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 CHIMERICAS - 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 NBtBlich in pET 22b+). The 2.1 kb fusion chimera was PCR-amplified by deleting CBD 5-12 from NBtBlich, cloned and the construct was termed as pANE-NBtBlGH (fusion NBtBlGH 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 NBtBlich and 74 and 50 kDa with NBtBlGH,
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.
- Characterization 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 Bliegl showed 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 °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.