Cloning and characterization of chimeric Bacillus chitinases using domain swapping

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

by

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July 2009

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CERTIFICATE

This is to certify that Ms. Chilukoti Neeraja has carried out the research work embodied in the present thesis under supervision and guidance of Prof. Appa Rao Podile for a full period prescribed under the Ph. D ordinance of this University. We recommend her thesis **"Cloning and characterization of chimeric *Bacillus* chitinases using domain swapping"** for submission for the degree of Doctor of Philosophy of the University.

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DECLARATION

I hereby declare that the work embodied in this thesis entitled "**Cloning and characterization of chimeric *Bacillus* chitinases using domain swapping**" has been carried out by me under the supervision of Prof. Appa Rao Podile and this has not been submitted for any degree or diploma in any other University earlier.

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ACKNOWLEDGEMENTS

I wish to extend my deep sense of gratitude to my supervisor and mentor Prof. Dr. Appa Rao Podile, Department of Plant Sciences for his constant encouragement, timely suggestions, support and meticulous supervision throughout my doctoral research. His positive outlook and confidence in my research topic inspired me and gave me confidence to carry out successfully.

I also thank Prof. Dr. Bruno Moerschbacher, University of Muenster, Germany for allowing me to work in his laboratory during student exchange programme. His constant support, guidance and untiring discussions gave me a lot of strength to carry out the work.

My heartful thanks to my mentor Dr. Nouri Eddine Guedari, University of Muenster, Germany for his suggestions, meticulous supervision and all his help that lead to a fruitful stay in Muenstel.

I thank the present and former Dean, School of Life Sciences, Prof. A. S. Raghavendra and Prof. T. Suryanarayana for allowing me to use the school facilities.

I also thank the present and former Head, Department of Plant Sciences, Prof. A.R. Reddy, Prof. Appa Rao Podile and Prof. P. B. Kirti for allowing me to use the facilities of the department.

My heartful thanks to Dr. Rajagopal, Dept. of Biochemistry for helping me in CD data analysis and also thanks are due to his student Mahesh for his timely help. My special thanks to Mr. Kiran (Dept. of Biochemistry) for his initial help in making my mind on CD analysis. I also thank Prof. Sheshagiri Rao, Dept. of Plant Scienes for his timely help.

My special thanks to Prof. Appa Rao Podile, Mrs. Padma Podile, Prof. Bruno Moerschbacher, Dr. Uschi Windmueller, Ms. Uschi Beike and Ms. Monika Domin for their elderly help and the wonderful environment they have given me during my doctoral tenure.

I thank Council of Scientific and Industrial Resources (CSIR) for the research fellowship. Infrastructural support provided by UGC-SAP and DST-FIST to the Dept. of Plant Sciences are highly acknowledged.

Thanks to DST-DAAD, DAAD and EU-NBS for funding my stay at University of Muenster, Germany to carry out part of my doctoral work. PURSE-travel grant provided by University of Hyderabad to attend 9th International conference of European chitin society in Italy is highly acknowledged.

I wish to thank all my seniors Dr. Sripriya, Dr. Tripura, Dr. Madhuri and Dr. Sashidhar who have helped me in laying stepping stones in the lab. Thanks to my best friends Dr. Aparna, Dr. Aruna, Saritha, Haritha, Srilatha, Padmalatha, Sruthi, Suma, Uma, Swaroopa, Swarnalee,Suprava,for their affection and fun through out my stay in the university.

I thank all my labmates Debasish, Vasudev, Anil, Purushotham, Sippy, Nandakishore, Subhnarayan, Sarma, Azra, Srinivas, Mahesh and Harika for their cooperation and for creating a lively atmosphere in and out of the lab.

I thank all my colleagues Andi, Frank, Carolin, Ritu, Jenni, Christian, Roberta, Malathi, Mareike in Univ. of Muenster, Germany for being very helpful and friendly during my stay in Germany.

My special thanks to Alisha, Chavi and Madhu prakash for their timely help and restless work in some of my experiments.

Thanks are also due to all the faculty members of the School of Life Sciences whose inspiration in my MSc led me to carry out doctoral research. I thank all the research scholars of the school of life sciences for their cooperation, for making my stay in HCU memorable.

The help and cooperation of the non-teaching staff in the school and at CIL is deeply acknowledged. I also thank our lab assistants Satyanarayana, Madhu, Narasimha and Srinivas.

I thank my best friend, husband, Dr. Vinod Kumar Verma for being my strength and helping me with his untiring discussions in and out of the subject always. I thank my parents, grand parents and in-laws for their love and support. Without their blessings, I would not have been successful in making this endeavor possible. Finally I dedicate the thesis to my living god, my mother for her love, blessings, support and struggle whose result is my life. Thanks to the almighty for giving me enough strength and placing me amidst of good people always.....

Neeraja

CONTENTS

ABBREVIATIONS

^{o}C	**degree centigrade/degree Celsius**
C-terminal	**carboxy terminal**
CBD	**carbohydrate binding domain**
ChBD	**chitin binding domain**
CeBD	**cellulose binding domain**
dNTPs	**deoxy nucleotide triphosphates**
DNA	**deoxy ribonucleic acid**
DA	**degree of acetylation**
EDTA	**ethylene diamine tetra acetic acid**
g	**gram**
GlcNAc	**N-acetyl glucosamine**
h	**hour (s)**
IPTG	**isopropyl β-D-thiogalactoside**
Km	**Michaelis Menton constant**
kb	**kilobase pair**
kDa	**kilodalton**
LB	**Luria-Bertani**
lit	**litre**
M	**molar**
mg	**milligram**
min	**minute**
ml	**milliliter**
mM	**millimolar**
mm	**millimeter**
ng	**nanogram**
Ni-NTA	**nickel-nitroacetic acid agarose**
nm	**nanometer**
nM	**nanomolar**
N-terminal	**amino terminal**
OD	**optical density**
PAGE	**polyacrylamide gel electrophoresis**
PCR	**polymerase chain reaction**
rpm	**revolutions per minute**
SDS	**sodium dodecyl sulphate**
Tris	**tris-(Hydroxymethyl) aminoethane**
U	**Unit**
µg	**microgram**
µl	**microlitre**
µmol	**micromole**

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Dedicated to
Almighty and my beloved parents

INTRODUCTION

1.1 Chitin is a structurally complex and abundant polymer rich of carbon and nitrogen

Chitin is a major structural polysaccharide consisting of N-acetyl-D-glucosamine (GlcNAc) monomeric units linked with β-1, 4-glycosidic bonds present abundantly in nature next to cellulose (Fig. 1.1). Chitin represents a potential source of renewable biomass and is structurally identical to cellulose except that the hydroxyl (–OH) group on C2 of glucose in cellulose is replaced by an acetamide group (NH.CO.CH3). The annual production of cellulose and chitin is around one trillion and 100 billion tons, respectively (Tharanathan and Kittur, 2003; Kim *et al.*, 2006). Chitin is abundantly present in fungal cell walls, insect exoskeletons and the shells of the crustaceans. In the aquatic biosphere alone, it is estimated that more than 10^{11} metric tons of chitin is produced annually. This enormous quantity of chitin, full of insoluble carbon and nitrogen, if not converted to biologically useful material, would deplete the oceans of these elements in a few decades (Keyhani and Roseman, 1999). Hence, the natural recycling of chitin by marine and terrestrial bacteria is of enormous ecological significance. Such bacteria can be expected to be ideal sources for enzymes suitable for the biotechnological recycling of chitin from wastes of the seafood industry which is of significant economic and environmental impact. Chitin in nature is available in two conformations, arrangement of poly GlcNAc as anti parallel strands (α chitin) or as parallel strands (β chitin). Its degree of crystallinity or its context with other polysaccharides or proteins that lead to the evolution of numerous hydrolytic enzymes and proteins that needs to specifically recognize it.

1.1.1 Basic physico chemical properties of chitin

Chitin is crystalline, intractable, highly hydrophobic, and insoluble in water and organic solvents. It is soluble in hexafluoroisopropanol, hexafluoroacetone, chloroalcohols in conjugation with aqueous solutions of mineral acids (Madhavan, 1992) and dimethylacetamide containing 5 % lithium chloride. Chitin is a non-toxic, non-allergenic, anti-microbial and biodegradable polymer. Current methods to modify chitin and transform it to useful carbohydrate products employ harsh chemical treatments that incur problems of undesirable by-products and limit further usage of the products. The

limitation in using the chemical treatments lead to the development of efficient bioconversion process based on the exploitation of chitinases (Waldeck *et al*., 2006). Chitin indeed is a highly versatile and promising biopolymer with numerous industrial, medical, agricultural and commercial uses.

1.1.2 Deacetylated form of chitin is chitosan

Chitosan (Fig. 1.1), a fully or partially deacetylated derivative of chitin, is soluble in dilute acids like acetic acid and formic acid. In nature chitosan is present in the cell walls of a limited group of fungi belonging to the order Mucorales, as well as during endophytic development of some biotrophic plant pathogenic fungi (El Gueddari *et al*., 2002). Functional derivatives of chitosan, by chemical modifications (Kim *et al*., 1994; Toffey *et al*., 1996; Crini *et al*., 1997) enabled preparation of a few chitosans soluble in general organic solvents (Sakamota, 1995; Knaul and Creber, 1997) and some in binary solvent systems (Tseng *et al*., 1995). Chemical modification of chitin and chitosan resulted in improved solubility in general organic solvents (Kubota, 1997).

1.2 Several enzymes and substrate binding proteins are used by microorganisms in catabolism of chitin

The crystalline and inaccessible nature of chitin compelled the chitin degrading organisms to develop a tool box of enzymes and accessory proteins to ensure efficient degradation (Suzuki *et al*., 2002). Chitin degradation in a marine environment comprises at least four major steps: sensing of chitin either by random collisions or by chemotaxis; attachment to the chitin to stay in close proximity to the nutrient; expression of a magnitude of enzymes and other proteins required for catabolism of the polymer; and finally, uptake and catabolism of the hydrolysis products of the glycosidases (West and Colwell, 1984). The catabolism typically occurs in two steps involving the initial cleavage of the chitin polymer by chitinases (endo- and exo-chitinases) into chitin oligosaccharides, and then further cleavage to N-acetylglucosamine monomers by N-acetylglucosaminidases and chitobiases. In *Vibrio cholerae*, the chitin catabolic operon (VC0611– VC0622 genes) was proposed to be regulated by chitoligosaccharides based

on micro array expression profiling studies (Li and Roseman, 2004, Meibom *et al*., 2004). In the chitin catabolic system, chitin sensor (ChiS, VC0622) has an important role in regulating the gene cluster. The gene cluster was also found to be available in the revealed genomic sequences of other *Vibrio* species (Ruby *et al*., 2005).

1.3 Chitinases are the primary enzymes involved in breakdown of chitin

Chitinases are produced by different organisms for diverse purposes based on their own physiology and use. In plants, they play a defensive role against pathogenic or pestiferous organisms (Jung *et al*., 2008). In arthropods, chitinases are involved in cuticle turnover and mobilization, and in nutrient digestion (Reynolds and Samuels, 1996). The role of chitinase in vertebrates is uncertain; however, it may be involved in resistance to fungal infections (Malaguarnera *et al*., 2004). For fungi, chitinases apparently help to degrade and mobilize organic matter and probably to antagonize the growth of competitors (Adams, 2004) apart from their developmental role during cell wall formation. In yeasts, chitinases are important for cell separation (Sakuda *et al*., 1990). Bacteria secrete chitinases for processing and digestion of GlcNAc-containing macromolecules, which form their nutrient source during their saprophytic phase (Cohen-Kupiec and Chet, 1998). Chitinases play an important role in the ecology of many marine bacteria.

1.3.1 Chitinases are grouped under two families of glycosyl hydrolases

Chitinases are classified into two families, family 18 and 19 chitinases, on the basis of the amino acid sequence of their catalytic domains according to the classification of glycosyl hydrolases by Henrissat and Bairach (1993). Family 19 chitinases have been isolated mostly from plants and were subdivided into classes I, II and IV (Neuhaus *et al*., 1996). Family 18 chitinases include most of the chitinases from bacteria, fungi, insects, plants (class III and V chitinases), and animals. Family 19 chitinases have also been found in prokaryotic organisms such as *Streptomyces* species and *Aeromonas* sps. (Ueda *et al*., 2003). Chitinases can be further classified into two major categories; endochitinases and exochitinases based on their mode of action (Sahai and Manocha,

1993). Karlsson and Stenlid (2009) analyzed the diversity, domain structure and phylogenetic relationships between family 18 chitinases based on the complete genome sequences of bacteria, archaea, viruses, fungi, plants and animals and family 18 chitinases are divided into three main clusters, A, B and C. Clusters A and B both contain family 18 chitinases from bacteria, fungi and plants, suggesting that the differentiation of cluster A and B chitinases preceded the appearance of the eukaryotic lineage.

1.4 Bacterial chitinases are heterogeneous and inducible

Chitinase-producing bacteria were isolated from different environments including soil (Wang *et al*., 1997), garden and park waste compost (Poulsen *et al*., 2008), shellfish waste (Wang and Hwang, 2001) and from hot springs (Yuli *et al*., 2004). Bacteria typically possess multiple, usually inducible chitinases and chitobiases. Chitinases isolated from a single bacterial strain often appear to be heterogeneous with respect to substrate specificity and molecular size, and may, in addition, be subject to post-translation modification including proteolytic processing (Alam *et al*., 1996). Chitinases are reported in bacterial systems like *Aeromonas, Arthrobacter, Beneckea, Bacillus, Chromobacterium, Clostridium, Klebsiella, Pseudomonas, Serratia, Streptomyces and Vibrio* (Deshpande, 1986).

Genes encoding chitinases (*chi*) from *Serratia marcescens* strains 2170 (Suzuki *et al*., 2002), QMB 1466 (Jones *et al*., 1986), KCTC2172 (Gal *et al*., 1998) and BJL200 (Bruberg *et al*., 1994) have been cloned. Chitinolytic activity of *S. marcescens* Nima was found to be 43 times higher than those produced by other *S. marcescens* strains. This strain produced an endochitinase (Chi60), an exochitinase (Chi50), and *N*-acetylglucosaminidase (Ruiz-Sanchez *et al*., 2005). The *ChiA* gene encoding chitinase in *S. marcescens* was efficiently over expressed, purified and also crystallized (Vorgias *et al*., 1992). In addition to four different chitinases, a 21-kDa protein with chitin-binding ability was found in the culture supernatant of *S. marcescens*. The chitin- binding protein (Cbp21) potentiates chitinase action by disrupting the structure of the β-chitin substrate suggesting possible synergistic effects (Vaaje Kolstad *et al*., 2005). Among various biocontrol agents antagonistic to many fungal phytopathogens, *Enterobacter* species are

the most prominent. Antagonism could be attributed to the chitinolytic ability as reported in *E. cloacae* and *E. agglomerans* (Chernin *et al*., 1995). *Enterobacter* sp. NRG4 chitinase reduced the viscosity of glycol chitin significantly due to cleavage of chitin long chains by the chitinase at 45 $^{\circ}$C (Dahiya *et al*., 2005). Two bifunctional chitinolytic enzymes (chitinases/lysozyme activity) were purified from *Pseudomonas aeruginosa* K-187 (Wang and Chang, 1997) and also from many other microorganisms; their enzymatic properties have been investigated (Toharisman *et al*., 2005).

Few thermostable chitinolytic enzymes from bacteria have been reported (Gomes *et al*., 2001, Wen *et al*., 2002, Toharisman *et al*., 2005). Thermostable chitinases are reported from very few organisms namely *Pyrococcus furiosus, Thermococcus kodakaraensis, Thermococcus chitonophagaus* and *Rhodothermus marinus* from different environments. The chitinase from the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1, tk-ChiA has a multi domain structure containing dual catalytic domains and triple chitin binding domains, and the significance of each catalytic domain and their synergistic activity in the degradation of chitin was studied (Tanaka *et al*., 2001). Bendt *et al.* (2001) isolated chitinase from a marine psychrotolerant bacterium *Vibrio* sp. strain Fi: 7, found in the Antarctic Ocean, and the strain showed high chitinolytic activity, even at 5 $^{\circ}$C. Although it had an optimum activity at a temperature of 35 $^{\circ}$C, it still showed 40 % of optimum activity at 5 $^{\circ}$C. The enzymatic activity of ChiA from the same species could be enhanced two-fold by the addition of Ca^{2+}. Chitinases from different organisms and their characterization has added a lot of new aspects in chitinase research. The rich diversity of chitinolytic organisms present in the nature opens more windows to identify novel chitinases with enhanced functional properties.

1.4.1 Bacillus has several species that produce efficient chitinases

Bacillus spp. is regarded as a group of bacteria particularly efficient in the break down of chitin (Driss *et al*., 2005). *B. circulans, B. subtilis, B. alvei, B. lentus, B. licheniformis, B. cereus* and *B. thuringiensis* secrete chitinases (Suginta *et al*., 2004). Sequence analysis of *Bacillus* chitinases revealed that this group has the glycosyl hydrolase domain at the N- terminus with one or more carbohydrate binding domains.

Chitinases from *B. thuringiensis* and *B. cereus* share amino acid homology up to 98 %. Structural features of chitinase from *B. circulans* and *B. cereus* exhibit the complexity in elucidating the structure of multi domain proteins like chitinase. The characterization of *Bacillus* chitinases showed activity in the temperature range of 50-60^{0}C whereas, optimum pH varied across the species (Huang and Chen, 2005; Barboza Corona *et al*., 2003; Chuang *et al*., 2008; Driss *et al*., 2005; Barboza Corona *et al*., 2008). The chitinase system of *B. circulans* WL-12 provided comprehensive information on biochemical properties, structure-function relationships, the identification of essential amino acid residues for catalytic activity, and the mechanism by which multiple chitinases are generated (Alam, 1995).

B. circulans secretes six distinct chitinases into growth medium, of which the chitinase A1 was responsible for efficient chitin degradation (Watanabe *et al*., 1990). Barboza-Corona *et al*. (1999) identified three strains of *B. thuringiensis* native to Mexico, which produced endochitinases, chitobiases, and *N*-acetyl- *β*-glucosaminidases in a medium containing colloidal chitin as a main source of carbon. They later cloned the endochitinase gene *chiA74* from *B. thuringiensis* sv. *kenyae* strain LBIT-82 in *E. coli* DH5*α*. The expressed ChiA74 enzyme was active within a wide pH range (4.0–9.0), while the optimal temperature was 57.2 $^{\circ}$C (Barboza-Corona *et al*., 2003). This enzyme could potentially be used as a synergistic factor along with Cry proteins to increase the larvicidal activity of *B. thuringiensis* (Regev *et al*., 1996). Post-translational modification of chitinase precursor(s) occurs either at the N-terminus (Takayanagi *et al*., 1991; Blaak and Schrempf, 1995) or C-terminus (Suginta *et al*., 2004).

Three enzymatically active chitinases (Chi50, Chi62 and Chi68) were present in young cultures of *B. licheniformis* TP-1 (Tantimavanich, 1998). Khiyami and Masmali (2008) showed that the chitinase of *B. licheniformis* A2 and A35 were different from each other. The chitinases from A2 and A35 were activated by Ca^{2+} similar to *Bacillus* MH-1 (Sakai *et al*., 1998), yet only chitinase form *B. licheniformis* A2 was activated by Mg^{2+}. On the other hand, both chitinases from A2 and A35 were inhibited by Mn^{2+} and Co^{2+}.

Yang *et al*. (2009) cloned the chitinase (chi18) from the constructed *B. subtilis* CHU26 genomic DNA library. The chi18 consisted of an open reading frame of 1791 nucleotides and encodes 595 amino acids with a deduced molecular weight of 64 kDa, next to a promoter region containing a 9 base pair direct repeat sequence (ATTGATGAA). The deduced amino acid sequence of the chitinase from *B. subtilis* CHU26 exhibits 62 % and 81 % similarity to those from *B. circulans* WL-12 and *B. licheniformis*, respectively.

1.5 Reaction mechanism of family 18 glycosyl hydrolase has been extensively studied

Knowledge of the enzymatic stereochemistry is an essential prerequisite to the further understanding of enzymatic action. It provides essential mechanistic information allowing the prediction of side reactions, such as the transglycosylation catalysed by retaining enzymes. Davies and Henrissat (1995) described how the shape of the active site cleft of glycoside hydrolases is reflected in their mode of action. The catalytic residues of these enzymes are normally found in one of three locations which were named pocket, cleft and tunnel. True exo-enzymes, specific for polysaccharide chain ends, tend to have their active site located within a pocket. The depth and shape of this pocket reflects the number of sub-sites that contribute to binding and to the length of the leaving group (Davies *et al*., 1997). True endo-enzymes have their catalytic machinery located in an open cleft that allows a random binding action within the polymer chain (Sulzenbacher *et al*., 1996). The catalytic domains of family 18 chitinases have $(\beta\alpha)_8$-barrel folds (Perrakis *et al*., 1994) exhibiting a substrate-assisted catalytic mechanism (Fig. 1.2) whereby the anomeric configuration of the substrate is retained (Davies and Henrissat, 1995; Tews *et al*., 1997; Brameld *et al*., 1998) while the catalytic domain of family 19 chitinases has a lot of α-helices exhibiting a general acid-and-base catalytic mechanism whereby the anomeric configuration is inverted (Garcia-Casado *et al*., 1998). The stereochemistry of catalysis is dictated by the spatial arrangement of the catalytic groups in the active site of the enzyme. The β-strand four of the TIM-barrel contains a characteristic DXDXE sequence motif including a glutamate residue that protonates the oxygen in the scissile glycosidic bond. Catalysis in family 18 chitinases involves the *N*-

acetyl group of the sugar bound in the -1 subsite of the enzyme. Protonation of the glycosidic bond and leaving group departure are accompanied by nucleophilic attack by the carbonyl oxygen of the *N*-acetyl group on the anomeric carbon, thus yielding an oxazolinium ion intermediate (Vaaje-Kolstad *et al*., 2004). Enzymatic glycoside hydrolysis requires the presence of two catalytic groups, which in most cases are observed to be aspartate and glutamate residues. These catalytic groups are separated by approximately 5 Å in the case of retaining enzymes and approximately 9-10 Å in the case of inverting enzymes, which must accommodate the nucleophilic water between the base and the anomeric carbon (McCarter and Withers, 1994).

1.6 Processivity adds new dimension to polysaccharide-degrading enzymes.

Enzymes hydrolysing carbohydrate polymers can do so by three principally different mechanisms, as first described by Robyt and French (1967, 1970): (a) A multiple-chain mechanism, where the enzyme-substrate complex dissociates after each reaction. (b) A single-chain mechanism, where the enzyme remains associated with the substrate until every cleavable linkage in the chain has been hydrolyzed. (c) A multiple attack mechanism, where a given average number of attacks are performed after the enzyme-substrate complex has been formed. The latter two mechanisms are often referred to as ‘processive’. Processive cellulases or chitinases have long and deep active site clefts, or even tunnels (Rouvinen *et al*., 1990). Single polymer chains are threaded through these clefts or tunnels while disaccharides are being cleaved off at the catalytic center. This mechanism is considered to be beneficial for enzyme efficiency because the enzyme remains closely associated with the detached single polymer chain in between hydrolytic steps. Furthermore, the detached chain is prevented from re-associating with the crystalline material, which would make it less accessible.

Chitosans are very valuable substrates for in-depth studies of processivity in family 18 chitinases (Sikorski *et al*., 2006). Sørbotten *et al*. (2005) studied the degradation of the soluble heteropolymer chitosan, to obtain further insight into catalysis in ChiB and experimentally assessed the proposed processive action of this enzyme. The

degradation showed biphasic kinetics: the faster phase is dominated by cleavage on the reducing side of two acetylated units (occupying subsites -2 and -1), while the slower kinetic phase reflects cleavage on the reducing side of a deacetylated and an acetylated unit (bound to subsites -2 and -1, respectively). The results show that nonproductive binding events are not necessarily followed by substrate release but rather by consecutive relocations of the sugar chain. Horn *et al*. (2006) studied the link between enzyme efficiency and processivity by analyzing the effects of mutating aromatic residues in the substrate-binding groove of a processive chitobiohydrolase and chitinase B from *Serratia marcescens*. Mutation of two tryptophan residues (Trp-97 and Trp-220) close to the catalytic center (subsites -1 and -2) led to reduced processivity and a reduced ability to degrade crystalline chitin, suggesting that these two properties are linked.

1.7 Chitin binding domain (ChBD) facilitates chitin hydrolysis

Cellulases and chitinases have one or more carbohydrate binding domains (CBDs) that are beneficial to the enzyme because they adhere to and sometimes disrupt the substrate (Watanabe *et al*., 1994; Carrard *et al*., 2000; Lehtio *et al*., 2003; Boraston *et al*., 2004; McCartney *et al*., 2004). ChBDs are found mainly at the N-termini of plant chitinases, but in bacterial or fungal chitinases they can be located either at the C–terminal or N-terminal end. The three aromatic amino acids tryptophan, phenylalanine and/or tyrosine are found among the residues conserved in the bacterial and plant ChBDs, and are also present in cellulose binding domains (CeBDs). These aromatic amino acids have been implicated in the binding of chitinases to chitin (Zeltin and Schrempf, 1997) and cellulases to cellulose (Linder *et al*., 1995). Another common feature of ChBDs and CeBDs is that they are connected to the catalytic domains of the enzyme by a linker region that is Gly/Pro-rich in plant chitinases (Raikhel and Lee, 1993) and Thr/Ser/Pro rich in fungal cellulases (Abuja *et al*., 1988). Family 19 chitinase from *Aeromonas* sp. No.10S-24 (72.6 kDa) reported to be composed of two chitin-binding domains (ChBDs), two proline- and threonine-rich (PT-rich) linkers, and a catalytic domain. The hydrolytic activities toward insoluble and soluble substrates were significantly reduced by the truncation of two ChBDs. In addition, antifungal activity determined from the digestion rate of haustoria of powdery mildew was reduced by the ChBD truncation (Kojima *et al*.,

2005). The differences in efficiency of chitinases toward various forms of chitin substrates may be due in part to different types of auxiliary CBD within these chitinase molecules.

Like other enzymes involved in the degradation of polysaccharides, most chitinases have carbohydrate-binding domain (CBD), which assist in specific association to insoluble substrates. Some CeBDs have affinity towards chitin (Goldstein *et al*., 1993) and some chitinases have affinity for cellulosic materials (Morimoto *et al*., 1997). This is explained by the structural similarities of chitin and cellulose. The binding efficiency of the cellulases is enhanced in the presence of CeBD which clearly seems to correlate with better activity towards insoluble cellulose (Kruus, *et al*., 1995). Analogous to cellulases, removal of ChBDs of the plant and bacterial chitinases decrease their activities on insoluble but not on soluble substrates (Blaak and Schrempf, 1995). The carbohydrate binding and catalytic modules of both enzymes seem to generate a continuous substrate–binding site formed by a surface of aromatic residues, which guide the substrate into the enzyme's active site (van Aalten *et al*., 2001)

1.8 Structural details of few chitinases are known

Structural data on individual domains of glycoside hydrolases has accumulated over the past few years. There are only a few determined structures of full-length modular glycoside hydrolases, probably because the flexibility of the interdomain linker is an obstacle to protein crystallization (Bourne and Henrissat, 2001). The known 3D structure of three bacterial family 18 chitinases, all belonging to subfamily A, are chitinase A (ChiA) and chitinase B (ChiB) from *S. marcescens* QMB1466 and chitinase A1 (ChiA1) from *Bacillus circulans* WL-12 (Perrakis *et al*., 1994). The catalytic domain of family 18 chitinases has a TIM barrel fold and includes a conserved glutamate residue that presumably acts as an acid during catalysis (Mc carter *et al*., 1994). *Serratia* ChiA comprises three domains: an N-terminal domain, a catalytic $(\beta/\alpha)_8$ barrel domain and a small $(\alpha+\beta)$ domain inserted within the $(\beta/\alpha)_8$ barrel domain. C-terminal chitin-binding domain (ChBD), in addition to the catalytic $(\beta/\alpha)_8$ barrel, is present in *Serratia* ChiB. In *ChiA* of *S. marcescens*, the deep substrate-binding cleft seems accessible, whereas the

substrate-binding cleft of ChiB is more 'tunnel-like'. On the basis of structural characteristics and enzymological work it was suggested that ChiA and ChiB are exochitinases which degrade chitin chains from opposite ends, *ChiA* from the reducing end and ChiB from the nonreducing end. The structure of ChiC is not known yet, but its amino acid sequence shows that it consists of a catalytic domain and two putative ChBDs, which are located C-terminally in the sequence (Horn *et al*., 2006). The catalytic domain of ChiC lacks the α + β domain which makes up a wall in the substrate binding grooves of ChiA and ChiB. This suggests that ChiC has a much more open substrate-binding groove, as observed in the crystal structure of the endochitinase of hevamine (Terwisscha Van Scheltinga *et al*., 1994).

B. circulans ChiA1 is composed of the catalytic domain (CatD), two fibronectin type III (FnIII)-like domains and the C-terminal ChBD. The 3D structure of the CatD of *B. circulans* ChiA1 (CatDChiA1) is similar to that of *Serratia* ChiA. Two small insertion domains located on top of the $(\beta/\alpha)_8$ barrel of *B. circulans* ChiA1 provide a deep cleft for substrate binding (Matsumoto *et al*., 1999). 1.9-Å resolution structure of the complete ChiB was elucidated from *S. marcescens*. The structure is one of the few in which both the catalytic core and the ChBD are observed. The complete structure provides insight into the catalytic function of ChiB and reveals how the ChBD relates to the catalytic domain and may contribute to substrate binding (van Aalten *et al*., 2000). Chitinase (ChiNCTU2) from *Bacillus cereus* with molecular weight 36 kDa has been crystallized using the hanging-drop vapour-diffusion method (Kuo *et al*., 2006). The full-length structure of ChiA1 of *B. circulans* was studied by small angle X-ray scattering. The obtained low-resolution structure showed that ChiA1 is an elongated molecule with a length of ~145 A° composed of a large globular head and a rod-like tail. The domains of ChiA1 are connected by linker peptides rich in Pro, Gly and Thr, which are probably highly flexible (Toratani *et al*., 2006). Chitinase C (ChiC) from *Streptomyces griseus* HUT6037 is the first non-plant family 19 chitinase whose 3D structure has been determined. It is composed of an N-terminal ChBD and a C-terminal catalytic domain, with the two domains connected by a ten-residue linker peptide (Kezuka *et al*., 2006).

1.9 Chitinases were altered/improved by *in vitro* manipulation

Homology-based three-dimensional modelling of the enzymes offers wide range of possibilities to engineer enzyme with improved properties. Site directed mutagenesis (SDM), a commonly employed strategy to investigate the importance of individual amino acids, has been extensively exploited on bacterial chitinases. A lot of information about the importance of specific amino acid residues either in the catalytic domain or in the CBD of chitinases was generated. Studies on *Vibrio carchariae*, using HPLC-MS suggested that the binding site of chitinase A was composed of an array of six binding subsites. Point mutations were introduced into two active site residues Glu315 and Asp392. The D392N mutant retained significant chitinase activity in the gel activity assay and showed 20 % residual activity towards chitooligosaccharides and colloidal chitin in HPLC-MS measurements. The complete loss of substrate utilization with the E315M and E315Q mutants suggested that Glu315 is an essential residue in enzyme catalysis. The D392N mutant displayed strikingly greater efficiency in oligosaccharide synthesis than the wild-type enzyme (Suginta *et al*., 2005).

Hardt and Laine (2004) developed a fluorescent binding assay to investigate the effects of mutagenesis on the binding affinity and substrate specificity of the ChBD of chitinase A1 from *B. circulans* WL-12. Substitution of tryptophan residue (W687) abolished chitin-binding affinity and lowered chitosan binding, while retaining the original level of curdlan binding. Double mutation E668K/P689A had significantly altered the specificity for several substrates and also impaired chitin binding significantly. Other substitutions in the binding site altered substrate specificity but had little effect on overall affinity for chitin. Interestingly, mutation T682A led to a higher specificity towards chitinous substrates than the wildtype.

Mavromatis *et al*. (2003) reported SDM of a cold-adapted chitinase from *Arthrobacter* sp.TAD20, their study aimed at testing theories related to stability and activity of psychrophilic enzymes which in turn can provide insights for re-engineering their stability and activity parameters. Molecular sequence alignment of the available chitinases followed by molecular modelling could reveal the residues involved in the

adaptation and stability in extreme environments that can be exploited to tailor the mesophilic counterparts using mutational analysis. Studies on the thermodynamic stability of small proteins and their mutated variants have provided some fundamental insights into factors that determine protein stability (Vielle and Zeikus, 2001). Stability is a beneficial parameter for biocatalysis and the prolonged lifetime of the catalyst reduces process costs. Mutations involved in the stability determining parts will have noticeable stability effects, as observed in protein engineering work on proteases and amylases (Machius *et al*., 2003). Extensive mutational analysis together with computational backbone has generated a wide scope to improve the existing properties of chitinases.

Advances in protein engineering have come from creating multi-functional chimeric proteins containing modules from various proteins and these modules are typically joined via an oligopeptide linker, the correct design of which is crucial for the desired function of the chimeric protein (George and Heringa, 2003). Multiple approaches allowed the identification of mutant enzymes possessing desirable qualities such as increased activity, modified specificity, selectivity or cofactor binding. The functional and structural significance of the C-terminal region of *Bacillus licheniformis* chitinase was explored using C-terminal truncation mutagenesis (Chuang *et al*., 2008).

1.10 Chromatographic techniques facilitate analysis of chitooligosaccharides formed during chitin hydrolysis.

TLC analysis allows a qualitative assessment of product formation, but is relatively insensitive and not quantitative. More advanced and much more sensitive analysis can be performed to better characterize the products released by chitin–degrading enzymes using more sophisticated analytical instrumentation, most notably mass spectrometry. Analyses of degradation products of tk-chiA from *N*-acetyl-chitooligosaccharides and their chromogenic derivatives with TLC indicated that the N-terminal catalytic domain mainly hydrolyzed the second glycosidic bond from the nonreducing end of the oligomers, whereas the C-terminal domain randomly hydrolyzed glycosidic bonds other than the first bond from the nonreducing end (Tanaka *et al*., 2001).

Chitinases from *Serratia marcescens* have a characteristic catalytic mechanism which depends on participation of the N-acetyl group of the sugar unit bound to the -1 subsite (Synstad *et al*., 2004). This substrate-assisted catalytic mechanism implies that family 18 chitinases are expected to have an absolute preference for A-units in the -1 subsite and that the presentation of a deacetylated sugar (D-unit) to the -1 subsite would represent nonproductive binding. Crude chitinase from *Burkholderia cepacia* TU09 and *Bacillus licheniformis* SK-1 were used to digest α and β chitin powder. Chitinase from *B. cepacia* TU09 produced GlcNAc in greater than 85 % yield from β and α chitin within 1 and 7 days, respectively. *B. licheniformis* SK-1 chitinase completely hydrolyzed β chitin within 6 days, giving a final GlcNAc yield of 75 %, along with 20 % of chitobiose. However, only a 41 % yield of GlcNAc was achieved from digesting α chitin with *B. licheniformis* SK-1 chitinase (Pichiyangkura *et al*., 2002). Several methods that employ HPLC have been described for the separation and quantification of chito-oligosaccharides. Mass spectrometry and NMR were utilized to determine the size of chito-oligosaccharides produced by the activity of chitinases or chemical depolymerisation of chitin (Howard *et al.,* 2003). Specific combinations of chitinolytic enzymes with high levels of endo-chitinase, exo-chitinase and low activity of N-acetyl-glucosaminidase would be necessary to obtain desired chain length of the oligomers.

1.11 Chitinases and chitooligosaccharides play important role in plant disease control

Safety and environmental concerns regarding chemical plant protectants has led to a search for alternative biocontrol agents that can replace synthetic fungicides efficiently (Wilson *et al*., 1991) for pre- and post-harvest diseases. Suitable targets for biocontrol agents should be widespread and indispensable in plant pathogens but absent in the host plants themselves. Chitin serves as a fibrous strengthening element through strong hydrogen bonding and crystal formation between adjacent polymers, thus contributing to cell wall rigidity. On the other hand, chitin is absent in plant cells that use as the fibre forming polysaccharide in their cell walls. Enzymatically breaking the glycosidic bond in

chitin, thus, will destroy the morphology of fungal hyphae and lead to the leakage of the cell contents without affecting plant cells.

Antagonism of fungal plant pathogens by microorganisms specifically by the production of chitinases plays a major role in biological disease control (Chernin *et al.*, 1995). *Fusarium chlamydosporum* produces an endochitinase that inhibits germination of uredospores of the rust fungus *Puccinia arachidis* (Mathivanan *et al.*, 1998). Chitinolytic enzymes of *T. harzianum*, the most intensely studied mycoparasitic fungus were inhibitory to a wide range of deleterious fungi (Lorito *et al.*, 1993). Sampson and Gooday (1998) reported the chitinolytic activity of two strains of *Bacillus thuringiensi*s, inhibition of activity by allosamidin and the effect of addition of exogenous chitinse or allosamidin on their insecticidal activity. When *Chi*A was combined with *B. thuringenesis* δ – endotoxin, a synergistic toxic effect was seen on insect larvae (Regev *et al.*, 1996; Sampson and Gooday, 1998). Selective enzymolysis of cell walls of *Pyricularia oryzae* by single and combined actions of glucanases and chitinases produced by *Bacillus circulans* was reported by Tanaka and Watanabe (1995). Wang *et al*. (2002) showed inhibitory activities of two chitinases from *Bacillus amyloliquefaciens* V656 on the growth of *F. oxysporum*.

Serratia marcescens controls *Botrytis cinerea, Rhizoctonia solani* and *Fusarium oxysporum* (Someya *et al*., 2000). *S. marcescens* controlled growth of *Sclerotinia minor*, the causal agent of basal drop diseases, in lettuce (El-Tarabily *et al*., 2000). Chitinase *Chi*A from *S. marcescens* partially purified after cloning into *E. coli*, was found to reduce disease caused by *Sclerotium rolfsii* in beans and *R. solani* in cotton (Shapira *et al.*, 1989). The *Chi*A and *Chi*B genes from *S. marcescens* have been transformed into other bacterial species like *P. fluorescens* and *E. coli* in an attempt to improve their ability to control fungal plant pathogens (Koby *et al.*, 1994). Field-testing of products that were based on compounds of biological origin including *Bacillus thuringiensis* subsp. *israelensis* (BACTICIDE®) and *Streptomyces avermitilis* metabolites (PHYTOVERM®) were used against *Thomasiniana theobaldi* (a general member of the midge blight) and CHITINASE was used against fungi (mainly *Didymella applanata*) to control raspberry midge blight in Siberia (Shternshis *et al.*, 2002). Clearly, bio-control agents can be

effectively used to control plant diseases, partly because bacterial enzymes like glucanases, chitinases and chitosanases target and degrade the cell walls of phytopathogenic fungi. A simple and rapid application of the hydrolytic enzyme preparations might serve the purpose of plant disease control in a more effective manner. Patil *et al*. (2000) reviewed the biotechnological applications of chitin and chitinolytic enzymes. The use of bacterial biocontrol agents for the control of post-harvest diseases of fruits has gained considerable attention and has moved from the laboratory to commercial application (Wilson and Wisniewski, 1994), and more recently success has been achieved for foliar disease control as well (Kishore *et al*., 2005a). Chitinolytic *Serratia marcescens* GPS5 reduced the severity of late leaf spot disease of ground nut, and when supplemented with 1 % colloidal chitin, a substrate for chitinase, disease control efficiency of the formulation increased (Kishore *et al*., 2005b). *Aeromonas caviae*, a chitinolytic soil isolate, was shown to control *Rhizoctonia solani, Sclerotium rolfsii* and *Fusarium oxysporum* f.sp. *vasinfectum* in cotton (Inbar and Chet, 1991). Zhang and Yeun (2000) showed the role of chitinase production by *Stenotrophomonas maltophilia* strain C3 in biological control of leaf spot on tall fescue (*Festuca arundinacea*) caused by *Bipolaris sorokiniana.* I*n vitro* and *in vivo* studies suggested that chitinolysis is one of the mechanisms in biological control by strain C3, functioning in concert with other mechanisms. The success in using chitinases for different applications depends on the availability of highly active preparations at reasonable cost.

1.12 Chitin, chitosan and chitooligosaccharides have wide-ranging applications

It is generally believed that chitinases have a dual mode of action, firstly by attacking the chitin in fungal cell walls, thus weakening the pathogens, and secondly, by generating biologically active chito-oligosaccharides that may trigger resistance reactions, thus strengthening the host. Apart from the use of chitinase in biological control, these enzymes could be exploited in converting the shrimp waste that contains chitin as a major component into chitin oligomers that have wide usage in various fields. Knowledge on the mode of cleavage by the chitinases can be used to explore the

possibilities of producing defined chitooligosaccharide mixtures by degrading partially acetylated chitosans and such chitooligosaccharides have a number of potential applications (Akiyama *et al*., 1995; Denarie *et al*., 1996; Vander *et al*., 1998).

In the last two decades, there has been a lot of focus on the production of N-acetyl-D-glucosamine through enzymatic hydrolysis of chitin. *N*-acetyl-D-glucosamine (GlcNAc) and D-glucosamine (GlcN) have recently been promoted as a treatment or as nutriceutical agents for patients with osteoarthritis and inflammatory bowel disease (Talent and Gracy, 1996; Salvatore *et al*., 2000). In contrast to GlcN hydrochloride or sulfate, both of which have a bitter taste, GlcNAc has a sweet taste that facilitates its use in daily consumption. Chitin oligosaccharides and chitosan oligosaccharides have recently attracted much attention for their various physiological activities, including antifungal activity, antitumor activity, and immuno-enhancing effects (Nam *et al*., 2001; Prashanth and Tharanathan, 2005).

Various applications of chitin and chitosan were reviwed by Ravi Kumar (2000). Chitin gels with pre sizes ranging from 100 to 500 mm were obtainable (Chow *et al*., 2001) and mouse and human fibroblast cell cultures exposed to these chitin matrixes were found to be growing and proliferating indicating the feasibility of using these porous chitin matrices for cell transplantation applications to regenerate tissues. The transfection efficiency when using chitosan as a gene delivery vehicle has been studied by Sato *et al.* (2001). Lee (2009) reviewed several studies that evaluated immunologic effects of chitin *in vivo* and *in vitro* and revealed new aspects of chitin regulation of innate and adaptive immune responses. Exogenous chitin activates macrophages and other innate immune cells and also modulates adaptive type 2 allergic inflammations. These studies further demonstrate that chitin stimulates macrophages by interacting with different cell surface receptors such as macrophage mannose receptor, toll-like receptor 2 (TLR-2), C-type lectin receptor Dectin-1, and leukotriene B4 receptor (BLT1). Chitin and chitosan also have fungicidal and fungistatic properties (Oliveira *et al*., 2008). Chitosan is the only natural cationic gum that becomes viscous on being neutralized with acid. These materials are used in creams, lotions and permanent waving lotions and several derivatives have also been reported as nail lacquers (Mark *et al*., 1985).

1.13 Significance of present study

Chitinases are an exceptionally productive area to explore. Due to the important applications of chitinases there has been a lot of research in the recent years for enhanced production of chitinase from microbial sources. Several studies have reported on the characterization and modification of chitinases using recent molecular technologies. Several bacterial chitinases have been reported in literature and there is an ample scope to clone and construct hybrid chitinases. Natural bacterial enzymes have been improved using techniques of protein engineering and directed protein evolution to maximize the effectiveness and efficiency of hydrolytic enzymes. A better understanding of the genetics of chitinase expression and the advent of recombinant technologies has led to a large increase in chitinase production. The aim of these studies is to effectively use the large insoluble chitin biomass by converting it into high added-value products using the various chitinolytic enzymes reported till date. However, to exploit fully the potential of specific chito-oligosaccharides, novel chitinases with defined substrate specificities, cleavage mechanisms, and product patterns will be required. Construction of chimeric chitinases based on domain swapping may add a new facet to chitinase research. The availability of diverse domains in chitinases ranging from mesophilic to thermophilic bacteria may pave the way to rearrange the domains and design chimeric chitinases that can most likely be called as novel chitinase.

1.14 Objectives

The present work on cloning and characterization of chitinase chimeras using domain swapping and on studying the properties of the recombinant enzymes has the following objectives:

- Cloning, expression, purification, and characterization of *Bacillus thuringiensis* (Bt) and *Bacillus licheniformis* (Bli) chitinases
- Construction and characterization of *B. thuringiensis* and *B. licheniformis* chitinase chimeras by
  - ✓ domain swapping, i.e. by replacing the ChBD of Bt and Bli chitinases by CeBd of endoglucanase and exchange of ChBD in Bt and Bli (internal CBD exchange)
  - ✓ domain fusion, i.e. by N-terminal fusion of Blichi with CBD of Btcbp and N-terminal fusion of BliGH with CBD of Btcbp
  - ✓ domain deletion, i.e. deletion of ChBD from both Bt and Bli chitinase

MATERIALS AND METHODS

2. 1. 1 Bacterial cultures

The details of the various bacterial isolates and list of plasmids used in the present study are described in Table 2.1 & 2.2

2. 1. 2 Media used

2.1.2.1 Luria Bertani medium (LB medium)

To 900 ml of water, 10 g tryptone, 10 g NaCl, 5 g yeast extract and 15 g agar was added and pH was adjusted to 7.1 and volume was made up to 1 litre.

2.1.2.2 Chitinase detection agar medium (Chih *et al*., 2002)

Colloidal chitin-10 g, Na_2HPO_4-0.065 g, KH_2PO_4-1.5 g, NaCl-0.25 g, NH_4Cl-0.5 g, $MgSO_4$-0.12 g, $CaCl_2$-0.005 g and the pH was adjusted to 6.5 and the volume was made up to 1 liter.

2.1.3 Chemicals

Isopropyl-β-D-thiogalactoside (IPTG) and X-gal were procured from Amersham biosciences (UK). Ampicillin (100 µg/ml working conc. for cloning and expression vectors) was from Sigma-Aldrich (USA). All chemicals used were purchased either from Sigma-Aldrich, Merck or Hi-media labs.

2.1.4 Enzymes

All restriction enzymes, T4 DNA ligase and *Pfu* DNA polymerase used for molecular cloning were procured either from NEB (USA) or MBI Fermentas (Germany) and used as per the manufacturers instructions.

2.1.5 Kits

Plasmid isolation kit (Amersham and Biosciences, Sigma), Gel extraction kit (QIAgen), DNeasy kit for genomic DNA isolation (Qiagen), PCR cloning kit (Invitrogen) were used.

2.2 SELECTION OF CHITINOLYTIC BACTERIA

Screening of chitinolytic bacteria on solid media

About 5 µl of the over night grown culture of 18 different bacterial cultures (Table 2.1) was spotted on to the chitinase detection agar plates and the plates were incubated at 30 °C for 4-5 days to observe the clear zone around the bacterial colonies.

2.3 CLONING AND CHARACTERIZATION OF CHITINASE FROM *B. THURINGIENSIS* SV. *KURSTAKI* AND *B. LICHENIFORMIS* DSM 13

2.3.1 PCR based cloning of chitinase (*chi*) from chitinolytic bacteria

The molecular biology methods such as PCR, agarose gel electrophoresis, competent cells preparation, plasmid isolation, restriction digestion, ligation and transformation were done according to the protocols described by Sambrook *et al*. (1989). Oligonucleotide primers synthesis and sequencing of the amplicons was done by MWG Biotech (Germany). *Pfu* polymerase was used all through for PCR. The details of primers and templates used in the PCR for amplification are listed in Table 2.3.

2.3.1.1 Genomic DNA isolation from bacterial cultures

Genomic DNA (gDNA) was isolated using Dneasy kit (Qiagen) from *Bacillus thuringiensis kurstaki* (Bt) and *Bacillus licheniformis* DSM 13 (Bli).

2.3.1.2 PCR amplification and cloning of chitinase (c*hi*) from *B. thuringiensis* and *B. licheniformis*

Chitinase encoding gene (*chi*) was amplified from gDNA of chitinolytic *B. thuringiensis* and *B. licheniformis* at annealing temperature of 58 and 56 °C, respectively with 2 min of polymerization using primers BtchiFpmodnew and BtchiRpmod for *Btchi* and BlchiFpmodnew and BlchiRpmod for *Blichi*. Expression vector pET 22b+ and the amplicon were double digested either with *Eco*R I and *Hind* III for *Btchi* or *Bam*H I and *Xho* I for *Blichi*, gel purified and ligated using T4 DNA ligase at 16 °C for 16 h. The

recombianant plasmids were designated as pANE-Btchi and pANE-Blichi, respectively for *B. thuringiensis* and *B. licheniformis.*

2.3.2 Heterologous expression and purification of Btchi and Blichi

2.3.2.1 Heterologous expression

Highly efficient competent cells of *E. coli* BL21 (DE3) were transformed using the recombinant plasmids pANE-Btchi and pANE-Blichi, and selected on ampicillin plates. The cells harboring positive plasmids were grown at 37 $^{\circ}$C and 1 mM IPTG was used to induce the cells at an OD_{600} of 0.6 and continued for 3 h. The cells were harvested and processed further to extract periplasmic fraction. The expressed recombinant protein from plasmids pANE-Btchi and pANE-Blichi was designated as Btchi and Blichi, respectively. Preparation of periplasmic fraction, PAGE analysis, western blotting etc. were carried out at room temperature (~28 $^{\circ}$C), unless specified.

2.3.2.2 Preparation of periplasmic fraction

Periplasmic fraction was prepared according to the protocol (pET expression system manual, Novagen). Forty ml of the induced culture was centrifuged at 8000 rpm for 5 min at 4 $^{\circ}$C. Cells were resuspended in 30 ml of buffer (30 mM TrisCl pH 8, 20 % sucrose and 1 mM EDTA) with slow stirring for 10 min at ~28 $^{\circ}$C, followed by centrifugation at 4 $^{\circ}$C for 10 min at 10,000 rpm. The supernatant was discarded and the pellet was further resuspended in 30 ml of 5 mM $MgSO_4$ followed by slow stirring at 4 $^{\circ}$C for 10 min, centrifued at 10,000 rpm for 10 min at 4 $^{\circ}$C. Using amicon filters (Millipore, cut off 10 kDa) periplasmic fraction was concentrated with buffer exchange.

2.3.2.3 SDS-PAGE analysis

Protein fractions were resolved on 12 % polyacrylamide vertical slab gels with SDS according to Laemmli (1970). The gels were stained with coomassie blue stain (0.25 g of coomassie brilliant blue G250 in 90 ml of methanol: H_2O (1:1 v/v) and 10 ml glacial acetic acid) for 1 h and washed repeatedly in the destaining solution (45 ml methanol, 10

ml glacial acetic acid and the final volume made up to 100 ml with water) till the protein bands were visible against a clear background.

2.3.2.4 Western Blotting

Concentrated periplasmic fractions of the recombinant Btchi and Blichi were loaded on 12 % SDS-PAGE, the proteins were transferred on to nitrocellulose membrane using semi-dry method and transfer buffer (25 mM Tris base, 150 mM glycine and 20 % methanol pH 8.3). Membrane was washed twice for 10 min each time with TBS buffer (10 mM Tris Cl pH 7.5 and 150 mM NaCl) at ~28 $^{\circ}$C and was incubated for 1 h in blocking buffer (3 % BSA (w/v) in TBS buffer) at ~28 $^{\circ}$C. Second step involved washing the membrane twice for 10 min each time in TBS-tween/triton buffer (20 mM Tris Cl pH 7.5, 500 mM NaCl, 0.05 % (v/v) tween 20, 0.2 % (v/v) triton x-100) at ~28 $^{\circ}$C and the third wash was given with TBS for 10 min at ~28 $^{\circ}$C which was followed by incubation with anti-his antibody (1/1000–1/2000 dilution of antibody in blocking buffer) at ~28 $^{\circ}$C for 1 h. Membrane was washed twice for 10 min each time in TBS-tween/triton buffer at ~28 $^{\circ}$C. Subsequently membrane was washed for 10 min in TBS buffer at ~28 $^{\circ}$C and incubated with secondary antibody solution (diluted 1/10,000 in 10 % milk powder (w/v) in TBS) for 1 h at ~28 $^{\circ}$C. Membrane was washed 4 times each 10 min with TBS-tween/triton buffer at ~28 $^{\circ}$C and was developed using enhanced chemiluminiscence method of detection.

2.3.2.5 Batch purification of Btchi and Blichi using Ni-NTA chromatography

One ml of the 50 % Ni-NTA slurry was equilibrated with lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl and 10 mM imidazole (pH 8.0), concentrated periplasmic fraction of Btchi and Blichi were added to the equilibrated slurry based on the concentration of the recombinant protein present and was mixed gently on gel rocker at 4 $^{\circ}$C for 60 min. The lysate Ni-NTA was loaded onto the column with the bottom outlet cap closed. Flow through was collected by removing the bottom cap. The column was washed 4 times each with 1 ml of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl and 20 mM imidazole (pH 8.0) and wash fractions were collected. Recombinant protein was eluted 4 times each

with 0.5 ml of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl and 50 mM imidazole (pH 8.0) followed by elution with elution buffer containing 100 mM imidazole. All the fractions were loaded on 12 % SDS-PAGE and stained with coomassie staining solution.

2.3.2.6 Semi-Native PAGE analysis for Btchi and Blichi chitinase activity

In-gel assay was carried out by preparing 12 % polyacrylamide gel. The resolving gel was supplemented with 0.1 % glycol chitin. Other denaturing agents were not added to the protein in order to retain the activity. The periplasmic fractions were loaded and the gel was run at 4 °C, 80 V till the dye front enters the buffer in lower tank. The gel after run was washed twice with buffer (100 mM sodium acetate pH 5.5 and 1 % triton-x100**)** each for 20 min at 4 °C. Third wash was given with 100 mM sodium acetate pH 5.5 for 20 min at 4 °C. The gel was incubated at 37 °C for overnight. After the incubation, gel was rinsed with the same buffer for 5 min and was stained with 0.01 % calcoflour white in 0.5 M Tris-Cl pH 8.9. The gel was observed under trans-illuminator for dark bands indicating chitinase activity.

2.3.3 Characterization of recombinant Btchi and Blichi chitinases

2.3.3.1 Reducing end assay

Chitinase activity was measured by the method of Imoto and Yagishita (1971) using N-acetyl glucosamine as standard. Reaction mixture containing 5 µg of purified recombinant Btchi and Blichi were incubated with colloidal chitin at 37 °C for 1 h. After incubation, 1.5 ml of the reagent (0.5 M sodium carbonate, 0.05 % potassium ferricyanide) was added and boiled for 15 min in dark and centrifuged at 10,000 rpm for 10 min. Each sample was prepared in triplicates in the micro titer 96-well plates. The supernatant was measured for reducing ends at 420 nm using microtiter plate reader (Multiskan, Labsystems). The reducing end assay was done as above, unless stated other wise.

2.3.3.1.1 Kinetic analysis

Chitinase activity of the purified Btchi and Blichi was measured by incubating 5 µg of the enzyme with different concentrations of colloidal chitin (0-35 mg/ml) at 37 °C for 1 h. Reducing end assay was done at 420 nm as described above. One katal unit of activity was defined as the release of one micromole of NAG equivalent per sec under standard experimental conditions. Specific activity in nkat/mg of protein was calculated to plot the Lineweaver-Burk reciprocal plot to determine V_{max}, K_m and k_{cat} of the chitinases.

2.3.3.1.2 Effect of temperature on Btchi and Blichi activity

2.3.3.1.2a Optimum temperature

Five µg of purified Btchi or Blichi was added to colloidal chitin (8 mg/ml) in 50 mM sodium acetate pH 5.2, incubated individually for 1 h at different temperatures 20, 40, 60, 80 and 100 °C to determine the optimum temperature of the purified chitinases using reducing end assay as described in 2.3.3.1.

2.3.3.1.2b Thermal stability

Purified Btchi or Blichi (5 µg) in 10 mM sodium acetate pH 5.2 was incubated individually at different temperatures ranging from 30-100 °C for 1 h followed by reducing end assay using colloidal chitin as described in 2.3.3.1 to determine the thermal stability of the Btchi and Blichi.

2.3.3.1.2c Stability at optimum temperature

Purified Btchi or Blichi (5 µg) in 50 mM sodium acetate pH 5.2, was incubated at 60 °C for 1-4 h followed by reducing end assay using colloidal chitin as explained in 2.3.3.1 to determine the stability of the Btchi and Blichi at 60 °C.

2.3.3.1.3 Optimum pH

Purified Btchi or Blichi (5 µg) and colloidal chitin (8 mg/ml) was incubated individually at 37 °C for 1 h in 50 mM of buffers with various pHs (2.0-12.0) to determine the optimal

pH of the recombinant proteins using reducing end assay. The buffers used were: 50 mM each of sodium citrate (pH 2.0-5.0), sodium acetate (pH 5.0-6.0), sodium phosphate (pH 6.0-8.0), glycine-NaOH (pH 8.0-10.0) and NaH_2PO_4-NaOH (pH 10.0-12.0).

2.3.3.1.4 Chitinase activity on chitosan with different DA

Reducing end assay (2.3.3.1) was performed using 5 µg of purified chitinase (Btchi or Blichi) incubated with 20 µl of 0.1 % chitosan with different degrees of acetylation (DA 66, 40 and 10 %) at 37 ºC for 1 h in 50 mM of sodium acetate, pH 5.2.

2.3.3.2 Circular Dichroism (CD)

CD measurements were obtained using Jasco spectropolarimeter (J-810, Japan) equipped with peltier thermostat for temperature control. Wavelength scans in the far UV region (190-260 nm) were performed in quartz precision cells of 0.1 cm path length. Each spectrum was obtained by averaging three successive accumulations with a wavelength step of 1 nm at a rate of 20 nm/min, response time 2 sec and band width 1 nm. Fifty µg of pure Btchi or Blichi in 10 mM sodium acetate pH 5.2 was used. The CD data was analyzed for secondary structure prediction using CDNN software.

2.3.3.2a Conformational changes with increase in temperature

Fifty µg of the purified protein in 400 µl of 10 mM sodium acetate pH 5.2 was set for CD spectrum and the temperature was gradually increased from 30 to 100 $^{\circ}$C using a peltier system connected to the instrument. At each temperature the CD spectrum was obtained to analyze the change in secondary structure with increase in temperature.

2.3.3.2b Effect of longer incubations at different temperatures on secondary structure of recombinant chitinases

Fifty µg of the purified Btchi or Blichi in 400 µl of 10 mM sodium acetate pH 5.2 was pre incubated at different temperatures ranging from 30-100 $^{\circ}$C for 1 h and stored on ice. CD spectrum was obtained at 30 $^{\circ}$C for the pre-incubated enzymes and analysed to

determine the thermal stability of the enzymes with reference to secondary structure conformation.

2.3.3.2c Conformational changes at optimum temperature

Purified chitinases (50 µg) of Btchi or Blichi in 10 mM sodium acetate pH 5.2 were incubated individually at 60 °C, followed by the CD spectrum at 30 °C to analyze the secondary structure stability.

2.3.3.3 Insoluble substrate binding of recombinant Btchi and Blichi

Different amounts of Btchi or Blichi (20-100 µg) were incubated with 1 mg (dry weight) of α or β chitin in 1 ml of 50 mM sodium acetate pH 5.2 for 1 h, in a gel shaker at 450 rpm at 4 °C. After incubation, reactions were stopped by centrifugation (13000 rpm, 10 min and 4 °C). Unbound protein was measured in a fluorescence spectrophotometer (Hitachi F3010) with excitation at 280 nm and emission at 350 nm. The bound protein was calculated as the total protein minus the unbound protein.

2.3.3.4 Soluble substrate binding of Btchi and Blichi using affinity electrophoresis

The binding of the recombinant Btchi and Blichi to soluble substrates was evaluated using affinity electrophoresis in 8 % polyacrylamide gels polymerized in the absence or presence of soluble polysaccharides (0.1 %) like laminarin, CM cellulose and glycol chitin. Electrophoresis was performed for 1.5 h at 4 °C at a constant voltage of 80 V. The gels were stained with coomassie blue to detect protein. Binding was assessed visually or, alternatively, the migration distances of the chitinases and reference proteins were measured directly on the resolving gels.

2.3.3.5 Thin layer chromatography (TLC)

The reaction mixture containing the hydrolyzed products was loaded onto silica gel coated TLC plates and carefully dried, run against the solvent (n-butanol, methanol, 25 % ammonia and water in a ratio of 41.6: 33.3: 16.6: 8.3) in a chromatography chamber till the solvent front 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 phosphoric acid (85 %)-750 µl) followed by heating at 180 °C (hot air gun, Black and Deacker, Germany) to develop the spots on the TLC plate.

2.3.3.5.1 Hydrolysis pattern of Btchi and Blichi on chito oligomers

Five µg of purified proteins were incubated with 1 mM chitin hexamer, chitin tetramer and chitin trimer each at 37 °C for 1 h. 10 µl of the reaction was stopped by adding equal volume of 0.1 M NaOH at different time points (0, 2, 5, 10, 15, 30, 60, 120 min and ON.) and the samples were stored on ice. Loading and developing of the TLC plate was done as described in 2.3.3.5.

2.3.3.5.2 Hydrolysis pattern of Btchi and Blichi on chitin polymers

Five µg of the purified Btchi and Blichi were incubated individually with 0.5 mg of α and β chitin and the samples were drawn as mentioned in 2.3.3.5.1. Loading and developing of the TLC plate was done as described in 2.3.3.5.

2.4 CONSTRUCTION AND CHARACTERIZATION OF CHITINASE CHIMERAS - REPLACING THE ChBD OF *B. THURINGIENSIS* AND *B. LICHENIFORMIS* CHITINASES BY CeBD OF ENDOGLUCANASE (DOMAIN SWAPPING)

2.4.1 PCR based construction and cloning of chimeric chitinases Btegln and Bliegln

Schematic representation of the overlap extension PCR and chimera construction is shown in Fig. 2.1 & 2.2. The details of primers and templates used in the PCR for amplification are listed in Table 2.3 & 2.4. Oligonucleotide primer synthesis and sequencing of the amplicons was done by MWG Biotech (Germany). *Pfu* polymerase was used in the PCR.

2.4.1.1 PCR amplification and cloning of endoglucanase (*egln*) from *Bacillus licheniformis* DSM 13

Endoglucanase encoding gene (*egln*) was amplified from gDNA of *B. licheniformis* DSM 13 at annealing temperature of 53 °C with 2 min of polymerization using gene specific primers BleglnFpGH5new and BleglnRpGH5. Expression vector pET 22b+ and the amplicon were double digested with *Bam*H I and *Xho* I, gel purified, and ligated using T4 DNA ligase at 16 °C for 16 h.

2.4.1.2 PCR amplification, fusion and cloning of chimeric chitinase *Btegln* and Bliegln

Glycosyl hydrolase (GH) encoding gene (*GH*) with C-terminal overhang (OH) was amplified from plasmid pANE-Btchi and pANE-Blichi at annealing temperature of 56 °C with 3 min of polymerization using primers BtchisalFor and BtGHOEglnRev for *Btchi* (*GH*) and BlchiFpmodnew and BlchiGHglnOERev for *Blichi* (*GH*). C-terminal *CeBD* from endoglucanase encoding gene (*egln*) was amplified from plasmid pANE-egln at annealing temperature of 56 °C with 2 min of polymerization using gene specific primers EglncbmOEFor and BleglnRpGH5. Equimolar concentration of *Btchi* (*GH*), *CeBD* and *Blichi* (*GH*) and *CeBD* was added as template to fuse the two and further amplified at annealing temperature of 56 °C with 4 min of polymerization using gene specific primers BtchisalFor and BleglnRpGH5 for *Btegln* and BlchiFpmodnew and BleglnRpGH5 for *Bliegln*. Expression vector pET 22b+ and the fused amplicon were double digested either with *Sal* I and *Xho* I for *Btegln* and *Bam*H I and *Xho* I for *Bliegln*, gel purified, and ligated using T4 DNA ligase at 16 °C for 16 h. The recombinant plasmids were designated as pANE-Btegln and pANE-Bliegln, respectively.

2.4.2 Heterologous expression and purification of the chimeric contructs

Heterologous expression of egln was done as described in 2.3.2 whereas Btegln and Bliegln were expressed with slight modifications; IPTG and post induction temperature was reduced to 0.4 mM and 18 °C, respectively and the induction time was extended to 20 h. All the other procedures till purification were as described in 2.3.2.

2.4.3 Characterization of chimeric chitinase Bliegln was as described in 2.3.3

2.5 CONSTRUCTION AND CHARACTERIZATION OF CHITINASE CHIMERAS - REPLACING THE ChBD OF *B. THURINGIENSIS KURSTAKI. CHI* WITH ChBD OF *B. LICHENIFORMIS DSM 13 CHI* AND *vice versa* (DOMAIN SWAPPING)

2.5.1 PCR based construction and cloning of chitinase chimera *Btswap* and *Bliswap*

The details of primers and templates used in the PCR for amplification are listed in Table 2.3 & 2.4. Schematic representation of the overlap extension PCR and chimera construction is shown in Fig. 2.1 & 2.3.

2.5.1.1 PCR amplification, fusion and cloning of *Btswap* and *Bliswap*

GH encoding gene (*GH*) overhang (OH) at the C-terminal end of the amplicon was amplified from plasmid pANE-Btchi and pANE-Blichi at annealing temperature of 56 $^{\circ}$C with 3 min of polymerization using the gene specific primers BtchiBamHIFor and BtGHOESbl for *Btchi* (*GH*) and BlchiFpmodnew and BlGHOESbt for *Blich*i (*GH*). Chitin binding domain including FN III encoding gene (*ChBD*) was amplified from plasmid pANE-Btchi and pANE-Blichi at annealing temperature of 56 $^{\circ}$C with 2 min of polymerization using gene specific primers BtcbmOE and BtchiRpmod for *Btchi* (*ChBD*) and BlcbmOE and BlchiRpmod for *Blichi* (*ChBD*). Equimolar concentration of *Btchi* (*GH*) and *Blichi* (*ChBD*) for *Btswap* and *Blichi* (*GH*) and *Btchi* (*ChBD*) for *Bliswap* was added to fuse the two and further amplified at annealing temperature of 56 $^{\circ}$C with 4 min of polymerization using gene specific primers BtchiBamHIFor and BlchiRpmod for *Btswap* and BlchiFpmodnew and BtchiRpmod for *Bliswap*. Expression vector pET 22b+ and the fused amplicon were double digested with either *Bam*H I and *Xho* I for *Btswap* and *Sal* I and *Xho* I for *Bliswap*, gel purified and ligated using T4 DNA ligase at 16 $^{\circ}$C for 16 h. The recombinant plasmids were designated as pANE-Bliswap and pANE-Btswap, respectively.

2.5.2 Heterologous expression and purification of chimeric Btswap and Bliswap chitinases were as described in 2.4.2

2.5.3 Characterization of recombinant Btswap and Bliswap was as described in 2.3.3

2.6 CONSTRUCTION OF CHITINASE CHIMERAS – FUSION OF CBD FROM CBP OF *B. THURINGIENSIS TO CHITINASE OF B. LICHENIFORMIS* (DOMAIN FUSION)

2.6.1 PCR based construction and cloning of chitinase chimeras *NBtBlichi* and *NBtBliGH*

The details of primers and templates used in the PCR for amplification are listed in Table 2.5. Schematic representation of chimera construction is shown in Fig. 2.4

2.6.1.1 PCR amplification and cloning of *cbp* from *B. thuringiensis* and *B. licheniformis*

Chitin binding protein encoding gene (*cbp*) was amplified from gDNA of *B. thuringiensis* and *B. licheniformis* at annealing temperature of 56 °C with 3 and $1^{1/2}$ min polymerization respectively using the gene specific primers BthcbpFp and BthcbpRp for *Btcbp* and BlicbpFp and BlicbpRp for *Blicbp*. Expression vector pET 22b+ and the amplicon were double digested with *Sal* I and *Xho* I for *Btcbp* and *Blicbp*, gel purified, and ligated using T4 DNA ligase at 16 °C for 16 h. The recombinant plasmids were designated as pANE-Btcbp and pANE-Blicbp respectively.

2.6.1.2 PCR amplification, fusion and cloning of *NBtBlichi* and *NBtBliGH*

N-terminal CBD and FN III encoding gene (*CBD*) overhang at the C-terminus was amplified from plasmid pANE-Btcbp at annealing temperature of 56 °C with 2 min of polymerization using gene specific primers BthcbpFp and BtcbpOERev. Chitinase encoding gene (*chi*) was amplified from plasmid pANE-Blichi at annealing temperature of 56 °C with 4 min of polymerization using the gene specific primers BliGHOEFor and BlchiRpmod. Equimolar concentration of the two amplicons was added to fuse the two

and further amplified at annealing temperature of 56 °C with 6 min of polymerization using gene specific primers BthcbpFp and BlchiRpmod for *NBtBlichi*. N-terminal *Btcbp* (*CBD*) fused to *BliGH* termed as *NBtBliGH* was amplified using pANE-NBtBlichi as the template at annealing temperature of 58 °C with 4 min of polymerization using gene specific primers and BthcbpBamHIFp and BlchiGHXhoIRev for *NBtBliGH*. Expression vector pET 22b+ and the amplicon were double digested with *Sal* I and *Xho* I for *NBtBlichi* and *Bam*H I and *Xho* I for *NBtBliGH*, gel purified, and ligated using T4 DNA ligase at 16 °C for 16 h. The recombinant plasmids were designated as pANE-NBtBlichi and pANE-NBtBliGH, respectively.

2.6.2 Heterologous expression of chitin binding proteins and chimeric contsructs

Expression of cbp (Btcbp, Blicbp) and chimeric constructs (NBtBlichi, NBtBliGH**)** was carried out as described in 2.3.2 and 2.4.2, respectively.

2.7 CONSTRUCTION AND CHARACTERIZATION OF CHITINASE CHIMERAS – DELETION OF ChBD FROM *CHITINASES* OF *B. THURINGIENSIS AND B. LICHENIFORMIS* (DOMAIN DELETION)

2.7.1 PCR based construction and cloning of chitinase chimeras *BtGH* and *BliGH*

The details of primers and templates used in the PCR for amplification are listed in Table 2.5. Schematic representation of chimera construction was shown in Fig. 2.5.

2.7.1.1 PCR amplification and cloning of *BtGH* and *BliGH*

GH encoding gene (*GH*) was amplified from plasmid pANE-Btchi and pANE-Blichi at annealing temperature of 56 °C with 3 min of polymerization using the gene specific primers BtchiFpmodnew and BtchiGHHindIIIRev for *BtGH* and BlchiFpmodnew and BlchiGH XhoI Rev for *BliGH*. Expression vector pET 22b+ and the amplicon was double digested with *Eco*R I and *Hind* III for *BtGH* and *Bam*H I and *Xho* I for *BliGH*, gel purified, and ligated using T4 DNA ligase at 16 °C for 16 h. The recombinant plasmids were designated as pANE-BliGH and pANE-BtGH, respectively.

2.7.2 Heterologous expression and purification of chimeric BtGH and BliGH chitinases as described in 2.4.2.

2.7.3 Characterization of recombinant BliGH was described in 2.3.3

RESULTS

3.1 SELECTION OF CHITINOLYTIC BACTERIA

3.1.1 Screening of chitinolytic bacteria on solid media

Chitinolytic ability of the 18 bacterial cultures listed in Table 2.1 was tested on solid media supplemented with colloidal chitin as the sole carbon and nitrogen source. Six cultures were chitinolytic (Fig. 3.1) with the clearance zone around the bacterial colony. Of the selected *S. marcescens*, *B. licheniformis* and *B. circulans* were highly chitinolytic when compared with *S. maltophilia*, *B. thuringiensis* and *B. amyloliquefaciens* which are comparatively less chitinolytic in terms of diameter of the clearance zone thus formed.

3.2 CLONING AND CHARACTERIZATION OF CHITINASE FROM *B. THURINGIENSIS* AND *B. LICHENIFORMIS*

3.2.1 PCR based amplification and cloning of chitinase (c*hi*) from *B. thuringiensis* and *B. licheniformis*

PCR with the gDNA of *B. thuringiensis* and *B. licheniformis* as templates using BtchiFpmodnew and BtchiRpmod for *Btchi* and BlchiFpmodnew and BlchiRpmod for *Blichi* (Table 2.3) resulted in 1.9 and 1.7 kb amplicon, respectively (Fig. 3.2A & C). The amplicon was cloned in the *Eco*R I and *Hind* III sites for *Btchi* and *Bam*H I and *Xho* I sites for *Blichi* in pET22b+. The resultant plasmids were designated as pANE-Btchi and pANE-Blichi, respectively. The inserts of 1.9 and 1.7 kb *chi* were released from pANE-*Btchi* and pANE-Blichi, respectively when digested with *Eco*R I and *Hind* III for *Btchi*, *Bam*H I and *Xho* I for *Blichi* (Fig. 3.2B & D). Sequencing of 1.9 and 1.7 kb insert of pANE-Btchi and pANE-Btchi further confirmed that the cloned amplicons were chitinase from *B. thuringiensis* and *B. licheniformis*.

3.2.2 Heterologous expression and purification of Btchi and Blichi

Periplasmic fractions were prepared for the induced *E. coli* BL21 (DE3) cultures harboring pANE-Btchi and pANE-Blichi. SDS-PAGE analysis of the proteins showed ~77 and 70 kDa corresponding to Btchi and Blichi (Fig. 3.3A). The soluble fraction of

the protein was resolved by 12 % SDS-PAGE, transferred on to nitrocellulose membrane and probed with anti-His antibody. A signal corresponding to ~77 and 70 kDa, using chemiluminiscent method confirmed Btchi and Blichi (Fig. 3.3B). The recombinant proteins were purified through Ni-NTA chromatography using elution buffers containing 50 and 100 mM imidazole (Fig. 3.3C). Flow through and wash fractions were devoid of the recombinant protein indicating the successful purification of the protein.

3.2.3 Semi-Native PAGE analysis for expressed chitinase activity

The periplasmic fractions of Btchi and Blichi were loaded on Semi-Native PAGE containing glycol chitin to detect chitinase activity, Btchi and Blichi expressed periplasmic fractions were active (Fig. 3.3B).

3.2.4 Characterization of recombinant Btchi and Blichi chitinases

3.2.4.1 Kinetic analysis

Reducing end assay with Btchi and Blichi (Fig. 3.4A & E) using colloidal chitin as a substrate showed a gradual increase in chitinase activity until the substrate concentration reached 15 mg/ml, a further increase in substrate concentration did not affect the chitinase activity much. Specific enzyme activities in nkat/mg of protein were calculated to plot Lineweaver-Burk plots (Fig. 3.4B & F), showing near perfect linearity for Btchi but less so for Blichi. Better linearity was seen when the last data point (corresponding to the lowest substrate concentration) was omitted, as is often the case. Values for V_{max} and K_m were determined directly from the Michaelis-Menten plot using computer aided line fitting (GRAPHPAD PRISM software), and indirectly from the two versions of the Lineweaver-Burk plot, for comparison. In this and all following kinetic analyses, the software based values were close to the values obtained from the corrected Lineweaver-Burk plot. In the following, thus, the software generated values were used for comparison between constructs, but the results are always documented in all versions described here. V_{max} for Btchi and Blichi were 0.05 and 0.022 nkat/mg of protein respectively. K_m for Btchi was 5.3 mg/ml, whereas Blichi 6.9 mg/ml, indicating that

Btchi has a slightly higher affinity to colloidal chitin than Blichi. The turn over number (k_{cat}) was higher in case of Btchi (0.014 sec^{-1}) than Blichi (0.005 sec^{-1}).

3.2.4.2 Effect of temperature on Btchi and Blichi activity

3.2.4.2a Optimum temperature

The effect of temperature (20-100 $^{\circ}$C) on the activity of Btchi (Fig. 3.5A) or Blichi (Fig. 3.6A) chitinases was examined using colloidal chitin (8 mg/ml) as the assay substrate. Optimum temperature of 60 $^{\circ}$C was observed with both the chitinases. The activity increased gradually from 20 to 60 $^{\circ}$C and suddenly decreased at 80 $^{\circ}$C by more than 50 %.

3.2.4.2b Thermal stability

The effect of pre-incubation (1h) of Btchi (Fig. 3.5B) at different temperatures followed by reducing end assay revealed that the specific activity decreased by 75 % from 30-40 $^{\circ}$C, (and also at 50 & 60 $^{\circ}$C) whereas pre-incubations at 70 to 100 $^{\circ}$C completely inactivated Btchi. Pre-incubation of Blichi (Fig. 3.6B) at various temperatures showed that the enzyme was not affected up to 50 $^{\circ}$C incubation. At 60-100 $^{\circ}$C incubation temperature there was a drastic reduction in the activity indicated that Blichi could resist the temperature changes up to 50 $^{\circ}$C compared to the temperature sensitive Btchi.

3.2.4.2c Stability at optimum temperature

The stability of both Btchi (Fig. 3.5C) and Blichi (Fig. 3.6C) at 60 $^{\circ}$C (optimum temperature) was observed for 1-4 h, followed by reducing end assay. Btchi activity was constant during 4 h incubation whereas Blichi had decreased activity after 2 h of incubation indicating the stability of Btchi was slightly higher than Blichi at optimum temperature.

3.2.4.3 Optimum pH

The effect of pH on the hydrolytic activity of Btchi and Blichi (Fig. 3.7) using colloidal chitin as the assay substrate was determined. Significant activity of Blichi was

served with sodium citrate buffer pH 4.0 & 5.0 whereas maximum activity of Btchi was seen at pH 5.0 & 6.0 when sodium acetate buffer was used. pH-profile of Blichi was different from that of Btchi. Maximum activity of Blichi was observed with sodium citrate buffer pH 4.0 & 5.0 and noticeable amount of activity was seen with sodium acetate buffer pH 5.0.

3.2.4.4 Chitinase activity on chitosan with different DA

Incubation of the two Bacillus chitinases, Btchi and Blichi with chitosan having different DA (66, 40 and 10 %) at 37 °C in pH 5.0 followed by reducing end assay suggested that these chitinases hydrolyze partially acetylated chitosan. Both the enzymes produced maximum reducing ends with chitosan DA 66 % followed by 40 %. Chitinases were less active on chitosan DA 10 % indicating that the lower degree of acetylation of chitosans was not preferred by Btchi and Blichi (Fig. 3.8).

3.2.5 Circular Dichroism (CD)

3.2.5.1 Conformational changes with increase in temperature

CD spectroscopy was employed to investigate and compare the effect of temperature on structural changes. CD experiments were carried out with gradual increase in temperature followed by secondary structure prediction. Relative change in secondary structure obtained at 30 °C, was considered as 100 %. The secondary structure of both Btchi (Fig. 3.5D) and Blichi (Fig. 3.6D) remain unaffected from 30 to 60 °C, whereas further increase in temperature resulted in the decrease of α helices, steady increase in β sheets and random coils.

3.2.5.2 Effect of longer incubations at different temperature on secondary structure of recombinant chitinases

Pre-incubation of Btchi and Blichi at various temperatures followed by CD analysis Secondary structure prediction at 30 °C was considered as 100 %. Secondary structure conformation of Btchi (Fig. 3.5E) changed with incubation at increased temperatures, which resulted in slow increase of β sheets and random coils at 40 °C

whereas α helices content decreased significantly which continued even at 60 °C. Incubations at 70 to 100 °C lead to increase in random coils in the secondary structure. Drastic change in secondary structure conformation was observed with Blichi (Fig. 3.6E) starting from 40 °C and steady increase in α sheets and random coils was observed with increase in temperature. Relative change in the secondary structure conformation of Btchi was not an abrupt change as seen in Blichi indicating that Btchi is a thermally stable protein by not loosing the conformation at different temperatures.

3.2.5.3 Conformational changes at optimum temperature

CD spectra were recorded with pre-incubated Btchi and Blichi at 60 °C for 1-4 h followed by secondary structure prediction. Both Btchi (Fig. 3.5F) and Blichi (Fig. 3.6F) retained its conformation only for 1 h and later showed altered conformation with decrease in α helices till 4 h indicating that both Btchi and Blichi are not stable in their conformation at 60 °C.

3.2.6 Insoluble substrate binding of recombinant Btchi and Blichi

Both Btchi and Blichi adhered to α chitin, binding capacity of the former being superior. Affinity to β chitin varied significantly in Btchi and Blichi, the latter showed negligible binding (Fig. 3.9).

3.2.7 Soluble substrate binding of Btchi and Blichi using affinity electrophoresis

Binding activity of Btchi and Blichi to some of the soluble polysaccharides was studied by native PAGE, with and without soluble polysaccharides. Electrophoretic mobility of the two proteins Btchi and Blichi (Fig. 3.10) decreased in the presence of glycol chitin in comparison to that of the presence of CM-cellulose, laminarin and without substrate (control). Btchi and Blichi bound to glycol chitin, the substrate of its catalytic domain, instead of CM-cellulose and laminarin, as expected.

3.2.8 Thin layer chromatography (TLC)

3.2.8.1 Hydrolysis pattern of Btchi and Blichi on chito oligomers

Hydrolysis products of chitooligosaccharides catalysed by Btchi and Blichi were analysed on TLC and presented in (Fig. 3.11). Btchi hydrolysed the chitohexose and chitotetraose to chitobiose and a trace of N-acetylglucosamine whereas Blichi liberated only chitobiose. The reaction velocity of Btchi was higher than Blichi in degrading chitohexaose. Higher oligomers than chitotetraose were observed with Btchi hydrolysis on chitotetraose indicating a transglycosylation ability of Btchi which was absent in Blichi. Both Btchi and Blichi were not active against chitotriose (data not shown).

3.2.8.2 Hydrolysis pattern of Btchi and Blichi on chitin polymers

Hydrolysis products of chitin polymers catalysed by Btchi and Blichi were analysed on TLC and presented in (Fig. 3.12). Chitin polymers such as β, colloidal, and α chitin were hydrolysed by Btchi to various oligomers ranging from hexamer to monomer. Blichi hydrolyzed α and colloidal chitin to chitobiose and β chitin to various oligomers, indicating that chitin conformation plays a vital role in the hydrolysis by the enzyme.

3.3 CONSTRUCTION AND CHARACTERIZATION OF CHITINASE CHIMERAS - REPLACING THE ChBD OF *B. THURINGIENSIS* AND *B. LICHENIFORMIS* CHITINASES BY CeBD OF ENDOGLUCANASE (DOMAIN SWAPPING).

3.3.1 PCR based domain swapping and cloning of chitinase chimeras Btegln and Bliegln

3.3.1.1 PCR amplification and cloning of endoglucanase (egln) from *B. licheniformis*

PCR with the gDNA of *B. licheniformis* as template using BleglnFpGH5 new and BleglnRpGH5 resulted in 1.6 kb amplicon, corresponsing to the expected size of *egln* of *B. licheniformis* (Fig. 3.13A). The amplicon was cloned in the *Bam*H I and *Xho* I sites of pET22b+ and the plasmid was designated as pANE-egln. Insert of 1.6 kb *egln* was

released from pANE-egln when digested with *Bam*H I and *Xho* I (Fig. 3.13B). Sequencing of the 1.6 kb insert of pANE-egln further confirmed that the cloned amplicon was *egln* from *B. licheniformis*.

3.3.1.2 PCR amplification, fusion and cloning of chimeric chitinase *Btegln* and *Bliegln*

PCR with the plasmid pANE-Btchi and pANE-Blichi as template using BtchisalFor and BtGHOEglnRev for *Btchi* (*GH*) and BlchiFpmodnew and BlchiGHglnOE Rev for *Blichi* (*GH*) resulted in 1.2 kb amplicon with a complementary overhang at the C- terminus. *CeBD* from *egln* was amplified with the plasmid pANE-egln using EglncbmOEFor and BleglnRpGH5 reulted in 0.6 kb (Fig. 3.14A & 3.15A). Fusion PCR carried out with the gel extracted amplicons of *GH* from *Btchi* or *Blichi* and *CeBD* from *egln* as templates using BtchisalFor and BleglnRpGH5 for *Btegln* and BlchiFpmodnew and BleglnRpGH5 for *Bliegln* resulted in 1.8 kb amplicon (Fig. 3.14B & 3.15B). The amplicon was cloned in the *Sal* I and *Xho* I sites for *Btegln* and *Bam*H I and *Xho* I for *Bliegln* in pET22b+ and the resultant plasmids were designated as pANE-Btegln and pANE-Bliegln, respectively. The 1.8 kb inserts each of *Btegln* and *Bliegln* were released from pANE-Btegln and pANE-Bliegln, respectively when digested with *Sal* I and *Xho* I for *Btegln*, *Bam*H I and *Xho* I for and *Bliegln* (Fig. 3.14C & 3.15C). Sequencing of the 1.8 kb insert of pANE-Btegln and pANE-Bliegln further confirmed the fusion of *CeBD* of endoglucanase to the C-terminus of Btchi or Blichi catalytic domain (*GH*).

3.3.2 Heterologous expression and purification

3.3.2.1 Endoglucanase

Periplasmic fraction was prepared for the induced *E.coli* BL21 (DE3) culture harboring pANE-egln. SDS-PAGE analysis of the proteins showed ~65 kDa corresponding endoglucanase (Fig. 3.16A). The soluble fraction of egln was resolved on 12 % SDS-PAGE, transferred onto nitrocellulose membrane and probed with anti-His

antibody. A signal corresponding to ~65 kDa, using chemiluminiscent method confirmed endoglucanase (Fig. 3.16B).

3.3.2.2 Chimeric contructs

Periplasmic fraction as well as soluble and insoluble cytoplasmic fractions were prepared for the induced *E.coli* BL21 (DE3) culture harboring pANE-Bliegln and pANE-Btegln. SDS-PAGE analysis of the proteins showed ~75 kDa corresponding to both Btegln and Bliegln (Fig. 3.17A & B). Expression profile of Bliegln showed soluble fraction of the recombinant protein in periplasmic fraction while most of the protein was present in the insoluble fraction. On the other hand, expression of Btegln led to inclusion bodies. The soluble and insoluble fraction of Bliegln and Btegln respectively were resolved by 12 % SDS-PAGE, transferred on to nitrocellulose membrane and probed with anti-His antibody. A signal corresponding to ~75 kDa, using chemiluminiscent method confirmed Btegln and Bliegln (Fig. 3.17C). Soluble fraction of Bliegln was purified (Fig. 3.17D) through Ni-NTA chromatography using elution buffers containing 50 and 100 mM imidazole. Flow through and wash fractions were devoid of the recombinant protein indicating the successful purification of the protein.

3.3.3 Characterization of recombinant Bliegln

3.3.3.1 Kinetic analysis

Reducing end assay with Bliegln (Fig. 3.18A) using colloidal chitin showed a gradual increase in chitinase activity till the substrate concentration reached 15 mg/ml and further increase in substrate concentration did not affect the chitinase activity. Specific activity in nkat/mg of protein was calculated to plot Lineweaver-Burk plot (Fig. 3.18B), V_{max} and K_m for Bliegln were 0.03 nkat/mg and 13.4 mg/ml indicating the reduced affinity to colloidal chitin than Blichi. The turn over number (k_{cat}) was high in case of Bliegln (0.009 sec^{-1}) than Blichi (0.005 sec^{-1}).

3.3.3.2 Effect of temperature on Bliegln activity

3.3.3.2a Optimum temperature

The effect of temperature (20-100 °C) on the activity of Bliegln (Fig. 3.19A) was examined using colloidal chitin (8 mg/ml) as the assay substrate and was compared with Blichi. Optimum temperature of 40 °C was observed with Bliegln. The activity increased from 20 to 40 °C and gradually decreased at 80 °C by more than 75 %.

3.3.3.2b Thermal stability

The effect of pre-incubation (1h) of Bliegln (Fig. 3.19B) at different temperatures followed by reducing end assay revealed that the specific activity decreased by 3-fold when compared with Blichi, but the enzyme activity was not affected from 40-100 °C.

3.3.3c Stability of Bliegln at optimum temperature of Blichi

Stability of Bliegln at 60 °C for 1-4 h was done by reducing end assay. Specific activity of Bliegln (Fig. 3.19C) reduced from 1-2 h by 50 % and remained unaffected with increase in time indicating the stability of Bliegln improved when compared with Blichi.

3.3.4 Optimum pH

The effect of pH on the hydrolytic activity of Bliegln (Fig. 3.20) using colloidal chitin as the assay substrate was determined. pH-profile of Bliegln was different from that of Blichi; significant activity was seen with sodium citrate buffer pH 3.0 & 4.0 whereas activity reduced in pH 5.0 & 6.0 when sodium acetate buffer was used.

3.3.5 Circular Dichroism (CD)

3.3.5.1 Conformational changes with increase in temperature

CD spectroscopy was employed to investigate and compare the effect of temperature on structural changes in Bliegln (Fig. 3.19D). CD experiments were carried out with gradual increase in temperature followed by secondary structure prediction.

Relative change in secondary structure was obtained by considering the secondary conformation at 30 °C, as 100 %. α helices gradually decreased from 30-100 °C, with a concomitant increase of β sheets and random coils with increase in temperature.

3.3.5.2 Effect of longer incubations at different temperature on secondary structure of recombinant chitinases

Pre-incubation of Bliegln at various temperatures followed by CD analysis and secondary structure prediction at 30 °C was considered as 100 %. Relative change in secondary structure of Bliegln (Fig. 3.19E) at different temperatures resulted in slow increase of β sheets and random coils at 40 °C, whereas α helices decreased significantly and remained the same till 100 °C. At different temperatures, change in the secondary structure conformation of Bliegln was not drastic indicating the thermal stability of the protein.

3.3.5.3 Conformational changes of Bliegln at optimum temperature of Blichi

CD spectra were recorded with pre-incubated Bliegln at 60 °C for 1-4 h followed by analysis of the relative change in the secondary structure. Bliegln (Fig. 3.19F) retained its conformation for 2 h and further increase in time affected the conformation with decreased α helices and continued till 4 h indicating that Bliegln is stable at 60 °C as the change in secondary structure with reference to time was negligible.

3.3.6 Insoluble substrate binding of recombinant Bliegln

Binding ability of Bliegln towards insoluble substrates revealed that Bliegln did not adhere to α and β chitin significantly (Fig. 3.21), binding capacity was less in lower concentrations followed by complete loss with increase in concentration of protein.

3.3.7 Soluble substrate binding of Bliegln using affinity electrophoresis

Binding activity of Bliegln to some of the soluble polysaccharides was investigated by native PAGE with and without soluble polysaccharides. Electrophoretic mobility of Bliegln (Fig. 3.22) decreased in the presence of glycol chitin in comparison to

CM-cellulose, laminarin and without substrate (control). Bliegln showed strong affinity to glycol chitin than CM-cellulose and laminarin, as expected.

3.3.8 Thin layer chromatography (TLC)

3.3.8.1 Hydrolysis pattern of Bliegln on chito oligomers

Hydrolysis products of chitooligosaccharides catalysed by Bliegln were analysed using TLC and presented in Fig. 3.23A. Bliegln hydrolysed chitohexose and chitotetraose to chitotriose and chitobiose. The reaction velocity of Bliegln was higher in hydrolyzing chitohexose and was less with chitotetrose. Bliegln was not active against chitotriose similar to that of Blichi.

3.3.8.2 Hydrolysis pattern of Bliegln on chitin polymers

Hydrolysis products of chitin polymers catalysed by Bliegln were analysed on TLC plates and presented in Fig. 3.23B. Bliegln hydrolyzed β chitin to various oligomers and was not effective on α chitin indicating the importance of the chitin conformation and the CBD replaced in the modified enzyme which imparts binding ability towards chitinous substrates.

3.4 CONSTRUCTION AND CHARACTERIZATION OF CHITINASE CHIMERAS - REPLACING THE ChBD OF *B. THURINGIENSIS WITH* ChBD OF *B. LICHENIFORMIS AND vice versa* (DOMAIN SWAPPING)

3.4.1 PCR based domain swapping and cloning of chitinase chimeras Btswap and Bliswap

PCR with the plasmid pANE-Btchi ord pANE-Blichi as template using BtchiBamHIFor and BtGHOESbl for *Btswap* and BlchiFpmodnew and BlGHOESbt for *Bliswap* resulted in 1.25 and 1.2 kb amplicon of *GH* with a complementary overhang at the C- terminus. ChBD including FN III from *Btchi* and *Blichi* was amplified with the plasmid pANE-Btchi and pANE-Blichi using BtcbmOE and BtchiRpmod for *Btchi* (*ChBD*) and BlcbmOE and BlchiRpmod for *Blichi* (*ChBD*) resulted in 0.65 and 0.5 kb, respectively (Fig. 3.24A & 3.25A). Fusion PCR carried out with the gel extracted

amplicons of Btchi (*GH*) and Blichi (*ChBD*) using BtchiBamHIFor and BlchiRpmod primers and amplicons Blichi (*GH*) and Btchi (*ChBD*) were fused using BlchiFpmodnew and BtchiRpmod, which resulted in 1.75 and 1.85 kb amplicons, respectively (Fig. 3.24B & 3.25B). The amplicon was cloned in the *Bam*H I and *Xho* I sites for Btswap, and *Bam*H I and *Hind* III sites for Bliswap in pET22b+ and the resultant plasmids were designated as pANE-Btswap and pANE-Bliswap, respectively. The inserts of 1.75 kb and 1.85 kb were released from pANE-Btswap and pANE-Bliswap, respectively when digested with *Bam*H I and *Xho* I for *Btswap* (Fig. 3.24C), *Bam*H I and *Hind* III for *Bliswap* (Fig. 3.25C). Sequencing of the 1.75 and 1.85 kb insert of pANE-Btswap and pANE-Bliswap further confirmed the fusion of ChBD to the C-terminus of GH from *Bacillus.*

3.4.2 Heterologous expression and purification of Btswap and Bliswap

Periplasmic, cytoplasmic fractions soluble and insoluble were prepared for the induced *E.coli* BL21 (DE3) cultures harboring pANE-Btswap and pANE-Bliswap. SDS-PAGE analysis of the proteins showed ~71 and 75 kDa corresponding to Btswap and Bliswap (Fig. 3.26A & B), respectively. The insoluble fraction also contained the recombinant protein suggesting the effect of gene modification at the expression level. The soluble fraction of the protein was resolved on 12 % SDS-PAGE, transferred on to nitrocellulose membrane and probed with anti-His antibody. A signal corresponding to ~71 and 75 kDa, using chemiluminiscent method confirmed Btswap and Bliswap (Fig. 3.26C), respectively. The recombinant proteins Btswap and Bliswap (Fig. 3.26D & E) were purified through Ni-NTA chromatography using elution buffers containing 50 and 100 mM imidazole. Flow through and wash fractions were devoid of the recombinant protein indicating the successful purification of the protein.

3.4.3 Characterization of recombinant Btswap and Bliswap chitinases

3.4.3.1 Kinetic analysis

Reducing end assay with Btswap and Bliswap (Fig. 3.27A & E) using colloidal chitin showed a gradual increase in chitinase activity till the substrate concentration reached 15 mg/ml and further increase in substrate concentration did not affect the chitinase activity. Specific activity in nkat/mg of protein was calculated to plot

Lineweaver-Burk plot (Fig. 3.27B & E), V_{max} for Btswap and Bliswap was 0.04 and 0.03 nkat/mg respectively. K_m for Btswap was 4.5 mg/ml whereas Bliswap 3.2 mg/ml indicating that Bliswap has more affinity to colloidal chitin than Btswap. The turn over number (k_{cat}) was same in case of Btswap (0.010 sec^{-1}) than Bliswap (0.010 sec^{-1}).

3.4.3.2 Effect of temperature on Btswap and Bliswap activity

3.4.3.2a Optimum temperature

The effect of temperature (20-100 °C) on the activity of Btswap (Fig. 3.28A) or Bliswap (Fig. 3.29A) chitinases was examined using colloidal chitin (8 mg/ml) as the assay substrate. Optimum temperature of 40 and 60 °C was observed with Btswap and Bliswap, respectively. The Btswap activity increased gradually from 20 to 40 °C and suddenly decreased at 60 °C by more than 50 % whereas Bliswap activity increased from 20 to 60 °C and reduced at 80 °C by more than 75 %.

3.4.3.2b Thermal stability

The effect of pre-incubation (1 h) of Btswap (Fig. 3.28B) at different temperatures followed by reducing end assay revealed that the specific activity remained unaffected from 30-40 °C, further the activity decreased drastically with pre-incubations at 50 to 100 °C. Pre-incubation of Bliswap (Fig. 3.29B) at various temperatures showed that specific activity was not affected from 30-50 °C and activity gradually decreased from 60 to 100 °C.

3.4.3.2c Stability at optimum temperature

Stability of both Btswap and Bliswap at 40 & 60 °C for 1-4 h was done by reducing end assay. Specific activity of Btswap (Fig. 3.28C) was unaffected with increase in incubation time whereas Bliswap (Fig. 3.29C) activity was reduced by 50 % after 2 h of incubation and continued to retain its activity till 4 h, indicating the increased stability of Btswap than Bliswap.

3.4.3.3 Optimum pH

The effect of pH on the hydrolytic activity of Btswap and Bliswap using colloidal chitin as the assay substrate was determined. Significant activity was obtained with sodium citrate buffer pH 5.0 whereas maximum activity of Btswap (Fig. 3.30) was obtained at pH 5.0 & 6.0 when sodium acetate buffer was used. pH-profile of Bliswap (Fig. 3.30) was different from that of Btswap, highest activity of Bliswap was observed with pH 5.0 of sodium citrate and sodium acetate buffers.

3.4.4 Circular Dichroism (CD)

3.4.4.1 Conformational changes with increase in temperature

CD spectroscopy was employed to investigate and compare the effect of temperature on structural changes. CD experiments were carried out with gradual increase in temperature followed by secondary structure prediction. The relative change in secondary structure of Btswap (Fig. 3.28D) remained unaffected at 30 & 40 $^{\circ}$C followed by decrease in α helices and increase in β sheets and random coils. Bliswap (Fig. 3.29D) retained its conformation at 30-60 $^{\circ}$C followed by gradual decrease in α helices suggesting that both Btswap and Bliswap retained secondary structure till 40 & 60 $^{\circ}$C, respectively.

3.4.4.2 Effect of longer incubations at different temperature on secondary structure of recombinant chitinases

Pre-incubation of Btswap and Bliswap at various temperatures was followed by CD analysis to obtain the relative change in secondary structure prediction at 30 $^{\circ}$C considered as 100 %. Secondary structure of Btswap (Fig. 3.28E) was unaffected up to 40 $^{\circ}$C followed by slow increase in β sheets, random coils and α helices decreased significantly. Bliswap (Fig. 3.29E) retained secondary structure conformation till 60 $^{\circ}$C followed by a significant increase in β sheets and random coils from 70-100 $^{\circ}$C. Secondary structures of Btswap and Bliswap were found to be temperature-dependent and not thermally stable.

3.4.4.3 Conformational changes at optimum temperature

CD spectra were recorded with pre-incubated Btswap and Bliswap at 40 & 60 °C, respectively for 1-4 h followed by relative secondary structure prediction. Btswap (Fig. 3.28F) retained its conformation for 1-4 h without undergoing drastic changes in the secondary structure composition. Similarly, secondary structure conformation of Bliswap (Fig. 3.29F) was unaffected till 2 h at 60 °C followed by slow increase of β sheets and random coils. Btswap was highly stable at 40 °C, whereas Bliswap was not stable at its optimum temperature indicating that swapping has brought conformational stability to Btswap.

3.4.5 Insoluble substrate binding of recombinant Btswap and Bliswap

Both Btswap and Bliswap bound to α and β chitin; binding capacity being similar at low concentrations of the protein, at high concentrations Btswap was superior. Affinity to β chitin gradually increased in Btswap and Bliswap with increase in concentration of the protein (Fig. 3.31).

3.4.6 Soluble substrate binding of Btswap and Bliswap using affinity electrophoresis

Binding activity of Btswap and Bliswap to some of the soluble polysaccharides were investigated by native PAGE with and without soluble polysaccharides. Electrophoretic mobility of the two proteins Btswap and Bliswap (Fig. 3.32) decreased in the presence of glycol chitin in comparison to that of the presence of CM-cellulose, laminarin and without substrate (control). Btswap and Bliswap bound to glycol chitin, the substrate of its catalytic domain, instead of CM-cellulose and laminarin, as expected.

3.4.7 Thin layer chromatography (TLC)

3.4.7.1 Hydrolysis pattern of Btswap and Bliswap on chito oligomers

Hydrolysis products of chitooligosaccharides catalysed by Btswap and Bliswap were analysed on TLC and presented in (Fig. 3.33A & B). Btswap hydrolysed chitohexose and chitotetraose to chitobiose. The reaction velocity of Btswap was lower

than Btchi in degrading chitohexose and chitotetraose. Longer oligomer, like chitohexose, was observed with Btswap hydrolysis on chitotetraose indicating the transglycosylation ability similar to that of Btchi. Bliswap hydrolyzed chitohexose to chitotetraose, chitotriose and chitobiose. The reaction velocity of Bliswap decreased significantly in hydrolyzing chitohexose than Blichi. Bliswap was not able to hydrolyze chitotetraose. Both Btswap and Bliswap were not active against chitotriose.

3.4.7.2 Hydrolysis pattern of Btswap and Bliswap on chitin polymers

Hydrolysis products of chitin polymers catalysed by Btswap and Bliswap were analysed on TLC and presented in Fig. 3.34A & B. Both Btswap and Bliswap hydrolysed β chitin to various oligomers ranging from hexamer to monomer. α chitin hydrolysis to chitobiose was observed with Btswap and Bliswap.

3.5 CONSTRUCTION OF CHITINASE CHIMERAS – FUSION OF CBD FROM CBP OF *B. THURINGIENSIS TO CHITINASE OF B. LICHENIFORMIS* (DOMAIN FUSION)

3.5.1 PCR based amplification and cloning of chitin binding protein from *B. thuringiensis* and *B. licheniformis*

PCR with the gDNA of *B. thuringiensis* and *B. licheniformis* as template using BtcbpFp and BtcbpRp for *Btcbp* and BlicbpFp and BlicbpRp for *Blicbp* resulted in 1.3 and 0.6 kb amplicon respectively, corresponding to the expected size of chitin binding protein (*cbp*) (Fig. 3.35A & B). The amplicons were cloned in the *Sal* I and *Xho* I sites of pET22b+ and the resultant plasmids were designated as pANE-Btcbp and pANE-Blicbp, respectively. The inserts of 1.3 and 0.6 kb *cbp* was released from pANE-Btcbp and pANE-Blicbp, respectively when digested with *Sal* I and *Xho* I for both Btcbp and Blicbp (Fig. 3.35C & D). Sequencing of the 1.3 and 0.6 kb insert of pANE-Btcbp and pANE-Blicbp further confirmed that the cloned amplicons were chitin binding proteins (*cbp*) from *B. thuringiensis* and *B. licheniformis*.

3.5.2 PCR based fusion and cloning of chitinase chimeras NBtBlichi and NBtBliGH

PCR with the plasmid pANE-Btcbp as template using BthcbpFp and BtcbpOERev resulted in 0.9 kb amplicon, corresponsing to the expected size of *CBD* (*cbp*) of *B. thuringiensis* (Fig. 3.36A) with a complementary overhang at the C- terminus. Chitinase (chi) of *B. licheniformis* was amplified with the plasmid pANE-Blichi using BliGHOEFor and BlchiRpmod resulted in 1.7 kb. Fusion PCR carried out with the gel extracted amplicons of *CBD* from *Btcbp* and *chi* from *Blichi* as templates using BthcbpFp and BlchiRpmod resulted in 2.6 kb amplicon (Fig. 3.36B). The amplicon was cloned in the *Sal* I and *Xho* I sites in pET22b+ and the resultant plasmid was designated as pANE-NBtBlichi. Insert of 2.6 kb *NBtBlichi* was released from pANE-NBtBlichi when digested with *Sal* I and *Xho* I (Fig. 3.36C). Sequencing of the 2.6 kb insert of pANE-NBtBlichi further confirmed the N-terminal fusion of *CBD* to *Blichi.* PCR with the plasmid pANE-NBtBlichi as template using BthcbpBamHIFp and BlchiGH XhoIRev resulted in 2.1 kb amplicon, corresponsing to the expected size of *NBtBliGH* (Fig. 3.37A). The amplicon was cloned in the *Bam*H I and *Xho* I sites in pET22b+ and the resultant plasmid was designated as pANE-NBtBliGH. Insert of 2.1 kb *NBtBliGH* was released from pANE-NBtBliGH when digested with *Bam*H I and *Xho* I (Fig. 3.37B). Sequencing of the 2.1 kb insert of pANE-NBtBliGH further confirmed the NBtBliGH.

3.5.3 Heterologous expression

3.5.3.1 Heterologous expression of Btcbp and Blicbp

Periplasmic fractions were prepared for the induced *E.coli* BL21 (DE3) cultures harboring pANE-Btcbp and pANE-Blicbp. SDS-PAGE analysis of the proteins showed ~55 and 21 kDa corresponding to Btcbp and Blicbp (Fig. 3.38A &B). The soluble fraction of the protein was resolved by 12 % SDS-PAGE, transferred on to nitrocellulose membrane and probed with anti-His antibody. A signal corresponding to ~55 and 21 kDa, using chemiluminiscent method confirmed Btcbp and Blicbp (Fig. 3.38C & D).

3.5.3.2 Heterologous expression of NBtBlichi and NBtBliGH

Periplasmic fraction was prepared for the induced *E.coli* BL21 (DE3) cultures harboring pANE-NBtBlichi and pANE-NBtBliGH. SDS-PAGE analysis of the proteins showed ~100 and 70 kDa corresponding to NBtBlichi (Fig. 3.39A) and ~83 and 52 kDa for NBtBliGH (Fig. 3.39B). The soluble fraction of the protein was resolved by 12 % SDS-PAGE, transferred on to nitrocellulose membrane and probed with anti-His antibody. Signal corresponding to ~100 and 70 kDa was observed with pANE-NBtBlichi (Fig. 3.39C). Similary, signal corresponding to ~83 and 52 kDa was obtained with plasmid pANE-NBtBliGH (Fig. 3.39C) indicating that the expressed proteins of each construct positive for western blotting were similar at C-terminus.

3.6 CONSTRUCTION AND CHARACTERIZATION OF CHITINASE CHIMERAS – DELETION OF ChBD FROM CHITINASES OF *B. THURINGIENSIS AND B. LICHENIFORMIS* (DOMAIN DELETION)

3.6.1 PCR based deletion and cloning of chitinase chimeras BtGH and BliGH

PCR with the plasmid pANE-Btchi and pANE-Blichi as template using BtchiFpmodnew and BtchiGH HindIII Rev for *Btchi* (*GH*) and BlchiFpmodnew and BlchiGH XhoI Rev for *Blichi* (*GH*) resulted in 1.2 kb amplicon, corresponsing to the expected size of *GH* (Fig. 3.40A & C). The amplicon was cloned in the *Eco*R I and *Hind* III sites for *Btchi* (*GH*) and *Bam*H I and *Xho* I for *Blichi* (*GH*) of expression vector pET22b+ and the plasmids were designated as pANE-BtGH and pANE-BliGH. Inserts of 1.2 kb *GH* were released from pANE-BtGH and pANE-BliGH, respectively when digested with *Eco*R I and *Hind* III for BtGH, *Bam*H I and *Xho* I for BliGH (Fig. 3.40B & D). Sequencing of the 1.2 kb insert of pANE-BtGH and pANE-BliGH further confirmed the deletion of ChBD from *GH* of *Bacillus* chitinases.

3.6.2 Heterologous expression and purification of BtGH and BliGH

Periplasmic, cytoplasmic fraction soluble and insoluble was prepared for the induced *E.coli* BL21 (DE3) culture harboring pANE-BtGH and pANE-BliGH. SDS-PAGE analysis of the proteins showed ~52 kDa corresponding to both BtGH and BliGH

(Fig. 3.41A & B). Expression profile of BliGH showed that small quantity of recombinant protein is in periplasmic fraction and most of the protein was present in the insoluble fraction whereas profile of BtGH expression showed that the recombinant protein was in insoluble fraction as a result of inclusion bodies. The soluble fraction of the protein was resolved by 12 % SDS-PAGE, transferred on to nitrocellulose membrane and probed with anti-His antibody. A signal corresponding to ~52 kDa, using chemiluminiscent method confirmed BtGH and BliGH (Fig. 3.41C). Soluble fraction of BliGH (Fig. 3.41D) was purified through Ni-NTA chromatography using elution buffers containing 50 and 100 mM imidazole. Flow through and wash fractions were devoid of the recombinant protein indicating the successful purification of the protein. Purified BliGH was characterized for enzymatic properties.

3.6.3 Characterization of recombinant BliGH

3.6.3.1 Kinetic analysis

Reducing end assay with BliGH (Fig. 3.42A) using colloidal chitin showed a gradual increase in chitinase activity till the substrate concentration reached 30 mg/ml and further increase in substrate concentration did not affect the chitinase activity. Specific activity in nkat/mg of protein was calculated to plot Lineweaver-Burk plot (Fig. 3.42B), V_{max} and K_m for BliGH was 0.06 nkat/mg and 68.3 mg/ml indicating the reduced affinity to colloidal chitin than Blichi. The turn over number (k_{cat}) was high in case of BliGH (0.011 sec^{-1}) than Blichi (0.005 sec^{-1}).

3.6.3.2 Effect of temperature on BliGH activity

3.6.3.2a Optimum temperature

The effect of temperature (20-100 $^{\circ}$C) on the activity of BliGH (Fig. 3.43A) chitinase was examined using colloidal chitin (8 mg/ml) as the assay substrate and was compared with Blichi. BliGH was optimally active at 40 $^{\circ}$C was observed with. The activity increased marginally from 20-40 $^{\circ}$C followed by gradual decrease up to 80 $^{\circ}$C, where more than 50 % was noticed.

3.6.3.2b Thermal stability

The effect of pre-incubation of BliGH for 1h (Fig. 3.43B) at different temperatures followed by reducing end assay revealed that the specific activity was unaffected at 30 to 40 °C followed by decrease at 50 °C by 50 % and the activity continued to decrease up to 100 °C.

3.6.3.2c Stability of BliGH at optimum temperature of Blichi

Stability of BliGH at 60 °C for 1-4 h was done using reducing end assay. Specific activity of BliGH (Fig. 3.43C) drastically decreased with increase in incubation time indicating that BliGH was not stable at 60 °C.

3.6.4 Optimum pH

The effect of pH on the hydrolytic activity of BliGH using colloidal chitin as the assay substrate was determined. pH-profile of BliGH (Fig. 3.44) was different from that of Blichi, maximum activity was obtained with sodium citrate buffer pH 4.0 whereas activity decreased in sodium acetate buffer pH 5.0 followed by increased activity in pH 6.0 of sodium acetate and phosphate buffers.

3.6.5 Circular Dichroism (CD)

3.6.5.1 Conformational changes with increase in temperature

CD spectroscopy was employed to investigate and compare the effect of temperature on structural changes. CD experiments were carried out with gradual increase in temperature followed by secondary structure prediction. Relative change in secondary structure was obtained by considering the secondary conformation at 30 °C as 100 %. Conformation of BliGH (Fig. 3.43D) retained at 30-50 °C followed by slight change observed at 60 °C continued with gradual decrease in α helices while β sheets and random coils increased with increase in temperature leading to loss in the integrity of secondary structure.

3.6.5.2 Effect of longer incubations at different temperature on secondary structure of recombinant chitinases

Pre-incubation of BliGH at various temperatures followed by CD analysis and secondary structure prediction at 30 °C considered as 100 %. Relative change in secondary structure of BliGH (Fig. 3.43E) with incubation at increased temperatures resulted in gradual increase of β sheets and random coils whereas α helices decreased significantly. Gradual changes in the secondary structure of BliGH at different temperatures reveal that BliGH is not a thermally stable protein.

3.6.5.3 Conformational changes at optimum temperature

CD spectra were recorded with pre-incubated BliGH at 60 °C for 1-4 h followed by predicting the relative change in the secondary structure. Conformation of BliGH changed with gradual decrease in α helices by 10 % which remained the same till 3 h (Fig. 3.43F) and further increase in incubation time showed increased α helices.

3.6.6 Insoluble substrate binding of recombinant BliGH

Binding capacity of BliGH was less with β than α chitin. Higher concentration of BliGH (Fig. 3.45) showed maximum binding to α chitin.

3.6.7 Soluble substrate binding of BliGH using affinity electrophoresis

Binding activity of BliGH to some of the soluble polysaccharides was studied by native PAGE with and without soluble polysaccharides. Electrophoretic mobility of BliGH (Fig. 3.46) decreased in the presence of glycol chitin in comparison of that in the presence of CM-cellulose, laminarin and without substrate (control). BliGH bound to glycol chitin than CM-cellulose and laminarin, as expected.

3.6.8 Thin layer chromatography (TLC)

3.6.8.1 Hydrolysis pattern of BliGH on chito oligomers

Hydrolysis products of chitooligosaccharides catalysed by BliGH were analysed on TLC and presented in (Fig. 3.47A). BliGH hydrolysed chitohexose to chitotriose and chitobiose, whereas, chitotetraose was cleaved to chitobiose. The reaction velocity of BliGH was higher in hydrolyzing chitohexose and chitotetraose. BliGH was not active against chitotriose similar to that of Blichi.

3.6.8.2 Hydrolysis pattern of BliGH on chitin polymers

Hydrolysis products of chitin polymers catalysed by BliGH were analysed on TLC and presented in (Fig. 3.47B). BliGH hydrolyzed β chitin to various oligomers and α chitin to chitobiose similar to that of Blichi.

Enzymatic properties of native (Btchi and Blichi) and chimeric chitinases (Bliegln, Btswap, Bliswap and BliGH) are summarized in Table 3.1.

DISCUSSION

4.1 SELECTION OF CHITINOLYTIC BACTERIA

Chitin is an abundant polymer present in nature after cellulose. The source of chitin being many, predominant is the aquatic biosphere. Chitinases are the hydrolyzing enzymes that degrade the β-1, 4-glycosidic bonds. Chitin containing organisms like fungi and insects produce chitinases for their growth and development, whereas non chitin containing organisms, including bacteria produce chitinases in their saprophytic phase to derive carbon and nitrogen from chitin. An efficient bioconversion of such large biomass into useful products led to the identification of chitinolytic microorganisms and chitinases. Screening of chitinolytic bacteria from different sources will enable identification of more chitinases and characterization of such chitinases could allow better understanding of chitin hydrolysis.

Several bacterial species have been isolated for the production of chitinases. Since chitinases are able to diffuse through agar, assays to identify chitinolytic bacteria producing chitin-degrading enzymes were performed by monitoring the degradation of polymeric chitin incorporated into an agar medium (Howard *et al*., 2003). The larger and clearer zone of chitin hydrolysis was attributed to the chitinolytic ability of the bacteria. Even though, the plate method was considered to be a preliminary qualitative test with limited sensitivity, still it represents simple and inexpensive method that would separate the chitinolytic microorganisms from the pool of bacterial diversity. In the present study, a total of 18 bacterial cultures were tested for their chitinolytic ability *in vitro* on chitinase detection agar medium. *Bacillus circulans* and *B. licheniformis, B. amyloliquefaciens, B. thuringiensis, Serratia marcescens* and *Stenotrophomonas maltophilia* were positive for chitin hydrolysis as a clear halo around the colony (Fig. 3.1). Cody (1989) used a similar method for detection of chitinolytic bacteria and selected 17 out of 52 strains representing species of *Bacillus* that were chitinolytic, of which *B. circulans, B. licheniformis* and some others were chitinolytic. Kishore *et al*. (2005b) screened 393 bacterial isolates from peanut plants and selected 95 potential chitinolytic bacteria for biological control of peanut diseases. Microorganisms isolated from kimchi juice, including strain KCK identified as *Serratia*, were selected based on

the clear zone on the colloidal chitin plate (Kim *et al*., 2007). *Bacillus* and *Serratia* sps. were highly predominant in such screening studies.

Liu *et al*. (2002) screened 70 *B. thuringiensis* strains and 38 strains had chitinase activity both on inducing plate and in inducing broth. Most of the chitinase producing strains secreted chitinase only at a low level, in comparison to *S. marcescens. Serratia marcescens* showed larger zones of clearance, compared to all other bacterial sources (including *Bacillus*) in all such efforts to screen bacteria including this work. *S. maltophilia* (Kobayashi *et al*., 2002) and *B. amyloliquefaciens* (Wang *et al*., 2002) were antagonistic to *M. poae* and *F. oxysporum*, respectively and produced chitinases; preliminary characterization of such chitinases was carried out.

Extensive research has been carried out on chitinases of *S. marcescens* and *B. circulans.* Information on gene structure and biocontrol traits of *S. maltophilia* (Kobayashi *et al*., 2002) and *Bacillus thuringiensis* (Zhong *et al*., 2005) were described, while little is known about chitinases of *B. amyloliquefaciens* and *B. licheniformis.* Though significant sequence information is known, detailed characterization of chitinases was not done. In the present study, we have selected *B. licheniformis* and *B. thuringiensis* for isolation of chitinase encoding genes and subsequently carried out domain swapping based on the domain architecture.

4.2 CLONING AND CHARACTERIZATION OF CHITINASE FROM *B. THURINGIENSIS KURSTAKI* AND *B. LICHENIFORMIS* DSM 13

The *chi* and its derived gene swapping sequences were cloned in expression vector pET 22b+ (Novagen), as this vector contains a *pelB* leader sequence that exports the protein into the periplasmic fraction and a 6x his tag that facilitates purification of the recombinant protein using Ni-NTA affinity chromatography.

BLAST search showed that Btchi (1.9 kb, 640 aa) sequence in the present study has high degree of amino acid similarity with chitinases of other *Bacillus* sps. Btchi is 99 % similar to both *B. thuringiensis* sv. *tochigiensis* (ZP_04143843.1) and *B. cereus*

(ZP_04282272.1), 95 % similar to the reported sequence *B. thuringiensis* (AAL71886) for which the primers were designed and 94 % similar to *B.t.* sv. *alesti* (AAR19092.1) indicating that different serovars of *B.thuringiensis* exhibit variation in the chitinase sequences as described earlier by Lin and Xiong (2004). In their study, the BLAST search of *B.t* sv. *alesti* showed amino acid sequence similarity with chitinases isolated from various strains such as ChiB (AB041932) from *B. cereus* (97.6%), ChiA74 (AF424979) from *B.t.* sv. *kenyae* strain LBIT-82 (98.1%), chitinase (U89796) from *B.t.* sv. *pakistani* (83%), chitinase (AY189740) from *B.t.* sv. *kurstaki* (97.8%), chitinase (AF526379) from *B.t.* sv. *israelensis* (97.6%), chitinase (AY074882) from *B.t.* strain (98.1%), and chitinase (AY129671) from *B.t.* sv. *sotto* (98.8%).

BLAST search of Blichi (1.7 kb, 563 aa) from the present study also showed high degree of amino acid similarity to the sequences of similar species, Blichi was 90 % similar to *B. licheniformis* (AAB47847) of the reported sequence for which primers were designed, 99 % to *B. licheniformis* (ACK44109.1), 94 % to *B. subtilis* (ABG57262.1) and 50 % to *B. thuringiensis* (AAL71886). Protein sequence alignment revealed 50 % identity between Btchi and Blichi, domain organization of both chitinases revealed N-terminal GH 18, fibronectin type III domain, and C-terminal carbohydrate binding domain CBD 2 and CBD 5-12 for Btchi and Blichi, respectively. Clearly, thus, Btchi and Blichi are different sequences and therefore, were selected for domain swapping.

Btchi and Blichi are the resultant proteins expressed from constructs pANE-Btchi and pANE-Blichi, respectively. Chitinases from *B. thuringiensis* are poorly studied (Barboza Corona *et al*., 1999). Chitinase could enhance the larvicidal process and might be used to improve the pesticidal activity of *B. thuringiensis* (Wiwat *et al*., 2000). Kinetic parameters were evaluated for full length Blchi1 using substrates 4MU-$(GlcNAc)_{2,}$ 4MU-$(GlcNAc)_3$ and α-chitin (Chuang *et al*., 2008), BlChi1 exhibited approximately two-fold higher K_m and k_{cat} values toward the insoluble α -chitin substrate compared to those of deletion derivatives. K_m and V_{max} of *B. thuringiensis* sv. *aizawai* and Chi72 of *B. licheniformis* were determined recently (de la Vega *et al*. 2006; Kudan and Pichiyangkura, 2009) using colloidal chitin and were significantly different from the present analysis of Btchi and Blichi using colloidal chitin. Kinetic analysis of Btchi and

Blichi using colloidal chitin revealed that the V_{max} and k_{cat} were high for Btchi and were significantly low with Blichi indicating that Btchi could be more efficient in degrading colloidal chitin than Blichi (Fig. 3.4). Btchi also exhibited greater affinity towards colloidal chitin than Blichi. The difference in the kinetic parameters of Btchi and Blichi could be attributed to the difference in the amino acid sequence. More specifically, the variation in the affinity of the two chitinases could be due to amino acid sequence variation that imparts a strong or weak binding affinity towards the polymeric substrate or a more or less efficient catalytic process.

Effect of temperature on the activity of Btchi and Blichi (Fig. 3.5 & 3.6) further revealed the thermal tolerance of these two proteins would facilitate upgrading the recombinant protein to the application level. An optimum temperature of 60 $^{\circ}$C for Btchi was similar to 57.2 $^{\circ}$C for chiA74 of *B. thuringiensis* (Barboza Corona *et al*., 2003), whereas *B. thuringiensis* subsp. *aizawai* displayed maximum activity at 50 $^{\circ}$C. On the other hand, Blichi was optimally active at 60 $^{\circ}$C similar to BlChi1 (Chuang *et al*., 2008) which had 50-60 $^{\circ}$C. Clearly, optimum temperature of Btchi and Blichi was in the same temperature range reported for other chitinases from this species.

High-resolution structures of CatD, FnIIID-2, and ChBD of ChiA1 have been determined separately (Matsumoto *et al*., 1999; Jee *et al*., 2002; Ikegami *et al*., 2000). Structural information on a full-length glycoside hydrolase containing FnIIID between ChBD and CatD of *B. circulans* was reported by Toratani *et al*. (2006). Chuang *et al*. (2008) explained about the thermal stability of Blchi1 using CD analysis and concluded that the deletion derivatives were more thermo stable than the full length protein. In the present study, an attempt was made to predict the effect of temperature on the relative secondary structural changes in Btchi and Blichi using CD analysis that would further explain the structure-function relationship of these chitinases. Secondary structure prediction showed that α helices were predominant in Btchi and Blichi. Relative change in the secondary structure of Btchi and Blichi, with gradual temperature increase, revealed that both these proteins retained conformation up to 60 $^{\circ}$C; further increase in temperature disturbed the integrity of the protein conformation which directly affected the activity. Btchi did not retain activity at different temperatures as evident with loss of

specific activity with pre-incubations at different temperatures (Fig. 3.5B). At the same time, Btchi was thermally stable in its secondary structure as observed from the CD analysis, probably due to the temperature-dependent changes in the active site of the enzyme that affected only the activity but not the secondary structure. Blichi was thermally active while it could not retain conformational stability leading to proportionate increase in random coils, probably due to the unaffected active site at different temperatures that made Blichi thermally stable.

The activity of Btchi and Blichi at 60 $^{\circ}$C revealed that former was superior in stablility while later was not stable as it could retain its activity only for 2 h, similar to the Chi72 of *B. licheniformis* described by Kudan and Pichiyangkura (2009). Both Btchi and Blichi responded differently at 60 $^{\circ}$C, probably because of the difference in the amino acid sequence. Both Btchi and Blichi were unstable in their protein conformation, as both of them exhibited similarity in the relative change in secondary structure at 60 $^{\circ}$C. Both Btchi and Blichi retained the integrity with gradual increase in temperature up to 60 $^{\circ}$C but they were not stable at this temperature. The specific activity of the two chitinases at 60 $^{\circ}$C was higher when incubated together with substrate than when the pre-incubations of enzyme alone was followed by the enzyme assay, suggesting that the substrate conferred stability to the enzyme. CD analysis-driven secondary structure prediction, to study the influence of temperature on bacterial chitinases, described in the present study opens a new angle in chitinase research.

Barboza Corona *et al*. (2003) reported chiA74 from *B. thuringiensis* to be active in a wide pH range (4 to 9), whereas, *B.t.* sv. *aizawai* was optimally active at pH 5.0 (de la Vega *et al*., 2006) similar to Blchi1 of *B. licheniformis* (Chuang *et al*., 2008). Btchi and Blichi (Fig. 3.7) exhibited different pH-activity profile with colloidal chitin, where both showed best activity in the pH range of 4.0-6.0. Btchi displayed maximum activity in sodium acetate buffer, Blichi responded to sodium citrate and acetate buffer suggesting an influence of buffer on the activity of the two chitinases. Additionally, both chitinases showed significant activity at pH 3.0 and 9.0 indicating that the assumption made by Kawase *et al*. (2006) holds good in this case as well. Extremely low or high pH could lead to conformational changes which make the top of the catalytic cleft wider or the cleft

structure more flexible, which in turn results in long chain soluble substrate easily entering into the catalytic cleft and the chance of hydrolysis thereby increases.

Chitinases are reported to hydrolyze chitosan, the deacetylated form of chitin. Chitosans are very valuable substrates for in depth studies of processivity in family 18 chitinases (Sikorski *et al*., 2006). Acetylation could be random or blockwise in chitosan. Hydrolyzing efficiency of Btchi and Blichi towards chitosan with varying DA was determined for the first time in *Bacillus* spp. Both the native chitinases preferred higher DA chitosan (Fig. 3.8), and their activity readily decreased with decrease in DA.

Binding studies are limited in chitinases of *B. thuringiensis*, while Chuang *et al*. (2008) explained that the full length Blchi1 and the deletion derivatives were similar in their hydrolysis of insoluble chitin substrates. In the present study, Both Btchi and Blichi bound to α chitin; the binding affinity of the later being superior(Fig. 3.9).The difference in the binding ability could be due to the substrate binding domains that belonged to different CBD families in these two chitinases. Both Btchi and Blichi showed affinity to glycol chitin when compared with the control (Fig. 3.10). The substrate binding domain (ChBD) of Btchi belongs to CBD 2 family according to CAZY database; their characteristic features include the ability of cellulose binding which is overruled in Btchi as retarded mobility was not seen in CM-cellulose containing gels similar to *B. thuringiensis* chitinase that did not hydrolyse soluble substrates like, CM-cellulose and laminarin other than gycol chitin (de la Vega *et al*., 2006). The chitin binding domain (ChBD) of Blichi belongs to CBD 5-12, that is found in most of the bacterial chitinases. Affinity of Blichi towards glycol chitin, and not to CM-cellulose and laminarin indicated the specificity of Blichi towards chitinous substrates.

Chitooligosaccharides with degree of ploymerisation (DP) of 4 & 6, *i.e*., chitotetraose and chitohexaose are good substrates of both chitinases (Fig. 3.11) although Btchi showed higher reaction velocities with these two substrates, while chitotriose was not hydrolysed by both these chitinases. When Btchi hydrolysed chitotetraose, formation of chitohexaose pointed to a transglycosylation activity, a phenomenon not known for

chitinase of *B. thuringiensis*. Extensive product analysis would allow understanding the detailed mechanism(s) behind transglycosylation.

Btchi hydrolysed chitin polymers (colloidal chitin, β and α chitin) into oligomers ranging from 1-6 (monomer to hexamer) indicating the endo-type of hydrolysis, similar pattern was observed with Blichi on β chitin. Chitobiose and N-acteylglucosamine to a smaller extent were released from colloidal chitin and α chitin revealing the exo-type of hydrolysis with Blichi. The hydrolysis products of Btchi and Blichi (Fig. 3.12) revealed that Btchi is an endo-chitinase and Blichi is an exo-chitinase except on β chitin where it is endo-chitinase. de la Vega *et al*. (2006) characterized chitinase from *B. thuringiensis* that hydrolyses colloidal chitin and releases $(GlcNAc)_2$ as the major end product explaining the exo-mode of action.

While the domain organization of Btchi and Blichi was similar, these two enzymes exhibit properties that were significantly different due to the different CBD present as per CAZY database. Major differences in the properties of the two enzymes prompted us to investigate the role of individual domains and focus on the compatibility of the domains by adopting domain swapping as a strategy.

4.3 CONSTRUCTION AND CHARACTERIZATION OF CHITINASE CHIMERAS - REPLACING THE ChBD OF *B. THURINGIENSIS* AND *B. LICHENIFORMIS* CHITINASES BY CeBD OF ENDOGLUCANASE (DOMAIN SWAPPING).

Structurally, cellulose and chitin are very similar, and the CBD present in the hydrolyzing enzymes was either ChBD or CeBD that share several features in common. There are some CeBDs that have affinity to chitin (Linder *et al*., 1996). Chitinases were generated with strong chitin binding capacity by fusing a ChBD from *Nicotiana tabacum* and a CeBD from the cellobiohydrolase II gene of *Trichoderma reesei* to *T. harzianum* chitinase (Limon *et al*., 2001). Limon *et al*. (2004) showed increased antifungal and chitinase activities of *T. harzianum* by addition of CeBD to the chitinases that lack a ChBD. Bacterial hybrid chitinases based on the addition of CeBD were not known to

explain the role of ChBD and to what extent CeBD can substitute ChBD in bacterial chitinases.

In the present study, an endoglucanase encoding gene was amplified from *B. licheniformis* and cloned in expression vector pET22b+, sequence analysis followed by domain analysis showed that endoglucanase consists of a GH 5 catalytic domain and cellulose binding domain (CeBD) that belongs to family CBD 3. Multiple alignment of CBD 3, CBD 2 and CBD 5-12 of Btchi and Blichi showed that ChBD of chitinases and CeBD do not share homology except for few amino acid residues. CeBD essentially binds to cellulose (Linder *et al*., 1995). Due to structural similarity of chitin and cellulose, CeBD could also impart binding ability towards chitin. Hence we have selected CeBD as an alternative binding module from endoglucanase for domain swapping. Chimeric chitinases were constructed with CeBD from endoglucanse fused to the C-terminus of GH from Btchi or Blichi. Btegln and Bliegln are the resultant proteins expressed from the constructs pANE-Btegln and pANE-Bliegln, respectively.

The V_{max} and k_{cat} for Bliegln increased while specific activity of Bliegln decreased compared to Blichi, possibly due to the reduced affinity of Bliegln as a result of domain swapping and also the presence of CeBD as substrate binding module. Temperature tolerance of Bliegln would be helpful to understand the modifications and structural features of Bliegln. There was a shift in the optimum temperature of Bliegln while Blichi showed 60 $^{\circ}$C as its optimum suggesting that these two enzymes bahaved differently. The specific activity of Bliegln was less compared to Blichi. Further, secondary structure prediction using CD analysis showed that α helices were predominant in Bliegln; the ratio being less when compared with Blichi. Domain swapping brought structural changes that affected the catalysis and substrate binding. Relative change in the secondary structure of Bliegln with gradual temperature increase resulted in slow and gradual decrease in α helices that could be attributed to the stability of the enzyme. Bliegln was thermally more stable than Blichi as it could retain the activity with pre-incubations at different temperatures. Bliegln exhibited difference in protein folding due to the modifications and also retained 50 % activity at longer time points. A steady decrease in α helices did not affect the enzymatic activity during pre incubations or in

prolonged incubations indicating the stablility of Bliegln. The reduced specific activity of Bliegln over Blichi explains the lower affinity due to the presence of CeBD 3. In contrast to Blichi, Bliegln proves to be resistant to the pre-incubations. Hence, domain swapping made Bliegln thermally stable than Blichi.

Kawase *et al*. (2006) stated that extremely low pH leads to conformational changes which make the top of the catalytic cleft wider or the cleft structure more flexible, which in turn results in long chain soluble substrate easily entering into the catalytic cleft and the chance of hydrolysis hence increases. Bliegln exhibited a different pH-activity profile compared to native chitinases (Fig. 3.20). Significant activity was obtained at pH 3.0 and 4.0. Blichi had significant activity in alkaline range also, which was not seen with Bliegln, indicating that the swapping resulted in protein folding that was different from Blichi, which narrowed down the pH range of Bliegln.

Bliegln bound to α chitin similar to Blichi, affinity being higher at low concentrations, whereas it showed lower affinity to β chitin (Fig. 3.21). The difference in the binding ability of Bliegln and Blichi could be due to the substrate binding domains that belonged to different CBD families present in these chitinases. Limon *et al*. (2004) reported increase in the activity of the chitinases by the addition of CeBD. Bliegln (Fig. 3.22) showed affinity to glycol chitin and not to CM-cellulose and laminarin, similar to that of Blichi, indicating that the cellulose binding ability of CBD 3 in Bliegln did not affect the binding properties of the chimeric chitinase.

Chitotriose was not hydrolysed by Bliegln similar to Blichi. Bliegln hydrolysed chitohexose with high reaction velocity than Blichi, whereas the later proved to hydrolyse chitotetraose more efficiently. Bliegln hydrolyses β chitin polymer into oligomers ranging from 1-6 (monomer to hexamer) indicating the endo-type of hydrolysis, similar to Blichi whereas chitobiose was the end product of α chitin hydrolysis revealing the exo-type of Bliegln hydrolysis. Addition of cellulose binding domain to GH of Blichi resulted in a thermostable enzyme with reduced velocity of reaction in hydrolyzing chitooligomers and binding ability to soluble substrates remain not affected.

4.4 CONSTRUCTION AND CHARACTERIZATION OF CHITINASE CHIMERAS - REPLACING THE ChBD OF *B. THURINGIENSIS WITH* ChBD OF *B. LICHENIFORMIS AND vice versa* (DOMAIN SWAPPING).

Novel approaches in protein engineering have emerged in recent years to improve the existing properties of enzymes. Chimeric constructs, between the enzymes from two different plants, formed hybrids despite the fact that they were separated by speciation (Boehlein *et al*., 2005). Domain swapping between a cyanobacterial and a plant subunit ADP-glucose pyrophosphorylase was initiated by Iglesias *et al*. (2006). Btchi and Blichi are well characterized and different in several enzymatic properties. Domain swapping was adopted to alter/improve the existing enzyme properties. Chimeric chitinases, Btswap (1.8 kb) and Bliswap (1.7 kb) were constructed by exchanging the ChBD between *Btchi* and *Blichi*. Btswap and Bliswap are the resultant proteins expressed from constructs pANE-Btswap and pANE-Bliswap, respectively.

Kinetic analysis of Btswap and Bliswap using colloidal chitin revealed high V_{max} and k_{cat} for Btswap and low with Bliswap (Fig. 3.27) indicating that Btswap is more efficient in hydrolysing colloidal chitin than Bliswap. Bliswap showed higher affinity to colloidal chitin than Btswap. On the contrary, Btswap having CBD 5-12 from Blichi had decreased affinity to colloidal chitin.

The specific activity of Btswap and Bliswap showed significant differences from native chitinases (Fig. 3.28 & 3.29). Bliswap was similar to Blichi in responding to different temperatures incubated with the substrate or to pre-incubations followed by activity analysis. On the other hand, Btswap showed maximum activity in pre-incubations compared to enzyme incubated with substrate, indicating that Btswap had acquired relatively new protein fold different from Btchi. Bliswap showed an elevated activity when compared with Blichi probably due to the different structure obtained due to swapping. While Bliswap showed an optimum temperature similar to that of Blichi, that of Btswap showed decreased to 40 $^{\circ}$C. The secondary structure of Btswap and

Bliswap were predominant with α helices similar to that of Btchi and Blichi. Gradual increase in temperature revealed that Btswap retained its structural integrity up to 40 °C and Bliswap up to 60 °C indicating that the later is similar to Blichi and the former is different in its secondary structure. Btswap responded to pre-incubations different from Btchi. Bliswap retained its structural integrity up to 60 °C and sudden drop in α helices was similar to Btchi. Bliswap is active at its optimum temperature for longer period than Blichi. Btswap was stable in its activity and the secondary structure was unaffected at 40 °C for long periods indicating that domain swapping has affected the protein fold. Bliswap retained its secondary structure at 60 °C for 2 h and the activity decreased different from that of Blichi. The two chimeric chitinases retained their secondary structure and activity at optimum temperatures and hence are stable when compared with the native chitinases.

Exchange of the ChBD between the two enzymes did not change the pH profile (Fig. 3.30) for Bliswap, while the pH optimum for Btswap clearly shifted to a pH range of 5.0 to 8.0. The most dramatic effect was the total loss of activity of Btswap at pH 4.0. Btswap had an optimum pH of 6.0 which is quite different from Btchi whereas Bliswap responded equally well to sodium citrate and acetate buffers, while Blichi showed significant difference in both the buffers.

The most striking effect was observed in the ability of the chimeric proteins to bind to insoluble substrates (Fig. 3.31). While the affinity towards α chitin decreased slightly, both chimeric enzymes bound to β chitin more efficiently. The exchange of the ChBD resulted in chitinase chimeras with different protein fold as evident from their response to different temperatures. The new combination of catalytic and substrate binding domain resulted in a different conformation that explains the increased binding ability to insoluble substrates. The reasons why these chitinase chimeras are effective to β chitin and not to α is yet to be investigated, probably chitin conformation plays a role which makes them less effective on α chitin. Both Btswap and Bliswap have strong affinity (Fig. 3.32) to glycol chitin and the swapping did not bring affinity towards laminarin or CM-cellulose indicating that the two chitinase chimeras are specific towards chitinous substrates.

Chitotetraose and chitohexaose are good substrates of Btswap, while Bliswap was effective on chitohexaose only (Fig. 3.33 & 3.34). Bliswap did not hydrolyse chitotetraose and chitotriose, possibly swapping has altered the conformation of the subsite present in the catalytic cleft. Btswap had slow velocity of reaction on chitohexaose similar to Blichi. Btswap hydrolysed chitotetraose with slow velocity of reaction similar to Blichi, formation of chitohexaose further indicates that Btswap has transglycosylation activity similar to Btchi. Domain swapping did not disturb the GH 18 domain of either of the chitinase chimeras, it did not affect transglycosylation with both Btswap and Btchi while Bliswap and Blichi had no such activity. Btswap hydrolysed chitotetraose with cleavage specificity different from Btchi as the trimer and monomers were not detected on TLC plates.

Both Btswap and Bliswap cleaved β chitin in endo- mode similar to native chitinases. Bliswap hydrolysed α chitin in exo-mode similar to Blichi while Btswap cleaved in endo-mode similar to Btchi. The intensity of the spots on TLC plates further indicated that Bliswap is similar to Btchi in α chitin hydrolysis, while Btswap is similar to Blichi. Domain swapping has resulted in two chitinase chimeras that are superior to native chitinases indicating that swapping could be a powerful tool to tailor the enzymes that would result in novel enzymes.

4.5 CONSTRUCTION OF CHITINASE CHIMERAS – FUSION OF CBD FROM CBP OF *B. THURINGIENSIS TO CHITINASE OF B. LICHENIFORMIS* (DOMAIN FUSION).

A bifunctional domain organization, consisting of a catalytic domain and a substrate binding domain connected by an extended linker region, is typical for enzymes degrading solid substrates. Linder *et al*. (1996) reported significant increase in affinity by the linkage of two CBDs as compared with either of the single CBDs. In this work, CBD was fused to the N-terminus of Blichi, or ChBD from Blichi and then the CBD was fused at the N-terminus. These were constructed to study the change in enzymatic properties of the fusion proteins. The additional CBD including FN3 to be fused was obtained from a chitin binding protein (CBP) of *Bacillus thuringiensis* which is a non catalytic protein

produced by chitinolytic organisms expected to fine-tune the insoluble substrate in a way that chitinases can effectively act on the solid chitinous substrates (Vaaje Kolstad *et al*., 2005).

cbp of 1.3 and 0.6 kb from *B. thuringiensis* and *B. licheniformis* gDNA resulted in constructs pANE-Btcbp and pANE-Blicbp, respectively. The domain organization of Btcbp is different from Blicbp, sequence analysis showed that Btcbp comprises of two CBDs belonging to different families *i.e*., ChBD 3 and CBD 5, inter-connected with two FN3 modules while Blicbp has ChBD3 alone.

The 2.6 and 2.1 kb fusion constructs resulted in pANE-NBtBlichi and pANE-NBtBliGH. Expression in *E. coli* using standard conditions with slight modifications resulted in the formation of two recombinant proteins with each construct. 12 % SDS-PAGE and Western blot showed two protein bands each with NBtBlichi and NBtBliGH with MW of 100, 74 and 74, 50 kDa, respectively, having similar C-terminus but different N-terminus, when compared with a standard protein weight marker (Fig. 3.39). Both the fusion constructs were added with a CBD and FN3 next to it at the N-terminus. The occurrence of two proteins from one fusion construct could be because of linker incompatibility. Linker engineering studies have shown that the length of the linker is important for the activity of some cellulases (Srisodsuk *et al*., 1993). The use of long linkers may result in low yield of active fusion protein since unprotected and flexible regions are often susceptible to proteolytic cleavage during recombinant protein production. Use of a shorter linker might overcome problems associated with protease degradation; short linkers could bring the modules too close to each other, resulting in loss of function (Gustavsson *et al*., 2001). The reasons being many that can explain the appearence of two proteins in the expression profile of the construct, a focused investigation could lead to a better understanding and perhaps add a little to the problem of linker engineering. Subsequent fusions at C-terminus has not resulted in the same phenomenon indicating that the linker compatibility holds good in the later case (unpublished data).

4.6 CONSTRUCTION AND CHARACTERIZATION OF CHITINASE CHIMERAS – DELETION OF ChBD FROM CHITINASES OF *B. THURINGIENSIS AND B. LICHENIFORMIS* (DOMAIN DELETION).

Deletion of the domain was adopted to understand the importance of the domain deleted (Lin *et al*., 2001; Chuang *et al*., 2008). We have also adopted a similar approach to understand and relate the properties of the chimeric constructs, previously made, to give more insights into the structure function relationship with reference to each domain. The 1.2 kb *GH* of *B. thuringiensis* and *B. licheniformis* were expressed as earlier and the expressed proteins were named as BtGH and BliGH, respectively. Kinetic analysis of BliGH using colloidal chitin revealed V_{max} and k_{cat} (Fig. 3.42) to be significantly high when compared to those of Blichi. Specific activity of BliGH was less than Blichi, this could be attributed to the reduced affinity of BliGH as a result of domain deletion. Domain deletion has lowered the affinity of BliGH and increased the V_{max} when compared with Blichi and Bliegln. Chuang *et al*. (2008) showed that deletion derivative Blchi4 had higher affinity towards α chitin than Blchi1 and k_{cat} was higher for native Blchi1 than Blchi4.

Temperature dependent secondary structure of BliGH would be helpful to understand the modifications undergone due to domain deletion. Optimum temperature of 40 $^{\circ}$C was observed with BliGH similar to Bliegln, while Blichi shows 60 $^{\circ}$C revealing the nature of the former chitinase being different from Blichi. Chuang *et al*. (2008) showed that the deletion derivatives had the same optimum temperature and were more thermo-stable compared to the full length sequences. The specific activity of BliGH was less than that of Blichi. Secondary structure prediction using CD analysis showed that α helices are predominant in BliGH, the ratio (α, β and random coil) being similar when compared with Blichi indicating that domain deletion has not disturbed the secondary structure but has affected the catalysis and substrate binding (Fig. 3.43). BliGH was different from Blichi in the specific activity with pre-incubations at different temperatures. Steady decrease in α helices in pre-incubations suggested that BliGH is temperature sensitive and is not stable at 60 $^{\circ}$C unlike Bliegln and Blichi to some extent. BliGH was not stable at 60 $^{\circ}$C as evident by the loss of activity, while Blichi could retain its activity for 2 h at

60 °C possibly due to a different protein fold upon deletion of CBD. The decreased specific activity of BliGH compared to Blichi explains the lowered affinity to substrate due to deletion of CBD.

The pH-activity profile of BliGH using colloidal chitin was different from native chitinases (Fig. 3.44). Significant activity of BliGH was obtained at pH 4.0 and 7.0, while Chuang *et al*. (2008) observed an optimum at pH 5.0 with both deletion and full length chitinases. Domain deletion has resulted in protein folding different from Blichi, which narrows down the pH range of BliGH.

The hydrolyzing ability of Blchi1 and deletion derivatives towards insoluble substrates was similar (Chuang *et al*., 2008). BliGH bound to α chitin similar to Blichi, affinity being maximum in high concentrations, and it showed stronger affinity to β chitin than to α chitin indicating that in spite of the deletion, BliGH could display minimum binding (Fig. 3.45). BliGH showed affinity to glycol chitin similar to Blichi. In spite of the deletion of CBD, BliGH showed similar properties to Blichi

BliGH hydrolysed chitohexaose and chitotetraose with higher reaction velocity than Blichi. The GH of chitinase was effective on oligomers and not on polymers due to the absence of CBD as reported by Chuang *et al*. (2008). Chitotriose was not hydrolysed by BliGH similar to Blichi indicating that the subsite in the catalytic cleft might not be able to accept the trimers. BliGH hydrolyses β chitin polymer into oligomers ranging from DP 1 to 6 (monomer to hexamer) indicating an endo-type of hydrolysis, similar to Blichi whereas chitobiose was the end-product with α chitin revealing the exo type of BliGH hydrolysis. Although the pattern of end-products seems to be similar even after deletion, there could be difference in the rate of reaction on other substrates as seen with colloidal chitin. As we have not further investigated the velocity of reaction on other polymers, there is a wide scope to understand the mechanism in detail. Deletion of CBD from Blichi had revealed that the enzyme was slow in its rate of reaction on chitinous polymers and lowered affinity was observed when compared with native chitinases, but both were equally effective on chito oligomers.

In spite of the fact that diverse domain organizations are exhibited by bacteria to efficiently degrade the polymer, too little is known about the chitinases that are exploited in the industries. Present study was focused on cloning and characterization of chitinases from *B. thuringiensis* and *B. licheniformis* to study substrate binding and hydrolysis that was subsequently extended into domain swapping studies. Chimeric chitinases were constructed based on swapping, fusion and deletion as a strategy. Further studies focusing on sequence to structure relationship would be highly interesting as it can lead to tailor the enzyme *In silico* with appropriate functional requirements according to its application. This approach which is new in chitinase engineering will probably allow designing novel chitinases specific to applications.

SUMMARY AND CONCLUSIONS

5.1 BACKGROUND AND OBJECTIVE

Chitin is a homopolymer consisting of N-acetylglucosamine units linked with β-1,4-glycosidic bonds, present abundantly in nature and next in total mass only to cellulose. Chitin is present in fungal cell walls as well as insect exoskeleton and it is also a major component in other systems. Many micro-organisms are known to evolve a sophisticated chitinolytic system in order to effectively degrade the available chitin biomass, thereby generating products that are useful for the human well-being. Chitinases are the hydrolyzing enzymes that degrade chitin, multiple chitinases are characteristically produced to convert chitin into useful oligomers. The chitinolytic system of bacteria also includes chitin binding proteins which are non-catalytic proteins, helping chitinases in a synergistic manner for efficient hydrolysis of chitin. Many bacterial genera have evolved chitinases with different structure and function. The present study aims at identifying chitinolytic bacteria, cloning the chitinase encoding genes from selected *Bacillus* sps, domain based swapping of the chitinase encoding genes followed by characterization in order to understand the role of individual domain and also add note on the compatibility of the domains in the multi-domain protein of chitinases.

5.2 SELECTION OF EFFICIENT CHITINOLYTIC BACTERIA

Bacterial cultures procured from IMTECH or from our lab collection were screened for chitinolytic ability on CHDA medium supplemented with colloidal chitin. Among 18 cultures tested, six cultures were chitinase positive as evident from the formation of clearance zones. Chitinolytic *Bacillus licheniformis* and *Bacillus thuringiensis* strains were selected for the present work.

5.3 CLONING AND CHARACTERIZATION OF CHITINASE FROM *B. THURINGIENSIS KURSTAKI* AND *B. LICHENIFORMIS* DSM 13

Chitinase encoding gene (*chi*) was cloned from *Bacillus licheniformis* (Blichi) and *Bacillus thuringiensis* (Btchi). Amplicons of 1.9 kb (Btchi) and 1.7 kb (Blichi) were

cloned into the expression vector pET 22b+ resulting in pANE-Btchi and pANE-Blichi. Expression of the above plasmids in *E. coli* strain BL21 (DE3) resulted in 74 and 70 kDa proteins of Btchi and Blichi, respectively. Subsequent characterization revealed that the two proteins were similar in optimum temperature, secondary structure (predominant with α helices), preference for high DA chitosan and affinity to glycol chitin, while, the pH profile, product profile, stability and kinetic parameters were dissimilar.

The differences in the properties of Btchi and Blichi prompted us to analyze the domain organization to see if the properties that were varying among the two could be linked to domains and their organization.

5.4 CONSTRUCTION AND CHARACTERIZATION OF CHITINASE CHIMERAS - REPLACING THE ChBD OF *B. THURINGIENSIS* AND *B. LICHENIFORMIS* CHITINASES BY CeBD OF ENDOGLUCANASE (DOMAIN SWAPPING)

The 1.8 kb fusion of the hydrolytic domain (*GH*) from *chi* of *B. thuringiensis*, and CeBD (CBD)3, the cellulose binding domain from the endoglucanase *egln*, was PCR-amplified, fused, cloned and the construct was termed as pANE-Btegln (fusion Btegln in pET 22b+). The 1.8 kb fusion *chi* of *B. licheniformis* (GH) and CeBD was PCR-amplified, fused, cloned and the construct was termed as pANE-Bliegln (fusion Bliegln in pET 22b+). Expression of the above plasmids in BL21 (DE3) resulted in 74 kDa each of Btegln and Bliegln, respectively.

Characterization of chimeric protein Bliegln and its comparison with native Blichi revealed that the former had different kinetic properties, optimum pH and temperature, structural characteristics as revealed by CD analysis and insoluble substrate binding abilities when compared with Blichi. Bliegln showed a similar product profile from the hydrolysis of oligomers and polymers, although significant deviation in the velocity of reaction was observed indicating that upon domain swapping, CeBD has brought considerable changes in the structure and function of Bliegln.

5.5 CONSTRUCTION AND CHARACTERIZATION OF CHITINASE CHIMERAS - REPLACING THE CBD OF *B. THURINGIENSIS WITH* CBD OF *B. LICHENIFORMIS AND vice versa* (DOMAIN SWAPPING)

The 1.8 kb fusion *chi* of *B. thuringiensis* (GH) and the chitin binding domain ChBD (CBD 5-12) from Blichi was PCR-amplified, fused, cloned and the construct was designated as pANE-Btswap (fusion Btswap in pET 22b+). The 1.7 kb fusion *chi* of *B. licheniformis* (GH) and ChBD (CBD 2) from Btchi was PCR-amplified, fused, cloned and the construct was designated as pANE-Bliswap (fusion Bliswap in pET 22b+). Expression of the above plasmids in BL21 (DE3) resulted in 74 and 70 kDa each of Btswap and Bliswap, respectively.

Characterization of Btswap and Bliswap in comparison with native chitinases (Btchi and Blichi) revealed that Btswap and Bliswap showed improved kinetic properties, pH profile, optimum temperature, and secondary structure, as well as increased binding to β chitin different from the native chitinases indicating that exchange of ChBD between Btchi and Blichi resulted in Btswap and Bliswap enhanced binding to insoluble substrates which was low in the native chitinases.

5.6 CONSTRUCTION OF CHITINASE CHIMERAS – FUSION OF CBD FROM CBP OF *B. THURINGIENSIS TO CHITINASE OF B. LICHENIFORMIS* (DOMAIN FUSION)

The 2.6 kb fusion *chi* of *B. licheniformis* and the chitin binding domain ChBD 3 including FN III from the chitin binding protein Btcbp was PCR-amplified, fused, cloned and the construct was termed as pANE-NBtBlichi (fusion NBtBlichi in pET 22b+). The 2.1 kb fusion chimera was PCR-amplified by deleting CBD 5-12 from NBtBlichi, cloned and the construct was termed as pANE-NBtBliGH (fusion NBtBliGH in pET 22b+). Expression of the above plasmids in *E. coli* BL21 (DE3) resulted in two proteins in both cases, with MW of 100 and 74 kDa with NBtBlichi and 74 and 50 kDa with NBtBliGH,

having similar C-terminus but different N-terminus. The reasons behind these degradations resulting in two proteins need to be investigated.

5.7 CONSTRUCTION AND CHARACTERIZATION OF CHITINASE CHIMERAS – DELETION OF CBD FROM CHITINASES OF *B. THURINGIENSIS AND B. LICHENIFORMIS* (DOMAIN DELETION)

The 1.2 kb *GH* of *B. thuringiensis* (chi) was PCR-amplified, cloned and the construct was named as pANE-BtGH (GH of BtGH in pET 22b+). The 1.2 kb *GH* of *B. licheniformis* was PCR-amplified, cloned and the construct was named as pANE-BliGH (GH of BliGH in pET 22b+). Expression of the above plasmids in *E. coli* BL21 (DE3) resulted in 50 kDa each of BtGH and BliGH, respectively.

Characterization of chimeric protein BliGH and its comparison with native Blichi revealed that the two differed in kinetic properties, optimum pH and temperature. BliGH had increased velocity of reaction towards hydrolysis of oligomers, while its ability to bind to insoluble substrates was lost, as expected, due to the deletion of ChBD.

5.8 MAJOR FINDINGS IN THE PRESENT WORK

Summary of the cloning, expression and characterization of native and chimeric chitinases of *Bacillus*, is summarized in Table 3.1.

Cloning and characterization of Btchi and Blichi chitinases

- Chitinase and chitin binding protein encoding genes were cloned from *B. thuringiensis* and *B. licheniformis.*
- Chracterization of Btchi and Blichi revealed that both these enzymes had similar optimum temperature, activity on high DA chitosan, proportion of α helices and capability to bind to glycol chitin and α chitin.
- Affinity to colloidal chitin, transglycosylation ability, pH profile, thermostability and TLC product profile were different for Btchi and Blichi.

Domain swapping of Btchi and Blichi using CeBD of endoglucanase

- Cloning and characterization of Bliegln resulted in decreased affinity to colloidal chitin, different pH profile and optimum temperature, thermally stable protein that had similar product profile as that of Blichi.

Domain swapping by exchanging the ChBD of Btchi and Blichi

- Cloning and characterization of Btswap and Bliswap showed that Bliswap had increased affinity to colloidal chitin, Btswap was stable at 60 $^{\circ}$C for 4 h and both of them had increased binding to β chitin.

Domain fusion by adding the CBD of Btcbp to Blichi

- N-terminal addition of CBD from Btcbp to Blichi and BliGH resulted in two proteins, that had the same C-terminus but different N-terminus.

Domain deletion of CBD in Btchi and Blichi

- Cloning and characterization of BliGH resulted in decreased affinity to colloidal chitin and increased velocity of reaction towards hydrolysis of oligomers.

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Name of the organism	Purpose	Source
Gram-negative isolates		
Pseudomonas aeruginosa	Screening for chitinolysis	Kishore *et al*., 2004
Pseudomonas fluorescens	,,	MTCC, Chandigarh
Enterobacter asburiae	,,	Prof. G. Naresh kumar
Serratia marcescens	,,	Kishore *et al*., 2004
Serratia proteamaculans	,,	MTCC, Chandigarh
Stenotrophomonas maltophilia	,,	,,
Vibrio harveyi	,,	,,
Aeromonas hydrophila	,,	,,
Streptomyces griseus	,,	,,
E. coli (Top 10) [F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ^-]	Cloning host	Invitrogen
E. coli BL21 (DE3) [F^- ompT gal dcm lon hsdS_B(r_B^- m_B^-) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]	Expression host	Novagen
Gram-positive isolates		
Bacillus circulans	Screening for chitinolysis	MTCC, Chandigarh
Bacillus megaterium	,,	,,
Bacillus thuringiensis sv. *kurstaki*	screening for chitinolysis & isolation of *chi* and *cbp*	Prof. Aparna Dutta Gupta
Bacillus amyloliquifaciens	,,	MTCC, Chandigarh
Bacillus licheniformis DSM 13	screening for chitinolysis & isolation of *chi, egln* and *cbp*	Prof. Meinhardt, Muenster
Bacillus subtilis	,,	,,
Bacillus cereus	,,	,,
Bacillus circulans	,,	Kishore *et al*., 2004
Bacillus megaterium	,,	,,

Table 2.1: Details of the bacterial cultures screened for chitinolysis, used for the isolation of *chi, cbp, egln* genes and/or cloning and expression hosts

Plasmid	Size (kb)	Purpose/Description	Source
pET 22b+	5.5	Cloning & expression	Novagen
pANE-Blichi	7.2	*B. licheniformis chi* in pET 22b+	Present study
pANE-Btchi	7.4	*B. thuringiensis chi* in pET 22b+	,,
pANE-Blicbp	6.1	*B. licheniformis cbp* in pET 22b+	,,
pANE-Btcbp	6.8	*B. thuringiensis cbp* in pET 22b+	,,
pANE-egln	6.9	*B. licheniformis egln* in pET 22b+	,,
pANE-Bliegln	7.3	chimeric Bliegln in pET 22b+	,,
pANE-Btegln	7.3	chimeric Btegln in pET 22b+	,,
pANE-Bliswap	7.2	chimeric Bliswap in pET 22b+	,,
pANE-Btswap	7.3	chimeric Btswap in pET 22b+	,,
pANE-BliGH	6.7	*B. licheniformis GH* in pET 22b+	,,
pANE-BtGH	6.7	*B. thuringiensis GH* in pET 22b+	,,
pANE-NbtBlichi	8.1	chimeric NBtBlichi in pET 22b+	,,
pANE-NbtBliGH	7.6	chimeric NBtBliGH in pET 22b+	,,

Table 2.2: Details of the plasmids used in the present work

S.No	Name of the primer	Sequence 5'-3'	Restriction sites	Name of the gene	Name of the clone	Size of amplicon (~ kb)	Expected Protein Size (~ kDa)
1	BlchiFpmodnew	cgc gga tcc g gat tcc gga aaa aac tat aaa atc atc	*Bam*H I	*chi* from Bli	pANE-Blichi	1.7	70
2	BlchiRpmod	cg ctc gag ttc gca gcc tcc gat cag ccg	*Xho* I				
3	BtchiFpmodnew	cg gaa ttc g gat tca cca aag caa agt caa aaa att g	*Eco*R I	*chi* from Bt	pANE-Btchi	1.9	77
4	BtchiRpmod	cc aag ctt gtt ttc gct aat gac ggc att taa aag	*Hind* III				
5	BtchisalFor	cg gtc gac gat tca cca aag caa agt caa aaa att g	*Sal* I				
6	BleglnFpGH5 new	cgc gga tcc g gct tct aaa aca ccc gtt gct gta aac (37)	*Bam*H I	*egln* from Bli	pANE-egln	1.6	65
7	BleglnRpGH5	cg ctc gag ttt agg ttc agt gcc cca aat cag ctt tcc (38)	*Xho* I				
8	BlicbpFp	cg gtc gac atg gcc gcg tat gtt ttc ttt ttg cat c (36)	*Sal* I	*cbp* from Bli	pANE-Blicbp	0.6	21
9	BlicbpRp	cg ctc gag ttt gtt cac tag atc aac atc aat cac (35)	*Xho* I				
10	BthcbpFp	cg gtc gac atg aat aat cga tta tta aaa caa cta c (36)	*Sal* I	*cbp* from Bt	pANE-Btcbp	1.3	55
11	BthcbpRp	cg ctc gag cac tgt ttt cca taa tga taa ggc (32)	*Xho* I				

Table 2.3: Cloning details of *chi, cbp* and *egln* using gene specific primers

S. No	Name of the primer	Sequence (5'-3')	Template used for PCR	Name of the gene amplified	Size of the amplicon (Kb)	Primer combination for fusion	Rest. sites	Name of the plasmid	protein size (~kDa)
1	BtchisalFor	cg gtc gac gat tca cca aag caa agt caa aaa att g	pANE-Btchi	BtGH with overhang of egln	1.2	BtchisalFor BleglnRpGH5 (1.8 kb)	*Sal* I *Xho* I	pANE-Btegln	75
	BtGHOEglnRev	gcc tgg gtt taa agc atc ccc gct aag ctc							
2	EglncbmOEFor	gct tta aac cca ggc gcc tct aaa aac gga	pANE-egln	CBD from egln	0.6				
	BleglnRpGH5	cg ctc gag ttt agg ttc agt gcc cca aat cag ctt tcc							
3	BlchiFpmodnew	cgc gga tcc g gat tcc gga aaa aac tat aaa atc atc	pANE-Blichi	BliDSMGH with overhang of BliDSM egln	1.2	BlchiFpmodnew BleglnRpGH5 (1.8 kb)	*Bam*H I *Xho* I	pANE-Bliegln	75
	BlchiGHglnOE Rev	gcc tgg gtt taa agc atc gcc gct gaa atc							
4	EglncbmOEFor	gct tta aac cca ggc gcc tct aaa aac gga	pANE-egln	CBD from egln	0.6				
	BleglnRpGH5	cg ctc gag ttt agg ttc agt gcc cca aat cag ctt tcc							
5	BtchiBamHIFor	cg gga tcc g gat tca cca aag caa agt caa aaa att g	pANE-Btchi	BtGH with overhang of Blichi CBD	1.25	BtchisalFor BlchiRpmod (1.75 kb)	*Bam*H I *Xho* I	pANE-Btswap	71
	BtGH OESbl	aag cgt ccg att gga atc ccc gct aag ctc							
6	Blcbm OE	tcc aat cgg acg ctt ctc aat aaa ttg gca	pANE-Blichi	CBD from Blichi	0.5				
	BlchiRpmod	cg ctc gag ttc gca gcc tcc gat cag ccg							
7	BlchiFpmodnew	cgc gga tcc g gat tcc gga aaa aac tat aaa atc atc	pANE-Blichi	BliGH with overhang of Btchi CBD	1.2	BlchiFpmodnew BtchiRpmod (1.85 kb)	*Bam*H I *Hind* III	pANE-Bliswap	75
	BlGH OESbt	tgg act tgt acg gca atc gcc gct gaa atc							
8	Btcbm OE	tgc cgt aca agt cca aaa tat agt tgt agt	pANE-Btchi	CBD from Btchi	0.65				
	BtchiRpmod	cc aag ctt gtt ttc gct aat gac ggc att taa aag							

Table 2.4: List of primers used to construct chitinases using domain swapping

S. No	Name of the primer	Sequence (5'-3')	Template used for PCR	Name of the gene amplified	Size of the amplicon (Kb)	Primer combination for fusion	Rest. sites	Name of the plasmid	protein size (~kDa)
1	BthcbpFp	cg gtc gac atg aat aat cga tta tta aaa caa cta c	pANE-Btcbp	CBD from Btcbp with overhang of Blichi GH	0.9	Bthcbp Fp BlchiRpmod (2.6 kb)	*Sal* I *Xho* I	pANE-NBtBlichi	100
	BtcbpOE Rev	act ttc ttt cga tac att tcc agc tgc gtc							
2	BliGH OE For	gta tcg aaa gaa agt gat tcc gga aaa aac	pANE-Blichi	CBD from Blichi	1.7				
	BlchiRpmod	cg ctc gag ttc gca gcc tcc gat cag ccg							
3	BthcbpBamHI Fp	cg gga tcc g atg aat aat cga tta tta aaa caa cta c	pANE-NBtBlichi	C-terminal truncation of CBD from NBtcbpBlichi	2.1	Bthcbp Fp BlchiGH *Xho* I Rev (2.1 kb)	*Bam*H I *Xho* I	pANE-NBtBliGH	83
	BlchiGHXhoI Rev	cg ctc gag atc gcc gct gaa atc cca							
4	BtchiFpmodnew	cg gaa ttc g gat tca cca aag caa agt caa aaa att g	pANE-Btchi	BtGH	1.2	--	*ECoR I* *Hind* III	pANE-BtGH	52
	BtchiGHHindIII Rev	cc aag ctt atc ccc gct aag ctc cca							
5	BlchiFpmodnew	cgc gga tcc g gat tcc gga aaa aac tat aaa atc atc	pANE-Blichi	BliGH	1.2	--	*Bam*H I *Xho* I	pANE-BliGH	52
	BlchiGHXhoI Rev	cg ctc gag atc gcc gct gaa atc cca							

Table 2.5: List of primers used to construct chitinase chimeras using domain fusion and deletion

Properties	Btchi			Blichi			Bliegln			Btswap			Bliswap			BliGH		
V_{max} (nkat mg^{-1} protein)	0.051			0.022			0.034			0.042			0.035			0.064		
K_m (mg/ml)	5.36			6.92			13.47			4.57			3.23			68.38		
k_{cat} (sec^{-1})	0.014			0.005			0.009			0.010			0.10			0.011		
Temp. optima (°C)	60			60			40			40			60			40		
pH optima	5-6			4-7			3-4			5-6			4-6			3-4		
CD analysis	Thermo stable α- 52%, β-24%, RC-25%			Not thermo stable α- 64%, β-20%, RC-17%			Thermo stable α- 45%, β-27%, RC-26%			Stable at 40°C α- 56%, β-22%, RC-21%			Thermo stable α- 36%, β-31%, RC-31%			Not thermo stable α- 62%, β-18%, RC-20%		
Binding-Insoluble sub	β chitin ✓ α chitin			β chitin ✓α chitin			X β chitin ✓α chitin			✓β chitin ✓α chitin			✓β chitin ✓α chitin			X β chitin ✓ α chitin		
Affinity-Soluble sub	GC			GC			GC			GC			GC			GC		
TLC -oligomers	3mer -	4mer *	6mer +	3mer -	4mer +	6mer +	3mer _	4mer +	6mer +	3mer -	4mer *	6mer +	3mer -	4mer -	6mer +	3mer _	4mer + +	6mer + +
TLC-polymers (mode of action)	Exo- on α chitin Endo- on β chitin			Exo- on α chitin Endo- on β chitin			No hydrolysis on α chitin Endo- on β chitin			Exo- on α chitin Endo- on β chitin			Exo- on α chitin Endo- on β chitin			Exo- on α chitin Endo- on β chitin		

* Trans glycosylation, ++ increased velocity of reaction

Table 3.1: Summary of the properties of native and chimeric *Bacillus* chitinases

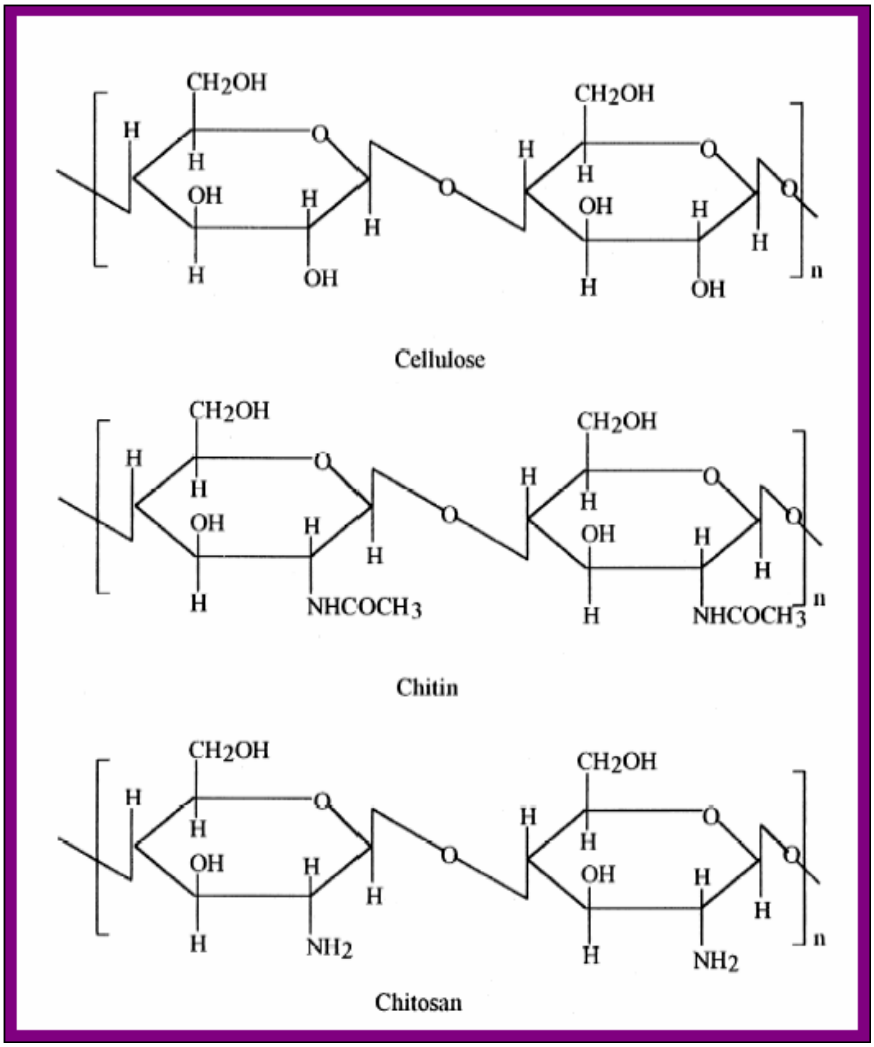


Fig. 1.1: Molecular structure of cellulose, chitin and chitosan (Ravi kumar, 2000)

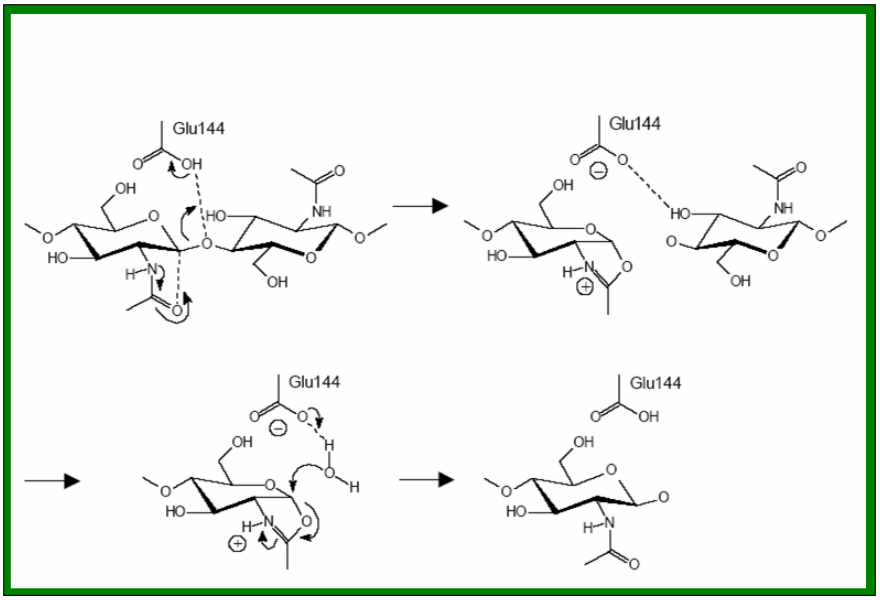


Fig. 1.2: Catalytic mechanism of family 18 chitinases (Brurberg *et al*., 2000)

Legend for Fig. 2.1

An initial round of PCR included the amplification of two individual amplicons from different templates one with the overhang complementary to the second to be fused either to N or C terminal of the first amplicon. Equimolar concentration of the two amplicons were used as the template for second round of PCR using the gene specific primers for the fused length of the gene.

Legend for Fig. 2.2

An initial round of PCR amplified *BtGH* and *BliGH* with C-terminal overhang and *CBD* amplicons individually from templates pANE-Btchi, pANE-Blichi and pANE-egln, respectively using gene specific primers described in Table 2.4. Equimolar concentration of the amplicons *BtGH* with C-terminal overhang and *CBD3* for *Btegln* and *BliGH* with C-terminal overhang and *CBD* for *Bliegln* were used as the template for the second round of PCR using the primers in Table 2.4 for the fused length of the gene.

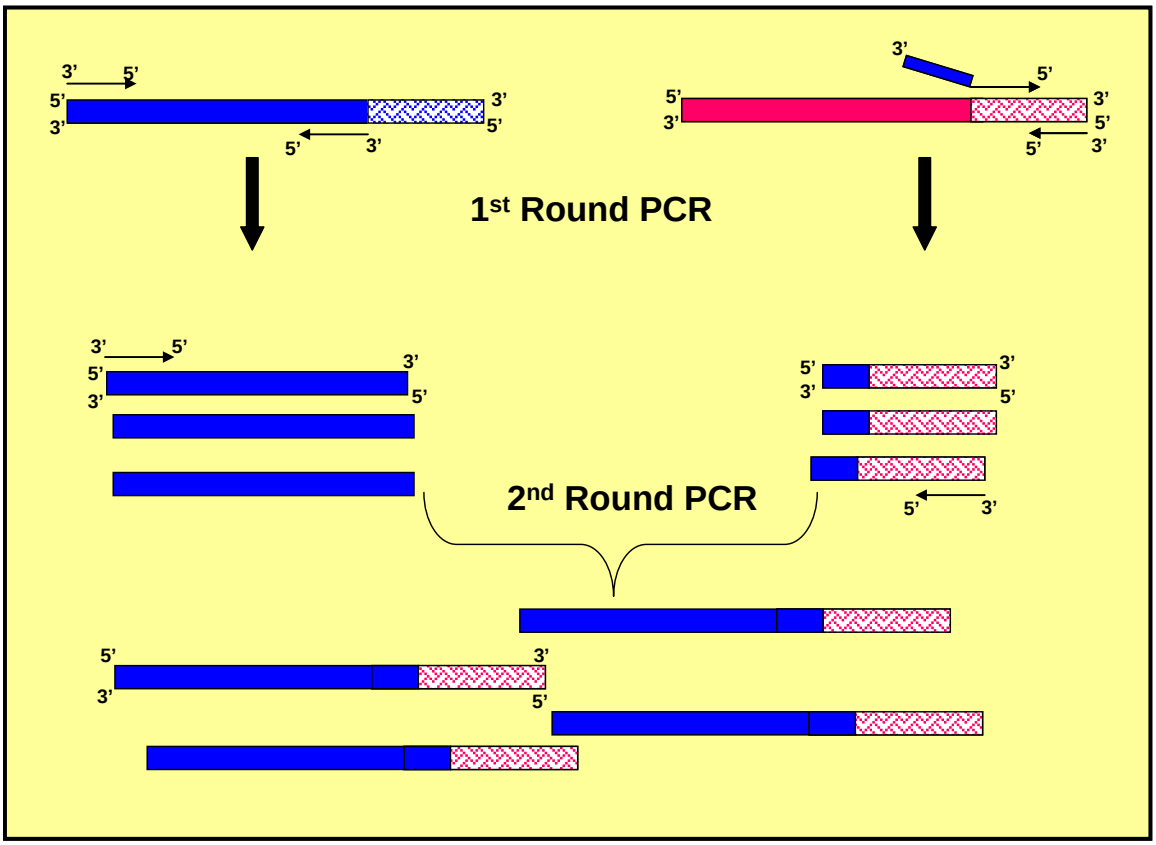


Fig. 2.1: Schematic representation of the protocol used to generate fusion chimera

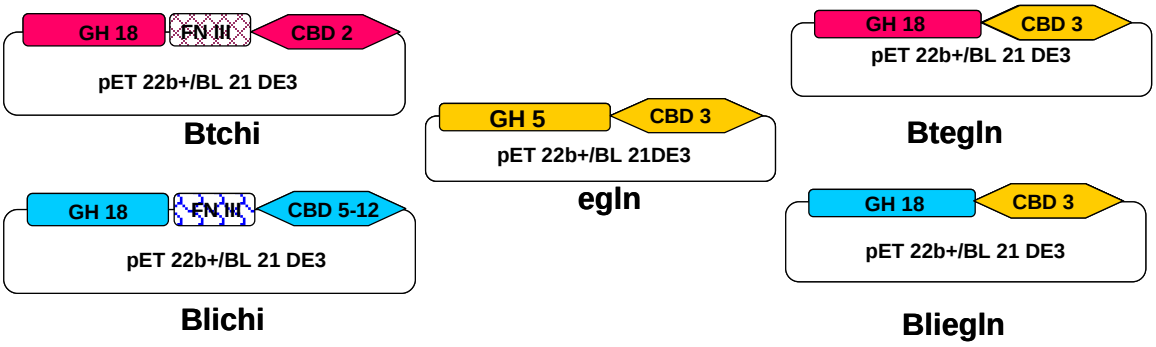


Fig. 2.2: Schematic representation of chimera construction by replacing the FN III and ChBD with CeBD

Legend for Fig. 2.3

An initial round of PCR amplified *GH* with C-terminal overhang and *CBD* from templates pANE-Btchi and pANE-Blichi using gene specific primers described in Table 2.4. Equimolar concentration of the amplicons *BtGH* with C-terminal overhang and *BliCBD* for *Btswap* and *BliGH* with C-terminal overhang and *CBD* for *Bliswap* were used as the template for the second round of PCR using the primers in Table 2.4 for the fused length of the gene.

Legend for Fig. 2.4

An initial round of PCR amplified N-terminal *CBD* (*NBtcbp*) and *Blichi* with N-terminal overhang from templates pANE-Btcbp and pANE-Blichi using gene specific primers described in Table 2.5. Equimolar concentration of the amplicons *NBtcbp* and *Blichi* with N-terminal overhang were used as the template for the second round of PCR using the primers in Table 2.5 for the fused length of *NBtBlichi*. Deletion of C-terminal CBD from *NBtBlichi* was amplified from template pANE-NBtBlichi resulting in *NBtBliGH* using primers in Table 2.5.

Legend for Fig. 2.5

GH was PCR amplified from templates pANE-Btchi and pANE-Blichi using gene specific primers described in Table 2.5 for the deletion constructs.

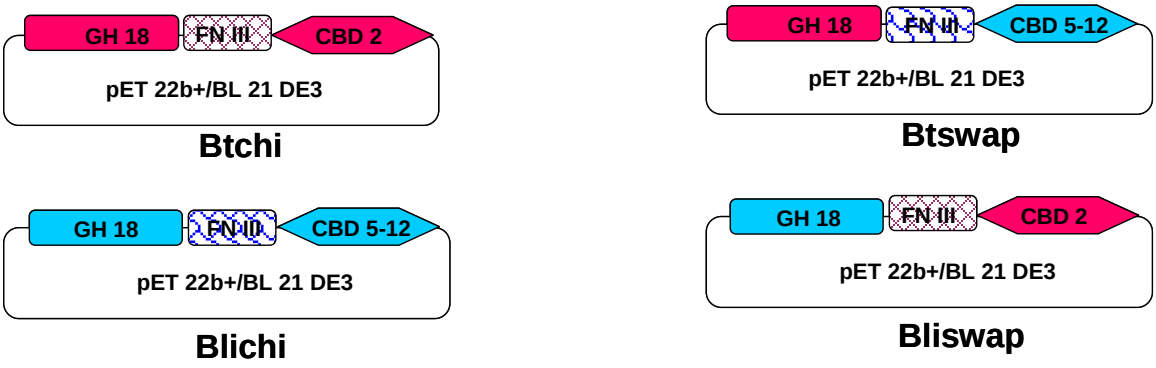


Fig. 2.3: Schematic representation of chimera construction by exchanging the FN III and CBD between Bt and Blichi

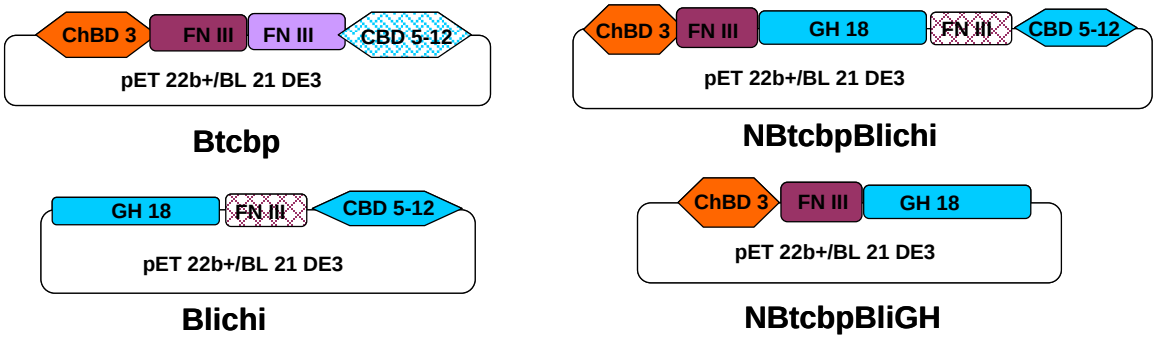


Fig. 2.4: Schematic representation of chimera construction by fusing the CBD and FN III to Blichi

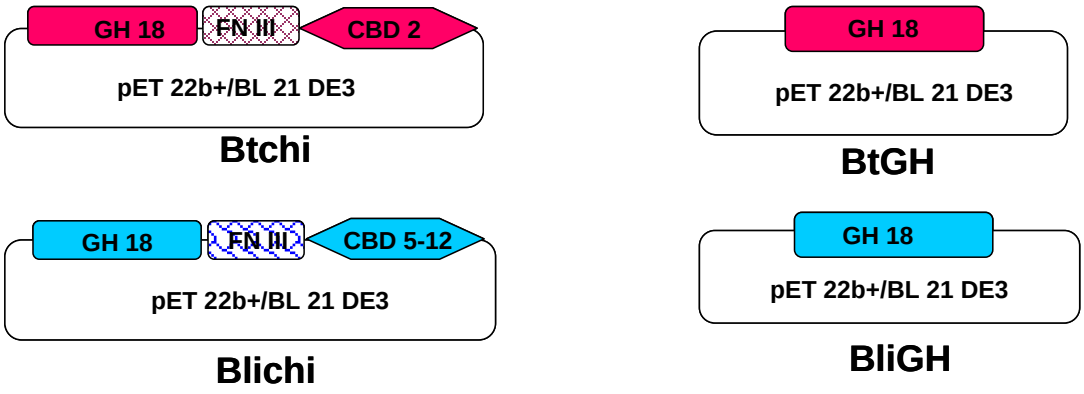


Fig. 2.5: Schematic representation of chimera construction by deleting the FN III AND CBD in Bt and Blichi

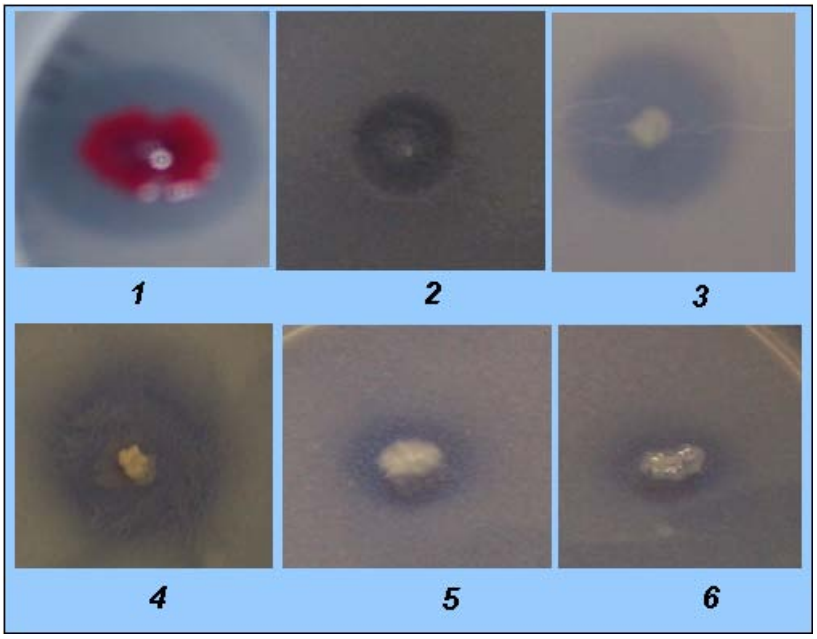


Fig. 3.1: Screening of chitinolytic bacteria on solid media

Bacterial cultures (1. *S. marcescens*, 2. *Stenotrophomonas maltophilia*, 3. *B. licheniformis*, 4. *B. circulans*, 5. *B. amyloliquefaciens*, 6. *B. thuringiensis*) were grown on colloidal chitin containing M9 minimal medium for 5 days. Zone of clearance was observed around the bacterial colony indicating the chitinolytic ability of the cultures.

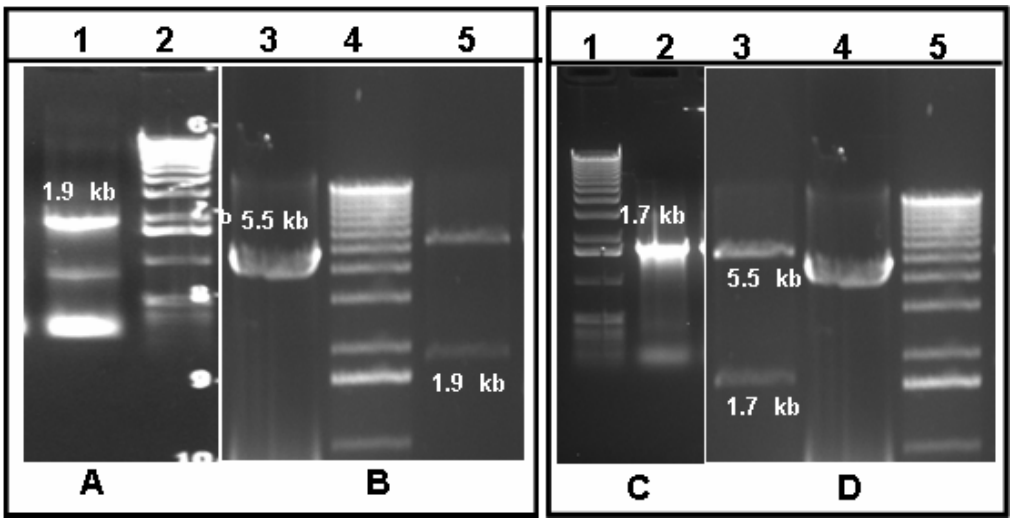
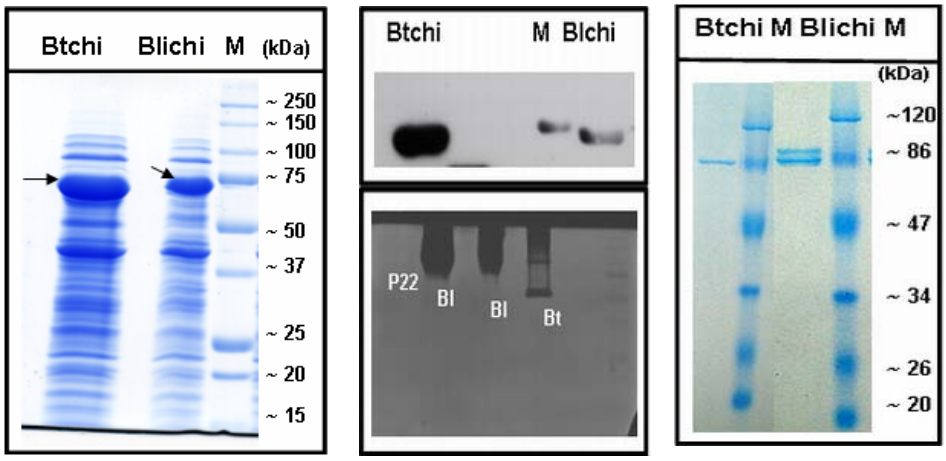


Fig. 3.2: PCR based amplification and cloning of chitinase (*chi*) from *B. thuringiensis* and *B. licheniformis*

A & C) Amplification of *chi* from gDNA of *B. thuringiensis* and *B. licheniformis* as template using primers BtchiFpmodnew and BtchiRpmod for Btchi (Lane A1), and BlchiFpmodnew and BlchiRpmod for Blichi (Lane C2). The amplicons were compared with 100 bp ladder (Lanes A2 & C1).

B & D) The amplicons were amplified and ligated to *Eco*R I and *Hind* III sites for *Btchi*, *Bam*H I and *Xho* I for *Blichi* in pET22b+ (Novagen) resulting in pANE-Btchi and pANE-Blichi, respectively. Double digestion of pANE-Btchi with *Eco*R I and *Hind* III and pANE-Blichi with *Bam*H I and *Xho* I released fragments of 1.9 (Lane B5) and 1.7 kb (Lane C3), respectively confirming the presence of the correct insert. *Eco*R I single digest of pET 22b+ (Lanes B3 & D4) served as control.



A **B** **C**

Fig. 3.3: Heterologous expression and purification of Btchi and Blichi using Ni-NTA chromatography

A) IPTG (1 mM) was used to induce the *E. coli* BL21 (DE3) at 37 °C harboring the vector pANE-Btchi or pANE-Blichi. Concentrated periplasmic fractions were loaded on 12 % SDS-PAGE and compared with the standard protein weight marker (M).

B) **Upper panel:** The periplasmic fractions were transferred onto the nitrocellulose membrane. Using Anti-His-tag antibody, the His tag fused to the C-terminus of the expressed protein was detected by chemiluminescent method. The His-tagged protein marker (M) has a molecular weight of 75 kDa. **Lower panel**: The periplasmic fractions were loaded on 12 % Semi-Native PAGE containing 0.1 % glycol chitin as a substrate in the resolving gel and were labeled as Bt, Bl (applied twice) and p22 (negative control). After incubation, washing and calcofluor staining, the gel was observed under UV light to detect chitinase activity.

C) Ni-NTA chromatography was used to purify the recombinant protein. Elution buffers with 50 and 100 mM imidazole was used to elute the protein. Fractions loaded on 12 % SDS-PAGE followed by coomassie staining showed the successful purification of Btchi and Blichi in the elution fractions.

Legend for Fig. 3.4

Reducing end assay was done to determine the chitinase activity of purified Btchi and Blichi using colloidal chitin as substrate at 37 $^{\circ}$C for 1 h. Mean of the OD at 420 nm for each sample in triplicates was plotted on y-axis.

Increased concentration of colloidal chitin (0-35 mg/ml), incubated with 5 µg of Btchi and Blichi was used for the assay. Specific activity in nkat/mg of protein was plotted against the substrate concentration for Btchi (A) and Blichi (E). Graph pad prism software was used to calculate the V_{max}, Michaelis-Menten constant (K_m) and K_{cat} values using non linear regression analysis.

Software calculated V_{max}, K_m and K_{cat} of Btchi (B) and Blichi (F) plotted on the graph to visualize and compare the data points obtained from Lineweaver-Burk (LB) plots. LB reciprocal plot of Btchi (C) and Blichi (G) was plotted by considering all the data points, Btchi (D) and Blichi (H) except last data point.

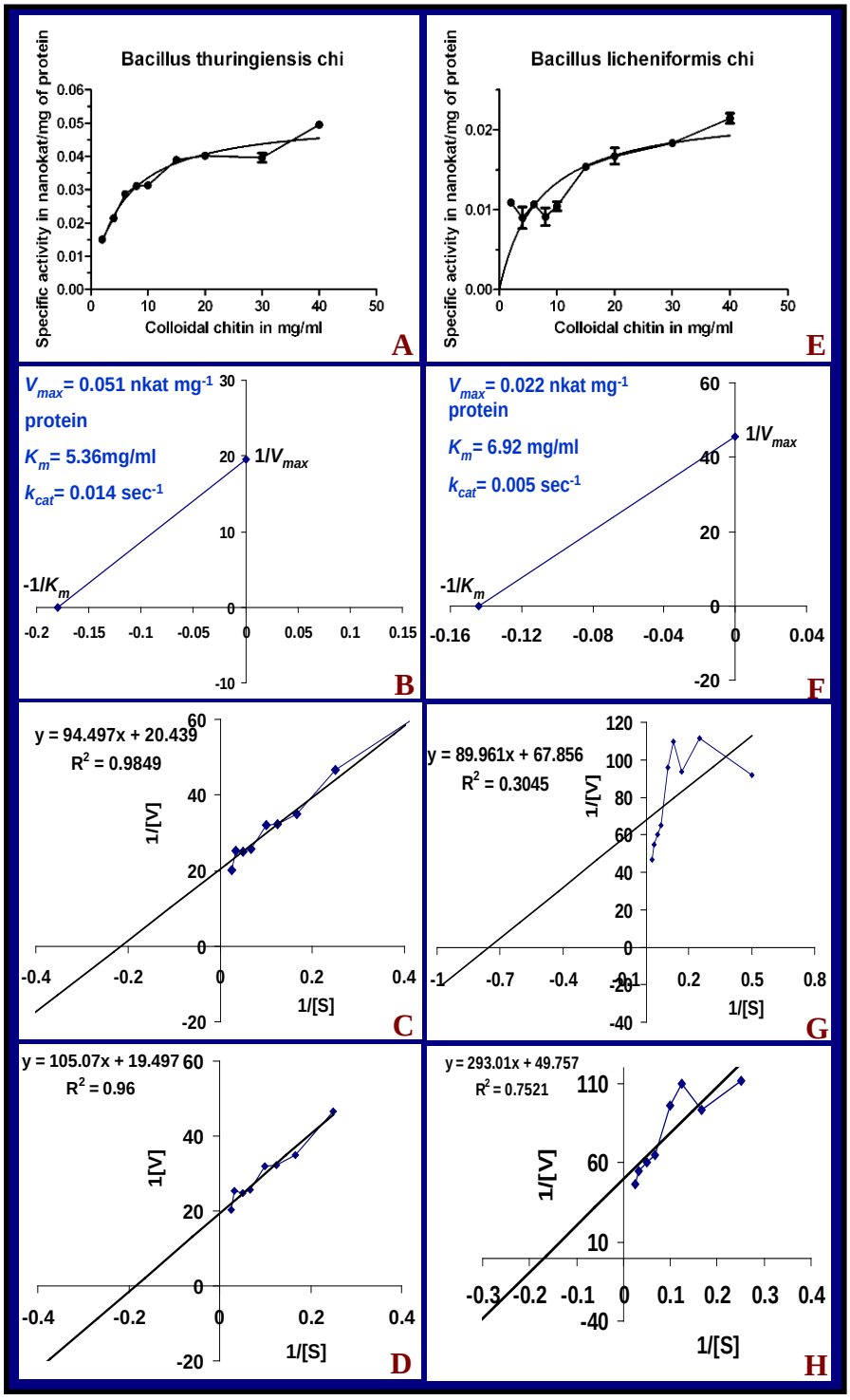


Fig. 3.4: Kinetic analysis of Btchi and Blichi chitinases

Legend for Fig. 3.5

Reducing end assay was done using colloidal chitin (8 mg/ml) and purified Btchi (5 µg), the incubations were performed in triplicates. Specific activity was calculated using mean of the OD at 420 nm. Vertical bars indicate standard deviation.

A) Optimum temperature: Incubations were done at different temperatures (20, 40, 60, 80 and 100 $^{\circ}$C) for 1 h to determine the optimum temperature of Btchi.

B) Thermal stability: Reducing end assay was done at standard conditions using colloidal chitin and Btchi pre-incubated in triplicates at temperatures (30-100 $^{\circ}$C) for 1 h to determine the thermal stability of Btchi.

C) Stability at optimum temperature: Purified Btchi was pre-incubated in triplicates at 60 $^{\circ}$C for 1-4 h followed by reducing end assay using colloidal chitin under standard reaction conditions to determine the stability of Btchi at 60 $^{\circ}$C . Fifty µg of purified Btchi in 10 mM sodium acetate pH 5.2 was analysed for CD spectrum followed by secondary structure prediction. At each temperature CD spectrum was recorded to plot the relative change in secondary structure by considering the CD spectrum at 30 $^{\circ}$C as 100 %.

D) Changes in secondary structure with increase in temperature: CD spectrum of Btchi was obtained at 30 $^{\circ}$C followed by gradual increase in temperature till 100 $^{\circ}$C to determine the change in the relative secondary structure of Btchi with increase in temperature.

E) Effect of pre incubation at different temperatures on secondary structure: Btchi pre-incubated at different temperatures for 1 h was analysed for CD spectrum to determine the thermal stability of Btchi.

F) Conformational stability at optimum temperature: Btchi was pre-incubated at 60 $^{\circ}$C for 1-4 h and analysed for CD spectrum at 30 $^{\circ}$C to determine the conformational stability of Btchi at 60 $^{\circ}$C.

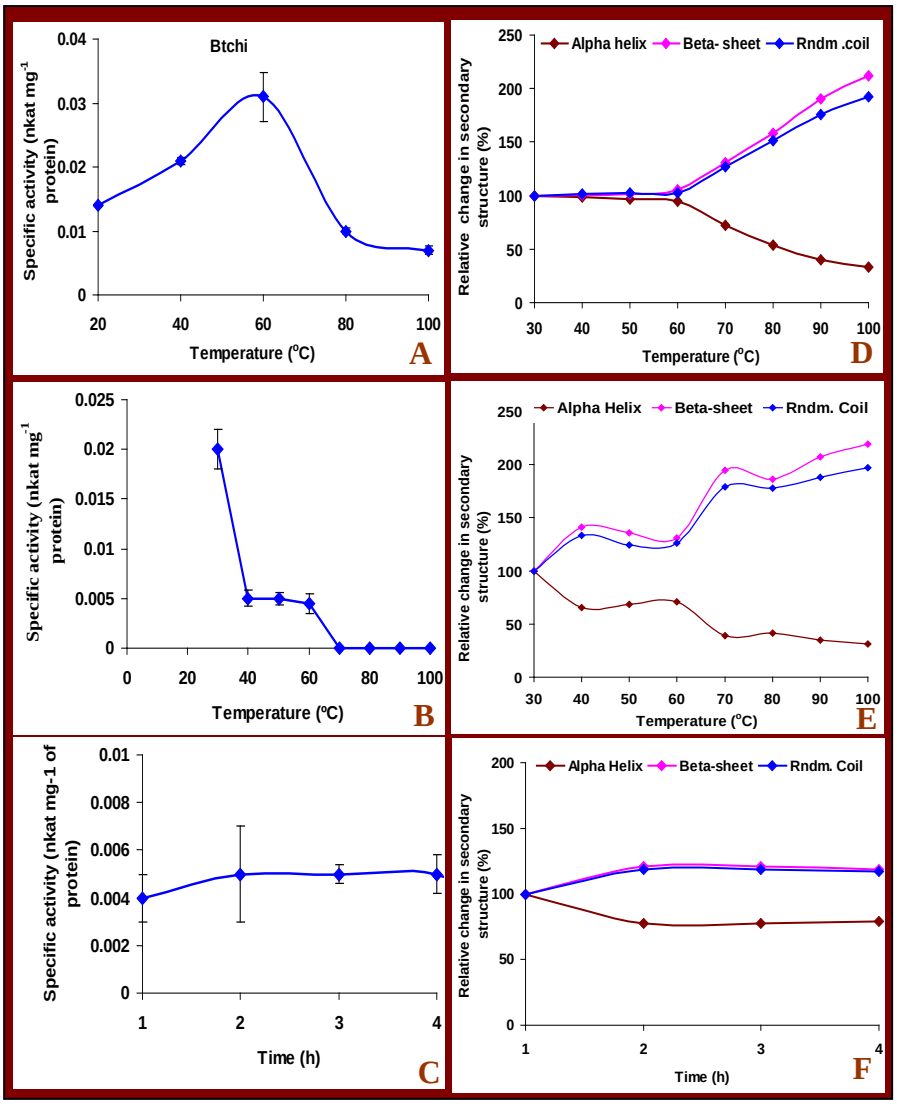


Fig. 3.5: Effect of temperature on the activity and secondary structure of Btchi

Legend for Fig. 3.6

Reducing end assay was done using colloidal chitin (8 mg/ml) and purified Blichi (5 µg), the incubations were performed in triplicates. Specific activity was calculated using mean of the OD at 420 nm. Vertical bars indicate standard deviation.

A) Optimum temperature: Incubations were done at different temperatures (20, 40, 60, 80 and 100 °C) for 1 h to determine the optimum temperature of Blichi.

B) Thermal stability: Reducing end assay was done at standard conditions using colloidal chitin and Blichi pre-incubated in triplicates at temperatures (30-100 °C) for 1 h to determine the thermal stability of Blichi.

C) Stability at optimum temperature: Purified Blichi was pre-incubated in triplicates at 60 °C for 1-4 h followed by reducing end assay using colloidal chitin under standard reaction conditions to determine the stability of Blichi at 60 °C . Fifty µg of purified Blichi in 10 mM sodium acetate pH 5.2 was analysed for CD spectrum followed by secondary structure prediction. At each temperature CD spectrum was recorded to plot the relative change in secondary structure by considering the CD spectrum at 30 °C as 100 %.

D) Changes in secondary structure with increase in temperature: CD spectrum of Blichi was obtained at 30 °C followed by gradual increase in temperature till 100 °C to determine the change in the relative secondary structure of Blichi with increase in temperature.

E) Effect of pre incubation at different temperatures on secondary structure: Blichi pre-incubated at different temperatures for 1 h was analysed for CD spectrum to determine the thermal stability of Blichi.

F) Conformational stability at optimum temperature: Blichi was pre-incubated at 60 °C for 1-4 h and analysed for CD spectrum at 30 °C to determine the conformational stability of Blichi at 60 °C.

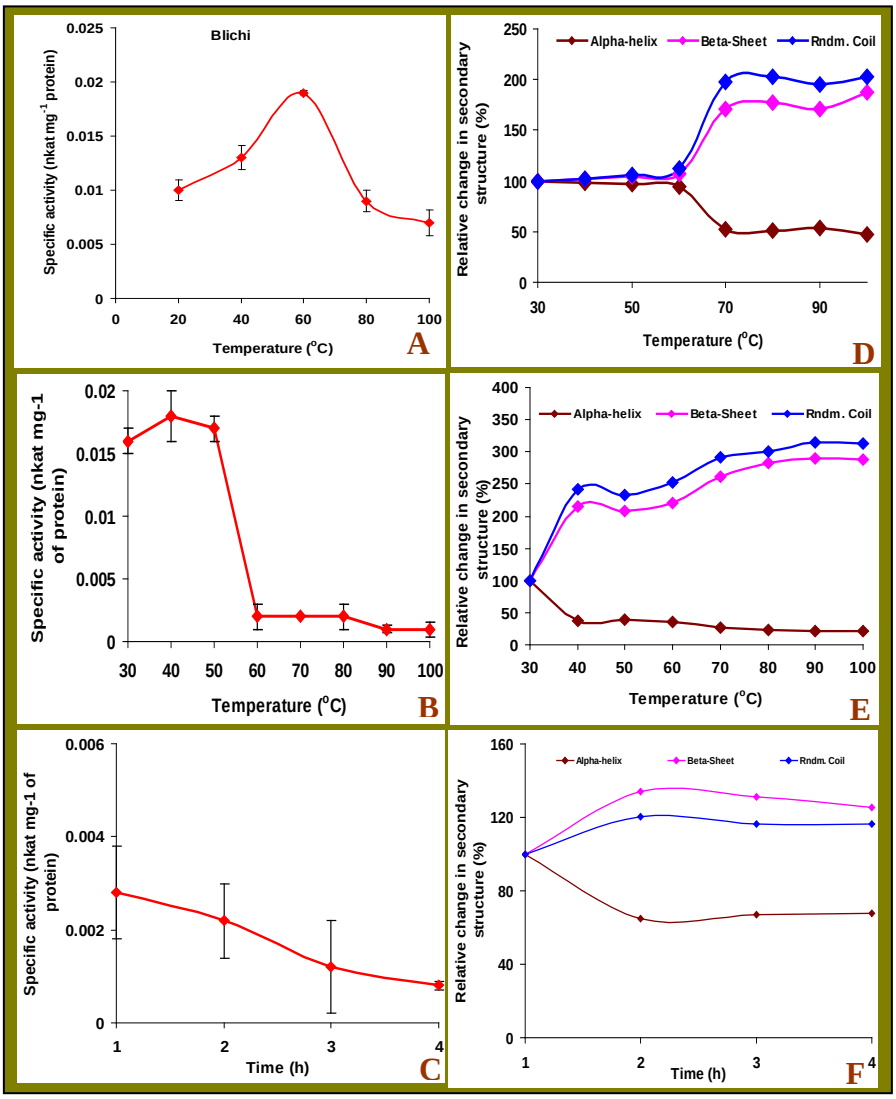


Fig. 3.6: Effect of temperature on the activity and secondary structure of Blichi

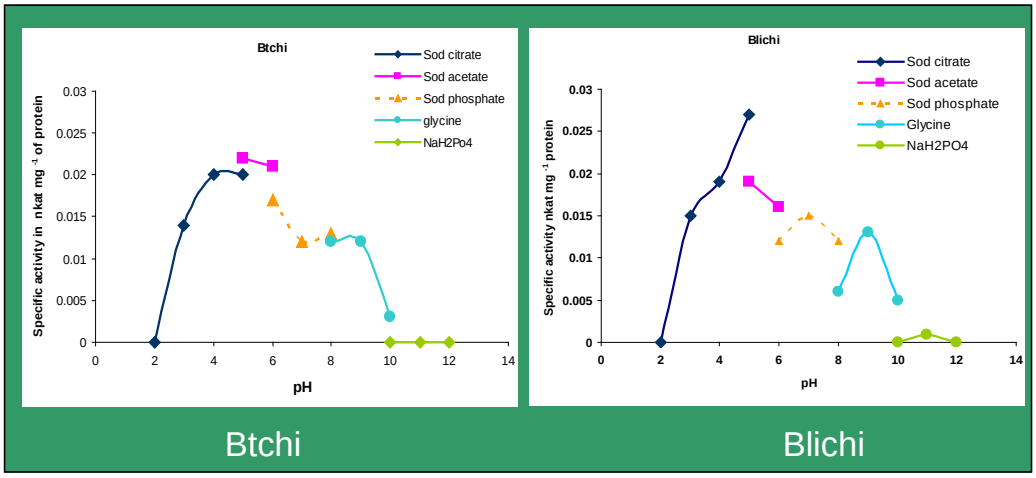


Fig. 3.7: Effect of pH on the activity of Btchi and Blichi using colloidal chitin as substrate

Reducing end assay was done in triplicates using colloidal chitin and 5 µg of purified Btchi and Blichi, the incubations were performed at different pH (2-12) for 1 h at 37 °C to determine the optimal pH of the two chitinases. Specific activity was calculated using mean of the OD at 420 nm.

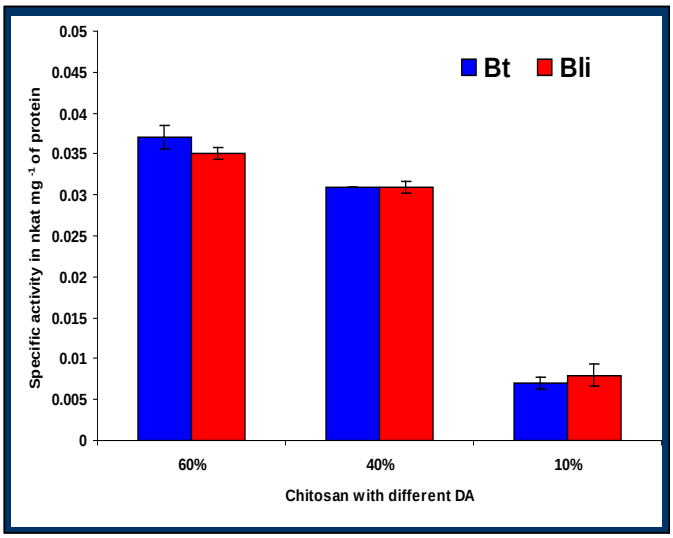


Fig. 3.8: Effect of acetylation on the chitinase activity of Btchi and Blichi using chitosan with different DA

Reducing end assay was done in triplicates using chitosan with different DA (66, 40 and 10 %) and purified Btchi and Blichi (5 µg) chitinase, using standard reaction conditions to determine the chitinase activity on chitosan. Specific activity was calculated using mean of the OD at 420 nm. Vertical bars indicate standard deviation.

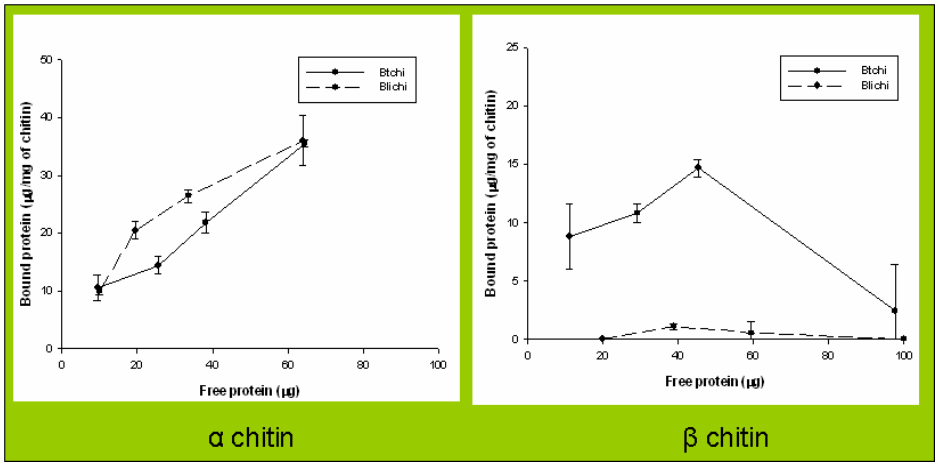


Fig. 3.9: Binding ability of Btchi and Blichi chitinases towards insoluble α and β chitin

Adsorption of Btchi and Blichi to insoluble α and β chitin was determined in triplicates. Different amounts of proteins ranging from 20-100 µg were incubated with 1 mg of chitin (dry weight) in 50 mM sodium acetate buffer pH 5.2 for 1 h in a gel shaker at 450 rpm at 4 °C. Free protein was measured using spectrofluorimeter. Bound protein=total protein-free protein. Vertical bars indicate standard deviation.

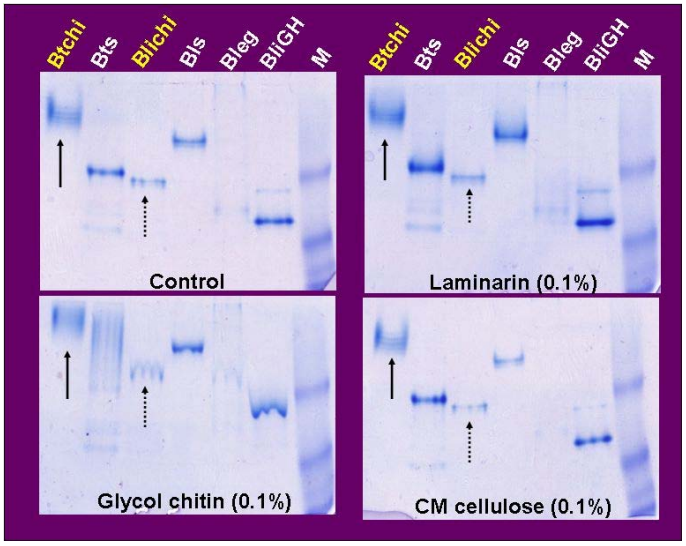


Fig. 3.10: Soluble substrate binding ability of Btchi and Blichi using affinity electrophoresis

Btchi and Blichi (20 µg) were loaded on 8 % native-PAGE with and without substrates (0.1 % of glycol chitin, CM cellulose and laminarin) followed by coomassie staining to observe the retardation in the electrophoretic mobility in order to determine the binding capacity of the two chitinases.

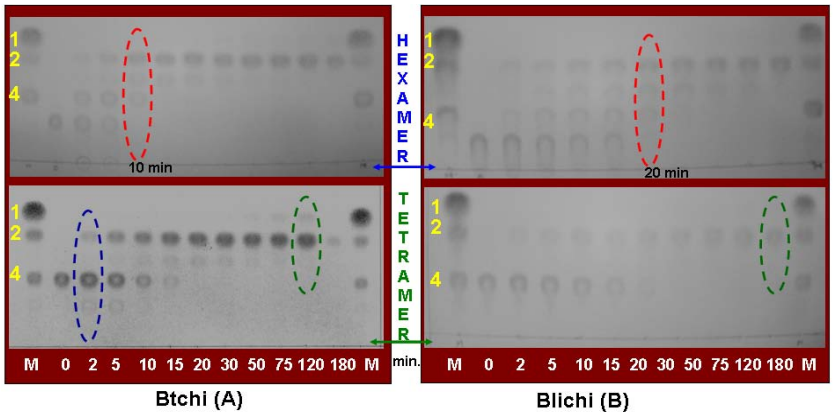
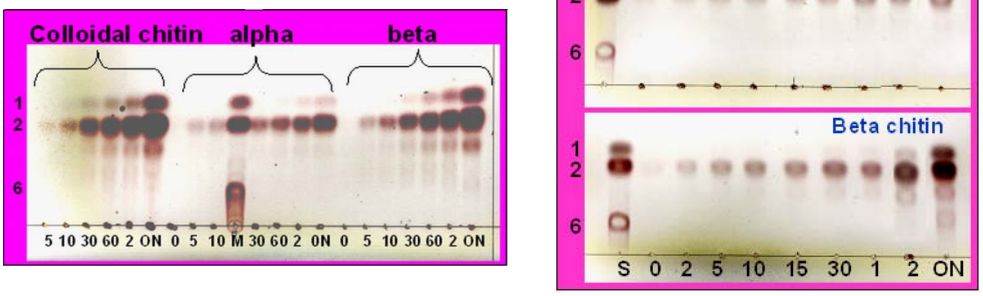


Fig. 3.11: Thin layer chromatography showing hydrolysis products of Btchi and Blichi using soluble substrates

Soluble substrates (1 mM each of chitin hexamer, chitin tetramer and chitin trimer were incubated with 5 µg of Btchi (A) and Blichi (B). The products were loaded on TLC plates, visualized by spraying (aniline-DPA solution) followed by heating and compared with the standard.



A **B**

Fig. 3.12: Thin layer chromatography showing hydrolysis products of Btchi and Blichi using insoluble substrates

Insoluble substrates 0.5 mg of colloidal chitin, α chitin and β chitin were incubated with 5 µg of Blichi (A) and Btchi (B). The products were loaded on TLC plates, visualized by spraying (aniline-DPA solution) followed by heating and compared with the standard.

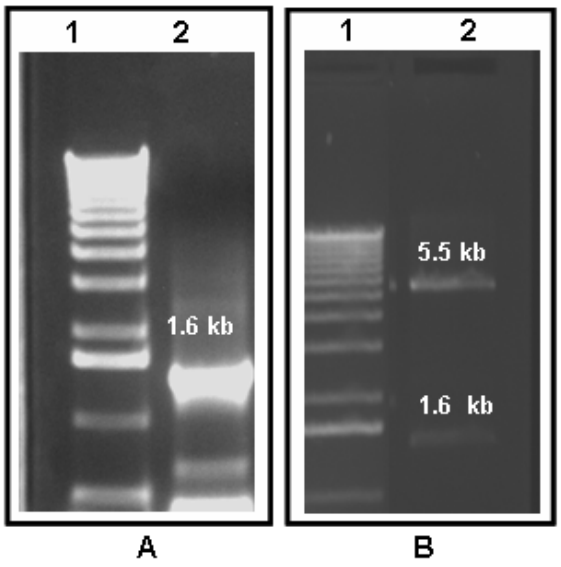


Fig. 3.13: Amplification and cloning of endoglucanase (*egln*) from *Bacillus licheniformis* DSM 13

A) Amplification of *egln* from gDNA of *B. licheniformis* as template using primers BleglnFpGH5 new and BleglnRpGH5. The amplicon was compared with 100 bp ladder. Lane 1: 100 bp DNA ladder, lane 2: 1.6 kb *egln* of *B. licheniformis*

B) *egln* was amplified and ligated to *BamH I* and *Xho I* sites of pET22b+ (Novagen) resulting in pANE-egln. Double digestion of pANE-egln with *Bam*H I and *Xho* I released fragment of 1.6 kb, confirming the presence of correct insert. Lane 1: 100 bp DNA ladder, lane 2: *Bam*H I and *Xho* I digest of pANE-egln released 1.6 kb.

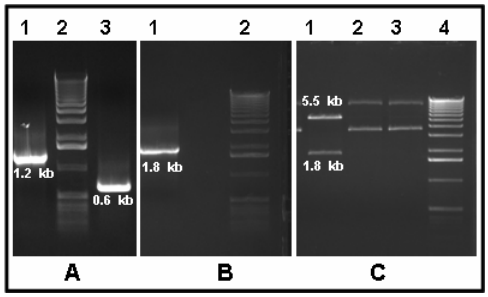


Fig. 3.14: Amplification, fusion and cloning of *Btegln*

A) Amplification of *GH* (*chi*) with C-terminal complementary overhang from plasmid pANE-Btchi as template using primers BtchisalFor and BtGHOEglnRev. *CeBD* was amplified using primers EglncbmOEFor and BleglnRpGH5 and pANE-egln as template. Lane 1: 1.2 kb of *GH* of pANE-Btchi, lane 2: 100 bp DNA ladder, lane 3: 0.6 kb of *CeBD* of pANE-egln

B) Amplification of the fusion PCR product *Btegln* using *GH* and *CeBD* as templates, primers BtchisalFor and BleglnRpGH5. Lane 1: 1.8 kb of fused amplicon, lane 2: 100 bp DNA ladder

C) *Btegln* was amplified and ligated to *Sal* I and *Xho* I sites of pET22b+ (Novagen) resulting in pANE-Btegln. Double digestion of pANE-Bliegln with *Sal* I and *Xho* I released fragment of 1.8 kb confirming the presence of insert. Lane 1: *Sal* I and *Xho* I digest of pANE-Btegln released 1.8 kb, lane 2 & 3: digestion resistant recombinant pANE-Btegln clones, lane 4: 100 bp DNA ladder.

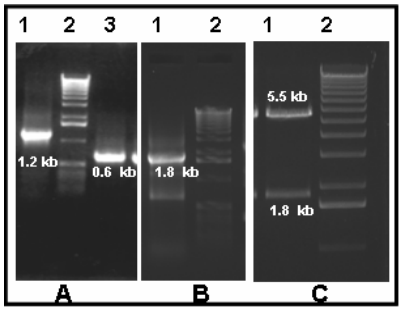


Fig. 3.15: Amplification, fusion and cloning of *Bliegln*

A) Amplification of *GH* (*chi*) with C-terminal complementary overhang from plasmid pANE-Blichi as template using primers BlchiFpmodnew and BlchiGHglnOERev. *CeBD* was amplified using primers EglncbmOEFor and BleglnRpGH5 and pANE-egln as template. Lane 1: 1.2 kb of *GH* of pANE-Blichi, lane 2: 100 bp DNA ladder, lane 3: 0.6 kb of *CeBD* of pANE-egln

B) Amplification of the fusion PCR product *Bliegln* using *GH* and *CeBD* as templates, primers BlchiFpmodnew and BleglnRpGH5. Lane 1: 1.8 kb of fused amplicon, lane 2: 100 bp DNA ladder

C) *Bliegln* was amplified and ligated to *Bam*H I and *Xho* I sites of pET22b+ (Novagen) resulting in pANE-Bliegln. Double digestion of pANE-Bliegln with *Bam*H I and *Xho* I released fragment of 1.8 kb confirming the presence of insert. Lane 1: *Bam*H I and *Xho* I digest of pANE-Bliegln released 1.8 kb, lane 2: 100 bp DNA ladder.

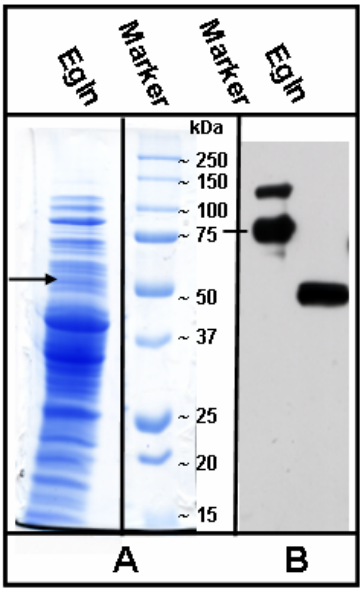


Fig. 3.16: Heterologous expression of endoglucanase (egln) in *E. coli*

A) IPTG (1 mM) was used to induce the *E. coli* BL21 (DE3) at 37 °C harboring the vector pANE-egln for 3 h. Periplasmic fraction (egln) was loaded on 12 % SDS-PAGE and compared with the standard protein weight marker (Marker).

B) The periplasmic fraction was transferred onto the nitrocellulose membrane. Using Anti-His tag antibody, the His tag fused at the C-terminal of the expressed protein was detected by chemiluminescent method. The His-tagged marker protein (M) has a molecular weight of 75 kDa.

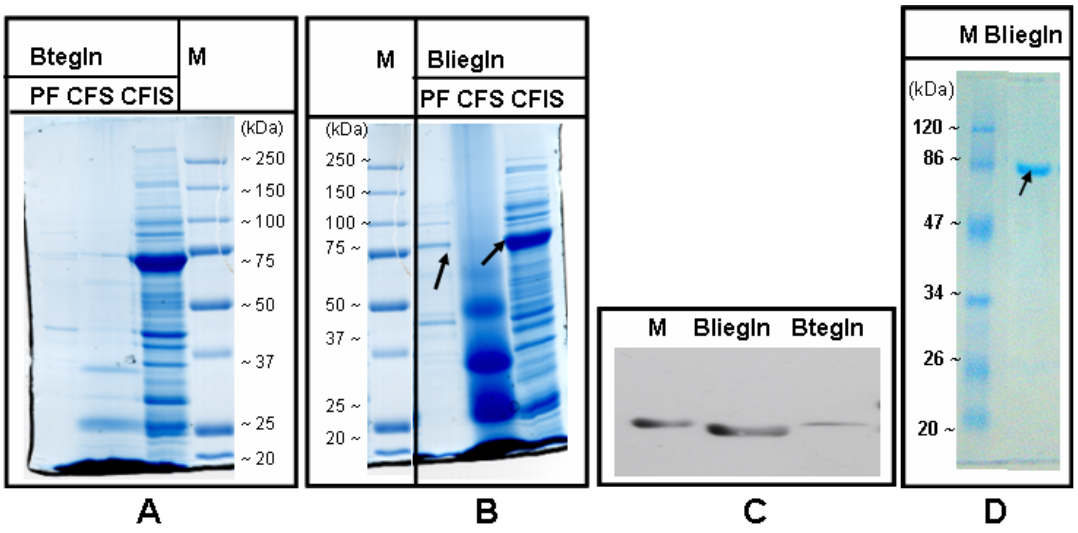


Fig. 3.17: Heterologous expression of Btegln and Bliegln in *E. coli*

A & B) 0.4 mM IPTG was used to induce the *E. coli* BL21 (DE3) at 37 °C harboring the vector pANE-Btegln or pANE-Bliegln, for 20 h at 18 °C. Periplasmic (PF), cytoplasmic soluble (CFS) and insoluble (CFIS) fractions were loaded on 12 % SDS-PAGE and compared with the standard protein weight marker (M).

C) The periplasmic fractions of both Btegln and Bliegln were transferred onto the nitrocellulose membrane. Using Anti-his tag antibody, the His tag fused at the C-terminal of the expressed protein was detected by chemiluminescent method. The His-tagged marker protein (M) has a molecular weight of 75 kDa.

D) Ni-NTA chromatography was used to purify the recombinant protein Bliegln. Elution buffers with 50 and 100 mM imidazole was used to elute the protein. Fractions loaded on 12 % SDS-PAGE followed by coomassie staining showed the successful purification of Bliegln in the elution fractions.

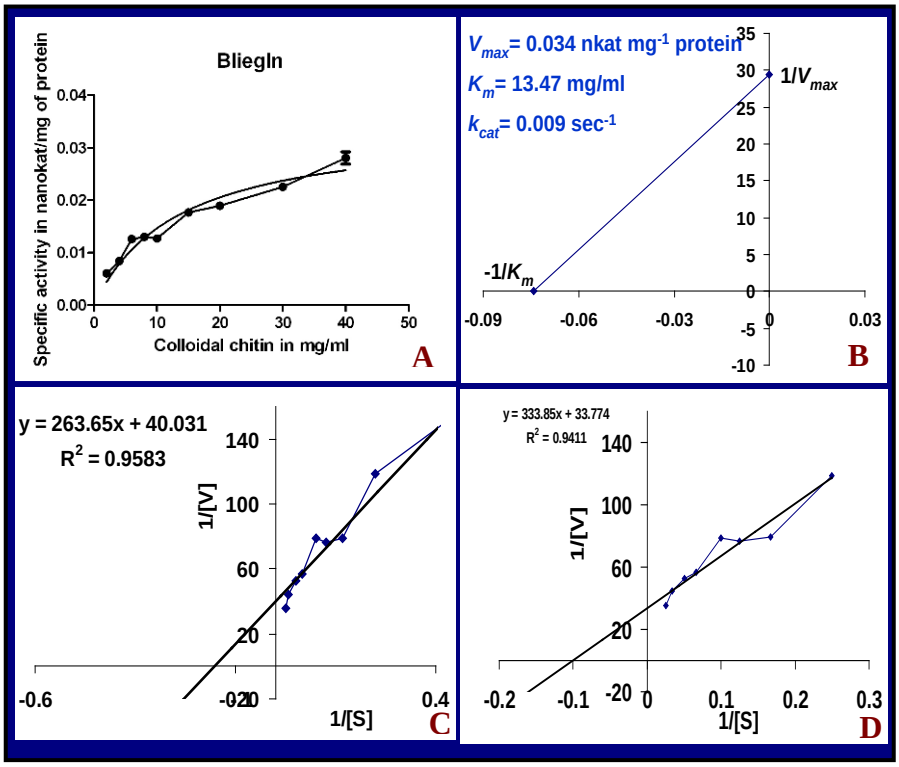


Fig. 3.18: Kinetic analysis of chimeric chitinase Bliegln

Reducing end assay was done to determine the chitinase activity of purified Bliegln using colloidal chitin as substrate at 37 °C for 1 h. Mean of the OD at 420 nm for each sample in triplicates was plotted on y-axis.

A) Increased concentration of colloidal chitin (0-35 mg/ml), incubated with 5 µg of Bliegln was used for the assay. Specific activity in nkat/mg of protein was plotted against the substrate concentration. Graph pad prism software was used to calculate the V_{max}, Michaelis menten constant (K_m) and K_{cat} values using non linear regression analysis. Software calculated V_{max}, K_m and K_{cat} plotted on the graph to visualize and compare the data points obtained from Lineweaver-Burk (LB) plots (B). LB reciprocal plot was plotted by considering all the data points (C) and by omitting last data point (D).

Legend for Fig. 3.19

Reducing end assay was done using colloidal chitin (8 mg/ml) and purified Bliegln (5 µg), the incubations were performed in triplicates. Specific activity was calculated using mean of the OD at 420 nm. Vertical bars indicate standard deviation.

A) Optimum temperature: Incubations were done at different temperatures (20, 40, 60, 80 and 100 °C) for 1 h to determine the optimum temperature of Bliegln.

B) Thermal stability: Reducing end assay was done at standard conditions using colloidal chitin and Bliegln pre-incubated in triplicates at temperatures (30-100 °C) for 1 h to determine the thermal stability of Bliegln.

C) Stability at optimum temperature: Purified Bliegln was pre-incubated in triplicates at 60 °C for 1-4 h followed by reducing end assay using colloidal chitin under standard reaction conditions to determine the stability of Bliegln at 60 °C . Fifty µg of purified Bliegln in 10 mM sodium acetate pH 5.2 was analysed for CD spectrum followed by secondary structure prediction. At each temperature CD spectrum was recorded to plot the relative change in secondary structure by considering the CD spectrum at 30 °C as 100 %.

D) Changes in secondary structure with increase in temperature: CD spectrum of Bliegln was obtained at 30 °C followed by gradual increase in temperature till 100 °C to determine the change in the relative secondary structure of Bliegln with increase in temperature.

E) Effect of pre incubation at different temperatures on secondary structure: Bliegln pre-incubated at different temperatures for 1 h was analysed for CD spectrum to determine the thermal stability of Bliegln.

F) Conformational stability at optimum temperature: Bliegln was pre-incubated at 60 °C for 1-4 h and analysed for CD spectrum at 30 °C to determine the conformational stability of Bliegln at 60 °C.

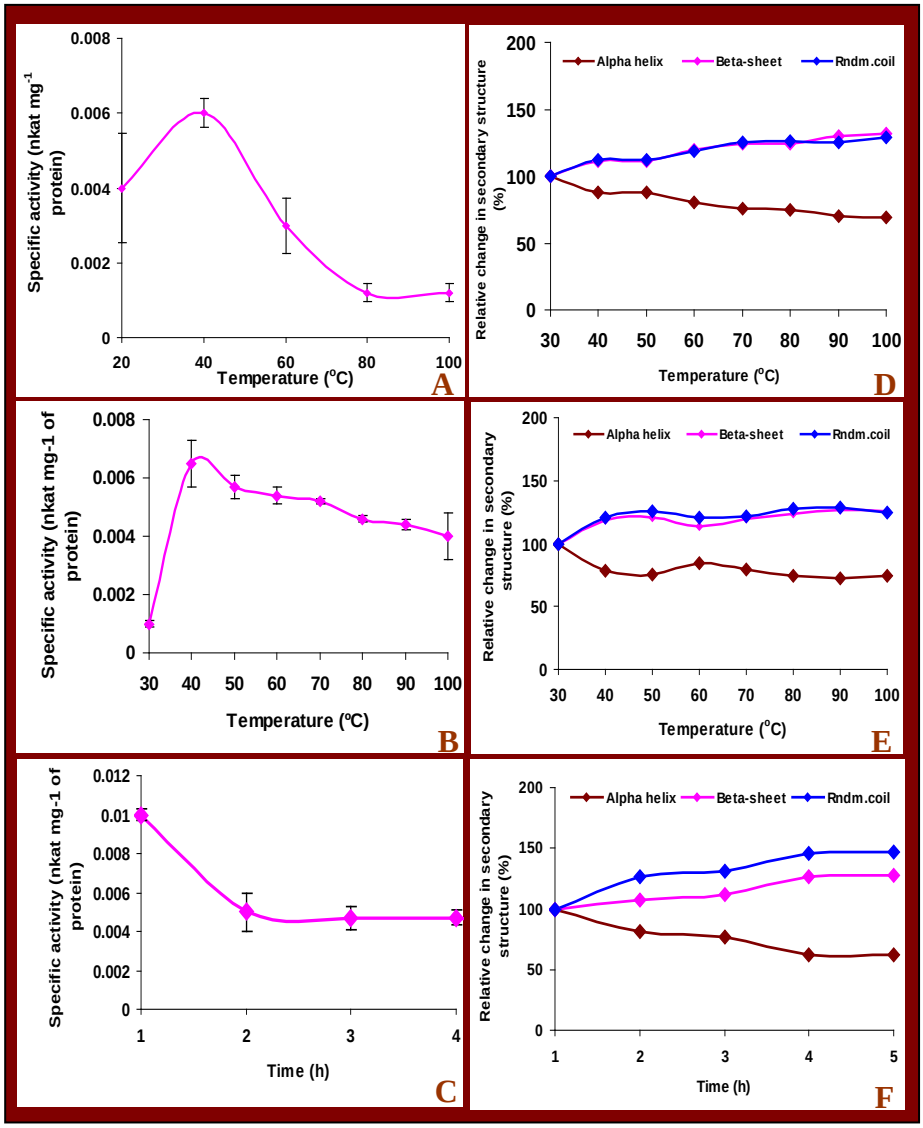


Fig. 3.19: Effect of temperature on the activity and secondary structure of Bliegln

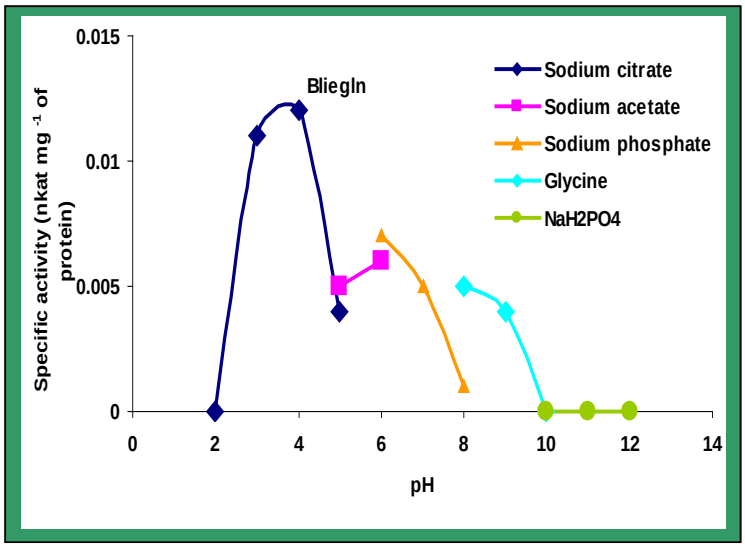


Fig. 3.20: Effect of pH on the activity of Bliegln using colloidal chitin as substrate

Reducing end assay was done in triplicates using colloidal chitin and purified 5 µg of Bliegln chitinase, the incubations were performed at different pH (2-12) for 1 h at 37 °C to determine the optimal pH of Bliegln.

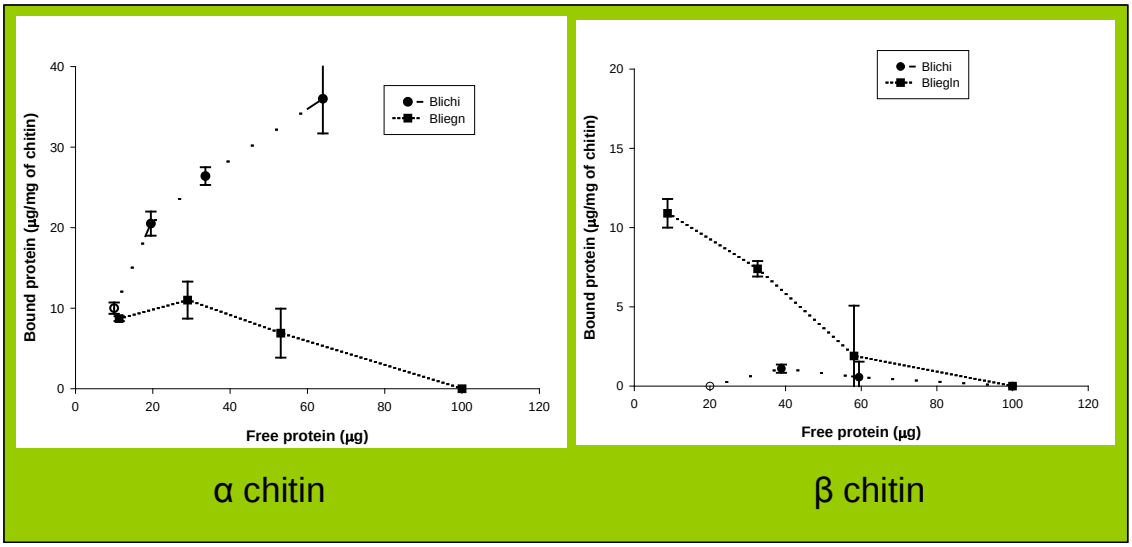


Fig. 3.21: Binding ability of Bliegln towards insoluble α and β chitin

Adsorption of Bliegln to insoluble α and β chitin was determined in triplicates. Different amounts of proteins ranging from 20-100 µg were incubated with 1 mg of chitin (dry weight) in 50 mM sodium acetate buffer pH 5.2 for 1 h in a gel shaker at 450 rpm at 4 °C. Free protein was measured using spectrofluorimeter. Bound protein=total protein-free protein. Vertical bars indicate standard deviation.

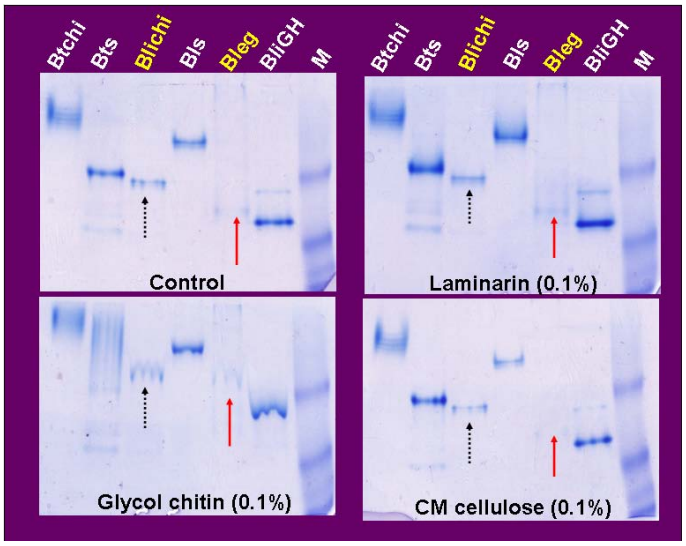
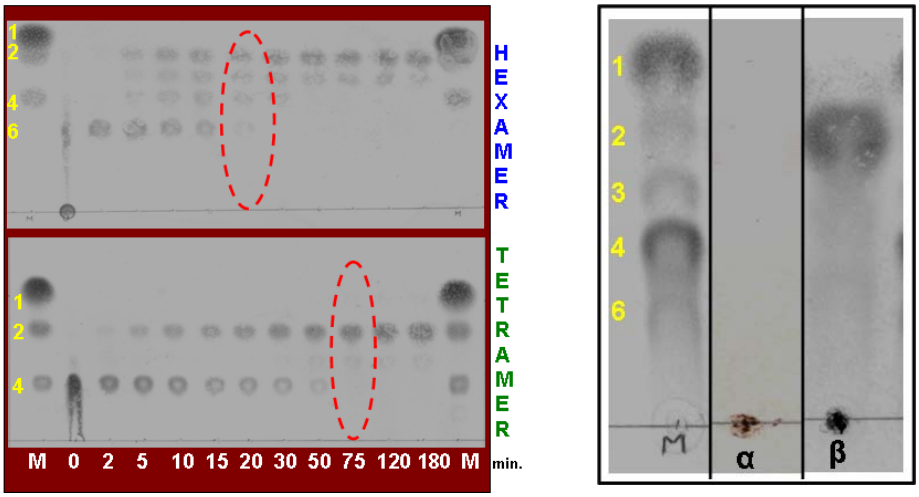


Fig. 3.22: Soluble substrate binding ability of Bliegln using affinity electrophoresis

Bliegln (20 µg) was loaded on 8 % native-PAGE with and without substrates (0.1 % of glycol chitin, CM cellulose and laminarin) followed by coomassie staining to observe the retardation in the electrophoretic mobility in order to determine the binding capacity of the two chitinases.



A **B**

Fig. 3.23: Thin layer chromatography showing hydrolysis products of Bliegln using soluble and insoluble substrates

Bliegln (5µg) was incubated with soluble substrates (1 mM each of chitin hexamer and chitin tetramer) (A) and insoluble substrates (0.5 mg of α and β chitin) ((B). The products were loaded on TLC plates, visualized by spraying (aniline-DPA solution) followed by heating and compared with the standard.

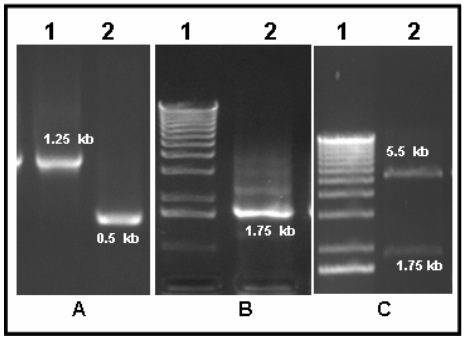


Fig. 3.24: Amplification, fusion and cloning of Btswap

A) Amplification of *GH* (*chi*) (Lane 1) with C-terminal complementary overhang from plasmid pANE-Btchi as template using primers BtchiBamHIFor and BtGHOESbl. *ChBD* (Lane 2) was amplified using primers BlcbmOE and BlchiRpmod and pANE-Blichi as template.

B) Amplification of the fusion PCR product Btswap (Lane 2) using *GH* and *ChBD* as templates, BtchiBamHIFor and BlchiRpmod primers. Lane 1: 100 bp DNA ladder.

C) *Btswap* was amplified and ligated to *Bam*H I and *Xho* I sites of pET22b+ (Novagen) resulting in pANE-Btswap. Double digestion of pANE-Btswap with *Bam*H I and *Xho* I released 1.75 kb confirming the presence of insert (Lane 2). Lane 1: 100 bp DNA ladder.

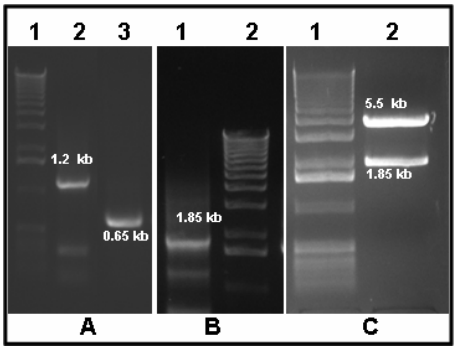


Fig. 3.25: Amplification, fusion and cloning of Bliswap

A) Amplification of *GH* (*chi*) (Lane 2) with C-terminal complementary overhang from plasmid pANE-Blichi as template using primers BlchiFpmodnew and BlGHOESbt. *ChBD* (Lane A3) was amplified using primers BtcbmOE and BtchiRpmod and pANE-Btchi as template. Lane 1: 100 bp DNA ladder.

B) Amplification of the fusion PCR product *Bliswap* (Lane 1) using *GH* and *ChBD* as templates, BlchiFpmodnew and BtchiRpmod primers. Lane 2: 100 bp DNA ladder.

C) *Bliswap* was amplified and ligated to *Bam*H I and *Xho* I sites of pET22b+ (Novagen) resulting in pANE-Bliswap. Double digestion of pANE-Bliswap with *Bam*H I and *Xho* I released 1.85 kb confirming the presence of insert (Lane 2). Lane 1: 100 bp DNA ladder.

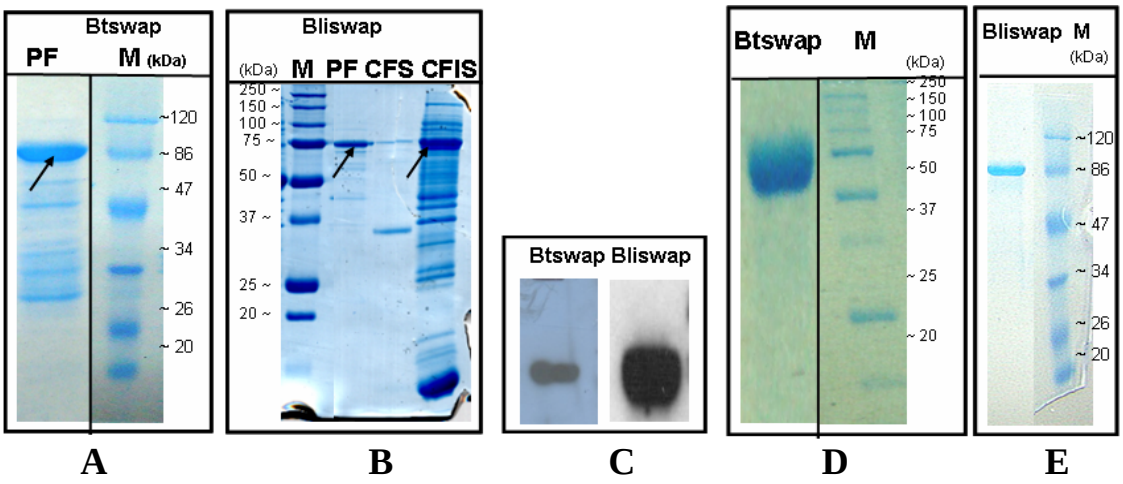


Fig. 3.26: Heterologous expression and purification of Btswap and Bliswap using Ni-NTA chromatography

A & B) 0.4 mM IPTG was used to induce the *E. coli* BL21 (DE3) at 37 °C horbouring the pANE-Btswap or pANE-Bliswap. The induction period was extended to 20 h at 18 °C. Concentrated periplasmic fractions were loaded on 12 % SDS-PAGE and compared with the standard protein weight marker (M).

C) The periplasmic fraction of Btswap and Bliswap were transferred onto the membrane. Using Anti-His tag antibody, the His tag fused at the C-terminal of the expressed protein was detected by chemiluminescent method.

D &E) Ni-NTA chromatography was used to purify both Btswap and Bliswap. Elution buffers with 50 and 100 mM imidazole was used to elute the protein. Fractions loaded on 12 % SDS-PAGE followed by coomassie staining showed the successful purification of Btswap and Bliswap in the elution fractions.

Legend for Fig. 3.27

Reducing end assay was done to determine the chitinase activity of purified Btswap and Bliswap using colloidal chitin as substrate at 37 $^{\circ}$C for 1 h. Mean of the OD at 420 nm for each sample in triplicates was plotted on y-axis.

Increased concentration of colloidal chitin (0-35 mg/ml), incubated with 5 µg of Btswap and Bliswap was used for the assay. Specific activity in nkat/mg of protein was plotted against the substrate concentration for Btswap (A) and Bliswap (E). Graph pad prism software was used to calculate the V_{max}, Michaelis menten constant (K_m) and K_{cat} values using non linear regression analysis. Software calculated V_{max}, K_m and K_{cat} of Btswap (B) and Bliswap (F) plotted on the graph to visualize and compare the data points obtained from Lineweaver-Burk (LB) plots. LB reciprocal plot of Btswap (C) and Bliswap (G) was plotted by considering all the data points and by omitting last data point Btswap (D) and Bliswap (H).

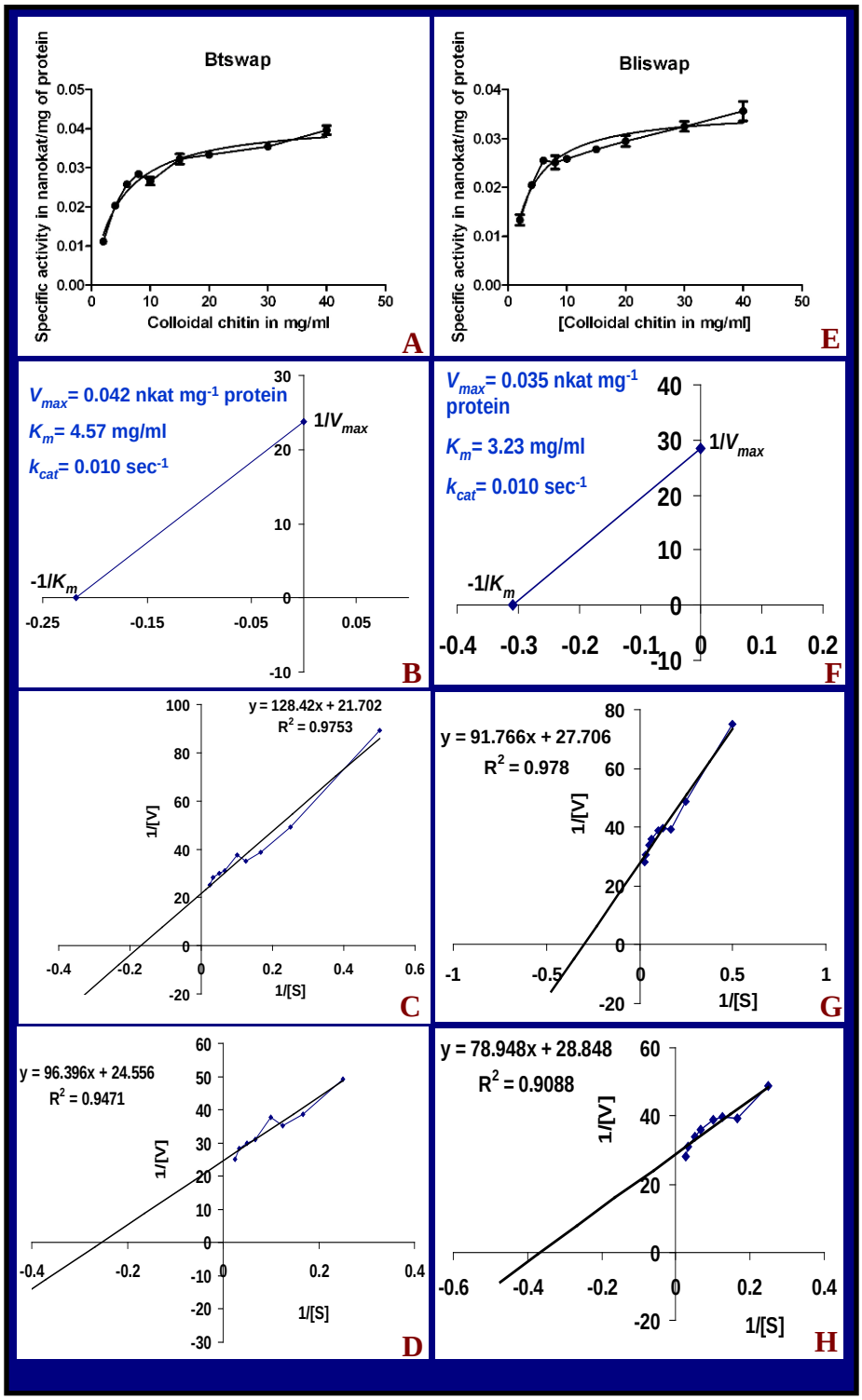


Fig. 3.27: Kinetic analysis of Btswap and Bliswap chitinases

Legend for Fig. 3.28

Reducing end assay was done using colloidal chitin (8 mg/ml) and purified Btswap (5 µg), the incubations were performed in triplicates. Specific activity was calculated using mean of the OD at 420 nm. Vertical bars indicate standard deviation.

A) Optimum temperature: Incubations were done at different temperatures (20, 40, 60, 80 and 100 °C) for 1 h to determine the optimum temperature of Btswap.

B) Thermal stability: Reducing end assay was done at standard conditions using colloidal chitin and Btswap pre-incubated in triplicates at temperatures (30-100 °C) for 1 h to determine the thermal stability of Btswap.

C) Stability at optimum temperature: Purified Btswap was pre-incubated in triplicates at 60 °C for 1-4 h followed by reducing end assay using colloidal chitin under standard reaction conditions to determine the stability of Btswap at 60 °C . Fifty µg of purified Btswap in 10 mM sodium acetate pH 5.2 was analysed for CD spectrum followed by secondary structure prediction. At each temperature CD spectrum was recorded to plot the relative change in secondary structure by considering the CD spectrum at 30 °C as 100 %.

D) Changes in secondary structure with increase in temperature: CD spectrum of Btswap was obtained at 30 °C followed by gradual increase in temperature till 100 °C to determine the change in the relative secondary structure of Btswap with increase in temperature.

E) Effect of pre incubation at different temperatures on secondary structure: Btswap pre-incubated at different temperatures for 1 h was analysed for CD spectrum to determine the thermal stability of Btswap.

F) Conformational stability at optimum temperature: Btswap was pre-incubated at 60 °C for 1-4 h and analysed for CD spectrum at 30 °C to determine the conformational stability of Btswap at 60 °C.

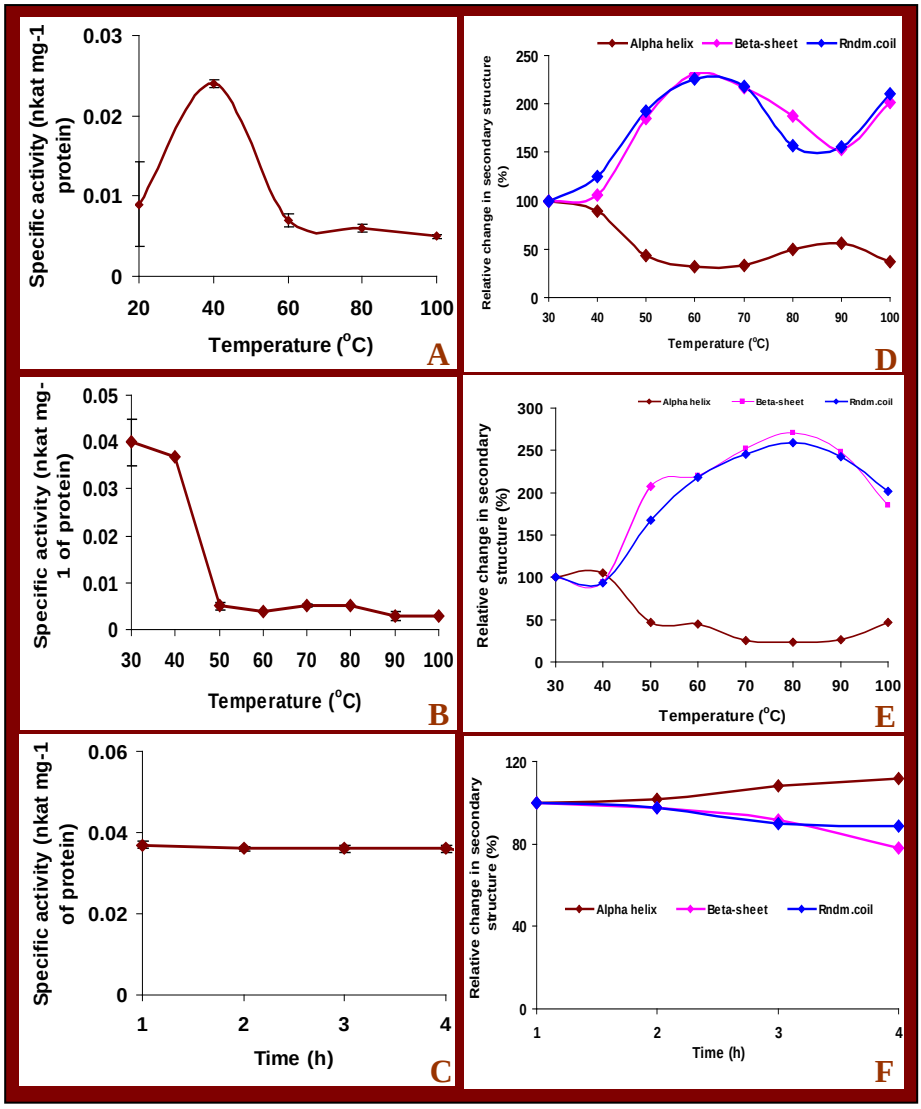


Fig. 3.28: Effect of temperature on the activity and secondary structure of Btswap

Legend for Fig. 3.29

Reducing end assay was done using colloidal chitin (8 mg/ml) and purified Bliswap (5 µg), the incubations were performed in triplicates. Specific activity was calculated using mean of the OD at 420 nm. Vertical bars indicate standard deviation.

A) Optimum temperature: Incubations were done at different temperatures (20, 40, 60, 80 and 100 °C) for 1 h to determine the optimum temperature of Bliswap.

B) Thermal stability: Reducing end assay was done at standard conditions using colloidal chitin and Bliswap pre-incubated in triplicates at temperatures (30-100 °C) for 1 h to determine the thermal stability of Bliswap.

C) Stability at optimum temperature: Purified Bliswap was pre-incubated in triplicates at 60 °C for 1-4 h followed by reducing end assay using colloidal chitin under standard reaction conditions to determine the stability of Bliswap at 60 °C . Fifty µg of purified Bliswap in 10 mM sodium acetate pH 5.2 was analysed for CD spectrum followed by secondary structure prediction. At each temperature CD spectrum was recorded to plot the relative change in secondary structure by considering the spectrum at 30 °C as 100 %.

D) Changes in secondary structure with increase in temperature: CD spectrum of Bliswap was obtained at 30 °C followed by gradual increase in temperature till 100 °C to determine the change in the relative secondary structure of Bliswap with increase in temperature.

E) Effect of pre incubation at different temperatures on secondary structure: Bliswap pre-incubated at different temperatures for 1 h was analysed for CD spectrum to determine the thermal stability of Bliswap.

F) Conformational stability at optimum temperature: Bliswap was pre-incubated at 60 °C for 1-4 h and analysed for CD spectrum at 30 °C to determine the conformational stability of Bliswap at 60 °C.

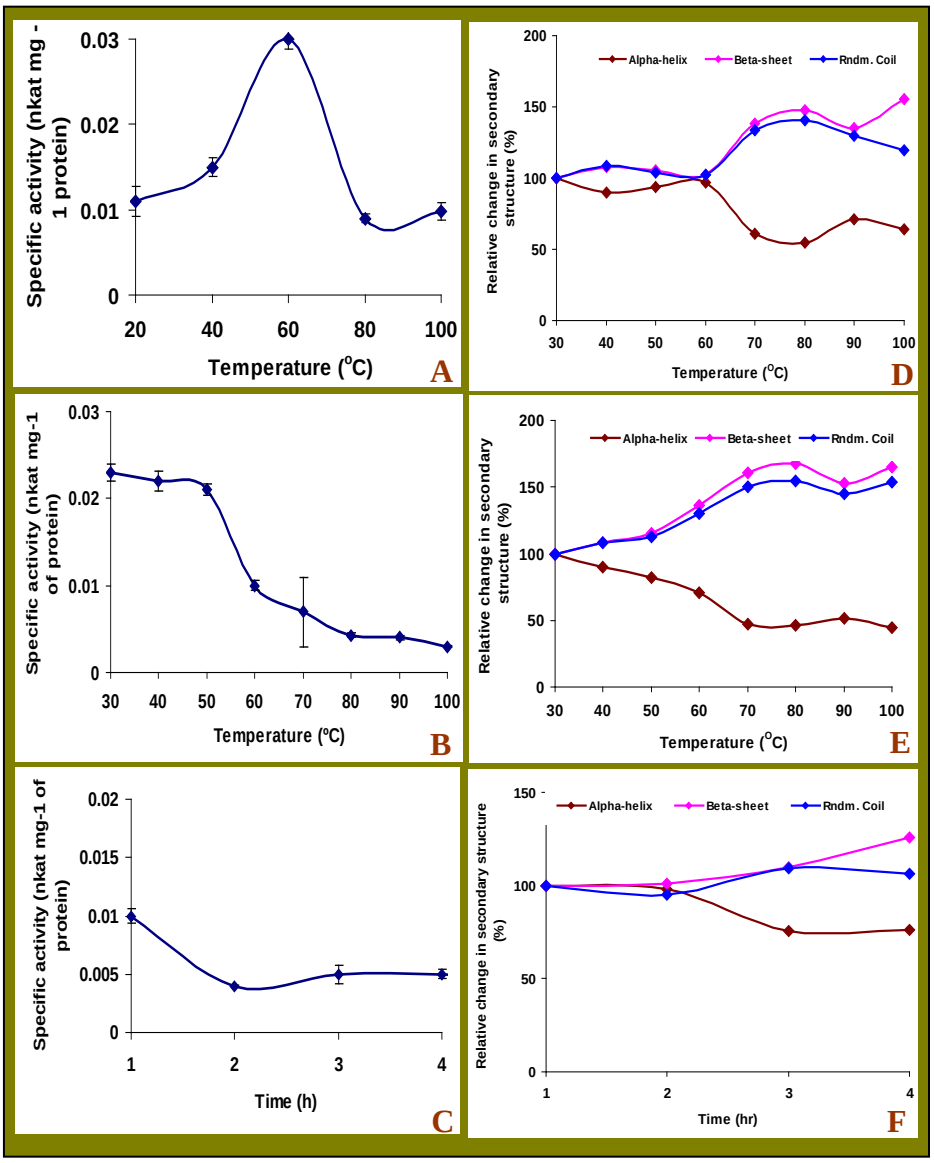


Fig. 3.29: Effect of temperature on the activity and secondary structure of Bliswap

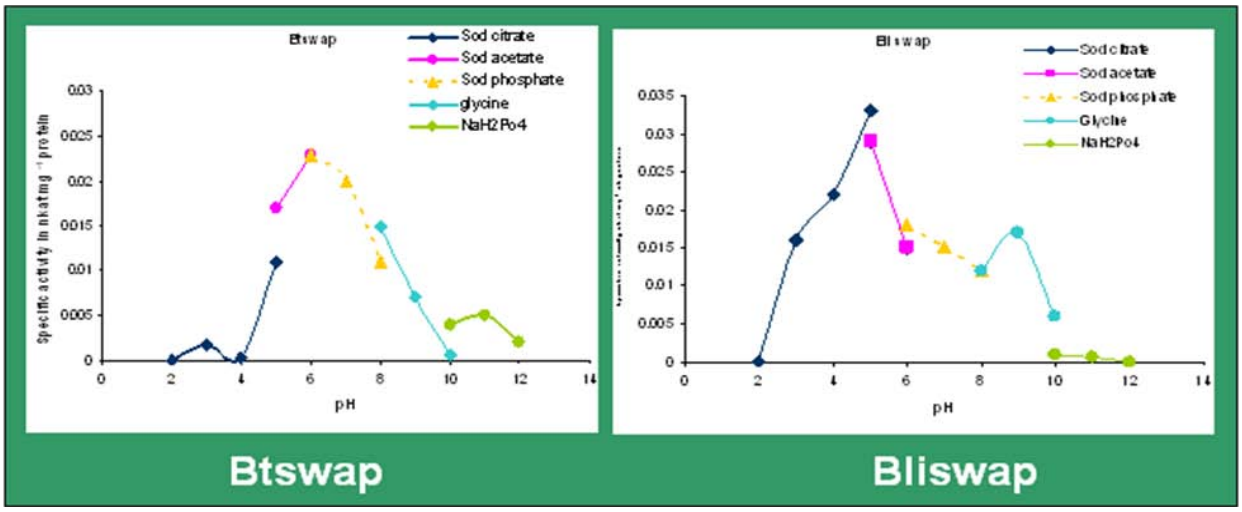


Fig. 3.30: Effect of pH on the activity of Btswap and Bliswap using colloidal chitin as substrate

Reducing end assay was done in triplicates using colloidal chitin and purified 5 µg of Btswap and Bliswap chitinase, the incubations were performed at different pH (2-12) for 1 h at 37 °C to determine the optimal pH of the two chimeric chitinases.

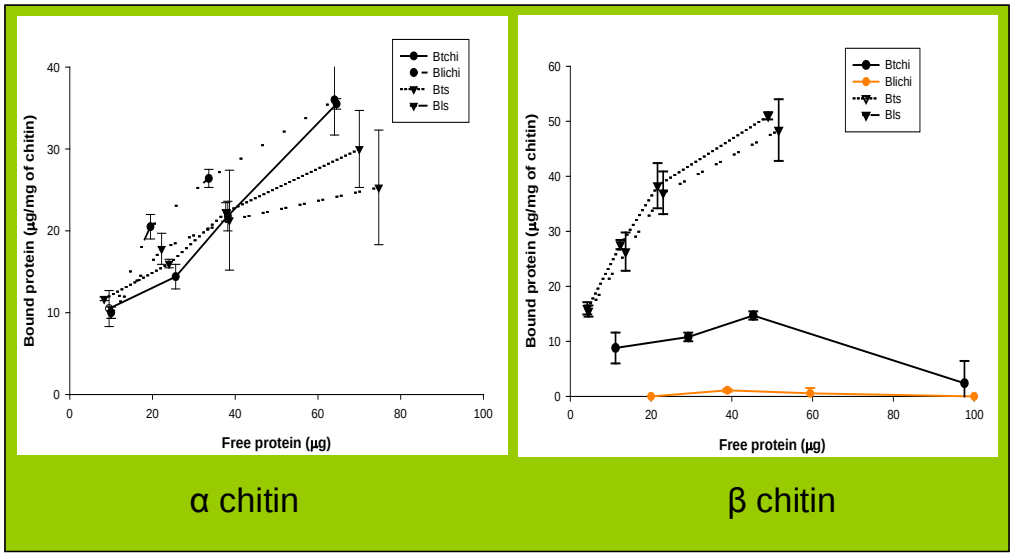


Fig. 3.31: Binding ability of Btswap and Bliswap chitinases towards insoluble α and β chitin and their comparison with native chitinases

Adsorption of Btswap and Bliswap to insoluble α and β chitin was determined in triplicates. Different amounts of proteins ranging from 20-100 µg were incubated with 1mg of chitin (dry weight) in 50 mM sodium acetate buffer pH 5.2 for 1 h in a gel shaker at 450 rpm at 4 °C. Free protein was measured using spectrofluorimeter. Bound protein=total protein-free protein. Vertical bars indicate standard deviation.

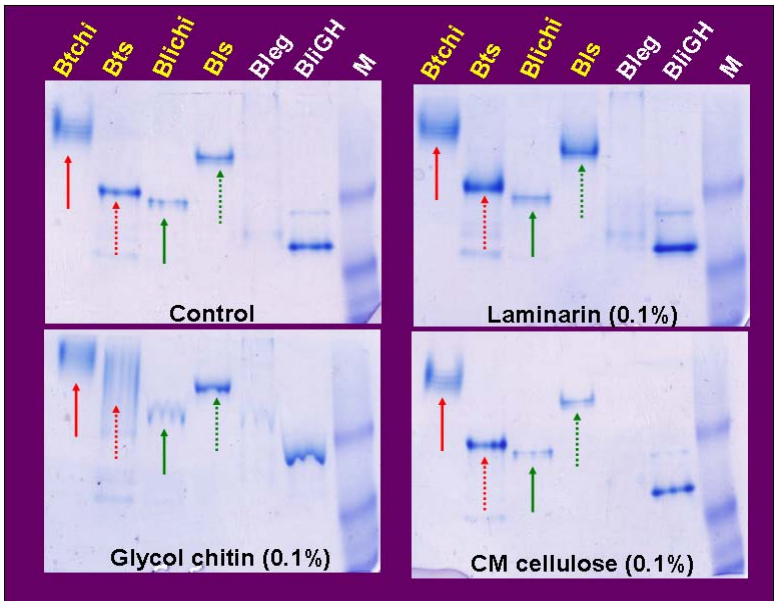
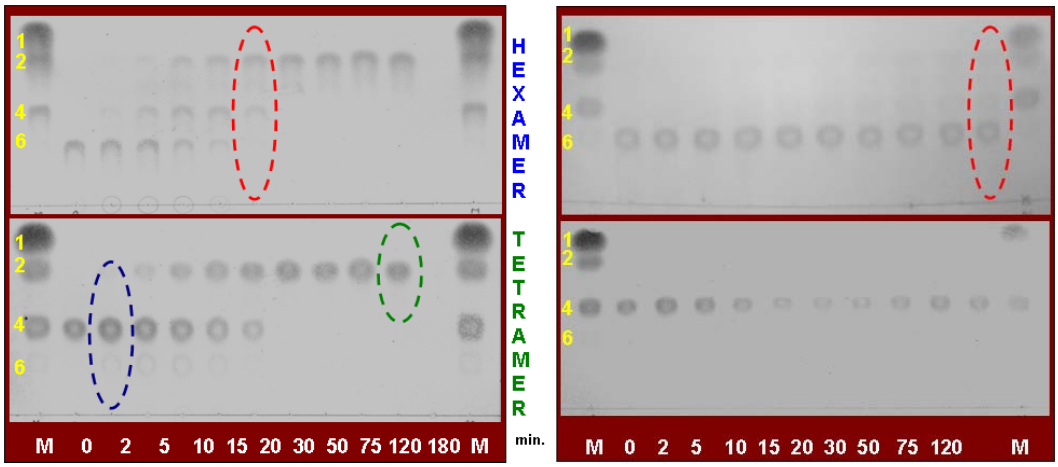


Fig. 3.32: Soluble substrate binding ability of Btswap and Bliswap using affinity electrophoresis and their comparison with native chitinases

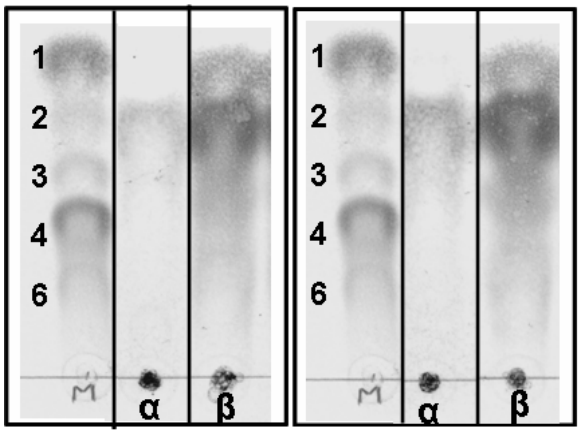
Bliswap and Btswap (20 µg) were loaded on 8 % native-PAGE with and without substrates (0.1 % of glycol chitin, CM cellulose and laminarin) followed by coomassie staining to observe the retardation in the electrophoretic mobility in order to determine the binding capacity of the two chitinases.



A **B**

Fig. 3.33: Thin layer chromatography showing hydrolysis producs of Btswap and Bliswap using soluble substrates

Soluble substrates (1 mM each of chitin hexamer, chitin tetramer and chitin trimer) incubated with 5 µg of Btswap (A) and Bliswap (B). The products were loaded on TLC plates, visualized by spraying (aniline-DPA solution) followed by heating and compared with the standard.



A **B**

Fig. 3.34: Thin layer chromatography showing hydrolysis products of Btswap and Bliswap using insoluble substrates

Insoluble substrates (0.5 mg) of α chitin and β chitin incubated with 5 µg of Btswap (A) and Bliswap (B). The products were loaded on TLC plates, visualized by spraying (aniline-DPA solution) followed by heating and compared with the standard.

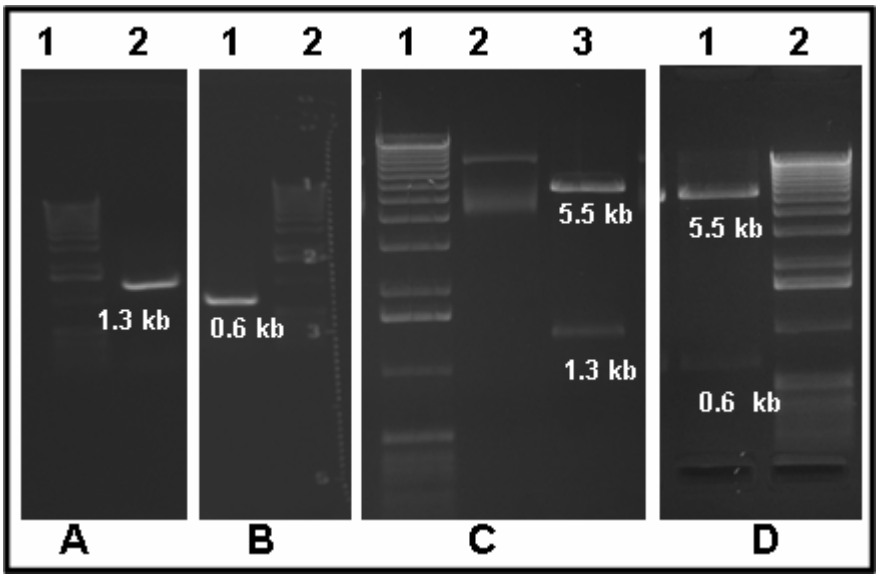


Fig. 3.35: PCR based amplification and cloning of chitin binding protein (*cbp*) from *B. thuringiensis* and *B. licheniformis*

A & B) Amplification of *cbp* from gDNA of *B. thuringiensis* and *B. licheniformis* as template using primers BthcbpFp and BthcbpRp for *Btcbp* (Lane A2), BlicbpFp and BlicbpRp for *Blicbp* (Lane B1). The amplicons were compared with 100 bp ladder (Lane A1 & B2).

C & D) *Btcbp* and *Blicbp* were amplified and ligated to *Sal* I and *Xho* I sites in pET22b+ (Novagen) resulting in pANE-Btcbp and pANE-Blicbp. Double digestion of both pANE-Btcbp and pANE-Blicbp with *Sal* I and *Xho* I released fragment of 1.3 (Lane C3) and 0.6 kb (Lane D1) confirming the presence of correct insert. Lane C1 & D2: 100 bp DNA ladder, lane 6: *Sal* I and *Xho* I digestion resistant plasmid.

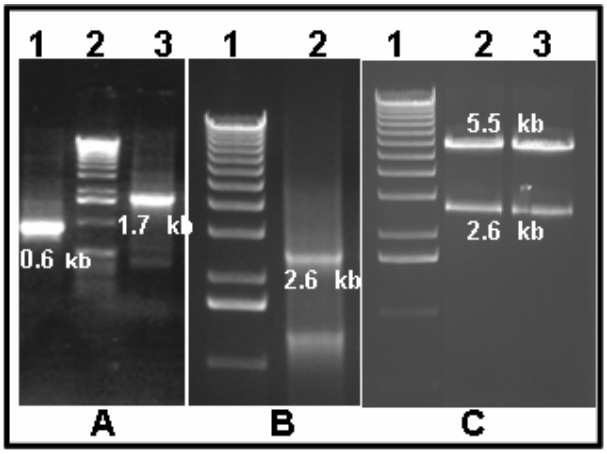


Fig. 3.36: Amplification, fusion and cloning of *NBtBlichi*

A) *CBD* was amplified (Lane 1) with C-terminal complementary overhang using primers BthcbpFp and BtcbpOERev and pANE-Btcbp as template. *Chi* was amplified (Lane 3) from plasmid pANE-Blichi as template using primers BliGHOEFor and BlchiRpmod when compared with 100 bp DNA ladder (Lane 2).

B) Amplification of the fusion PCR product *NBtBtlichi* (Lane 2) using *chi* and *CBD* as templates, BthcbpFp and BlchiRpmod primers. Lane 1: 100 bp DNA ladder.

C*)* *NBtBtlichi* was amplified and ligated to *Sal* I and *Xho* I sites in pET22b+ (Novagen) resulting in pANE-NBtBtlichi. Double digestion of pANE-NBtBtlichi with *Sal* I and *Xho* I released fragment of 2.6 kb confirming the presence of correct insert (Lanes 2 & 3). Lane 1: 100 bp DNA ladder.

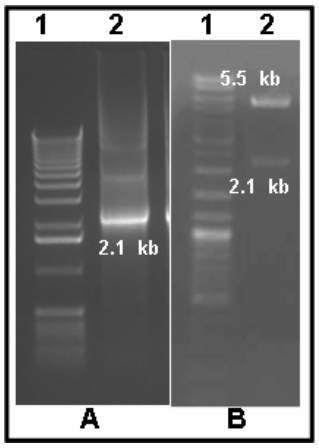


Fig. 3.37: Amplification and cloning of *NBtBliGH*

A) Amplification of *NBtBliGH* (Lane 2) using pANE-NBtBliGH as template using BthcbpBamHIFp and BlchiGH XhoIRev. The amplicon was compared with 100 bp ladder (Lane 1).

B) *NBtBliGH* was amplified and ligated to *Bam*H I and *Xho* I sites in pET22b+ (Novagen) resulting in pANE-NBtBliGH. Double digestion of pANE-NBtBliGH with *Bam*H I and *Xho* I released fragment of 2.1 kb confirming the presence of insert (Lane 2). Lane 1: 100 bp DNA ladder.

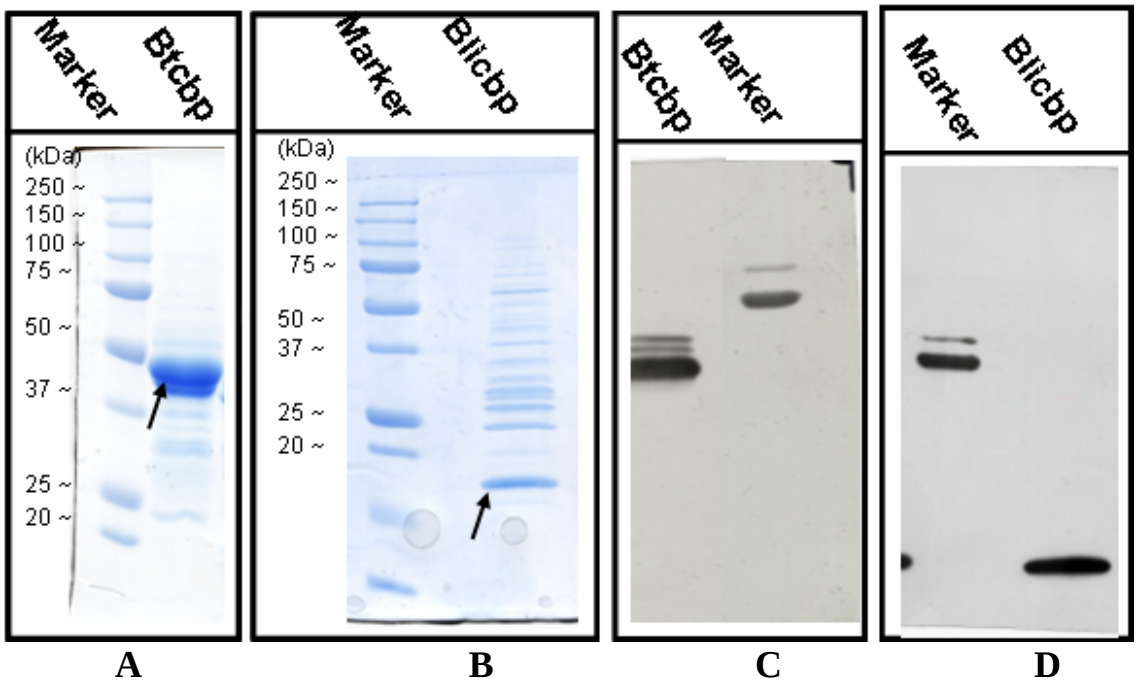


Fig. 3.38: Heterologous epression of Btcbp and Blicbp in *E.coli*

A & B) IPTG (1 mM) was used to induce the *E. coli* BL21 (DE3) at 37 °C harboring the vectors pANE-Btcbp or pANE-Blicbp. Concentrated periplasmic fractions were loaded on 12 % SDS-PAGE and compared with the standard protein weight marker.

C & D) The periplasmic fraction of Btcbp and Blicbp were transferred onto the nitrocellulose membrane. Using Anti-His tag antibody, the His tag fused at the C-terminus of the expressed protein was detected by chemiluminescent method**.** The His-tagged marker protein (M) has a molecular weight of 75 kDa.

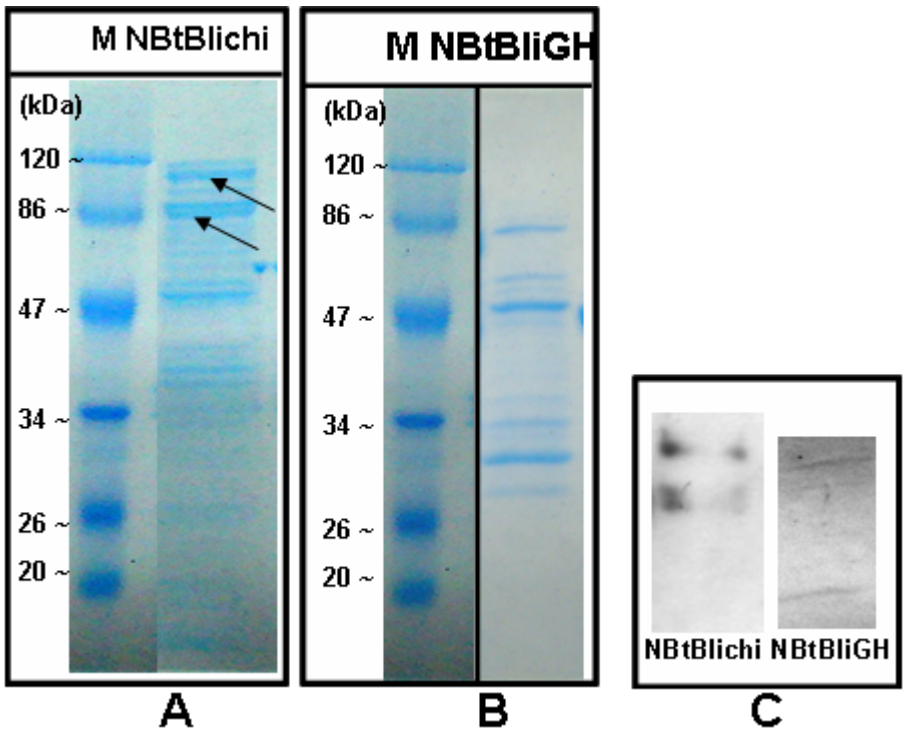


Fig. 3.39: Heterologous expression of NBtBlichi and NBtBliGH in *E. coli*

A) 1 mM IPTG was used to induce the *E. coli* BL21 (DE3) at 37 °C horbouring the vector pANE-NBtBlichi or pANE-NBtBliGH. Concentrated periplasmic fractions were loaded on 12 % SDS-PAGE and compared with the standard protein weight marker (M).

B) The periplasmic fractions of NBtBlichi and NBtBliGH were transferred onto the nitrocellulose membrane. Using Anti-His tag antibody, the His tag fused at the C-terminal of the expressed protein was detected by chemiluminescent method**.**

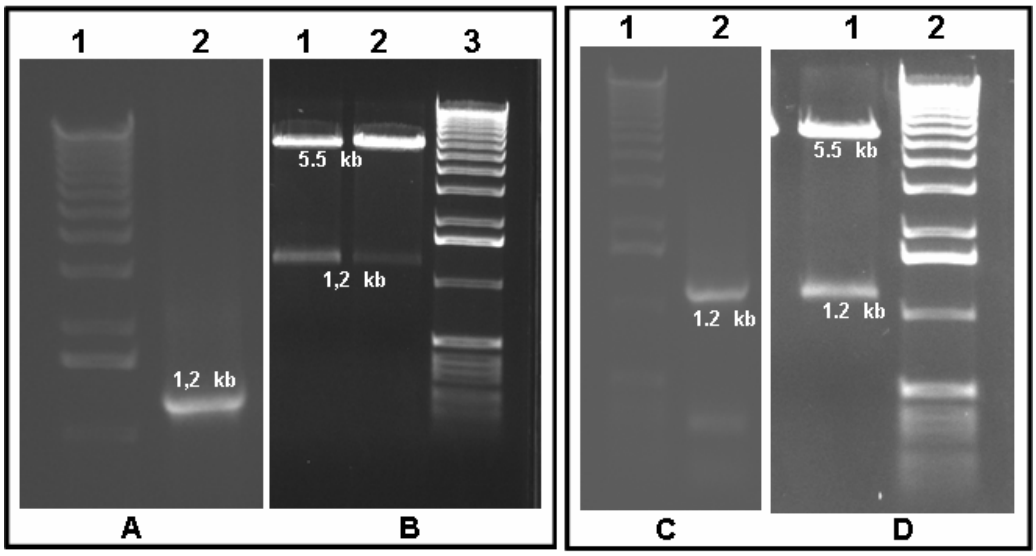


Fig. 3.40: PCR based amplification and cloning of glycosyl hydrolase (*GH*) from *B. thuringiensis* and *B. licheniformis* chitinase

A &C) Amplification of *GH* with the plasmid of pANE-Btchi and pANE-Blichi as template using primers BtchiFpmodnew and BtchiGH HindIII Rev for BtGH (Lane A2) BlchiFpmodnew and BlchiGH XhoI Rev for BliGH (Lane C2). The amplicon was compared with 100 bp ladder (Lanes A1 & C1).

B & D) *GH* was amplified and ligated to *Eco*R I and *Hind* III for *BtGH*, *Bam*H I and *Xho* I for *BliGH* sites in pET22b+ (Novagen) resulting in pANE-BtGH and pANE-BliGH. Double digestion of pANE-BtGH (Lanes B1 & 2) with *Eco*R I and *Hind* III and pANE-BliGH (Lane D1) with *Bam*H I and *Xho* I released fragment of 1.2 kb confirming the presence of insert, 100 bp DNA ladder (Lanes B3 & D2).

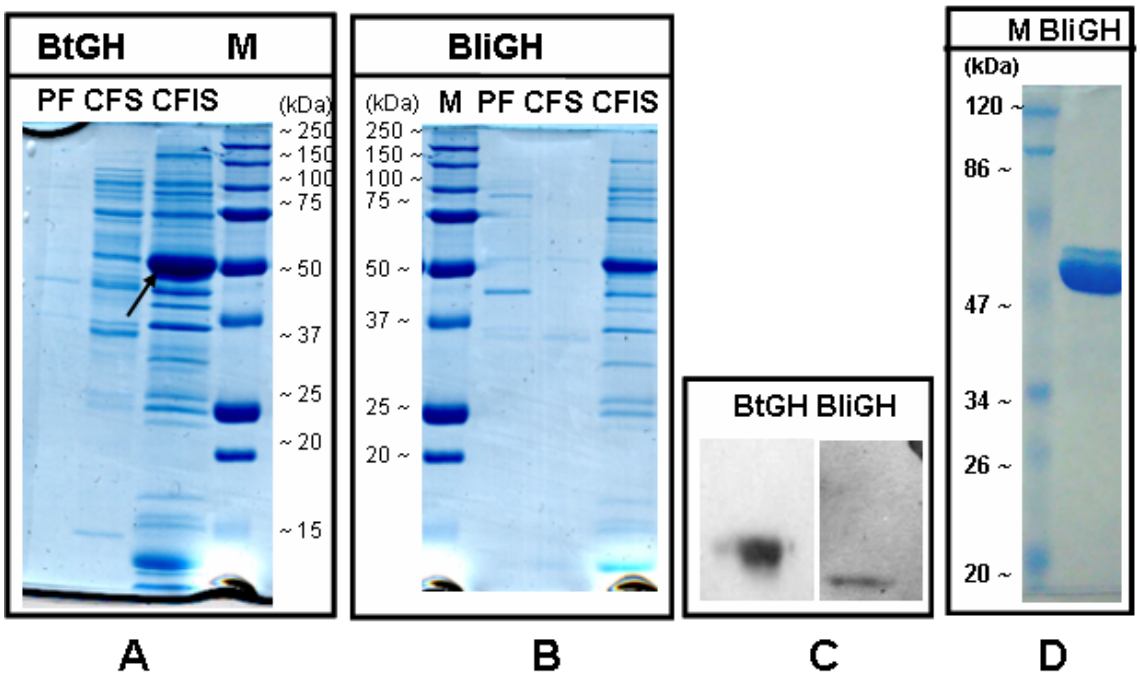


Fig. 3.41: Heterologous expression and purification of BtGH and BliGH

A & B) 0.4 mM IPTG was used to induce the *E. coli* BL21 (DE3) at 37 °C harboring vector pANE-BtGH or pANE-BliGH and the induction period was continued for 20 h at 18 °C. Concentrated periplasmic fractions (PF), cytoplasmic fraction soluble (CFS) and insoluble (CFIS) were loaded on 12 % SDS-PAGE and compared with the standard protein weight marker (M).

C) The periplasmic fraction of BtGH and BliGH were transferred onto the nitrocellulose membrane. Using Anti-His tag antibody, the His tag fused at the C-terminal of the expressed protein was detected by chemiluminiscent method.

D) Ni-NTA chromatography was used to purify the recombinant protein. Elution buffers with 50 and 100 mM imidazole was used to elute the protein. Fractions loaded on 12 % SDS-PAGE followed by coomassie staining showed the successful purification of BliGH in the elution fractions.

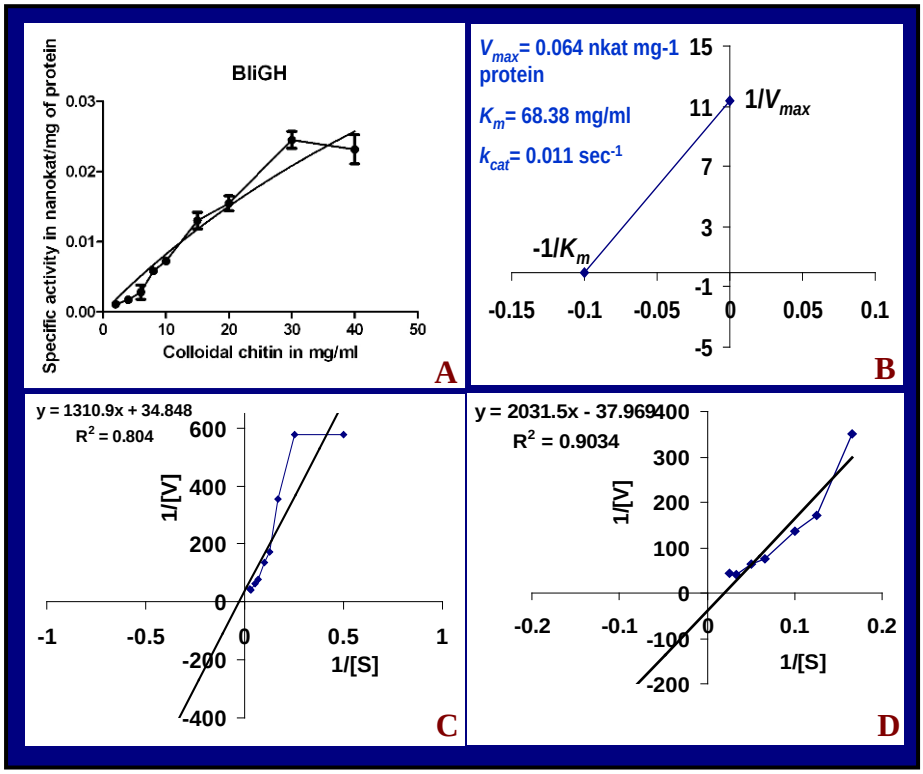


Fig. 3.42: Kinetic analysis of BliGH chitinase

Reducing end assay was done to determine the chitinase activity of purified BliGH using colloidal chitin as substrate at 37 °C for 1 h. Mean of the OD at 420 nm for each sample in triplicates was plotted on y-axis.

A) Increased concentration of colloidal chitin (0-35 mg/ml), incubated with 5 µg of BliGH was used for the assay. Specific activity in nkat/mg of protein was plotted against the substrate concentration. Graph pad prism software was used to calculate the V_{max}, Michaelis menten constant (K_m) and K_{cat} values using non linear regression analysis. Software calculated V_{max}, K_m and K_{cat} plotted on the graph to visualize and compare the data points obtained from Lineweaver-Burk (LB) plots (B). LB reciprocal plot was plotted by considering all the data points (C) and by omitting last two data points (D).

Legend for Fig. 3.43

Reducing end assay was done using colloidal chitin (8 mg/ml) and purified BliGH (5 µg), the incubations were performed in triplicates. Specific activity was calculated using mean of the OD at 420 nm. Vertical bars indicate standard deviation.

A) Optimum temperature: Incubations were done at different temperatures (20, 40, 60, 80 and 100 °C) for 1 h to determine the optimum temperature of BliGH.

B) Thermal stability: Reducing end assay was done at standard conditions using colloidal chitin and BliGH pre-incubated in triplicates at temperatures (30-100 °C) for 1 h to determine the thermal stability of BliGH.

C) Stability at optimum temperature: Purified BliGH was pre-incubated in triplicates at 60 ºC for 1-4 h followed by reducing end assay using colloidal chitin under standard reaction conditions to determine the stability of BliGH at 60 °C . Fifty µg of purified BliGH in 10 mM sodium acetate pH 5.2 was analysed for CD spectrum followed by secondary structure prediction. At each temperature CD spectrum was recorded to plot the relative change in secondary structure by considering the CD spectrum at 30 °C as 100 %.

D) Changes in secondary structure with increase in temperature: CD spectrum of BliGH was obtained at 30 °C followed by gradual increase in temperature till 100 °C to determine the change in the relative secondary structure of BliGH with increase in temperature.

E) Effect of pre incubation at different temperatures on secondary structure: BliGH pre-incubated at different temperatures for 1 h was analysed for CD spectrum to determine the thermal stability of BliGH.

F) Conformational stability at optimum temperature: BliGH was pre-incubated at 60 °C for 1-4 h and analysed for CD spectrum at 30 °C to determine the conformational stability of BliGH at 60 °C.

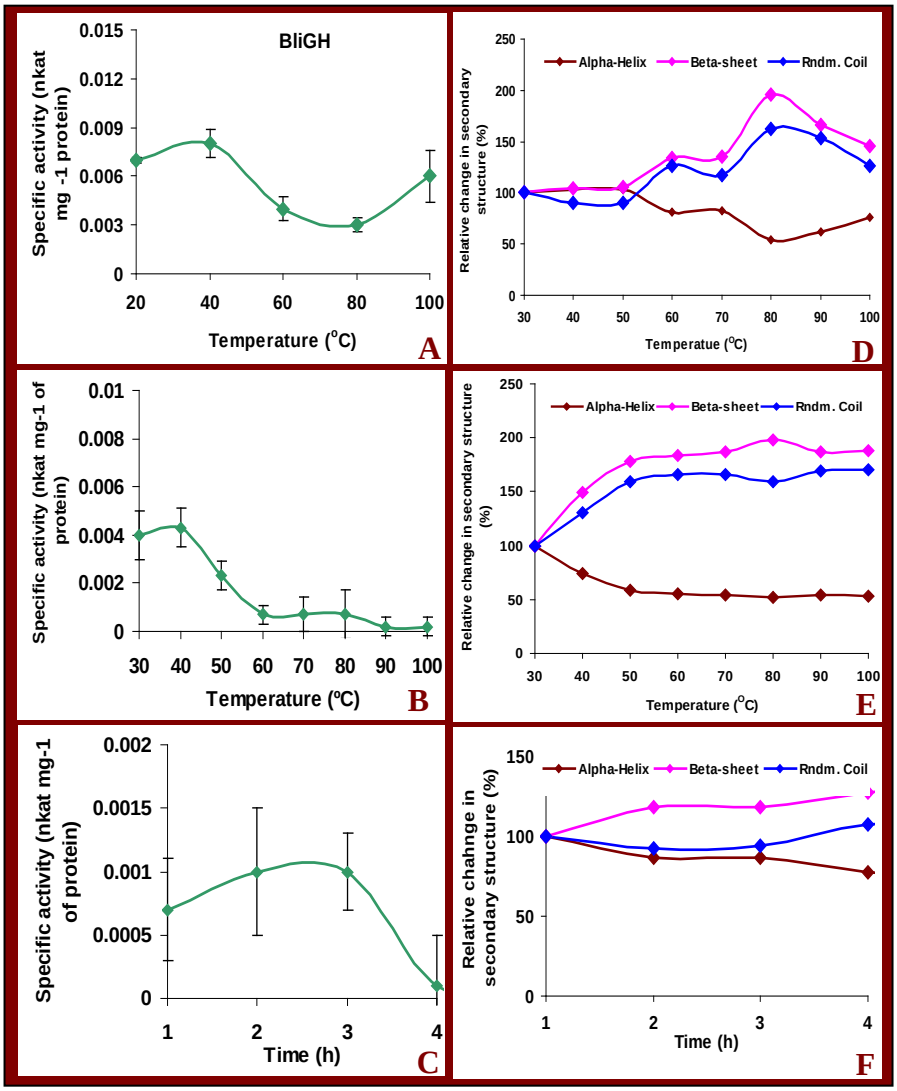


Fig. 3.43: Effect of temperature on the activity and secondary structure of BliGH

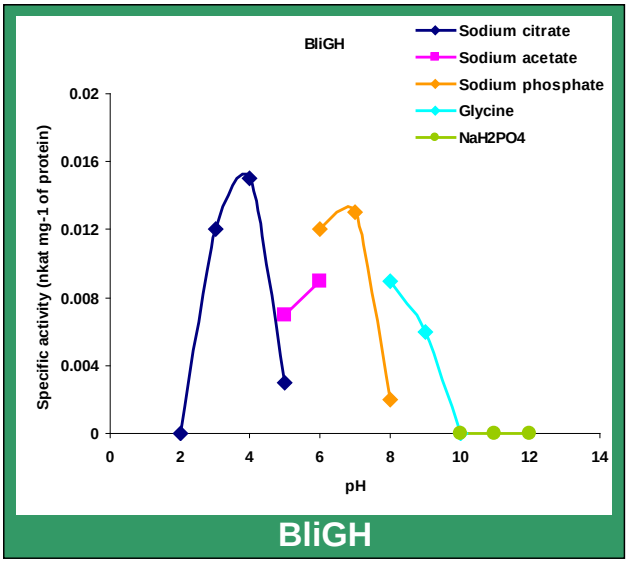


Fig. 3.44: Effect of pH on the activity of BliGH using colloidal chitin as substrate

Reducing end assay was done in triplicates using colloidal chitin and purified 5 µg of BliGH chitinase, the incubations were performed at different pH (2-12) for 1 h at 37 °C to determine the optimal pH of BliGH chitinase.

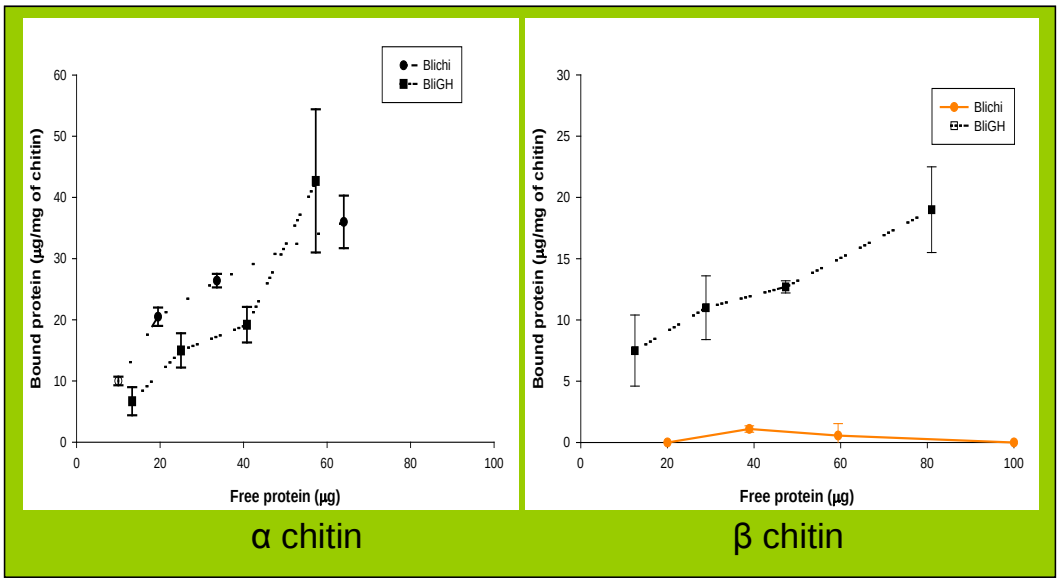


Fig. 3.45: Binding ability of BliGH towards insoluble α and β chitin

Adsorption of BliGH to insoluble α and β chitin was determined in triplicates. Different amounts of proteins ranging from 20-100 µg were incubated with 1mg of chitin (dry weight) in 50mM sodium acetate buffer pH 5.2 for 1 h in a gel shaker at 450 rpm at 4 °C. Free protein was measured using spectrofluorimeter. Bound protein=total protein-free protein. Vertical bars indicate standard deviation.

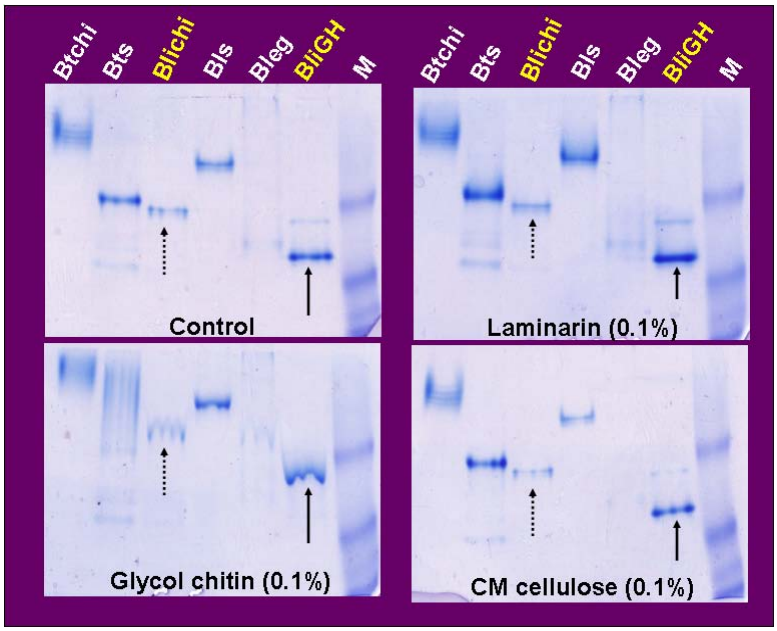
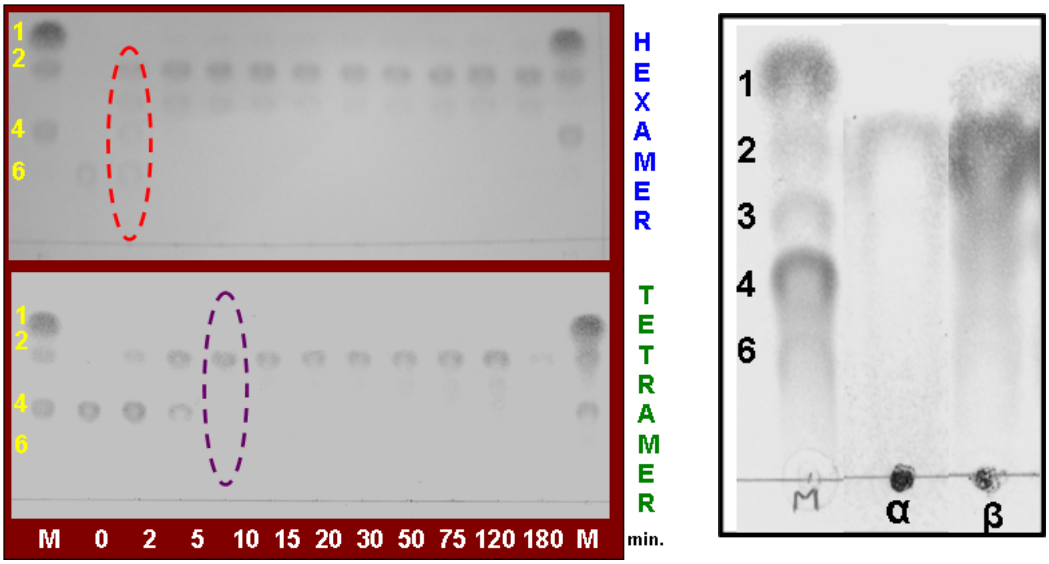


Fig. 3.46: Soluble substrate binding ability of BliGH using affinity electrophoresis

BliGH (20 µg) was loaded on 8 % native-PAGE with and without substrates (0.1 % of glycol chitin, CM cellulose and laminarin) followed by coomassie staining to observe the retardation in the electrophoretic mobility in order to determine the binding capacity of the two chitinases.



A **B**

Fig. 3.47: Thin layer chromatography showing hydrolysis products of BliGH using soluble and insoluble substrates

BliGH (5 µg) was incubated with soluble substrate (1 mM each of chitin hexamer, chitin tetramer and chitin trimer) (A) and insoluble substrates (0.5 mg of α and β chitin) (B). The products were loaded on TLC plates, visualized by spraying (aniline-DPA solution) followed by heating and compared with the standard.