Nano-ESI-q-TOF mass spectrometry was used to analyze the products generated by *Sp*CBP28 in concentrated supernatant. The instrument was operated in negative ion mode. For all experiments following parameters were used: the ion source temperature was set to 80°C, desolvation gas was used at a flow rate of 100 L/h, a potential of 100 V was applied to the capillary tip and the sampling cone voltage was set to 80 V. The MS and MS/MS signals were acquired over a range of *m/z* 100-5000 at a scan rate of 2.0 s/scan. All scans were displayed in a single spectrum. Spectra were analyzed using the MassLynx software (Waters Micromass, Manchester, UK).

2.19 Multiple sequence alignment

Sequence alignment was done using ClustalW2.

2.20 Phylogenetic analysis of *Sp*CBP28

Phylogenetic analysis was accomplished using online Protpars Phylip (the phylogeny interference package) program (Boc et al., 2012). The source and accession number of CBPs/LPMOs used to generate phylogenetic tree are listed in Table 2.3.

2.21 Homology modelling of *Sp*CBP28, *Bt*CBP and *Bli*CBP

Homology modeling was performed using modeler 9.12. *Sm*CBP21 (2BEM) was used as template to generate a model for *Sp*CBP28. For segment modeling structures were selected based on the short segment sequence similarity and secondary structure similarity (selected sequences were functionally not similar). Hundred models were generated using modeler 9.12. The best model was selected based on the DOPE score. Selected structure side chain was optimized, energy minimized and refined with 3D refine program. The best homologous structures identified for *Bli*CBP were 2YOX and 2YOY.pdb. Out of the two, 2YOY was

used as template structure. Thirty models were generated using modeler and the best model was selected based on the dope score. Post refinement was carried out using KoBAMin server. For *Bt*CBP, 4AO2, 2YOY, 1MFN and 4MB4 were used as template structures. Thirty models were generated using modeler and the best model was selected based on the dope score. Post refinement was carried out using KoBAMin server. Final quality of models was tested with molprobity.

The chitinases and CBPs used in this study were expressed as soluble proteins with C- terminal His-tag in *E. coli*, and purified to homogeneity using Ni-NTA affinity chromatography. The purified proteins, resolved on SDS-PAGE, showed a molecular mass of 75, 74, 52, 55, 50, 21 and 18.6 kDa for *Bt*Chi, *Bli*Chi, *Bli*GH, *Sp*ChiB, *Bt*CBP, *Bli*CBP and *Sp*CBP21, respectively (Figure 3.1).

We studied different aspects of chitin binding proteins (CBPs) from three different organisms: *Bacillus thuringiensis* (*Bt*CBP), *B. licheniformis* (*Bli*CBP) and *S. proteamaculans* (*Sp*CBP21 and *Sp*CBP28). The three CBPs (*Bt*CBP, *Bli*CBP and *Sp*CBP21) were characterized on the basis of pH and temperature by performing substrate binding assays under different conditions. Binding kinetics was studied for *Bt*CBP and *Bli*CBP on crystalline chitin substrates (α- and β-chitin). Synergistic effect of the three CBPs and chitinases was studied on isolated chitin substrates as well as fungal cell walls.

Role of different CBMs in *Bt*CBP was assessed by separately amplifying domains *Bt*CBM33/AA10 and *Bt*CBM5. The two domains were also characterized on the basis of pH and temperature. Oxido-hydrolytic activity of *Bt*CBP, *Bt*CBM33/AA10 and *Bt*CBM5 was tested by performing MALDI-TOF MS. Using fluorescence spectroscopy and circular dichroism we found that *Bt*CBP, *Bli*CBP, *Sp*CBP21, *Bt*CBM33/AA10 and *Bt*CBM5 bind to chito-oligosaccharides. We also studied the role of FnIII domains in *Bt*CBP by generating truncation mutants and by amplifying FnIII domains present in *Bt*CBP.

Site-directed mutants were generated for *Sp*CBP28 which does not bind to any chitin substrate, and binding assays were performed. Oxido-hydrolytic activity of *Sp*CBP28 and its triple mutant was studied using Nano-ESI-MS. Fluorescence spectroscopy, circular dichroism and MALDI-TOF MS revealed that *Sp*CBP28 also bind but does not cleave chito-oligosaccharides like *Bt*CBP, *Bli*CBP, *Sp*CBP21, *Bt*CBM33/AA10 and *Bt*CBM5. Phylogenetic analysis, homology modeling and circular dichroism indicated that SpCBP28 is similar to other CBPs

in structure. However, it is placed far from the CBPs which are known to bind to crystalline chitin substrates or show oxido-hydrolytic activity.

3.1 Sequence similarity, heterologous expression and affinity purification of *Bt*CBP, *Bli*CBP, *Sp*CBP21, *Bt*Chi, *Bli*Chi, *Sp*ChiB and *Bli*GH

The amino acid sequence of the chitinases and CBPs used in this study was analyzed using BLAST program available in NCBI database (http://blast.ncbi.nlm.nih.gov/). *Bt*Chi (ACW83014.1) showed 99 % identity with chitinase B from *B. cereus* (ADD91322.1), 98 % with chitinase from *B. anthracis* str. A2012 (ZP00390774.1), 95 % with chitinase CW from *B. cereus* (AAM48520.2), 54 % with chitinase from *Kurthia zopfii* (BAA09831.1) and 52 % with ChiS from *B. pumilus* (ABI15082.1). The BLAST search for *Bli*Chi displayed 94 % identity with chitinase from *B. circulans* (AAF23368.1), 87 % with chitinase from *B. subtilis* (AAC23715.1) and ChiS from *B. pumilus* (ABI15082.1), 65 % with chitinase A1 from *Paenibacillus* sp. HGF7 (ZP08507060.1) and 51 % to chitinase from *Geobacillus stearothermophilus* (AEO12133.1). *Bt*Chi and *Bli*Chi showed a sequence similarity of 47 %, whereas their GH18 domains were 58 % similar. *Sp*ChiB showed 94 % identity with *Sm*ChiB (ACX42072) and 36 % with ChiCW from *B. cereus* (AF416570).

*Bt*CBP (ACW 83015.1) showed 97 % identity with CBP from *B. cereus* (YP 003792526.1), 96 % to CBP from *B. anthracis* (NP845142.1), 67 % to CBP from *Lysinibacillus sphaericus* (YP001698788.1) and 55 % to CBP from *P. larvae* (ZP02329495.1). *Bli*CBP (ACW83017.1) showed 75 % identity to CDB3 protein from *B. cereus* (ZP04297922.1), 73 % similarity with CBD3 protein from *B. thuringiensis* serovar *kurstaki* (ZP04115187.1), 66 % similarity to CBP from *L. sphaericus* (YP001695749.1) and 58 % to CBP from *P. larvae* (ZP02329495.1). *Sp*CBP21 showed 93 % homology to CBP21 from *S. marcescens* (BAA31569), 57 % to CBP from *B. cereus* G9241 (EAL13960) and *B. thuringiensis* serovar *tochigiensis* BGSC 4Y1 and 44 % to CBP from *B. anthracis* str. CDC 684

(ACP12567). When CBM of *Bt*CBP, *Bli*CBP and *Sp*CBP21 were aligned using ClustalW2, the N- terminal CBM of *Bt*CBP showed 75 and 55 % homology with CBM of *Bli*CBP and *Sp*CBP21, respectively, whereas, CBM of *Bli*CBP and *Sp*CBP21 were 53 % similar. When whole sequence of *Bt*CBP was compared with *Bli*CBP or *Sp*CBP21 the homology was less, which may be because of the 3 extra domains (2 FnIII and 1 CBM) present in *Bt*CBP.

3.2 Characterization of *Bt*CBP, *Bli*CBP and *Sp*CBP21

3.2.1 Binding towards insoluble chitin substrates

3.2.1.1 Substrate binding assay

To study the binding preference, purified *Bt*CBP and *Bli*CBP were incubated with α- or β- chitin at pH 8.0. The binding efficiency of *Bt*CBP was high compared to *Bli*CBP towards both, α- and β- chitin. Both, *Bt*CBP and *Bli*CBP, showed preference to β- chitin (*Bt*CBP: 60, and *Bli*CBP: 44 %) followed by α- chitin (*Bt*CBP: 32 and *Bli*CBP: 15 %) as shown in Figure 3.2 A. This was further verified when bound protein was eluted by boiling the substrates with 1 % SDS and loaded on SDS-PAGE (Figure 3.2 B). More protein was eluted in elution fractions of *Bt*CBP as compared to *Bli*CBP. Also, more protein was observed in elution fractions when β- chitin was used as substrate. This observation further suggested that *Bt*CBP and *Bli*CBP prefer β- to α- chitin.

3.2.1.2 Time course binding of *Bt*CBP and *Bli*CBP

The time required by *Bt*CBP and *Bli*CBP to bind to the natural chitin variants, till saturation, was monitored as a function of time. The decrease in concentration of the free protein that remained in the supernatant, after incubation of *Bt*CBP or *Bli*CBP with chitin, was monitored up to 24 h. The binding of *Bt*CBP and *Bli*CBP to β- chitin reached equilibrium within 9 h, while binding towards α- chitin reached equilibrium by 15 h (Figure 3.2 C).

3.2.1.3 Determination of dissociation constants (K_d and B_{max})

Binding constants of *Bt*CBP and *Bli*CBP were estimated and plotted by varying the concentration of CBPs and keeping the substrate concentration constant (Figure 3.2 D). The dissociation constant (K_d) of the two CBPs towards α- and β-chitin was estimated by GraphPad Prism software using non-linear regression function. The K_d value of *Bt*CBP and *Bli*CBP for β- chitin (*Bt*CBP: 3.46 ± 1.3 µM; *Bli*CBP: 4.12 ± 1.6 µM) was lower than the K_d value for α- chitin (*Bt*CBP: 5.25 ± 1.4 µM; *Bli*CBP: 5.98 ± 2.1 µM). B_{max} values of *Bt*CBP (2.18 ± 0.53 µmol/g of α- chitin and 2.92 ± 0.29 µmol/g of β- chitin) were higher than *Bli*CBP (1.28 ± 0.007 µmol/g of α- chitin and 1.6 ± 0.18 µmol/g of β- chitin).

3.2.1.4 Characterization of *Bt*CBP, *Bli*CBP and *Sp*CBP21 on the basis of pH

*Bt*CBP, *Bli*CBP or *Sp*CBP21 were incubated with β-chitin for one hour on ice. Percent bound protein was calculated as mentioned above. *Bli*CBP and *Sp*CBP21 showed optimum binding at pH 6.0 (37%) and 7.0 (43%), respectively (Figure 3.3 A & B). However, *Bt*CBP showed optimum binding at two different pH i.e. pH 5.0 (56%) and 7.0 (58%) as shown in figure 3.3 C. Also, *Bt*CBP showed binding under extreme pH conditions (19% at pH 3.0 and 25% at pH 12). However, *Bli*CBP showed only 7% binding at pH 3.0 and 5% binding at pH 12.0. *Sp*CBP21 did not bind to β-chitin under extreme pH conditions.

3.2.1.5 Characterization of *Bt*CBP, *Bli*CBP and *Sp*CBP21 on the basis of temperature

*Bt*CBP, *Bli*CBP or *Sp*CBP21 were incubated with β-chitin for one hour at different temperatures (5-100 °C) and percent bound protein was calculated as above. *Bli*CBP and *Sp*CBP21, showed a constant binding pattern till certain temperature (*Bli*CBP showed 25% binding till 50°C; *Sp*CBP21 showed 70% binding till 40°C) after which binding declined. *Bli*CBP and *Sp*CBP21 did not bind to β-chitin after 65°C and 50°C, respectively (Figure 3.4). However, *Bt*CBP

did not follow a constant binding pattern. Maximum binding (74%) was observed at 20°C, after which binding declined and remained constant till 50°C (~58%). After 50°C, binding was much less; however, *Bt*CBP binds to chitin substrates till 85°C. After 90°C, *Bt*CBP lost binding ability. This indicated that *Bt*CBP binds to polymeric chitin substrates under extreme temperature conditions.

3.2.1.6 Variation in secondary structure of *Bt*CBP, *Bli*CBP and *Sp*CBP21 at different temperatures using CD

Changes in secondary structure of *Bt*CBP/*Bli*CBP/*Sp*CBP21 at different temperatures were anlayzed using CD. *Bli*CBP and *Sp*CBP21 showed similar spectra (Figure 3.5 A & B) i.e. hump (single maximum) in 230-240 nm region. However, *Bt*CBP showed different spectra (Figure 3.5 C).

Further, *Bli*CBP and *Sp*CBP21 showed spectral changes from 60 (increase in α-helices and decrease in β-sheets and random coils) and 50°C (decrease in α-helices and increase in β-sheets and random coils), respectively (Figure 3.6 A & B). *Bt*CBP displayed spectral changes at 20°C (increase in α-helices and decrease in β-sheets and random coils) after which it again reorganized and remained stable till 50°C. After 50°C, again conformational changes occurred, that were detected in terms of increase in α-helices and decrease in β-sheets and random coils (Figure 3.6 C).

3.2.2 Binding towards soluble CHOS

3.2.2.1 Quenching in fluorescent intensity indicated that *Bt*CBP, *Bli*CBP and *Sp*CBP21 bind to soluble CHOS

Binding of *Bt*CBP, *Bli*CBP and *Sp*CBP21 towards $(NAG)_3$ and $(NAG)_6$ was tested using fluorescence spectrometry. Quenching was observed in fluorescent spectra of *Bt*CBP, *Bli*CBP and *Sp*CBP21 in presence of $(NAG)_3$ as well as $(NAG)_6$ as shown in Figure 3.7 A-F.

The result indicated a perfect linear relationship (Figure 3.8 A-F). The number of binding sites for *Bt*CBP, *Bli*CBP and *Sp*CBP21 was close to one [*Bt*CBP: $(NAG)_3$-1.3, $(NAG)_6$-1.2; *Bli*CBP: $(NAG)_3$-0.94, $(NAG)_6$-1.0; *Sp*CBP21: $(NAG)_3$-1.0, $(NAG)_6$-1.1]. This observation suggests that *Bt*CBP, *Bli*CBP and *Sp*CBP21 bind to $(NAG)_3$ and $(NAG)_6$ in one to one ratio. The binding constants (K_B) calculated from the intercept for *Bt*CBP, *Bli*CBP and *Sp*CBP21 for $(NAG)_6$ were $1.5x10^6$, $6.7x10^5$ and $3.4x10^5$, and for $(NAG)_3$ were $3.0x10^6$, $1.2x10^5$ and $1.0x10^5$, respectively.

ΔG^o values for *Bt*CBP, *Bli*CBP and *Sp*CBP21 for $(NAG)_6$ were -8.4, -7.9 and -7.5, and for $(NAG)_3$ were -8.8, -6.9 and -6.8 kcal/mol, respectively at 25°C. K_B and ΔG^o values indicated that *Bli*CBP and *Sp*CBP21 have more affinity towards $(NAG)_6$ as compared to $(NAG)_3$. However, *Bt*CBP has more affinity towards $(NAG)_3$ as compared to $(NAG)_6$.

3.2.2.2 Changes in tertiary structure fingerprint further confirmed that *Bt*CBP, *Bli*CBP, and *Sp*CBP21 bind to soluble CHOS

Ligand binding studies were done using CD by analyzing secondary structure composition in peptide bond region (far UV region: 190-260 nm) or by looking into the tertiary structure fingerprint which arise from aromatic amino acid in near UV region: 260-320 nm. But, ligands should not have any intrinsic chirality. As CHOS showed ellipticity in far UV region (Figure 3.9 A-C), ligand binding studies were performed in near UV region (260-320 nm), where no ellipticity was observed for CHOS (Figure 3.9 D). Tertiary structure fingerprint of *Bt*CBP, *Bli*CBP and *Sp*CBP21 was recorded in CD. After recording CD spectra of proteins, $(NAG)_6$ was added to the protein (in two different ratios- 1:1 or 1:2) and spectrum was recorded after 5 min of incubation. Changes in the tertiary structure of *Bt*CBP, *Bli*CBP and *Sp*CBP21, after addition of $(NAG)_6$, further confirmed that *Bt*CBP, *Bli*CBP and *Sp*CBP21 bind to CHOS (Figure 3.10 A-C).

3.2.3 Homology modelling of *Bt*CBP and *Bli*CBP

Homology modeling was performed using modeler 9.12. The best homologous structures identified for *Bli*CBP were 2YOX and 2YOY.pdb. Out of the two, 2YOY was used as template structure. Thirty models were generated using modeler and the best model was selected based on the dope score. Post refinement was carried out using KoBAMin server. For *Bt*CBP, 4AO2, 2YOY, 1MFN and 4MB4 were used as template structures. Thirty models were generated using modeler and the best model was selected based on the dope score. Post refinement was carried out using KoBAMin server. Final quality of models was tested with molprobity. The tryptophan residues in *Bli*CBP and in different domains of *Bt*CBP were identified (Figure 3.11 A & B).

3.2.4 Dot blot assay for testing activity of *Bt*Chi, *Bli*GH and *Bli*Chi

To test activity of *Bt*Chi, *Bli*Chi and *Bli*GH purified proteins were spotted on glycol chitin containing PAGE. *Bt*Chi, *Bli*Chi and *Bli*GH showed activity zones which indicated that the three chitinases were active in glycol chitin (Figure 3.12 A).

3.2.5 Standardization of different conditions for chitinase assay

3.2.5.1 Chitinase concentration

The concentration of chitinase was standardized by performing chitinase assay using different concentrations of *Bt*Chi, till concentration of products reached saturation. Graph was plotted by taking different enzyme concentrations on X-axis and absorbance at 420 nm on Y-axis. Saturation was attained at an enzyme concentration of 14 µg/µL (Figure 3.12 B). Thus, for further experiments, 14 µg/µL of chitinase was used.

3.2.5.2 Stability of chitinases at 37°C

To test the stability of chitinase at 37°C, chitinase and dot blot assay for *Bt*Chi/*Bli*Chi/*Sp*ChiB were performed. For chitinase assay reaction mixtures were incubated at 37°C for different time points, up to 24 h and product concentration was measured. Concentration of products as detected by chitinase assay and activity of chitinases on glycol chitin containing PAGE were similar till 24 h of incubation indicating that chitinases were stable at 37°C up to 24 h (Figure 3.12 C).

3.2.6 Synergistic effect of chitinases and CBPs

3.2.6.1 On isolated chitin substrates

3.2.6.1.1 Chitinase assay

*Bt*Chi and *Sp*ChiB showed synergism in hydrolysis of pure chitin substrates with all the three CBPs, whereas *Bli*Chi did not show increase in product formation when CBPs were added to the reaction mixture (Figure 3.13 A-C). *Bt*Chi incubated with α- chitin and β- chitin produced 3-fold and 2-fold increase of products, respectively in the presence of *Bt*CBP, *Bli*CBP or *Sp*CBP21. When chitinase assay was performed with *Sp*ChiB, 1.5- fold increase in concentration of products was observed with α- chitin, whereas with β- chitin the increase was 2.7-folds in presence of *Sp*CBP21, *Bt*CBP or *Bli*CBP.

*Bli*Chi showed no significant increase in products formation in presence of *Bt*CBP, *Bli*CBP or *Sp*CBP21. The concentration of CHOS [including chitobiose and *N*-acetylglucosamine (data not shown)] produced by *Bli*Chi was equal to the concentration of CHOS produced by *Bt*Chi in the presence of CBPs (~0.6 µmol with β- chitin and ~0.1 µmol with α- chitin). The presence of CBP did not add to the chitin turn-over efficiency of *Bli*Chi. *Bli*GH was unable to act on α- chitin and showed 5-fold reduction in activity on β- chitin, when compared to native *Bli*Chi,

suggested that the accessory domains contributed to the activity of *Bli*Chi. Presence of any of the three CBPs restored little of *Bli*GH activity with only 0.02 µmol increase in products (Figure 3.13 D).

3.2.6.1.2 Visualization of products using HP-TLC

The hydrolytic products generated from chitin substrates (α-, β- or colloidal-chitin) when acted upon by purified *Bt*Chi and *Bli*Chi, in absence or presence of CBPs, were analyzed using HP-TLC. *Bt*Chi and *Bli*Chi generated DP1 and DP2 as final products after 24 h of incubation in absence of CBPs (Figure 3.14). In presence of *Bt*CBP/*Bli*CBP, the concentration of DP1 and DP2 products increased as observed by increase in intensity of DP1 and DP2. *Bli*GH produced only DP2 as final product and no monomers were observed after 24 h of incubation. Even in presence of *Bt*CBP/*Bli*CBP, only DP2 product was observed. Also, the intensity of DP2 product was almost similar in absence or presence of *Bt*CBP/*Bli*CBP, indicating very little increase in concentration of products upon addition of CBPs.

3.2.6.2 On fungal cell walls

3.2.6.2.1 PDA plate assay

The antifungal activity of purified chitinases and CBPs was tested against different fungal isolates *viz*., *Alternaria alternata*, *Fusarium oxysporum*, *Colletrotrichum falcatum*, *Rhizopus* sp., *Aspergillus* sp. and *Penicillium* sp., as hyphal extension inhibition assay. Both *Bt*Chi and *Sp*ChiB showed antifungal activity against *A. alternata* on PDA plates (Figure 3.15 A), while *Bli*Chi, *Bt*CBP, *Bli*CBP and *Sp*CBP21 did not show antifungal activity. To further quantify the antifungal activity of *Bt*Chi and *Sp*ChiB in absence or presence of CBPs, spores of *A. alternata* were used for further assays.

3.2.6.2.2 Isolation and staining of spores

Spores of *A. alternata* were isolated and tested for purity under microscope (Figure 3.15 B). Pure spores were then stained with calcofluor white and observed under confocal microscope (Figure 3.15 C). Presence of blue colour indicated presence of polymers with either β-1,4 or β-1,3 linkages. These purified spores were used for *in vitro* antifungal assays.

3.2.6.2.3 Evaluation of *in vitro* fungal growth in microtiter plates

To quantify the antifungal activity of *Bt*Chi and *Sp*ChiB, in presence or absence of the three CBPs, microtitre plate assay was performed. Growth of the germ tube/ mycelia of *A. alternata* was inhibited by *Bt*Chi or *Sp*ChiB from 12 h (Figure 3.16). The mycelial growth, in presence of *Bt*CBP, *Bli*CBP or *Sp*CBP21, was not inhibited. When *Bt*CBP, *Bli*CBP or *Sp*CBP21 was added with *Bt*Chi or *Sp*ChiB in reaction wells, the germination of spores was same as in presence of only *Bt*Chi or *Sp*ChiB (Figure 3.16). This observation indicated that *Bt*CBP, *Bli*CBP or *Sp*CBP21 did not show synergism with *Bt*Chi or *Sp*ChiB in inhibiting fungal growth. When germination of spores was studied at different time points under microscope, it was observed that *Bt*Chi or *Sp*ChiB slowed down the rate of germination of spores and did not affect spore morphology (Figure 3.17).

3.3 Cloning and characterization of deletion mutants of *Bt*CBP

3.3.1 Amplification and cloning of *Bt*CBM33/AA10 and *Bt*CBM5

Deletion mutants of *Bt*CBP (*Bt*CBM33/AA10 or *Bt*CBM5) were amplified using gene specific primers, with plasmid of *Bt*CBP as template. *Bt*CBM33/AA10 and *Bt*CBM5 were amplified as fragments of size 657 and 192 bp, respectively (Figure 3.18 A & B). The amplicons of 657 and 192 bp were cloned in *Nco*I and *Xho*I sites of pET-22b (+) expression vector. The clones were confirmed by

double digestion with *Nco*I and *Xho*I enzymes (Figure 3.18 C & D). Sequence of the insert was further confirmed by automated DNA sequencing (Eurofins, India).

3.3.2 Expression and purification of *Bt*CBM33/AA10 and *Bt*CBM5

*Bt*CBM33/AA10 and *Bt*CBM5 were over expressed with a C-terminal His-tag in *E. coli*. Periplasmic fraction of all proteins was isolated and purified using Ni-NTA chromatography. The PelB signal sequence in pET-22b (+) directs the expressed *Bt*CBM33/AA10 and *Bt*CBM5 towards periplasmic space (Figure 3.18 E & F). SDS-PAGE analysis of the purified *Bt*CBM33/AA10 and *Bt*CBM5 revealed approximate molecular weight of 26 and 7 kDa, respectively (Figure 3.18 G & H).

Amino acid sequences of CBM33/AA10 were analyzed using BLAST program. *Bt*CBM33/AA10 showed 75% identity to *Bli*CBP and 55% to *Sp*CBP21. When aligned using ClustalW2, *Bt*CBM33 was 49.39% identical to *Ef*CBM33A (AAO80225.1), 65.48% to *Ba*AA10A (CBI42985.1), 47.62% to GbpA (AAF96709.1), 22.02% to cellulose binding protein of *Thermobifida fusca* (AAZ55700.1) and 32.74% to chitin binding domain of *Burkholderia peudomallei* 1710b (ABA49030.1). *Bt*CBM5 showed 32.61% identity to CBM5 of *Bli*Chi from *Bacillus licheniformis*, 100% to CBM5 of chitinase from *B. anthracis* str CDC 684 (YP_002814404.1) and CBM5 of *B. cereus* CBP (WP_000795728.1) and 12% to GbpA of *Vibrio cholerae* (AAF96709.1).

3.3.3 Characterization of *Bt*CBM33/AA10 and *Bt*CBM5

3.3.3.1 Binding towards insoluble chitin substrates

3.3.3.1.1 Substrate binding assay

*Bt*CBM33/AA10 or *Bt*CBM5 were incubated with α- or β-chitin, for 24 h at 37°C. After incubation, protein was measured to assess the percent bound protein. *Bt*CBM5 showed 82 and 50 % binding towards β and α-chitin, respectively.

However, *Bt*CBM33/AA10 showed 32 % binding towards β- chitin and its activity was completely lost on α-chitin (Figure 3.19 A & B). When we compare binding profiles of these two domains to full length protein *Bt*CBP, *Bt*CBM33/AA10 showed reduced binding and *Bt*CBM5 showed increased binding. Also, when protein was eluted using 1% SDS, *Bt*CBM5 showed protein even in second elution (Figure 3.19 C). This means *Bt*CBM5 bound more tightly to chitin as compared to *Bt*CBP as well as *Bt*CBM33/AA10.

3.3.3.1.2 Characterization of *Bt*CBM33/AA10 and *Bt*CBM5 on the basis of pH

*Bt*CBM33/AA10 or *Bt*CBM5 was incubated with β-chitin for one hour on ice. Percent bound protein was calculated as mentioned above. *Bt*CBM33/AA10 showed optimum binding (39 %) at pH 7.0 (Figure 3.20 A), which is one of the pH where its wild type showed optimum binding. Also, it showed binding at extreme pH conditions, 14% binding at pH 3.0 and 17% binding at pH 12.0. After pH 3.0, binding was continuously increased and drop down after pH 7.0. *Bt*CBM5 also binds under extreme pH conditions. However, when binding of *Bt*CBM5 was studied at different pH, *Bt*CBM5 showed binding in broad range of pH conditions and (Figure 3.20 B). Maximum binding (43%) was observed in tris-Cl buffer pH 7.0, followed by 42% binding in NaH_2PO_4-NaOH buffer pH 10.0, 40 % binding in sodium phosphate pH 7.0 and glycine buffer pH 10. Thirty nine % binding was seen at pH 3, 4 and 5 (sodium citrate buffer).

3.3.3.1.3 Characterization of *Bt*CBM33/AA10 and *Bt*CBM5 on the basis of temperature

*Bt*CBM33/AA10 or *Bt*CBM5 was incubated with β-chitin for one hour at different temperatures (5-100°C). Percent bound protein was calculated as above. Like *Bli*CBP and *Sp*CBP21, which consist of only CBM33/AA10 domain, *Bt*CBM33/AA10 followed a constant binding pattern till 60°C (34%), after which

binding declined (Figure 3.21 A). However, *Bt*CBM5 did not follow a constant binding pattern like *Bt*CBP (Figure 3.21 B). *Bt*CBM5 showed 42% binding at 10°C, after which it declined and again increased at 40°C (45%). After 40°C, binding was less and there was no binding after 70°C.

3.3.3.1.4 Variation in secondary structure of *Bt*CBM33/AA10 and *Bt*CBM5 at different temperatures using CD

CD spectrum of *Bt*CBM33/AA10 was similar to the CD spectra of other *Bt*CBM33/AA10 domains (as mentioned above). However, CD spectrum of *Bt*CBM5 was different as compared to *Bt*CBM33/AA10 domain proteins (Figure 3.22 A & B). The hump in 230-240 nm region of *Bt*CBM33/AA10 proteins was inverted in case of *Bt*CBM5. *Bt*CBM33/AA10 showed decrease in α-helices and increase in β-sheets and random coils from 60°C (Figure 3.22 C). However, *Bt*CBM5 showed increase in α-helices and decrease in β-sheets and random coils from 60°C onwards (Figure 3.22 D).

3.3.3.1.5 Oxidohyrolytic activity of *Bt*CBM33/AA10 and *Bt*CBM5 and comparison with *Bt*CBP (WT)

*Bt*CBP was tested for its oxido-hyrolytic activity on β-chitin. *Bt*CBP generated oxidized CHOS ranging from DP2-DP10 (Figure 3.23 A). To further understand whether *Bt*CBP has acquired oxido-hydrolytic activity from CBM33/AA10 or CBM5 or both, we tested products generated by *Bt*CBM33/AA10 and *Bt*CBM5. Both, *Bt*CBM33/AA10 and *Bt*CBM5 generated oxidized CHOS ranging from DP3-DP8 and DP3-DP17, respectively (Figure 3.23 B & C). These results suggested that both *Bt*CBM33/AA10 and *Bt*CBM5 are oxido-hydrolytic.

3.3.3.1.6 Quenching in fluorescent intensity indicated that *Bt*CBM33/AA10 and *Bt*CBM5 bind to soluble CHOS

Binding of *Bt*CBM33/AA10 and *Bt*CBM5 towards $(NAG)_3$ and $(NAG)_6$ was tested using fluorescence spectrometry. Quenching was observed in fluorescent spectra of *Bt*CBM33/AA10 and *Bt*CBM5 in presence of $(NAG)_3$ as well as $(NAG)_6$ (Figure 3.24 A-D). Modified Stern-Volmer equation was used to analyze fluorescence quenching mechanism,

$$\text{Log } [(F_0\text{-}F/F)] = \log K_S + n * \log (Q)$$

where, n is the slope and corresponds to the number of binding sites, K_S is the binding constant and Q is the quencher concentration.

The result indicated a good linear relationship (Figure 3.25 A-D). The number of binding sites for *Bt*CBM33/AA10 and *Bt*CBM5 was close to one [*Bt*CBM33/AA10: $(NAG)_3$-1.7, $(NAG)_6$1.3; *Bt*CBM5: $(NAG)_3$-1.1, $(NAG)_6$-1.2]. This suggests that *Bt*CBM33/AA10 and *Bt*CBM5 bind to $(NAG)_3$ and $(NAG)_6$ in one to one ratio. The binding constants (K_B) calculated from the intercept for *Bt*CBM33/AA10 and *Bt*CBM5 in case of $(NAG)_3$ were $3.2x10^7$ and $4.0x10^6$, respectively and in case of $(NAG)_6$ were 3.7×10^6 and $3.1x10^6$, respectively. ΔG^o values for *Bt*CBM33/AA10 and *Bt*CBM5 for $(NAG)_6$ were -8.9 and -8.8 and for $(NAG)_3$ were -10.2 and -8.9 kcal/mol, respectively at 25°C. K_B and ΔG^o values indicated that *Bt*CBM33/AA10 and *Bt*CBM5, have more affinity towards $(NAG)_3$ as compared to $(NAG)_6$. This further indicated that the two domains bind differentially to CHOS.

3.3.3.1.7 Changes in tertiary structure fingerprint further confirmed that *Bt*CBM33/AA10 and *Bt*CBM5 bind to soluble CHOS

Ligand binding studies were done by analyzing the tertiary structure fingerprint which arises from aromatic amino acid(s) in near UV region: 260-320 nm.

Tertiary structure fingerprint of *Bt*CBM33/AA10 and *Bt*CBM5 was recorded in CD. After recording CD spectra of proteins, $(NAG)_6$ was added to the protein (in two different ratio- 1:1 or 1:2) and incubated for 5 min. After incubation, spectrum was recorded. Changes in the tertiary structure of *Bt*CBM33/AA10 and *Bt*CBM5 after addition of $(NAG)_6$ further confirmed that the above mentioned proteins bind to CHOS (Figure 3.26 A & B).

3.3.3.1.8 Activity of *Bt*CBP, *Bt*CBM33/AA10 and *Bt*CBM5 towards CHOS

*Bt*CBP, *Bt*CBM33/AA10 and *Bt*CBM5 showed binding to $(NAG)_6$. To test whether they hydrolyze/oxido-hydrolyze $(NAG)_6$, *Bt*CBP, *Bt*CBM33/AA10 and *Bt*CBM5 were incubated with $(NAG)_5$/$(NAG)_6$ for 24 h in presence or absence of reductant (ascorbic acid). After incubation, products were analyzed using MALDI-TOF. *Bt*CBP, *Bt*CBM33/AA10 and *Bt*CBM5 did not hydrolyze/oxido-hydrolyze $(NAG)_5$/$(NAG)_6$. Representative graph of *Bt*CBP is shown in Figure 3.27 A-F. This indicated that CBPs/AA10/CBMs bind to CHOS but do not cleave.

3.3.3.1.9 Antifungal activity of *Bt*CBM33/AA10 and *Bt*CBM5

To know whether small size of protein makes it more efficient to enter into the fungal hyphae and make them antifungal, we tested the antifungal activity of truncation mutants of *Bt*CBP (*Bt*CBM33/AA10 and *Bt*CBM5). *Bt*CBM33/AA10 and *Bt*CBM5 were not antifungal against *Alternaria alternata* like full length *Bt*CBP (Figure 3.28 A & B). Spores treated with *Bt*CBM5 showed increased rate of germination as compared to control.

3.4 Cloning and characterization of truncation mutants *Bt*CBP

To know the role of FnIII domains in *Bt*CBP, truncation mutants of *Bt*CBP were generated. The three mutants were named as *Bt*CBPΔCIII (C-terminal FnIII deleted), *Bt*CBPΔNIII (N-terminal FnIII deleted), *Bt*CBPΔCΔNIII (both FnIIIs

deleted) and *Bt*CBPFnIII(N+C) where both N- and C- terminal FnIII domains were PCR amplified and cloned.

3.4.1 Amplification and cloning of *Bt*CBPΔCIII, *Bt*CBPΔNIII, *Bt*CBPΔCΔNIII and *Bt*CBPFnIII(N+C)

Truncation mutants of *Bt*CBP (*Bt*CBPΔCIII, *Bt*CBPΔNIII, *Bt*CBPΔCΔNIII and *Bt*CBPFnIII(N+C)) were amplified using gene specific primers with plasmid of *Bt*CBP as template. *Bt*CBPΔCIII, *Bt*CBPΔNIII and *Bt*CBPΔCΔNIII were first amplified as N-terminal and C- terminal fragments (Figure 3.29 A). These fragments were then fused in second round of PCR. The amplicons generated from second round of PCR for *Bt*CBPΔCIII, *Bt*CBPΔNIII and *Bt*CBPΔCΔNIII were of size 951, 990 and 696 bp, respectively (Figure 3.29 B). *Bt*CBPFnIII(N+C) was amplified as amplicon of size 600 bp from single round of PCR (Figure 3.29 D). The amplicons of *Bt*CBPΔCIII, *Bt*CBPΔNIII, *Bt*CBPΔCΔNIII and *Bt*CBPFnIII(N+C) were cloned in *Nco*I and *Xho*I sites of pET-22b (+) expression vector. The clones were confirmed by double digestion with *Nco*I and *Xho*I (Figure 3.29 C & E). Sequence of the insert was further confirmed by automated DNA sequencing (Eurofins, India).

3.4.2 Expression and purification of *Bt*CBPΔCIII, *Bt*CBPNCIII, *Bt*CBPΔCΔNIII and *Bt*CBPFnIII(N+C)

*Bt*CBPΔCIII, *Bt*CBPΔNIII, *Bt*CBPΔCΔNIII and *Bt*CBPFnIII(N+C) were over expressed with a C-terminal His-tag in *E. coli*. Periplasmic fraction of all proteins was isolated and purified using Ni-NTA chromatography. The PelB signal sequence in pET-22b (+) directs the expressed *Bt*CBPΔCIII, *Bt*CBPΔNIII, *Bt*CBPΔCΔNIII and *Bt*CBPFnIII(N+C) towards periplasmic space. SDS-PAGE analysis of the purified *Bt*CBPΔCIII, *Bt*CBPΔNIII and *Bt*CBPΔCΔNIII revealed approximate molecular weight of 35, 36 and 27 kDa, respectively (Figure 3.30 A). *Bt*CBPΔNIII was highly unstable protein as it formed several other protein bands

visible on SDS-PAGE (Figure 3.30 B), and thus was not used for further binding experiments. SDS-PAGE analysis of the purified *Bt*CBPFnIII(N+C) revealed approximate molecular weight of 21 kDa (Figure 3.30 C).

3.4.3 Binding of *Bt*CBPΔCIII, *Bt*CBPΔCΔNIII and *Bt*CBPFnIII(N+C) to insoluble chitin substrates

*Bt*CBPΔCIII, *Bt*CBPΔCΔNIII and *Bt*CBPFnIII(N+C) were incubated with α- or β-chitin, for 24 h at 37°C. After incubation protein estimation was performed to estimate the percent bound protein. *Bt*CBPΔCIII showed slight increase in binding (α-chitin: 3%; β-chitin: 7%) as compared to full length *Bt*CBP. *Bt*CBPΔCΔNIII showed equal binding towards α- as well as β- chitin as that of *Bt*CBP (WT). *Bt*CBPFnIII(N+C) did not bind to either α- or β- chitin (Figure 3.31 A). Also, when protein was eluted using 1% SDS, *Bt*CBPΔCIII showed protein even in second elution; *Bt*CBPΔCΔNIII had equal amount of protein in elution fractions as that of *Bt*CBP (WT). In case of *Bt*CBPFnIII(N+C), no protein was eluted after boiling with SDS. This indicated that FnIII domains do not bind to either α- or β- chitin (Figure 3.31 B).

3.4.4 CD of FnIIIs

3.4.4.1 Variation in secondary structure of FnIIIs at different temperatures and pH

Conformational changes in FnIIIs (*Bt*CBPFnIII(N+C) at different temperatures (10-100 °C) were analyzed in far UV region (260-190 nm) using CD. Spectrum was totally different from that of CBPs (Figure 3.32 A). The conformational changes in FnIII domains started from 60°C from where there was a sudden increase in α-helices (Figure 3.32 B).

When subjected to different pH conditions, *Bt*CBPFnIII(N+C) showed secondary structural changes at pH 4.0 (sodium acetate buffer), where there was an increase

in α-helices and decrease in β-sheets and random coils. At pH other than 4, secondary structure elements were almost constant and did not show variation in secondary structure (Figure 3.32 C & D).

3.4.4.2 Variation in tertiary structure fingerprint of FnIII domains in presence of $(NAG)_6$

Ligand binding studies were done by analyzing the tertiary structure fingerprint which arises from aromatic amino acid(s) in near UV region: 260-320 nm. Tertiary structure fingerprint of *Bt*CBPFnIII(N+C) was recorded in CD. After recording CD spectra of proteins, $(NAG)_6$ was added to the protein (in different ratio- 1:1, 1:2, 1:3 or 1:4) and incubated for 5 min. After incubation, spectrum was recorded. Changes in the tertiary structure of *Bt*CBPFnIII(N+C) after addition of $(NAG)_6$, confirmed that the above mentioned proteins bind to CHOS (Figure 3.33).

3.4.5 Quenching in fluorescent intensity indicated that FnIII domains bind to $(NAG)_6$

Binding of *Bt*CBPFnIII(N+C) towards $(NAG)_6$ was tested using fluorescence spectrometry. Quenching was observed in fluorescent spectra of *Bt*CBPFnIII(N+C) in presence of $(NAG)_6$ (Figure 3.34 A). The result indicated a good linear relationship (Figure 3.34 B). The number of binding sites for *Bt*CBPFnIII(N+C) was close to one (n=1.1). This suggests that *Bt*CBPFnIII(N+C) bind to $(NAG)_6$ in one to one ratio. The binding constants (K_B) calculated from the intercept for *Bt*CBPFnIII(N+C) was 5.0×10^5 M^{-1}. ΔG^o value for *Bt*CBPFnIII(N+C) was -7.74 kcal/mol, at 25 °C.

3.5 Cloning and characterization of site-directed mutants of *Sp*CBP28

3.5.1 Sequence alignment of *Sp*CBP28 to other CBPs

Sequences of *Sp*CBP28, *Sp*CBP21, *Sp*CBP50, *Sm*CBP21, *Bli*CBP and *Bt*CBP were aligned using ClustalW2 (Figure 3.35). *Sp*CBP28 lacks three (two glutamic acid residues and one aspargine) out of five amino acid residues that were reported to be important for binding. These amino acids were mutated from *Sp*CBP28 and substituted with essential amino acids at respective positions.

3.5.2 Amplification and cloning of site-directed mutants of *Sp*CBP28

Site-directed mutants of *Sp*CBP28 (I79E, L84E, G263N, I79E+L84E, I79E+G263N, L84E+G263N and I79E+L84E+G263N) were amplified using gene specific primers with plasmid of *Sp*CBP28 as template. The amplicons, each of 750 bp (Figure 3.36 A), were cloned in *Nco*I and *Xho*I sites of pET-22b (+) expression vector. The clones were confirmed by double digestion with *Nco*I and *Xho*I enzymes, that generated a fragment of 750 bp (Figure 3.36 B). Sequence of the insert and site-directed mutants was further confirmed by automated DNA sequencing (Eurofins, India).

3.5.3 Expression and purification of *Sp*CBP28-I79E, *Sp*CBP28-L84E, *Sp*CBP28-G263N, *Sp*CBP28-I79E+L84E, *Sp*CBP28-I79E+G263N, *Sp*CBP28-L84E+G263N and *Sp*CBP28-I79E+L84E+G263N

Sequence analysis of *Sp*CBP28 was performed using BLAST program of NCBI database (http://blast.ncbi.nlm.nih.gov). *Sp*CBP28 shared 53.68% identity to CBP/LPMO of *Yokonella regensburgei* and 44.72% to *Pseudomonas pisci*. The sequence identity with CBP/LPMO from *S. marcescens*, *Enterococcus faecalis*, *Bacillus amyloliquefaciens* and *Vibrio cholerae* was 21.76, 24.1, 18.99 and 36.13%, respectively. CBPs/LPMOs from *Streptomyces coececolor* and *Str. olivaceoviridis* were 25.58 and 23.98 % identical, respectively.

SpCBP28 (WT), *Sp*CBP28-I79E, *Sp*CBP28-L84E, *Sp*CBP28-G263N, *Sp*CBP28-I79E+L84E, *Sp*CBP28-I79E+G263N, *Sp*CBP28-L84E+G263N and *Sp*CBP28-I79E+L84E+G263N were expressed as soluble proteins with C- terminal His-tag in *E. coli*, and purified to homogeneity using Ni-NTA affinity chromatography. The purified proteins, resolved on SDS-PAGE, showed a molecular mass of 28 kDa for SpCBP28 (WT), *Sp*CBP28-I79E, *Sp*CBP28-L84E, *Sp*CBP28-G263N, *Sp*CBP28-I79E+L84E, *Sp*CBP28-I79E+G263N, *Sp*CBP28-L84E+G263N and *Sp*CBP28-I79E+L84E+G263N (Figure 3.36).

3.5.4 Substrate binding assays

3.5.4.1 Binding towards insoluble chitin substrates

To study binding preference, purified *Sp*CBP28 (WT), *Sp*CBP28-I79E, *Sp*CBP28-L84E, *Sp*CBP28-G263N, *Sp*CBP28-I79E+L84E, *Sp*CBP28-I79E+G263N, *Sp*CBP28-L84E+G263N and *Sp*CBP28-I79E+L84E+G263N were incubated with α- or β-chitin at pH 8.0. *Sp*CBP28 (WT), single (*Sp*CBP28-I79E, *Sp*CBP28-L84E, *Sp*CBP28-G263N) and double mutants (*Sp*CBP28-I79E+L84E, *Sp*CBP28-I79E+G263N, *Sp*CBP28-L84E+G263N) did not bind to insoluble chitin substrates (α- or β-chitin). However, when triple mutant (*Sp*CBP28-I79E+L84E+G263N) was incubated with chitin substrates, it binds to insoluble chitin substrates and prefer α- to β-chitin (α- chitin: 15% and β-chitin: 6%) as shown in Figure 3.37 A. This was further verified by resolving the eluted protein (fractions E1 and E2) on SDS-PAGE (Figure 3.37 B & C). Protein was not present in elution fractions of *Sp*CBP28 (WT), single or double mutants (representative graph is shown in figure 3.37 B). However, elution fractions of *Sp*CBP28-I79E+L84E+G263N contain considerable amount of protein. Also, the amount of protein eluted in α-chitin fraction was more than that of β-chitin elution fraction. Some traces of protein was also observed in second elution of α-chitin fraction (E2).

3.5.4.2 Oxido-hydrolytic activity of *Sp*CBP28 (WT) and *Sp*CBP28-I79E+L84E+G263N towards insoluble chitin substrates

*Sp*CBP28 (WT) and *Sp*CBP28-I79E+L84E+G263N were tested for their oxido-hydrolytic activity on β-chitin (580# or 100-170 # mesh size) using Nano-ESI-MS. *Bt*CBP was used as positive control. *Sp*CBP28 (WT) and *Sp*CBP28-I79E+L84E+G263N did not generate oxidized CHOS when incubated with β-chitin (580# or 100-170 # mesh size); representative graph is shown in Figure 3.38 A and B. However, oxidized CHOS were observed in *Bt*CBP treated samples in both mesh size (Figure 3.38 C and D). This indicated that *Sp*CBP28 (WT) and *Sp*CBP28-I79E+L84E+G263N are not oxido-hydrolytic unlike *Bt*CBP.

3.5.4.3 Binding towards soluble chitin substrates

3.5.4.3.1 Quenching in fluorescent intensity indicated that *Sp*CBP28 (WT) bind to soluble CHOS

Binding of *Sp*CBP28 to $(NAG)_{2-6}$ was tested using fluorescence spectrometry. Quenching was observed in fluorescence spectra of *Sp*CBP28 in presence of $(NAG)_2$, $(NAG)_3$, $(NAG)_4$, $(NAG)_5$ as well as $(NAG)_6$ as shown in Figure 3.39 A-E. The result indicated a perfect linear relationship (Figure 3.40 A-E). The number of binding sites for *Sp*CBP28 was close to one [$(NAG)_2$-1.2, $(NAG)_3$-1.5, $(NAG)_4$-1.1, $(NAG)_5$-1.1 and for $(NAG)_6$-1.0). This suggests that *Sp*CBP28 binds to $(NAG)_2$, $(NAG)_3$, $(NAG)_4$, $(NAG)_5$ as well as $(NAG)_6$ in one to one ratio. The binding constants (K_B) calculated from the intercept for *Sp*CBP28 were $4.7x10^5$, $2.6x10^6$, $3.1x10^5$, $2.2x10^5$ and $1.9x10^5$ M^{-1} for $(NAG)_2$, $(NAG)_3$, $(NAG)_4$, $(NAG)_5$ and $(NAG)_6$, respectively. ΔG^o values for *Sp*CBP28 were -7.7, -8.7, -7.4, -7.2 and -7.1 kcal/mol for $(NAG)_2$, $(NAG)_3$, $(NAG)_4$, $(NAG)_5$ and $(NAG)_6$, respectively at 25°C. K_B and ΔG^o values indicated that *Sp*CBP28 binds to CHOS in following order:

$(NAG)_3>(NAG)_2>(NAG)_4>(NAG)_5>(NAG)_6$

3.5.4.3.2 Changes in tertiary structure fingerprint further confirmed that *Sp*CBP28 (WT) bind to soluble CHOS

In near UV region (250-320 nm), there are no peptide bond transitions of proteins and CD spectrum arises from aromatic amino acids (Trp: 290-305 nm; Tyr: 275-282 nm and Phe: 255-270 nm). Marked changes in near UV CD spectra of *Sp*CBP28 with addition of CHOS were largely due to changes in the environment of the three amino acids (predominately with Trp) as seen in Figure 3.41. These results revealed that *Sp*CBP28 (WT) showed conformational changes in presence of CHO with DP2/3/4/5/6.

3.5.4.4 Activity of *Sp*CBP28 (WT) towards soluble CHOS

*Sp*CBP28 (WT) showed binding to $(NAG)_2$, $(NAG)_3$, $(NAG)_4$, $(NAG)_5$ and $(NAG)_6$. To test whether *Sp*CBP28 (WT) hydrolyze/oxido-hydrolyze $(NAG)_5$ and $(NAG)_6$, *Sp*CBP28 (WT) was incubated with $(NAG)_5$ or $(NAG)_6$ for 24 h in presence or absence of reductant (ascorbic acid). After incubation, the products were analyzed using MALDI-TOF. *Sp*CBP28 (WT) did not hydrolyze/oxido-hydrolyze $(NAG)_5$ or $(NAG)_6$ as shown in Figure 3.42.

3.5.5 Effect of temperature and pH on secondary structure of *Sp*CBP28 (WT)

Conformational changes in *Sp*CBP28 (WT) at different temperatures (10-100 °C) were analyzed in far UV region (260-190 nm) using CD. Spectrum showed single negative and single positive (Figure 3.43 A). Secondary structure elements were analyzed using CDNN 2.1 software. Spectral changes in *Sp*CBP28 (WT) started at 60°C (Figure 3.43 B). There was an increase in β-sheets and random coils and decrease in α-helices.

Conformational changes in *Sp*CBP28 (WT) were studied at different pH conditions. *Sp*CBP28 (WT) does not show conformational changes at different

pH. The secondary structural elements and CD spectra remained unaltered when *Sp*CBP28 (WT) was subjected to buffers of different pH (Figure 3.43 C & D).

3.5.6 Homology modeling of *Sp*CBP28

Homology modeling was performed using modeler 9.12. *Sm*CBP21 (2BEM) was used as template to generate a model for *Sp*CBP28. For segment modeling, structures were selected based on the short segment sequence similarity and secondary structure similarity (selected sequences were functionally not similar). Hundred models were generated using modeler 9.12 and the best model was selected based on the DOPE score. Psiphered secondary structure of *Sp*CBP28 is shown in Figure 3.44 A, which shows position of α-helix, β-sheets and random coils present in *Sp*CBP28. The structure was energy minimized and refined with 3D refine program. Quality of final model generated is shown in Figure 3.44 B. Selected structure side chain was optimized, energy minimized and refined with 3D refine program. When structure of *Sp*CBP28 and *Sm*CBP21 were compared (Figure 3.45 A & B), *Sp*CBP28 was almost similar to *Sm*CBP21. The only difference found in secondary structure of *Sp*CBP28 was that it possesses three extra loop regions (represented in dark blue). The conserved amino acids mutated in present study are shown in the model.

3.5.7 Phylogenetic analysis

Phylogenetic analysis was accomplished using online Protpars Phylip program. Phylogenetic tree obtained from Protpars Phylip program clustered the CBPs sequences in two different clusters. Out of which *Sp*CBP28 was put far from the CBPs which have been classified till date and are known to bind to either α- and/or β-chitin or to oxido-hydrolyze them. *Sp*CBP28 was found to be very close to CBP of *Yokonella* sp., which is known to be pathogenic. E8, E7 and CBP from *Vibrio* sp. also clustered in the batch where *Sp*CBP28 was present (Figure 3.46).

4.1 Selection, heterologous expression and affinity purification of *Bt*CBP, *Bli*CBP, *Sp*CBP21, *Bt*Chi, *Bli*Chi and *Sp*ChiB

Chitin degradation is a key step in recycling of nutrients into the environment. However, the major problem is that chitin polymers form extremely dense and resilient bonds. During past several years, bacteria have been exploited for production of chitinases which have wide range of applications (Neeraja et al., 2010b). To completely replace the usage of fossil fuels, research efforts are on to find the most suitable source for production of biofuels. Crystalline cellulose and chitin have emerged as efficient sources that can be converted to soluble sugars by enzymatic hydrolysis for biofuel production. The discovery of oxido-hydrolase activity of CBP21 by Vaaje-Kolstad et al (2010) provided fresh impetus to research on CBPs, also known as lytic polysaccharide monooxygenases (LPMOs), to obtain more details. These LPMOs were previously placed in family 33 (CBM33) or glycosyl hydrolase 61 (GH61) of CAZy database. Due to their oxido-hydrolytic activity, CBM33 and GH61 have now been reclassified under auxiliary activity (AA) family of LPMOs. LPMOs are divided into three families and named as AA9, AA10 and AA11.

Conversion of crystalline chitin to soluble sugar molecules using LPMOs has opened new avenues for production of biofuels. To further exploit CBPs/LPMOs for production of soluble sugars from insoluble chitin substrates, we characterized three CBPs/LPMOs from *Bacillus thuringiensis* serovar *kurstaki* (*Bt*CBP), *B. licheniformis* DSM13 (*Bli*CBP) and *Serratia proteamaculans* 568 (*Sp*CBP21).

Genome analysis of chitinolytic bacteria indicates that family 33 CBPs are produced by most of the chitin-degrading microorganisms, but only a few have been characterized biochemically. The size of the CBPs varies from 14.9 kDa (Chb3 from *Streptomyces coelicolor*) to 50 kDa (*Sp*CBP50 from *S. proteamaculans*). *Bt*CBP, *Bli*CBP and *Sp*CBP21 showed a molecular mass of 50, 21 and 18.6 kDa, respectively. Variation in size was mainly due to the different

domains present in the CBPs. *Bt*CBP consists of four domains (one CBM33/AA10, two FnIIIs and one CBM5) and therefore was larger in size as compared to *Sp*CBP21 and *Bli*CBP which have a single domain (CBM33/AA10). Variation in size of *Sp*CBP21 and *Bli*CBP could be due to trans-membrane domain which is present at the N-terminus of *Bli*CBP. When expressed using auto-induction media, of the three CBPs, *Bt*CBP showed maximum expression in LBB (production: 12-15 mg/L) followed by *Bli*CBP and *Sp*CBP21, which we produced 5-7 mg/L. The chitinase (*Bt*Chi, *Bli*Chi and *Sp*ChiB) and CBP (*Bt*CBP, *Bli*CBP and *Sp*CBP21) encoding genes used in the present study were selected to test synergistic interactions among a combination of domain organization of GH18 family chitinases and CBM33 family CBPs. When the domain organization was same in *Bli*CBP and *Sp*CBP21, the sequence identity (53 %) was the factor for selection.

4.2 Characterization of *Bt*CBP, *Bli*CBP and *Sp*CBP21

4.2.1 Binding towards insoluble chitin substrates

4.2.1.1 Substrate binding assay

The binding efficiency of *Bt*CBP was high compared to *Bli*CBP, although their N-terminal CBM was 75 % similar. It could be mostly due to the three extra domains present in *Bt*CBP as observed by Mehmood et al., (2011). The two FnIII domains present in *Bt*CBP are rich in proline, serine and threonine residues (Figure 4.1), that are responsible for making bends in the polypeptide chains (Kay et al., 2000; Ballesteros et al., 2000). Thus, FnIII domains possibly help *Bt*CBP to change conformation in a way that binding towards the substrate could be increased. Also, the FnIII domains are known to be involved in unfolding and refolding of proteins (Fisher et al., 1999). Unfolding of proteins may expose the cryptic protein interaction sites and help in increasing the interaction of *Bt*CBP with substrates. *Sp*CBP21 (Purushotham et al., 2012) also preferred β- chitin as

substrate. This may be because of the open structure of β- chitin, which increases binding of CBPs (Saito et al., 2000, 2002).

Depending on the amino acid sequence and organization of the residues in CBPs, different CBPs prefer different substrates. Information from sequence, structure and mutational analysis showed that the surface of family 33 CBPs contains a patch of highly conserved, mostly polar residues (Tyr54, Glu55, Glu60, His114, Asp182, and Asn185) that are important for binding to chitin (Vaaje-Kolstad et al., 2005). The same conserved residues are also present in *Bt*CBP, *Bli*CBP, and *Sp*CBP21 contributing to the preference for β- chitin as substrate, like *Sm*CBP21. *Ll*CBP33A, which binds equally to α- and β-chitin, has two substitutions in the conserved surface patch; Ser63 occurs at a position at which CBP21 has a tyrosine (Tyr54) and Asn64 occurs instead of a glutamate residue (Glu55 in CBP21). Both these residues were important for functionality of CBP21 (Vaaje-Kolstad et al., 2005). CHB1 from *Streptomyces olivaceoviridis* binds strictly α-chitin, where Tyr54 is replaced by Trp57 (Zeltins and Schrempf, 1997). ChbB from *B. amyloliquefaciens* binds to both α- and β-chitin (Chu et al., 2001). Here Tyr54 and Glu55 are replaced by Asp62 and Asn63, respectively.

4.2.1.2 Time course binding of *Bt*CBP and *Bli*CBP

When binding of *Bt*CBP and *Bli*CBP was monitored as a function of time, *Bt*CBP and *Bli*CBP attained equilibrium relatively faster in the presence of β- chitin, when compared to α- chitin. Binding of *Sp*CBP21 and *Sp*CBP50 towards β- chitin reached equilibrium within 6 and 12 h, respectively, while binding saturation towards α- chitin was attained after 12 h (Purushotham et al., 2012). *Sm*CBP21 established binding equilibrium after 16 h of incubation (Vaaje-Kolstad et al., 2005). *Ll*CBP33A from *L. lactis* subsp. *lactis* established binding equilibrium by 24 h of incubation with both α- and β- chitin (Vaaje-Kolstad et al., 2009).

4.2.1.3 Determination of dissociation constants (K_d and B_{max})

The K_d value of *Bt*CBP and *Bli*CBP for β- chitin was lower than the K_d value for α- chitin similar to *Sp*CBP21 (Purushotham et al., 2012). The lower K_d values indicate that the three CBPs have high affinity binding towards β- chitin as compared to α- chitin. K_d values of all the three CBPs towards the β- chitin were relatively higher, while B_{max} values were lower when compared to the K_d and B_{max} of CBPs from *S. marcescens* and *B. thuringiensis* serovar *konkukian* (Vaaje-Kolstad et al., 2005; Mehmood et al., 2011).

4.2.1.4 Characterization of *Bt*CBP, *Bli*CBP and *Sp*CBP21 on the basis of pH

*Bli*CBP and *Sp*CBP21 showed optimum binding at pH 6 and 7, respectively (Figure 3.3 A & B). However, *Bt*CBP showed optimum binding at two different pHs i.e. pH 5.0 and 7.0 (Figure 3.3 C). If we compare the domain architecture of the three proteins, *Bli*CBP and *Sp*CBP21 consisted of only CBM33/AA10, while *Bt*CBP consisted of CBM33/AA10 and CBM5 tethered by two FnIII domains. There is a possibility that due to two CBMs, *Bt*CBP showed optimum binding at two different pHs. Other CBPs which possess only CBM33/AA10 domain for example, only one CBP (*Sm*CBP21) has been characterized so far on the basis of pH. *Sm*CBP21 also showed optimum binding at pH 7.0 (Suzuki et al., 1998). These observations together indicated that most of the CBM33/AA10 proteins optimally bind to polymeric chitin substrates in pH range 6-7. To further understand the difference in binding of *Bt*CBP and *Bli*CBP/*Sp*CBP21, we amplified CBM33/AA10 and CBM5 and characterized on the basis of pH.

4.2.1.5 Characterization of *Bt*CBP, *Bli*CBP and *Sp*CBP21 on the basis of temperature using substrate binding assay and CD

*Bli*CBP and *Sp*CBP21, consisting of only CBM33/AA10, showed a constant binding pattern till certain temperature (*Bli*CBP showed 25% binding till 50°C; *Sp*CBP21 showed 70% binding till 40°C) after which binding declined. However,

*Bt*CBP did not follow a constant binding pattern (Figure 3.4). *Bt*CBP binds to chitin substrates till 85°C. After 90°C, *Bt*CBP lost its binding ability. This indicated that *Bt*CBP binds to polymeric chitin substrates under extreme temperature conditions. *Bli*CBP and *Sp*CBP21 showed similar spectra (Figure 3.5 A & B) i.e. hump (single maximum) in 230-240 nm region. This kind of spectrum is specific for proteins which are rich in β-sheets and random coils, and lack α-helices (Manavalan and Johnson, 1983). Similar kind of spectrum was also observed for chitin binding protein CHB1 from *Streptomyces olivaceoviridis* (Zeltins and Schrempf, 1997). However, *Bt*CBP showed different spectra (Figure 3.5 C). *Bt*CBP consists of 4 different domains as compared to *Bli*CBP/*Sp*CBP21 which consist of single CBM33/AA10 domain. This difference in domain architecture of *Bt*CBP and *Bli*CBP/*Sp*CBP21 might be responsible for difference in the spectra. To further understand the reason for the difference in behaviour of *Bt*CBP at different temperature, we characterized the *Bt*CBM33/AA10 and *Bt*CBM5 on the basis of temperature and studied their secondary structure under CD.

4.2.1.6 Quenching in fluorescent intensity and changes in tertiary structure fingerprint indicated that *Bt*CBP, *Bli*CBP and *Sp*CBP21 bind to soluble CHOS

Binding of CBPs/LPMOs towards CHOS has not been studied in detail. We studied binding of *Bt*CBP, *Bli*CBP and *Sp*CBP21 towards CHOS using fluorescence spectrometry and CD. In both, fluorescence spectrometry and CD spectra generated by the aromatic amino acids. Quenching in fluorescence intensity and changes in tertiary structure fingerprint indicated that *Bt*CBP, *Bli*CBP and *Sp*CBP21 bind to soluble CHOS. K_B and ΔG values obtained from fluorescence spectroscopy indicated that CBPs bind differentially to CHOS as *Bt*CBP has more affinity towards $(NAG)_3$ as compared to $(NAG)_6$; whereas, *Bli*CBP/*Sp*CBP21 have more affinity towards $(NAG)_6$. The fluorescence spectrum of CHB1 recorded by Zeltins and Schrempf (1997) revealed the role of individual

tryptophan residues in CHB1 in chitin binding. Replacement of tryptophan residue with leucine, did not show spectral changes in fluorescence spectra of CHB1 in presence of $(NAG)_5$. GbpA, a colonization factor from *Vibrio cholerae* which possesses domains similar to *Bt*CBP, also indicated that GbpA binds selectively to CHOS. GbpA binds selectively to CHOS through domains 1 and 4 (Wong et al., 2012). This indicated that these two CBDs provide the protein an ability to bind to different types of CHOS. However, $ChBD_{ChiA1}$ from *Bacillus circulans* WL-12 was known to recognize only crystalline chitin substrates. NMR and ITC studies revealed that there was no interaction of $ChBD_{ChiA1}$ with CHOS (Hashimoto et al., 2000). Also, CBP21 from *S. marcescens* was known to bind to only β-chitin (Vaaje-Kolstad et al., 2005).

4.2.1.7 Homology modelling of *Bt*CBP and *Bli*CBP

*Bt*CBP (ACW 83015.1) showed 97 and 96% identity to *B. cereus* (YP 003792526.1) and *B. anthracis* (NP845142.1), respectively. *Bt*CBP consists of four different domains *viz*., N-terminal CBM33 domain, C- terminal CBM5 domain and both these domains are tethered by two FnIII domains. N-terminal CBM33 domain of *Bt*CBP (*Bt*CBM33) showed 75% identity to *Bli*CBP and 55% to *Sp*CBP21. When aligned using ClustalW2, *Bt*CBM33 was 49.39% identical to *Ef*CBM33A (AAO80225.1), 65.48% to *Ba*AA10A (CBI42985.1), 47.62% to GbpA (AAF96709.1), 22.02% to cellulose binding protein of *Thermobifida fusca* (AAZ55700.1) and 32.74% to chitin binding domain of *Burkholderia peudomallei* 1710b (ABA49030.1). C-terminal CBM5 domain of *Bt*CBP showed 32.61% identity to CBM5 of *Bli*Chi from *Bacillus licheniformis*, 100% to CBM5 of chitinase from *B. anthracis* str CDC 684 (YP_002814404.1) and CBM5 of *B. cereus* CBP (WP_000795728.1) and 12% to GbpA of *V. cholerae* (AAF96709.1). To model different domains *Bt*CBP, 4AO2, 2YOY, 1MFN and 4MB4 were used as template structures. Post refinement was carried out using KoBAMin server. Tryptophan residues present in different domains of *Bt*CBP were labeled and

identified whether they are buried or surface exposed. The Trp154, Trp84 and Trp95 were buried; Trp104, Trp200, Trp372 and Trp405 were surface exposed. Position of Trp412 and Trp294 was doubtful. Tryptophan is a rare amino acid residue as many proteins contain only few of them. Thus, fluorescence by tryptophan is considered to be a sensitive measurement for analysis of conformational states of a protein. This fluorescence also depends on whether the residue is buried or surface exposed. N-terminal CBM33 domain consists of only one surface exposed tryptophan residue; while C-terminal CBM5 domain possesses two surface exposed tryptophan residues.

*Bli*CBP showed 75% identity to CDB3 protein from *B. cereus* (ZP04297922.1), 46.34 % to *Ef*CBM33A, 58.93% to *Ba*AA10A, 43.45% to GbpA and 39.88% to chitin binding domain of *Burkholderia peudomallei* 1710b. The best homologous structures identified for *Bli*CBP were 2YOX and 2YOY.pdb. Out of the two, 2YOY was used as template structure. *Bli*CBP consisted of only one CBM33 domain. Tryptophan residues present in different domains of *Bli*CBP were labeled and identified whether they are buried or surface exposed. The Trp152, Trp84 and Trp95 were buried; Trp104 and Trp70 were surface exposed.

4.2.1.8 Synergistic effect of chitinases and CBPs

4.2.1.8.1 On isolated chitin substrates

4.2.1.8.1.1 Chitinase assay and HP-TLC

The bacterial chitinases used in this study showed synergism with CBPs from same or different organism on isolated pure substrates, suggesting that CBPs have a general effect as reported by Vaaje-Kolstad et al., (2005) and not specific for a chitinase-CBP combination. The *Sm*CBP21 strongly promoted hydrolysis of crystalline β- chitin by *Sm*ChiA and *Sm*ChiC, while it was essential for complete degradation by *Sm*ChiB (Vaaje-Kolstad et al., 2005). *Sp*CBP21 and *Sp*CBP50 enhanced the activity of *Sp* chitinases (Purushotham et al., 2012) in degradation of

natural chitin variants. Vaaje-Kolstad et al., (2009, 2012) also showed that the *Ll*CBP33A and *Ef*CBM33A increased the hydrolytic efficiency of *Ll*Chi18A and *Ef*Chi18A on both α- and β- chitin, respectively signifying the importance of CBPs in chitin turnover.

*Bli*Chi did not show synergism with the test CBPs (*Bt*CBP/ *Bli*CBP/ *Sp*CBP21). To understand the reason, we have compared GH18 domain and CBM present in *Bli*Chi and *Bt*Chi. GH18 domains of *Bt*Chi and *Bli*Chi were 58 % similar, whereas CBMs show only 10 % similarity. *Bli*GH, unable to act on α- chitin and less active on β- chitin, could restore little activity (0.02 µmol) in presence of CBPs. The activity of CBMs, therefore, may not be the same when they are attached to the GH18 domain as compared to their availability in the reaction mixture as CBPs. There is a possibility that FnIII domain is playing a role in changing the confirmation of *Bli*Chi. It was predicted by Fisher et al., (1999) that the FnIII domain might unfold and refold as protein executes mechanical functions. The proteins with FnIII might gain more access to the substrates and increase the efficiency of degradation as reported by Kataeva et al., (2002) for the cellobiohydrolase of *Clostridium thermocellum*. Deletion of C-terminal region of chitinases was reported for *B. circulans* WL-12 (Watanabe et al., 1990), *Str. olivaceoviridis* (Blaak and Schrempf, 1995), *Aeromonas caviae* (Wang et al., 2003), *A. hydrophila* (Chang et al., 2004), *Vibrio parahaemolyticus* (Chuang and Lin, 2007).The deletions resulted in reduced activity of chitinases towards insoluble chitin substrates. Chuang et al., (2008) made truncation mutants of *B. licheniformis* BCRC 11702 and reported the importance of C-terminal region of chitinases in chitin degradation. After 24 h of incubation, the native chitinases (*Bl*Chi1) hydrolyzed α- chitin more efficiently than the truncated mutants. In the present study, *Bli*GH was not active on α-chitin and the activity on β- chitin was reduced 5- folds.

4.2.1.8.2 On fungal cell walls

Antifungal assay revealed that CBPs did not work synergistically with chitinases in inhibiting the fungal growth, probably due to the tightly packed structure of fungal cell walls. The other polysaccharides like glucans and mannans, present in fungal cell wall, may also prevent the direct access of chitin to the CBPs. Chitinolytic bacteria *viz.*, *Enterobacter agglomerans* (Chernin et al., 1995), *S. marcescens* and *Xanthomonas maltophilia* (Kobayashi et al., 1995), *Stenotrophomonas maltophilia* (Zhang and Yuen, 2000) and *B. thuringiensis* serovar *konkukian* (Mehmood et al., 2011) are known for their antagonistic effect on fungal pathogens. The synergistic effect of enzymes and antifungal compounds has been reported for *S. marcescens* (Someya et al., 2001). We report here that the CBPs which aid chitinases in hydrolysis of isolated chitin substrates need not necessarily act synergistically in hydrolysis of the chitin present in the fungal cell walls.

4.3. Characterization of *Bt*CBM33/AA10 and *Bt*CBM5

4.3.1 Binding towards insoluble chitin substrates

4.3.1.1 Substrate binding assay

N- and C-terminal domains of *Bt*CBP, designated as *Bt*CBM33/AA10 and *Bt*CBM5, were amplified and cloned to know the differences in binding of CBMs belonging to two different families. *Bt*CBM33/AA10 did not show binding to α-chitin and also its binding was reduced towards β-chitin. *Bt*CBM33/AA10 showed 34 % binding towards β-chitin; whereas *Bt*CBP (WT) showed 62 % binding towards β-chitin. Same results were obtained when CBM5 domain was deleted from *Bli*Chi. The mutant of *Bli*Chi (*Bli*GH) lost its activity on α-chitin and showed 5- fold reduction in its activity on β-chitin (section 3.2.6.1). This indicated that CBM5 domain is important for binding or hydrolysis in CBPs as well as chitinases. Further, when binding of *Bt*CBM5 was studied, *Bt*CBM5

showed increased binding towards α- as well as β- chitin. This further suggested that *Bt*CBM5 has more affinity towards chitin substrates as compared to *Bt*CBM33/AA10. This could be due to smaller size of *Bt*CBM5, due to which it gains more access to the substrates and thus gets more tightly bound as compared to *Bt*CBM33/AA10.

4.3.1.2 Characterization of *Bt*CBM33/AA10 and *Bt*CBM5 on the basis of pH

As mentioned earlier, *Bt*CBP showed optimum binding at two different pHs i.e. pH 5.0 and 7.0 whereas *Bli*CBP and *Sp*CBP21 showed optimum binding at pHs 6.0 and 7.0, respectively. When domain architecture of the three proteins was compared, *Bli*CBP and *Sp*CBP21 consisted of only CBM33/AA10 and *Bt*CBP consisted of CBM33/AA10 and CBM5 tethered by two FnIII domains. There is a possibility that due to two CBMs, *Bt*CBP showed optimum binding at two different pH. To further understand the difference in binding of *Bt*CBP, we amplified CBM33/AA10 and CBM5 and characterized on the basis of pH. *Bt*CBM33/AA10 showed optimum binding at pH 7.0, which is one of the pHs where *Bt*CBP showed optimum binding. However, when binding of *Bt*CBM5 was studied at different pHs, *Bt*CBM5 showed binding in broad range of pH conditions. CBM5 domain of an alkaline chitinase from *Bacillus* sp. J813 also showed binding under extreme pH conditions (Fumiya et al., 2012) with maximum binding at pH 5.2. Our results, together with Fumiya et al., (2012) with *Bacillus* sp. J813 CBM5 indicated that CBM5 domains work under broad and extreme pH conditions. This also suggests that the two domains behave differently, when present independently and together in the same protein attached *via* FnIII domains.

4.3.1.3 Characterization of *Bt*CBM33/AA10 and *Bt*CBM5 on the basis of temperature using substrate binding assay and CD

As mentioned above the *Bli*CBP and *Sp*CBP21, consisting of only CBM33/AA10, showed a constant binding pattern till certain temperature.

However, *Bt*CBP did not follow a constant binding pattern. *Bt*CBP also showed different spectra as compared to CBPs with only CBM33 domain. To further understand the reason for different behaviour of *Bt*CBP at different temperature, we characterized the *Bt*CBM33/AA10 and *Bt*CBM5 on the basis of temperature and studied their secondary structure under CD. *Bt*CBM33/AA10 followed a constant binding pattern till 60°C after which the binding declined. However, *Bt*CBM5 did not follow a constant binding pattern like *Bt*CBP. Also, CD spectrum of *Bt*CBM33/AA10 was similar to the CD spectra of other *Bt*CBM33/AA10 domains. However, CD spectrum of *Bt*CBM5 was different as compared to *Bt*CBM33/AA10 domain proteins i.e., hump in 230-240 nm region of *Bt*CBM33/AA10 proteins was inverted in case of *Bt*CBM5. As behaviour of *Bt*CBM33 and *Bt*CBM5 does not correlate with *Bt*CBP, it may be possible that the two domains behave differently when they are independent as compared to when they are tethered *via* FnIII domains.

4.3.1.4 Oxido-hyrolytic activity of *Bt*CBM33/AA10 and *Bt*CBM5 and comparison with *Bt*CBP (WT)

*Bt*CBP generated oxidized CHOS ranging from DP2-DP10. To further understand whether *Bt*CBP has acquired oxido-hydrolytic activity from CBM33/AA10 or CBM5 or both, we tested the products generated by *Bt*CBM33/AA10 and *Bt*CBM5 using MALDI-TOF MS. Both, *Bt*CBM33/AA10 and *Bt*CBM5 generated oxidized CHOS ranging from DP3-DP8 and DP3-DP17, respectively. This indicated that both *Bt*CBM33/AA10 and *Bt*CBM5 are oxido-hydrolytic. CBP21 from *S. marcescens* (Vaaje-Kolstad et al., 2010), *Ef*CBM33A from *Enterococcus feacalis* (Vaaje-Kolstad et al., 2011), *Bl*AA10A from *B. licheniformis*, *Tf*AA10B from *Thermobifida fusca*, *Ba*AA10A from *B. amyloliquefaciens* (Hemsworth et al., 2014), *Hj*AA9B from *Hypocrea jecorina* (Karkehabadi et al., 2008), *Ta*AA9A from *Thermoascus auranticus* (Quinlan et al., 2011) and *Nc*LPMO9C from *Neurospora crassa* (Isaksen et al., 2014) were reported to be oxido-hydrolytic. Till now only CBM33/AA10 domains of CBPs/AA10 LPMOs were reported to

be oxido-hydrolytic. Here, we report that CBM33/AA10 and CBM5, both are oxido-hydrolytic.

4.3.1.5 Quenching in fluorescent intensity and variation in tertiary structure fingerprint indicated that *Bt*CBM33/AA10 and *Bt*CBM5 bind to soluble CHOS

Quenching in fluorescent intensity and changes in tertiary structure fingerprint indicated that *Bt*CBM33/AA10 and *Bt*CBM5 bind to soluble CHOS. K_B and $\Delta G°$ values obtained from fluorescence spectroscopy indicated that *Bt*CBM33/AA10 and *Bt*CBM5, have more affinity towards $(NAG)_3$ as compared to $(NAG)_6$, like *Bt*CBP. *Bt*CBM33/AA10 binds more efficiently to soluble chitin substrates as compared to *Bt*CBM5. GbpA from *V. cholerae* was screened for glycan binding against a library of 264 natural and synthetic glycans with amino linkers. This glycan screen revealed that GbpA binds selectively to CHOS through domains 1 and 4 (Wong et al., 2012). There are no other reports on binding of CBM33 and CBM5 to CHOS.

4.3.1.6 Activity of *Bt*CBP, *Bt*CBM33/AA10 and *Bt*CBM5 towards CHOS

To test whether *Bt*CBP, *Bt*CBM33/AA10 and *Bt*CBM5 hydrolyze/oxido-hydrolyze $(NAG)_6$, *Bt*CBP, *Bt*CBM33/AA10 and *Bt*CBM5 were incubated with $(NAG)_6$ for 24 h in presence or absence of reductant (ascorbic acid) and analyzed using MALDI-TOF. *Bt*CBP, *Bt*CBM33/AA10 and *Bt*CBM5 did not hydrolyze/oxido-hydrolyze $(NAG)_6$. This indicated that CBPs/AA10/CBMs bind to CHOS but did not cleave. *Sm*CBP21 also did not cleave $(NAG)_6$ (Vaaje-Kolstad et al., 2010), suggesting that these LPMOs might have alternative role in addition to binding and oxido-hydrolysis of insoluble chitin substrates.

4.3.1.7 Antifungal activity of *Bt*CBM33/AA10 and *Bt*CBM5

As mentioned earlier, *Bt*CBP, *Bli*CBP and *Sp*CBP21 were neither antifungal nor increased the antifungal activity of chitinases. Hevein, due to its small size, was known to penetrate through the fungal cell wall and reach the plasma membrane from where it may affect active sites of cell wall morphogenesis (Van Parijis et al., 1991). Plant CBP like GAFP from *Ginkgo biloba* (4.2 kDa) was antifungal and was believed to cause increased membrane permeabilization (Huang et al., 2000). To know whether small size of protein makes it more efficient to enter into the fungal hyphae and make them antifungal, we tested the antifungal activity of truncation mutants of *Bt*CBP (*Bt*CBM33/AA10 and *Bt*CBM5). *Bt*CBM33/AA10 and *Bt*CBM5 were not antifungal against *Alternaria alternata* like full length *Bt*CBP. Instead, spores treated with *Bt*CBM5 showed an abrupt increase in germination. The exact reason for increased germination was not known. When analyzed under microscope, *Bt*CBM5-treated spores showed longer germ tubes as compared to untreated ones; other morphological changes in spores were not seen. This indicated that *Bt*CBM5 has a positive effect instead of showing antifungal activity. This further indicates that, GAFP was antifungal because not necessarily due to small size.

4.4 Characterization of truncation mutants of *Bt*CBP

4.4.1 Binding of *Bt*CBPΔCIII, *Bt*CBPΔCΔNIII and *Bt*CBPFnIII(N+C) to insoluble chitin substrates

FnIII domains are common in bacterial carbohydrate active proteins. But, very little is known about their function. In eukaryotes, FnIIIs are involved in a variety of molecular recognition processes such as cell adhesion and also as cell surface hormone and cytokine receptors. The main function of FnIIIs is to facilitate protein-protein interactions and to act as a linker to get required biological function in the right space (Campbell and Spitzfaden, 1994). It is believed that the

FnIII domains also facilitate proper interaction between GHs and CBMs. FnIII-like repeat from *Clostridium thermocellum* cellobiohydrolase CbhA promotes hydrolysis of cellulose by modifying its surface (Kataeva et al., 2002). FnIII domains of ChiA1 from *Bacillus circulans* W12 have little role in chitin binding activity, but deletion of FnIII from ChiA1 caused significant decrease in hydrolysis of colloidal chitin (Watanabe et al., 1994). To further dig into the role of FnIII domains, we generated deletion mutants of *Bt*CBP which consisted of two FnIII domains. Substrate binding assays with *Bt*CBPΔCIII, *Bt*CBPΔCΔNIII and *Bt*CBPFnIII(N+C) indicated that FnIII domains did not bind to α- or β- chitin. This further suggests that FnIII domains do not play a role in either binding or hydrolysis of crystalline or insoluble chitin substrates.

4.4.2 Binding of *Bt*CBPFnIII(N+C) to CHOS using fluorescence spectrometry and CD

Since the FnIII domains do not play a role in either binding or hydrolysis of crystalline or insoluble chitin substrates, we tested binding of FnIII domains towards CHOS. Variation in tertiary structure fingerprint and quenching in fluorescence intensity indicated that FnIII domains bind to $(NAG)_6$. This suggests that FnIII domains which do not play a role in binding or hydrolyzing crystalline chitin, bind to CHOS. This is an indication towards role of FnIII domains in signalling. Fibrinogen-like recognition domain containing 1 (FIBCD1) which is a vertebrate acetyl group recognition receptor, is also known to bind to chitin. Two phosphorylation sites discovered in the cytoplasmic part indicate that FIBCD1 may play a role in signalling (Shrive et al., 2013).

4.4.3 Variation in secondary structure of FnIIIs at different temperatures and pH

FnIII domains possess only β-sheets, and the α-helices were rare. When FnIII domains were subjected to higher temperatures (> 60°C) there was a sudden

increase in α-helices. Also, when subjected to different pH conditions, *Bt*CBPFnIII(N+C) showed secondary structural changes at pH 4.0 (sodium acetate buffer), where there was an increase in α-helices and decrease in β-sheets and random coils. Thus, there is also a possibility that these FnIII domains play a role in changing conformation of proteins at higher temperatures.

4.5 Characterization of site-directed mutants of *Sp*CBP28

4.5.1 Substrate binding assays

4.5.1.1 Binding towards insoluble chitin substrates

Substrate binding assay revealed that *Sp*CBP28 (WT), single (*Sp*CBP28-I79E, *Sp*CBP28-L84E, *Sp*CBP28-G263N) and double mutants (*Sp*CBP28-I79E+L84E, *Sp*CBP28-I79E+G263N, *Sp*CBP28-L84E+G263N) did not bind to insoluble α- or β-chitin. However, when triple mutant (*Sp*CBP28-I79E+L84E+G263N) was incubated with chitin substrates, it binds to insoluble chitin substrates and prefer α- to β-chitin. *Sp*CBP28 (WT) was the only CBP which does not show binding towards insoluble chitin substrates (Purushotham et al., 2012). As already mentioned, when *Sp*CBP28 sequence was aligned with other CBPs, *Sp*CBP28 lacks three (two glutamic acid and one aspargine residue) out of five amino acid residues that were reported to be important for binding. We mutated these residues from *Sp*CBP28 and substituted with essential amino acids at respective positions to know whether *Sp*CBP28 lost its activity due to mutation in any or all of these amino acid residues. *Sp*CBP28-I79E+L84E+G263N showed very little binding to α- to β-chitin, suggesting a possibility that *Sp*CBP28 has a role other than binding. Also, *S. proteamaculans* produces three CBPs *viz*., *Sp*CBP21, *Sp*CBP50 and *Sp*CBP28. *Sp*CBP21 and *Sp*CBP50 bind equally well to different chitin substrates (Purushotham et al., 2012). An organism might not be producing three similar proteins with similar function. There is also a possibility that *Sp*CBP28 will show either oxido-hydrolytic activity or binding to CHOS like

other CBPs mentioned above. We, therefore, tested oxido-hydrolytic activity of *Sp*CBP28 towards insoluble chitin substrates and binding of *Sp*CBP28 to CHOS.

4.5.1.2 Oxido-hydrolytic activity of *Sp*CBP28 (WT) and *Sp*CBP28-I79E+L84E+G263N towards insoluble chitin substrates

CBPs/LPMOs bind to and oxido-hydrolyze insoluble and crystalline chitin substrates. *Sp*CBP28 is the only CBP which does not bind to chitin. *Sp*CBP28 (WT) and its triple mutant *Sp*CBP28-I79E+L84E+G263N were incubated with β-chitin (580# or 100-170 # mesh size) and products were analyzed using Nano-ESI-MS with *Bt*CBP as a positive control. Oxidized CHOS were observed in *Bt*CBP treated β-chitin of both mesh size. *Sp*CBP28 (WT) and *Sp*CBP28-I79E+L84E+G263N did not generate oxidized CHOS when incubated with β-chitin (580# or 100-170 # mesh size). This indicated that *Sp*CBP28 (WT) and *Sp*CBP28-I79E+L84E+G263N are not oxido-hydrolytic unlike *Bt*CBP. *Sp*CBP28-I79E+L84E+G263N which regained little of binding towards chitin substrates also did not oxido-hydrolyze either α- or β- chitin. Thus binding and oxido-hydrolytic activity of CBPs are not coupled. Also, there is a possibility that *Sp*CBP28 has a role other than binding and oxido-hydrolysis.

4.5.1.3 Binding and activity of *Sp*CBP28 towards CHOS

Quenching in fluorescence intensity and variation in tertiary structure fingerprint indicated that *Sp*CBP28 (WT) binds to soluble CHOS. As mentioned, other CBPs like *Bt*CBP, *Bli*CBP, *Sp*CBP21, *Bt*CBM33/AA10 and *Bt*CBM5 and colonization factor, GbpA (which possess domains similar to *Bt*CBP) also showed binding to CHOS. *Sp*CBP28 which neither binds nor oxido-hydrolyzes crystalline chitin, binds to CHOS ranging from $(NAG)_2$ to $(NAG)_6$. However, like *Bt*CBP, *Bt*CBM33/AA10 and *Bt*CBM5, *Sp*CBP28 did not hydrolyze/oxido-hydrolyze $(NAG)_5$ or $(NAG)_6$. As other CBPs and *Sp*CBP28 show a common feature of binding to CHOS, the CBPs may have a major role in binding/recognizing CHOS.

Binding to crystalline chitin could be a secondary function. CBPs thus may play a role in signaling like FnIII domains. Presence of FnIII domains with CBMs in chitinases and CBPs further indicate that presence of different domains with same function in a single protein endowed the ability to recognize different ligands to which protein or bacteria were exposed. All these domains together give the protein an ability to sense, bind and hydrolyze/oxido-hydrolyze different ligands.

4.5.2 Effect of temperature and pH on secondary structure of *Sp*CBP28

Conformational changes in *Sp*CBP28 at different temperatures (10-100°C) were analyzed in far UV region (260-190 nm) using CD. Spectral changes in *Sp*CBP28 started at 60°C. There was an increase in β-sheets and random coils and decrease in α-helices. *Sp*CBP28 did not show conformational changes at different pH. *Sp*CBP28, therefore, might have a role to play under extreme conditions of temperature. However, when binding was studied at different pH, *Sp*CBP28 did not show binding under extreme conditions. Thus, the *Sp*CBP28 had some role other than binding to crystalline chitin. *Sp*CBP28 showed single negative and single positive region in its spectrum, similar to other CBPs. This suggests that secondary structure of *Sp*CBP28 is similar to *Bt*CBM33/AA10, *Sp*CBP21 or *Bli*CBP. To further confirm whether *Sp*CBP28 is structurally similar to other CBPs, homology modelling was done.

4.5.3 Homology modeling of *Sp*CBP28

When NCBI blast program was run for *Sp*CBP28, it showed 52 % identity to chitin binding domain protein of *Yokonella regensburgei* (WP_006820597.1), 49 % identity to CBP of *Pseudomonas putida* (WP_023534296.1) and 37 % identity to CBP of *Shewenella woodyi* (WP_012325331.1), chitin binding domain containing protein of *Vibrio nigripulchritudo* (WP_004400380.1) and to CBP of *Burkholderia oklahomensis* (WP_010118051.1). *Sm*CBP21 (2BEM) was used as template to generate a model for *Sp*CBP28. *Sp*CBP28 was 23 % identical to

*Sm*CBP21. Thus, segment modelling was done. When structure of *Sp*CBP28 and *Sm*CBP21 were compared, *Sp*CBP28 was almost similar to *Sm*CBP21. This suggests that *Sp*CBP28 was similar to other CBPs but lacks binding and oxido-hydrolytic activity for crystalline chitin substrates, however, while retaining binding to CHOS.

4.5.4 Phylogenetic analysis

Phylogenetic tree clustered the CBPs in two different clusters. *Sp*CBP28 was put far from the CBPs which have been classified till date and are known to bind or oxido-hydrolyze α- and/or β-chitin. *Sp*CBP28 was found to be very close to CBP of *Yokonella* sp., which is a pathogenic organism. There is a possibility that CBPs from these organisms are serving as virulence factors. Bacterial CBMs serve as virulence factors and toxins (Frederiksen et al., 2013). CBPs are known to target glycoproteins and glycolipids in the hosts that contain GlcNAc. In addition to binding and oxido-hydrolysis of chitin, CBMs bind to a broad range of substrates like cellulose and mucin. GbpA from *V. cholerae* facilitated bacterial colonization in intestine where they bind to GlcNAc residues of mucin present in intestinal wall. Sugar binding properties of CBMs promote their adhesion to extracellular or intracellular targets which contribute to virulence (Guillen et al., 2010). E8, E7 and CBP from *Vibrio* sp. also clustered in the group where *Sp*CBP28 was present.

CBPs from *E. faecalis* (*Ef*CBM33), *B. thuringiensis* (*Bt*CBP), *B. licheniformis* (*Bli*CBP), *B. amyloliquefaciens* (*Bacamylo*), *Lactococcus lactis* (Lac lac), *S. proteamaculans* (*Sp*CBP21), *S. marcescens* (*Sm*CBP21), *Yersinia ruckeri* (Yer ruck), *B. cereus* (Baccer CBP), *Str. coelicolor* (Strcoe CBP) and *Str. olivaceoviridis* (Stroli CBP) formed another cluster. Out of these, *Ef*CBM33, Bacamylo and *Sm*CBP21 bind and oxido-hydrolyze crystalline chitin. Lac lac, *Sp*CBP21, Strcoe CBP and Stroli CBP bind to crystalline chitin substrates. This cluster does not include *Sp*CBP28 suggesting that *Sp*CBP28 was a distant relative to these CBPs. CBPs (other than E7, E8 and VibChol CB) in this cluster have not

been characterized and their role is still not known. This indicates that *Sp*CBP28 and CBPs which are clustered with *Sp*CBP28 may have some other role, whereas, CBPs which are clustered far from *Sp*CBP28 have primary role in chitin binding and oxido-hydrolysis. However, in present study *Bt*CBP, *Bli*CBP and *Sp*CBP21, which formed a separate cluster, and *Sp*CBP28 which formed another cluster have one thing in common i.e., all these CBPs bind to CHOS.

CBPs are known to bind to crystalline chitin substrates and to increase substrate accessibility for chitinases due to oxido-hydrolytic activity. Thus, when CBPs and chitinases are added to the reaction mixture, CBPs increase the hydrolytic activity of chitinases. This proves that CBPs and chitinases act in synergism with each other. Also, some small-sized CBPs have been reported to show antifungal activity. Due to small size, CBPs penetrate through the fungal cell wall and reach the plasma membrane. In present study, we found that there are CBPs which neither bind to nor oxido-hydrolyze crystalline chitin substrates (*Sp*CBP28). Also, there are chitinases which do not show synergism with CBPs (*Bli*Chi). When antifungal activity of CBPs was tested, CBPs neither showed antifungal activity nor did they increase antifungal activity of chitinases. Generation of truncation mutants also did not make CBPs antifungal.

Further, we report that *Bt*CBP, *Bli*CBP, *Sp*CBP21 and CBM33 and CBM5 domains of *Bt*CBP bind but did not cleave CHOS. Also *Sp*CBP28, which did not bind to crystalline chitin, binds to CHOS. Binding of CBPs to CHOS indicate that CBPs may play an alternate role (most probably in signaling) in addition to binding and oxido-hydrolytic activity.

5. Background and objectives

Chitin is high molecular weight, un-branched, obstinate, helical homo-polymer which is insoluble in most solvents. It is the second most abundant polymer on earth next to cellulose. It is predominantly found in fungal cell walls, insect exoskeleton, shrimps and squids pens. Thus chitin degradation is a key step in recycling of nutrients into the environment. In nature, chitin degradation is primarily mediated by chitinolytic micro-organisms. These organisms secrete chitinases as well as chitin binding proteins (CBPs). Hydrolysis of chitin produces chito-oligosaccharides (CHOS) which have application in agriculture, medicine, pharmaceuticals, cosmetics etc. Conversion of crystalline chitin using CBPs also referred to as lytic polysaccharide mono-oxygenases (LPMOs) opened new avenues for production of biofuels. In the present study, CBPs from *Bacillus thuringiensis* serovar *kurstaki* (*Bt*CBP), *B. licheniformis* DSM13 (*Bli*CBP) and *Serratia proteamaculans* 568 (*Sp*CBP21 and *Sp*CBP28) have been characterized. To understand differences in properties of CBM33 and CBM5 domains, we cloned and characterized the two domains of *Bt*CBP. Further, to know the role of FnIII domains in *Bt*CBP, truncation mutants of *Bt*CBP were generated and characterized. All CBPs are known to bind to crystalline chitin. However, *Sp*CBP28 does not bind to crystalline chitin substrates. To know whether *Sp*CBP28 lost its binding ability due to mutation in any or all the three conserved amino residues, site-directed mutants were generated and characterized.

5.1 Characterization of *Bt*CBP, *Bli*CBP and *Sp*CBP21

The three CBPs (*Bt*CBP, *Bli*CBP and *Sp*CBP21) in pET22b (+) expression vector were expressed in BL21(DE3) strain of *E. coli* using auto-induction and purified using Ni-NTA His-tagged column chromatography. Substrate binding assays towards insoluble substrates were performed. All three CBPs were characterized on the basis of pH and temperature. Synergistic effect of chitinases and CBPs was also studied on isolated chitin substrates and fungal cell walls. Binding of *Bt*CBP,

*Bli*CBP and *Sp*CBP21 towards soluble CHOS was studied using fluorescence spectroscopy and circular dichroism.

Substrate binding assays revealed that the three CBPs prefer β- to α-chitin. Binding of *Bt*CBP and *Bli*CBP towards α- and β- chitin attained equilibrium within 9 and 15 h, respectively. Determination of dissociation constants further confirmed that *Bt*CBP and *Bli*CBP prefer β-chitin as K_d value of *Bt*CBP and *Bli*CBP for β- chitin were lower than K_d values for α- chitin. Characterization of *Bt*CBP, *Bli*CBP and *Sp*CBP21 on the basis of temperature and pH revealed that proteins with only CBM33 domains behave in a similar manner, whereas proteins which possess multiple domains have different characteristics. *Bli*CBP and *Sp*CBP21 which possess only CBM33 showed optimum binding at pH 6 and 7, respectively and follow a constant binding pattern till certain temperature (*Bli*CBP: 50°C and *Sp*CBP21: 40°C) after which binding declined. However, *Bt*CBP showed optimum binding at two different pHs (5 and 7) and also did not follow a constant binding pattern. To confirm whether this behavior was because of two different CBMs in *Bt*CBP, CBM33 and CBM5 domains of *Bt*CBP were cloned and characterized.

When hydrolysis of isolated chitin substrates was studied in presence of CBPs and chitinases, we found that any CBP can be used with any chitinase, irrespective of genera or species, to improve chitin degradation. Also, some self efficient chitinases do not need CBPs for chitin degradation. Generation of deletion mutant of a self efficient chitinase revealed that C-terminal accessory domains were responsible for making it self-efficient. *Bt*CBP, *Bli*CBP or *Sp*CBP21 did not show synergism with chitinases in inhibiting fungal growth.

Fluorescence spectroscopy and circular dichroism studies revealed that the three CBPs bind to CHOS. Binding constant (K_B) and ΔG° values indicated that *Bli*CBP and *Sp*CBP21 have more affinity towards $(NAG)_6$ as compared to

$(NAG)_3$. However, *Bt*CBP has more affinity towards $(NAG)_3$ as compared to $(NAG)_6$.

5.2 Cloning and characterization of deletion mutants of *Bt*CBP

The two CBMs in *Bt*CBP *viz*., CBM33 (designated as *Bt*CBM33/AA10) and CBM5 (designated as *Bt*CBM5) were amplified, cloned in pET22b (+) expression vector, expressed in BL21(DE3) strain of *E. coli* using auto-induction and purified using Ni-NTA His tagged column chromatography. Substrate binding assays towards insoluble substrates were performed. Both the domains were characterized on the basis of pH and temperature. Oxido-hydrolytic activity of *Bt*CBM33/AA10 and *Bt*CBM5 was tested and compared with oxido-hydrolytic activity of full length *Bt*CBP using MALDI-TOF MS. Binding of *Bt*CBM33/AA10, *Bt*CBM5 and *Bt*CBP towards soluble CHOS was studied using fluorescence spectroscopy and circular dichroism.

Substrate binding assays revealed that both *Bt*CBM33/AA10 and *Bt*CBM5 prefer β- chitin as substrate like *Bt*CBP. However, *Bt*CBM33/AA10 lost its activity on α- chitin. *Bt*CBM5 showed increased binding towards both α- and β- chitin as compared to *Bt*CBP. This indicated that CBM5 domain is more efficient than CBM33 in binding towards insoluble substrates. When characterized on the basis of pH and temperature, *Bt*CBM33/AA10 showed characteristics similar to *Bli*CBP and *Sp*CBP21 which consists of only CBM33 domain. *Bt*CBM33/AA10 showed optimum binding at pH 7 and followed a constant binding pattern till 60 °C. CD spectrum of *Bt*CBM33/AA10 was also similar to *Bli*CBP and *Sp*CBP21 (only CBM33s). *Bt*CBM5 showed binding in a broad range of pH conditions and did not follow constant binding pattern like *Bt*CBP. CD spectrum of *Bt*CBM5 was different than *Bli*CBP and *Sp*CBP21.

MALDI-TOF MS revealed that *Bt*CBM33/AA10, *Bt*CBM5 and *Bt*CBP were oxido-hydrolytic towards β-chitin. *Bt*CBP generated oxidized CHOS ranging

from DP2-DP10. *Bt*CBM33/AA10 and *Bt*CBM5 generated oxidized CHOS ranging from DP3-DP8 and DP3-DP17, respectively. Till now only CBM33s were known to have oxido-hydrolytic activity. We now demonstrated that both CBM33 and CBM5 are oxido-hydrolytic towards β-chitin.

Fluorescence spectroscopy and CD studies revealed that *Bt*CBM33/AA10 and *Bt*CBM5 bind to CHOS. Binding constant (K_B) and ΔG° values indicated that *Bt*CBP, *Bt*CBM33/AA10 and *Bt*CBM5 have more affinity towards $(NAG)_3$ as compared to $(NAG)_6$.

5.3 Cloning and characterization of truncation mutants of *Bt*CBP

To know the role of FnIII domains in *Bt*CBP, truncation mutants of *Bt*CBP were generated. The three mutants were named as *Bt*CBPΔCIII (C-terminal FnIII deleted), *Bt*CBPΔCΔNIII (both FnIIIs deleted) and *Bt*CBPFnIII(N+C) where both N- and C- terminal FnIII domains were PCR amplified and cloned. The three mutants were cloned in pET22b (+) expression vector, expressed in BL21(DE3) strain of *E. coli* using auto-induction and purified using Ni-NTA His tagged column chromatography. Substrate binding assays towards insoluble chitin substrates were performed for these truncation mutants. Stability of *Bt*CBPFnIII(N+C) at different pH and temperature was studied using CD. *Bt*CBPFnIII(N+C) was tested for its binding towards CHOS using fluorescence spectroscopy and CD.

Substrate binding assays revealed that FnIII domains do not play a role in binding of *Bt*CBP towards insoluble chitin substrates. FnIII domains when amplified and present independently without CBMs in reaction mixture do not show binding to chitin. CD of *Bt*CBPFnIII(N+C) at different temperature and pH revealed that FnIII domains may play a role under extreme temperature conditions. Fluorescence spectroscopy and CD studies revealed that *Bt*CBPFnIII(N+C) bind to $(NAG)_6$.

5.4 Cloning and characterization of site-directed mutants of *Sp*CBP28

To know whether *Sp*CBP28 has lost its binding ability due to mutation in any or all the three conserved amino residues, site directed mutants were generated and characterized. The single (*Sp*CBP28-I79E, *Sp*CBP28-L84E, *Sp*CBP28-G263N), double (*Sp*CBP28-I79E+L84E, *Sp*CBP28-I79E+G263N, *Sp*CBP28-L84E+G263N) and triple (*Sp*CBP28-I79E+L84E+G263N) mutants of *Sp*CBP28 were cloned in pET22b (+) expression vector, expressed in BL21(DE3) strain of *E. coli* using auto-induction and purified using Ni-NTA His tagged column chromatography.

Substrate binding assay revealed that *Sp*CBP28 (WT), single and double mutants did not bind to insoluble α- or β-chitin. However, when triple mutant was incubated with chitin substrates, little binding was gained and it preferred α- to β-chitin. Also, Nano-ESI MS analysis revealed that *Sp*CBP28 (WT) and *Sp*CBP28-I79E+L84E+G263N are not oxido-hydrolytic, unlike other reported CBPs. Quenching in fluorescent intensity and variation in tertiary structure fingerprint indicated that *Sp*CBP28 (WT) binds to soluble CHOS. CD spectrum of *Sp*CBP28 showed single negative and single positive similar to other CBPs. Different studies indicated that *Sp*CBP28 was also similar to other CBPs but lacks binding and oxido-hydrolytic activity for crystalline chitin substrates. However, the *Sp*CBP28 binds to CHOS.

Phylogenetic analysis clustered *Sp*CBP28 and other CBPs which are characterized and are known to bind or oxido-hydrolyze crystalline chitin in two separate clusters. This indicates that *Sp*CBP28 and CBPs which are clustered with *Sp*CBP28 may have alternate role, whereas, CBPs which are clustered far from *Sp*CBP28 have primary role in chitin binding and oxido-hydrolysis.

Major findings of present work

- ✓ Binding assays of *Bt*CBP and *Bli*CBP revealed that both prefer β- to α-chitin. The K_d value of *Bt*CBP and *Bli*CBP for β- chitin (*Bt*CBP: 3.46 ± 1.3 µM; *Bli*CBP: 4.12 ± 1.6 µM) was lower than the K_d value for α- chitin (*Bt*CBP: 5.25 ± 1.4 µM; *Bli*CBP: 5.98 ± 2.1 µM).

- ✓ *Bli*CBP and *Sp*CBP21 showed optimum binding at pH 6.0 and 7.0, respectively. However, *Bt*CBP showed optimum binding at two pHs, 5 and 7. Also, *Bt*CBP showed binding under extreme pH conditions.

- ✓ *Bli*CBP and *Sp*CBP21 showed constant binding pattern till certain temperature after which binding declined. However, *Bt*CBP did not follow a constant binding pattern.

- ✓ CD analysis showed that *Bli*CBP and *Sp*CBP21 showed similar spectra which were different from *Bt*CBP, which consists of multiple domains.

- ✓ Fluorescence spectrometry and CD studies revealed that *Bt*CBP, *Bli*CBP and *Sp*CBP21 bind to CHOS.

- ✓ *Bt*Chi and *Sp*ChiB showed synergistic effect with *Bt*CBP, *Bli*CBP and *Sp*CBP21 suggesting that CBP from any organism will increase efficiency of a particular chitinase.

- ✓ *Bli*Chi did not show synergism with any of the CBPs, suggesting that *Bli*Chi was a self efficient chitinase and did not need support of CBPs.

- ✓ Generation of deletion mutant of *Bli*Chi indicated that C-terminal domains are essential for making *Bli*Chi self efficient.

- ✓ Antifungal assay proved that *Bt*CBP, *Bli*CBP and *Sp*CBP21 did not increase antifungal activity of chitinases.

- ✓ Characterization of CBM33 and CBM5 domains of *Bt*CBP suggested that these domains behave differently when they are present independently as compared to when connected *via* FnIII domains.

- ✓ *Bt*CBM5 showed increased binding towards α- (50%) and β-chitin (82%) as compared to *Bt*CBM33/AA10 (α- chitin: no binding and β-chitin: 32%).

- ✓ *Bt*CBM33/AA10 showed optimum binding at pH 7.0 and also showed binding under extreme pH conditions. *Bt*CBM5 showed binding under broad range of pH.

- ✓ Like *Bli*CBP and *Sp*CBP21, which consist of only CBM33 domain, *Bt*CBM33/AA10 followed a constant binding pattern. However, *Bt*CBM5 did not follow constant binding pattern.

- ✓ *Bt*CBM33/AA10 showed similar CD spectrum as showed by *Bli*CBP and *Sp*CBP21 i.e., hump at 230-240 nm region. This hump was inverted in case of *Bt*CBM5.

- ✓ *Bt*CBP, *Bt*CBM33/AA10 and *Bt*CBM5 generated oxidized CHOS from β-chitin and thus were oxido-hydrolytic.

- ✓ Fluorescence spectrometry and CD studies revealed that *Bt*CBM33/AA10 and *Bt*CBM5 bind to CHOS.

- ✓ MS analysis revealed that *Bt*CBP, *Bt*CBM33/AA10 and *Bt*CBM5 did not cleave CHOS.

- ✓ Deletion and amplification of FnIII domains from *Bt*CBP suggested that FnIII domains did not play a role in binding of *Bt*CBP towards insoluble chitin substrates.

- ✓ Fluorescence spectrometry and CD studies revealed that FnIII domains bind to CHOS.
- ✓ Site-directed mutants of *Sp*CBP28 revealed that when the three amino acid residues were mutated, *Sp*CBP28 regained very little binding ability.
- ✓ Nano-ESI MS further indicated that SpCBP28 and its triple mutant were not oxido-hydrolytic, this suggests that binding and oxido-hydrolytic activities are not coupled.
- ✓ Fluorescence spectrometry and CD studies revealed that *Sp*CBP28, which did not bind to insoluble chitin substrates, binds to CHOS.
- ✓ MS analysis revealed that *Sp*CBP28 did not cleave CHOS like *Bt*CBP, *Bt*CBM33/AA10 and *Bt*CBM5.

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