Characterization of unique chitinases from *Serratia marcescens* GPS5 and *Flavobacterium johnsoniae* UW101

Thesis submitted for the degree of
DOCTOR OF PHILOSOPHY

by

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CERTIFICATE

This is to certify that **Mr. PAPA RAO VAIKUNTAPU** has carried out the research work embodied in the present thesis under the supervision and guidance of Prof. Appa Rao Podile for a full period prescribed under the Ph.D. ordinances of this University. We recommend his thesis entitled **"Characterization of unique chitinases from *Serratia marcescens* GPS5 and *Flavobacterium johnsoniae* UW101"** for submission for the degree of Doctor of Philosophy of the University.

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DECLARATION

This is to declare that the work embodied in this thesis entitled "**Characterization of unique chitinases from *Serratia marcescens* GPS5 and *Flavobacterium johnsoniae* UW101**" has been carried out by me under the supervision of Prof. Appa Rao Podile, Department of Plant Sciences, School of Life Sciences. The work presented in this thesis is a bonafide research work and has not been submitted for any degree or diploma in any other University or Institute. A report on plagiarism statistics from the University Librarian is enclosed.

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PAPA RAO VIKUNTAPU

(పాపారావు వైకుంఠపు)

Dedicating

To

My beloved

Family

CONTENTS

ABBREVIATIONS

^{o}C	Degree centigrade/degree Celsius
CBD	Carbohydrate binding domain
CHOS	Chitooligosaccharides
ChBD	Chitin binding domain
CeBD	Cellulose binding domain
dNTPs	Deoxy nucleotide triphosphates
DNA	Deoxy ribonucleic acid
DDA	Degree of deacetylation
DA or FA	Degree of Acetylation or Fraction of Acetylation
DP	Degree of Polymerization
EDTA	Ethylene diamine tetra acetic acid
g	Gram
GlcNAc	N-acetyl glucosamine
h	Hour (s)
HPTLC	High Performance Thin Layer Chromatography
HPLC	High Performance Liquid Chromatography
IPTG	Isopropyl β-D-thiogalactoside
Km	Michaelis Menton constant
kb	Kilobase pair
kDa	Kilodalton
LB	Luria-Bertani
L	Litre
M	Molar
mg	Milligram
min	Minute
ml	Milliliter

mM	Millimolar
mm	Millimeter
MALDI-ToF	Matrix Assisted Laser Desorption/ionization–Time of Flight
ng	Nanogram
NCBI	National Centre For Biotechnological Information
Ni-NTA	Nickel-nitroacetic acid agarose
nm	Nanometer
nM	Nanomolar
OD	Optical density
PA	Pattern of Acetylation
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
SDS	Sodium dodecyl sulphate
PDB	Protein Data Bank
PKD	Polycystic Kidney Disease
Tris	Tris-(Hydroxymethyl) aminoethane
TG	Transglycosylation
TLC	Thin Layer Chromatography
UHPLC-ELSDESI-MS	Ultra-High Performance Liquid Chromatography-Evaporative Light-Scattering Detection-Electrospray Ionisation Mass Spectrometry
µg	Microgram
µL	Microlitre
µmol	Micromole

LIST OF FIGURES

LIST OF TABLES

Chapter-1

Introduction

1.1 Chitin: Braconnot discovered a treasure of world

Nature is gifted with innumerable materials which could be obtained from several plants and animal resources. The first discovery of chitin was made in 1811 by a French Professor **Henri Braconnot** (Khoushab and Yamabhai, 2010) and named this substance as "Fungine". Starch, cellulose, and chitin are naturally occurring abundant, biodegradable and renewable biopolymers. Chitin $(C_8H_{13}O_5N)_n$, a cellulose-like polysaccharide (Fig 1.1) is a linear, poly-β-(1,4)-*N*-acetyl-d-glucosamine and second most abundant biopolymer in nature next to cellulose (Gooday, 1990). Chitin is mainly found in fungal cell walls and in the outer shields, and exoskeleton of arthropods (approximately 75% of the total weight). Chitin is also part in cell walls of ciliates, chrysophytes amoebae, algae and in the spines of diatoms. Shrimp and crab shells are the main notable source of chitin polymers because of their availability and the availability of wastes from seafood processing industry (Hamed et al., 2016). Chitin is odorless and tasteless nitrogenous polysaccharide which is white or yellowish in color. Chitin is highly hydrophobic, and therefore insoluble in water.

In nature, chitin occurs as systematically arranged crystalline microfibrils. The X-ray diffraction studies unveiled that chitin present in three different polymorphic forms in nature: α chitin; β-chitin; and γ-chitin (Fig 1.2). α-Chitin is the most abundant, stable form among the three crystalline variations and chitin chains arranged anti-parallel. It serves as the vital structural component in insect cuticles, crab shells, shrimps, marine sponges, in yeast and fungal cell walls. β-Chitin is less stable than the α-chitin, where chitin chains arranged parallel and has weaker intermolecular forces. Chitin was found in the extracellular fibers of diatoms, the pens of squid (Lavall et al., 2007), and the spines of some annelids. γ-chitin is the least common form of chitin. It contains a combination of α and β- chitin structures. γ-chitin was reported to be present in the stomach of squid and in the cocoons of beetles (Rinaudo, 2006, Souza et al., 2011).

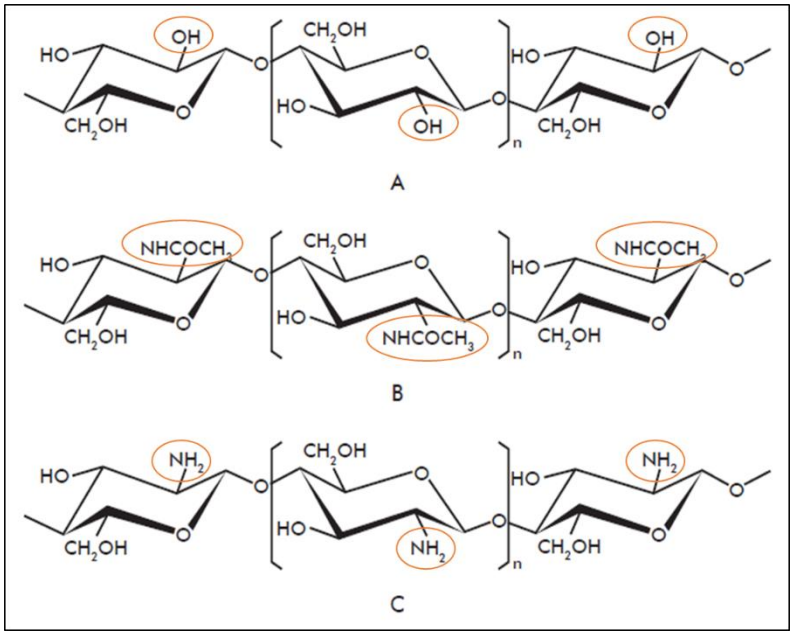


Fig 1.1: Structural representation: A) Cellulose, B) Chitin, C) Chitosan (Ramírez et al., 2010). Variations at C2 OH group of polymers shown in red circle.

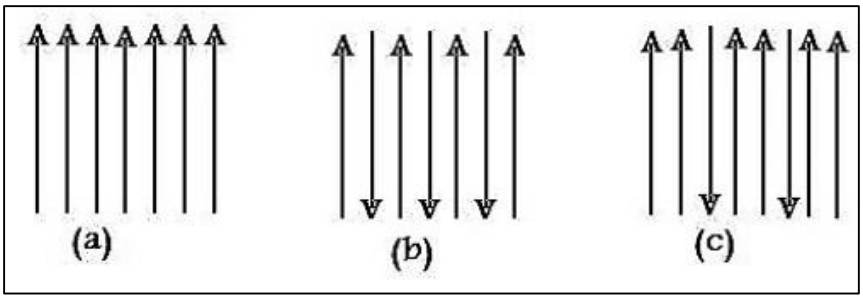


Fig 1.2: Polymorphic configuration: a) α-chitin, b) β-chitin, and c) γ-chitin

1.2 Chitosan: A natural do-it-all biopolymer

Chitosan is most important derivative of chitin. Pure chitin hardly exists in nature, instead, natural chitin products may have an acetylation degree of 80%-95%. Chitosan is a *N*-deacetylated derivative of chitin, which is obtained by deacetylation of chitin by either chemical or enzymatic methods. Marine crustacean shells are the primary source for the production of chitosan (Venkatesan and Kim, 2010) from raw chitin (Varum and Smidsrod, 2012). Unlike the chitin, chitosan is soluble in mild acid solutions.

Chitosans can be described and classified according to the degree of *N*-acetylation (DA), the degree of polymerization (DP) and the pattern of *N*-acetylation (PA). Because of its chemical nature, chitosan exhibits numerous interesting physicochemical and biological properties. Chitosans have received a great attention in recent years due to their immense biotechnological applications. Because of non-toxicity, low immunogenicity, biodegradability, and biocompatibility, chitosan grabbed more attention in cosmetics, agriculture, waste water treatment and pharmaceutical industries. The ratio of glucosamine (GlcN)/*N*-acetyl glucosamine (GlcNAc) defines the chitin/chitosan. When the percentage of *N*-acetyl glucosamine is more than glucosamine, the biopolymer is named as chitin and when the percentage of glucosamine exceeds *N*-acetyl glucosamine the compound is named as chitosan (Ramírez et al., 2010; Khor and Lim, 2003).

Unlike cellulose, chitin can be a source of both nitrogen (N) as well as carbon (C). The relatively high nutritional value of chitin, including both C and N components attracts many primary decomposers such as bacteria and fungi to produce chitin modifying enzymes, among which chitinases are predominant.

1.3 Glycoside hydrolases (GHs) and chitinases

Depending on the basis of their amino acid sequences, glycoside hydrolases (GH) have been classified into 135 families in the carbohydrate active enzyme (CAZy) database (http://www.cazy.org/Glycoside-Hydrolases.html). Among the 135 families, GH 4, GH 5, GH 7, GH 8, GH 18, GH 19, GH 20, GH 46, GH 75 and GH 80 are involved in chitin depolymerization (Fig 1.3) Speedy development of genome sequencing technologies enabled rapid bacterial

genomes sequencing. Many GH 18 chitinases were reported from several bacterial sources. To date, the number of GH 18, GH 19 and GH 20 chitinases from bacteria is reported to be more than eukaryota (http://www.cazy.org/Glycoside-Hydrolases.html) (Table 1.1).

Chitinases (E.C. 3.2.1.14) are glycosidase enzymes that specifically degrade chitin to release *N*-acetyl chitooligosaccharides (CHOS) and GlcNAc (Dahiya et al., 2006). Chitinases cleave the bond between C1 and C4 of two consecutive *N*- acetylglucosamine monomer. Chitinases are present in a wide range of organisms like viruses, bacteria, fungi, insects, plants, and animals (Park et al., 1997). Chitinases play diverse role in different organisms (Table 1.2).

In vertebrates, chitinases are directly secreted into the digestive tract. In insects and crustaceans, chitinases play a pivotal role in the developmental process by way of partial degradation of the old cuticle. Chitinases induce the plant innate immunity and inhibit the fungal pathogens *in vitro* (Das et al., 2015; Suma and Podile, 2013). Chitinases in fungi play role in autolysis, nutritional, and morphogenesis. In viruses, chitinases are known to be involved in the pathogenesis of baculovirus (Patil et al., 2000). Bacterial and fungal chitinases are more crucial for balancing the carbon and nitrogen present in chitin in nature (Dahiya et al., 2006).

1.4 Classification of chitinases

The enzymatic depolymerization of chitin depends on the chitinolytic system of the organism. Chitinases are classified into 2 major categories: exochitinases and endochitinases (Dahiya et al., 2006; Duo-Chuan, 2006). Endochitinases (EC 3.2.1.14) cleave chitin chain randomly at internal sites of polymer and generate low molecular mass chitin oligomers in the range between DP2-DP6. While, exochitinases are further classified into 2 sub categories, chitobiosidases (EC 3.2.1.30) and β-(1,4) *N*-acetyl hexosaminidases (EC 3.2.1.52). β- (1,4), *N*- acetyl hexosaminidases cleave the oligomeric products produced by endochitinases and chitobiosidases catalyze the progressive release of diacetyl chitobiose from nonreducing end of chitin chain. Chitobiase cleaves chitobiose to GlcNAc.

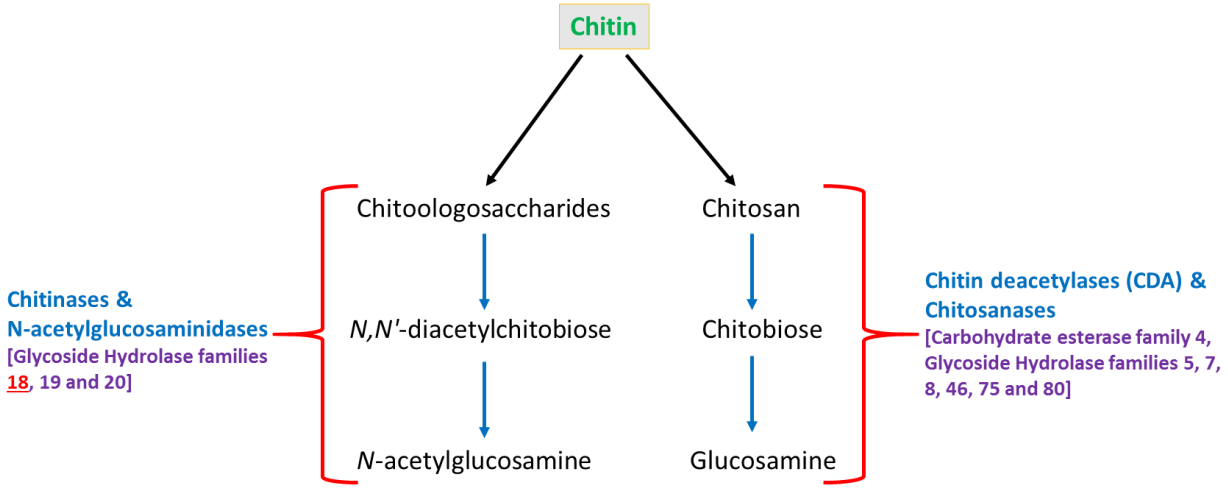


Fig 1.3: Enzymatic degradation of chitin by members of different glycoside hydrolase families.

GH family	Enzyme	Nomenclature (EC)	Source (prevalence)*
18	Chitinase	EC 3.1.1.14	**All (10018)** Achaea (59); Bacteria (6634); Eukaryote (2850); Viruses (233); Unclassified (242); Structure (74); Characterized (466). **Only 4.79% of GH18 chitinases were characterized till now**
	Endo-β-*N*-acetylglucosaminidases	EC 3.2.1.96	
	Lysozyme	EC 3.2.1.17	
	Xylanase inhibitor; Concanavalin B; Narbonin	non-catalytic proteins	
	Lysozyme	EC 3.2.1.17	
	Peptidoglycan hydrolase with endo-β-*N*-acetylglucosaminidase specificity	EC 3.2.1.-	
	Nod factor hydrolase	EC 3.2.1.-	
19	Chitinase	EC 3.2.1.14	**All (3472)** Bacteria (2309); Eukaryote (804); Viruses (356); Unclassified (3); Structure (15); Characterized (176).
	Lysozyme	EC 3.2.1.17	
20	β-hexosaminidase	EC 3.2.1.52	**All (2881)** Achaea (4); Bacteria (2570); Eukaryote (292); Viruses (1); Unclassified (14); Structure (18); Characterized (124).
	Lacto-*N*-biosidase	EC 3.2.1.140	
	β-1,6-*N*-acetylglucosaminidase	EC 3.2.1.-	
	β-6-SO3-*N*-acetylglucosaminidase	EC 3.2.1.-	

* CAZy database, Aug 1st, 2016.

Table 1.1: Glycoside hydrolases involved in chitin degradation.

(Source: http://www.cazy.org/Glycoside-Hydrolases.html)

Living being	Role of chitinases	References
Virus	Baculoviruses: Used for biological control of insect pests	(Patil et al., 2000)
Bacteria	Mineralization of chitin, in nutrition and parasitism	(Dahiya et al., 2006)
Fungi	Cell division, differentiation and nutrition	(Mellor et al., 1994)
Plants	Inhibit fungal growth, embryo and seed development, fruit ripening, pollination and sexual reproduction process	(Wu, 2003)
Insects	Throughout the developmental process by degrading the cuticle at different larval stages.	(Merzendorfer and Zimoch, 2003)
Protozoa	Malarial parasites life cycle	(Langer, 2002)
Human	Chitotriosidase is a new inflammatory marker in diabetes. It also used as a marker of a lysosomal Gaucher disease	(Di Rosa and Malaguarnera, 2016)
Yeast	Budding and Cell separation	(Kuranda and Robbins, 1991)

Table 1.2: Distribution and role of chitinase in different living beings.

1.5 Classification of chitinases based on amino acid sequences

Chitinolytic enzymes are divided into three families based on their similarity of amino acid sequence: GH 18, GH 19, and GH 20 (Henrissat and Bairoch, 1993). These three families are divided based on their primary structure. Enzymes that belong to the same family share common structural properties in terms of folding of the catalytic domain and substrate specificity. Each family differs in amino acid sequences, three-dimensional (3D) structures and molecular mechanisms of catalysis. Family GH 18 chitinases are diverse and present in bacteria, fungi, viruses, animals, and some plants. Family GH 19 chitinases are restricted to seed plants and some *Streptomyces* sp. The amino acid sequence similarity is different in family GH 18 and GH 19 chitinases. They have distinctly separate 3-D structures and catalytic mechanisms and therefore both the families probably evolved from two different ancestors (Suzuki et al., 1999). The family GH 20 chitinase, β-*N*- acetyl hexosaminidases are reported from bacteria and humans (Lemieux et al., 2006).

1.6 Classification of chitinases based on gene sequences

Based on gene sequences Iseli et al., (1996) grouped the chitinases into six classes. Grouping has been done based on: N-terminal sequence of the enzyme, localization of the enzyme, isoelectric pH, and signal peptide. Class I chitinases contains a cysteine rich N-terminal site, valine or leucine - rich signal peptide, and their localization (Flach et al., 1992). It is further subdivided into two sub Classes Ia and Ib based on their basic and acidic nature. The Class I chitinases include plants, whereas Class II chitinases are present in bacteria, fungi, and plants. Class II chitinases do not have the cysteine rich N- terminal domain, but they exhibit sequence similarity with Class I chitinases. Class I chitinases are endo chitinases, whereas Class II chitinases act in exo manner. Class II chitinases are majorly found in dicot plants and are secreted into the extracellular spaces. Class III chitinases don't show any sequence similarity with Class I or II chitinases (Collinge et al., 1993) but share good amino acid sequence similarities with lysozyme from *Hevea brasiliensis* (Meins et al., 1992). Class IV chitinases are similar to Class I chitinases. However, they are smaller in size than Class I chitinases because of four deletions in their catalytic domain (Collinge et al., 1993). Classes V and VI included single examples (Iseli et al., 1996). It was intimated that chitinases of class I, II, and IV are from plant origin and structurally and functionally similar to family GH 19

chitinases. Class III and V are similar to family 18 glycosyl hydrolases (Henrissat and Davies, 1997), which are structurally not related to family 19 chitinases.

1.7 Proposed catalytic mechanism of chitinases

Chitinases are classified based on the individual mode of action of the enzyme.The hydrolysis of the β-(1-4) glycosidic linkage is a nucleophilic replacement at the anomeric carbon, and can lead to either retention / inverting mechanism of the anomeric configuration (Koshland and Stein, 1954). In the retention / inversion mechanism, hydrolysis reaction occurs through general acid catalysis, and requires a pair of carboxylic amino acids at the enzyme's catalytic site. One carboxylic acid acts as a proton donor, facilitates leaving group departure, and the other carboxylic amino acid acts as either a base (inverting mechanism) or as a nucleophile (retaining mechanism).

The retaining mechanism (double displacement mechanism or substrate-assisted catalysis) is a two-step mechanism. The first step involves the protonation of the glycosidic oxygen by the catalytic acid and nucleophilic attack on the anomeric carbon by nucleophile, the second carboxylic acid. This attack causes to breakage of the glycosidic link and the formation of a covalent linkage between the anomeric carbon and the catalytic nucleophile. Eventually, this intermediate is hydrolyzed by a water molecule that leads to retention of the anomeric carbon configuration (Aam et al., 2010). Family GH 18 chitinases use this retaining mechanism (Fig 1.4a). Complete details of family GH 20 chitinases catalysis mechanism are not available.

The inverting mechanism (single displacement mechanism or acidic catalysis) is a "one-stop" mechanism, where the protonation of the glycosidic oxygen takes place simultaneously with a nucleophilic attack of an activated water molecule on the anomeric carbon. This water molecule is activated by the second carboxylic group that acts as a base. Because the water molecule attacks the anomeric carbon from the other side of the catalytic base, this mechanism leads to change in the anomeric configuration. Family GH 19 chitinases use this inverting mechanism (Iseli et al., 1996) (Fig 1.4b).

1.8 Transglycosylation (TG): Unique of some GHs

Most of the GHs favor the mechanistically controlled hydrolysis reaction. Transglycosylases are unusual GHs capable of catalyzing the formation of glycosidic bonds between carbohydrate oligomers. Transglycosylation (TG) takes place when the positively charged glycosyl intermediate reacts with an incoming alcoholic oxygen from a second sugar molecule during a catalysis which in turn leads to the generation of larger molecular weight products than the substrate used in the reaction. In general, positively charged glycosyl intermediate reacts with water to complete hydrolysis (Fig 1.5).

The TG activity of family 18 chitinases has been of special interest to researchers (Aguilera et al., 2003; Boer et al., 2004; Fukamizo et al., 2001; Purushotham and Podile, 2012; Suma and Podile, 2013; Zakariassen et al., 2011) because of the enormous beneficial applications for longer chain chitooligosaccharides (CHOS). Other chitinases exhibiting TG include a chitinase from *Nocardia orientalis* IFO 12806, which produced TG product GlcNAc7 (DP7) when incubated with GlcNAc4 and GlcNAc5 (Usui et al., 1987). An endochitinase purified from a *Bacillus* sp. also has been reported to perform TG (Ochiai et al., 2004). TG activity of β-*N*-acetylhexosaminidase from *Penicillium oxalicum* was used to synthesize drug carriers (Kadowaki et al., 1997). A new exochitosanase enzyme from *Aspergillus fumigatus* IIT-004 was characterized from and tested for TG (Sinha et al., 2016).

Site-directed mutagenesis (SDM) of crucial residues within the chitin binding domain of chitinase A and chitinase B from *Serratia marcescens* showed an improvement in TG activity (Zakariassen et al., 2011). TG improved by molecular modification of critical amino acids situated at different places in chitinase D from *Serratia proteamaculans* 568 (Madhuprakash et al., 2012). Mutagenesis at chitin binding site or chemical modification successfully improved the TG/hydrolysis (TG/H) ratio of several chitinases (Aronson et al., 2006; Fukamizo et al., 1989; Martinez et al., 2012; Nanjo et al., 1989; Sirimontree et al., 2014; Songsiriritthigul et al., 2008; Suginta et al., 2007; Taira et al., 2010; Umemoto et al., 2015, 2013).

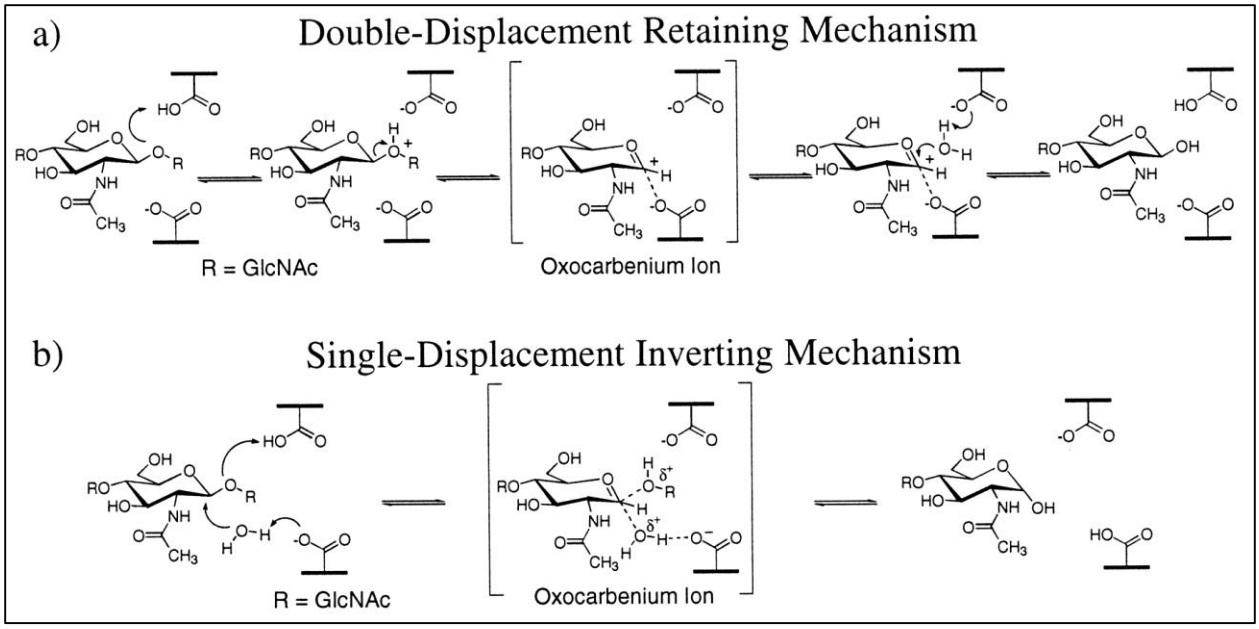


Fig 1.4: Mechanisms of hydrolysis by glycoside hydrolases: a) The double-displacement hydrolysis mechanism proposed for family 18 chitinases. b) The single-displacement hydrolysis mechanism proposed for family 19 chitinases (Dahiya et al., 2006).

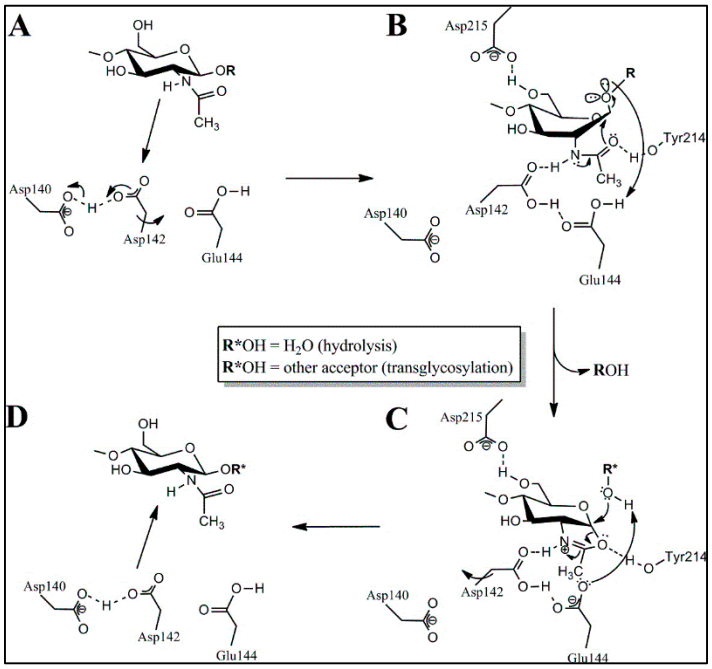


Fig 1.5: Catalytic mechanism of family 18 chitinases ChiB from *S. marcescens*. (A) Substrate binding the enzyme induces rotation of Asp142 toward Glu144. (B + C) Substrate binding causes distortion of the -1 sugar; Amino acid Glu144 functions as a general acid and helps leaving group departure by protonating the glycosidic oxygen. Simultaneously which will lead to the formation of an oxazolinium ion intermediate (C). (C + D) Glu144 acts as a general base and activates an acceptor molecule (water or another sugar acceptor) that attacks the oxazolinium intermediate. This results in a hydrolytic or TG product with leaving the same conformation at the anomeric carbon (D) (Zakariassen et al., 2011).

1.9 Industrial applications of chitin, chitosan, and chitooligosaccharides (CHOS)

Chitin and chitosan were extensively studied for versatile applications for a variety of reasons including bioavailability, biocompatibility, biodegradability, renewable, nontoxic, and inexpensive biomass (Szymańska and Winnicka, 2015). However, one of the main limitations in the use of the chitin biopolymer on a large-scale is poor solubility in water. Hence, water-soluble derivatives of chitin and chitosan have been produced.

Chitosan shows antimicrobial activity and wound healing properties which make chitosan based materials useful in the biomedical fields such as for artificial skin, bones, and cartilage regeneration (Fernández-de Castro et al., 2016; Parvez et al., 2012; Salah et al., 2013). Moreover, it was also used in agriculture as a plant elicitor, in food as stabilizer and thickener, and in waste water treatment (Das et al., 2015; Parvez et al., 2012). In addition, chitosan is readily processed into nanofibers, sponges, nanoparticles, gel, beads, scaffolds, membranes and standalone films (H.P.S et al., 2016; Song et al., 2016). Some industrial applications of chitin and its derivatives are listed in Table 1.3.

1.10 Discovery of new enzyme with better properties

Chitinases were identified in a number of biological systems. In addition to genomics and proteomics, new technologies hold great potential for exploring the richness of biodiversity in environments. Promising metagenomic or culturable technologies are helping in the screening of chitin-degrading genotypes of microbial communities from chitin rich soil (Das et al., 2010). Exploring the bacterial diversity in chitin degradation environments may also help in discover a novel enzymatic activities for hydrolysis of polymeric chitin.

Different oligo- and polysaccharides including β-glucan, chitin- and chitin oligomers, oligogalacturonide, alginate, fucan, carrageenan, and ulvan has been reported to be involved in inducing plant immunity (Trouvelot et al., 2014). Among them, chitin or chitin oligosaccharides (CHOS) have been reported to elicit defense response to a wide range of pathogens in many plants (van Aubel et al., 2016; Wang et al., 2015; Yin et al., 2016). However, the mechanism CHOS-induced immunity, especially the signaling processes are under active investigation. Plant hormones, especially JA, play a crucial role in CHOS triggered plant immunity (Yin et al., 2013).

Fields	Examples of application	References
Pharmaceuticals	-Pharmaceutical ingredients' carriers -Non-viral vectors for gene delivery -Gradual release of drugs	(Dash et al., 2011) (Bellich et al., 2016)
Biomedical engineering	-Tissue regeneration -Wound dressing of microbial infections/burns -Surgical stitches -Bioimaging	(Yang, 2011) (Rajitha et al., 2016)
Cosmetic and dermatological	-Active cosmetic product transporters -Skin, oral, and hair care products -Moisturizer and anti-aging agents	(Morganti et al., 2008) (Gautier et al., 2008)
Food	-Protective shield against food spoilage -Food preservative additives -Prebiotics (dietary fibers) ingredients	(Khoushab and Yamabhai, 2010)
Textiles	- Production of non-allergenic & antimicrobial fibers -Bioremediation	(Pillai et al., 2009) (Dutta et al., 2002)
Agriculture	-Seeds-coating agents -Enhancing plant immunity against pests and microbial pathogens -Elicitors -Promote seeds germination	(Das et al., 2015) (Madhuprakash et al., 2015a) (Malerba and Cerana, 2016)
Paper industry	-Strengthens paper against moisture -Biodegradable package for food wrapping	(Dutta et al., 2002)
Enzymes-immobilization	-Chitin-and chitosan-based materials used for enzymes immobilization -Biosensors manufacturing for *in situ* identification of environmental pollutants	(Krajewska, 2004)

Table 1.3: Application fields and potential uses of chitin and its derivatives.

1.11 Chitooligosaccharides (CHOS): natural plant immune regulators

Chitin and chitosan have been used for crop farming as seed coating formulation, biopesticides, biofertilizers, and agricultural film (Trouvelot et al., 2014). CHOS are the degradation products of chitin and chitosan, made up with *N*-acetylglucosamine (Chitin oligosaccharides, CTOS) or glucosamine (Chitosan oligosaccharides, CSOS) with a degree of polymerization (DP) from 2 to 10. The only difference is the presence of acetyl group on the C2 of the sugar ring. The acetyl group present on CHOS plays a crucial role in biological activity (Falcón et al., 2008; Maksimov et al., 2011). For example, the binding of CHOS with its plant receptor mainly depends on the acetyl group on the C2 of the sugar ring (Hayafune et al., 2014; Liu et al., 2012). In another point of view, CHOS, with an amino group on C2 of the sugar ring, are cationic oligosaccharides, which in turn makes them more attracted towards negatively charged biomaterials, such as plant cell membrane. Plant cells are known to recognize chitin and their derivatives to elicit immune response.

CHOS are the degradation products of chitin and chitosan, with different chemical and physical characteristics from the original polysaccharides, which are important for their activity (Jung and Park, 2014; Kim and Rajapakse, 2005). Chitin is not soluble in common solvents, whereas chitosan is soluble in aqueous acidic solutions. CTOS ranging from DP2 to DP6 can be dissolved easily in neutral water. However, the CTOS with DP > 6 cannot be dissolved easily in neutral water, which limits its application. On the other hand, all CSOS exhibit good solubility in neutral water. CHOS are known to act as elicitors, and priming agents by inducing the oxidative burst. Applications of chitin/chitosan as an elicitor of plant defense responses is listed in Table 1.4. **Elicitor** is the compound that induces defense responses in plants. **Priming** is induction of a physiological state that allows a plant to exhibit more rapid and stronger defense response than ever compared to a non-primed plant. **Oxidative burst** is a rapid accumulation of reactive oxygen species (e.g. O^{2-}, H_2O_2) with direct antimicrobial activity, but also implicated in plant signaling. It is expected that the commercial use of elicitors or priming agents in agriculture will help to decrease the pesticide application by using the plant's own defense system.

1.12 Synthesis of bioactive chitooligosaccharides (CHOS) from polymers

CHOS production is expensive and effective using several methods like chemical, physical, and biological techniques. Synthesis of CHOS by the environmentally friendly enzymatic approach interests many researchers because of production of different DP (Degree of polymerization), DA (Degree of acetylation) and PA (Pattern of acetylation) of the products with low-cost (Jung and Park, 2014). Even though many chitinases have been reported, enzymatic production of defined CHOS is a distant dream.

CHOS are being generated from polymers by using several different physical methods, like hydrothermal (Sato et al., 2003), microwave (Xing et al., 2005), ultra-sonication (Wu et al., 2008) and gamma-rays (Yoksan et al., 2004), but these approaches are not suitable for generating well-defined CHOS-mixtures. Chemical methods using acid (Einbu and Vårum, 2007), H_2O_2 (Lin et al., 2009) or $NaNO_2$ (Morris et al., 2009), can yield CHOS. But, chemical hydrolysis of polymers often gives low DP CHOS, up to hexamer (6>1), and often results in generating low yields of CHOS and produces high amount of *N*-acetyl glucosamine (Lodhi et al., 2014). However, due to the complication in controlling the reaction, these treatments always end with the formation of secondary compounds which are difficult to remove.

Enzymatic methods are environmentally friendly alternatives to produce CHOS with specific DP and DA. Along with chitinases and chitosanases, several non-specific enzymes such as pectinases, cellulases, lipases, and lysozymes have been used to produce CHOS chitosan (Kumar et al., 2004). The low specificity of these enzymes typically led to the production of low DP of CHOS. Hence, the use of chitin/chitosan specific enzymes would be a great step forward towards the production of CHOS mixture with specified chemical composition (DA, DP, PA) and desired biological activities.

1.13 CHOS recognition is universal

The mode of action of CHOS, particularly generation and transduction of signals of oligosaccharides are unclear (Trouvelot et al., 2014; Yin et al., 2010). Pioneering work in finding the role of CHOS in plant immunity started with the identification of the high-affinity binding protein for CHOS on the surface and microsomal membranes of rice cells (Shibuya et al., 1993). After that, several high-affinity CHOS-binding proteins were eventually identified in soybean,

rice, barley, wheat, and carrot by affinity labeling and advanced cross-linking of carbohydrate and protein techniques (Day, 2001; Kaku et al., 1997; Shibuya and Minami, 2001; Shibuya et al., 1996).

Purification and identification of a CHOS-binding protein (*Os*CEBiP) from plasma membrane of rice (*Oryza sativa*) cells contains an extracellular chitin binding lysin motif (LysM) domain, but doesn't have an intracellular domain (Kaku et al., 2006). *Os*CERK1, a LysM receptor-like kinase (RLK) has been identified as a molecule required for chitin signaling. Among the two OsCEBiP is the main receptor component for CHOS in rice, whereas *Os*CERK1 did not bind to either CHOS or colloidal chitin (CC). *Os*CEBiP and *Os*CERK1 were reported to form a heterodimeric receptor complex upon binding to a ligand which will lead to triggering downstream signaling. *N*-acetyl groups in the internal GlcNAc residues play a crucial role in binding of one CHOS from the opposite surface with two molecules of *Os*CEBiP to form a homodimer. Five internal GlcNAc residues are needed for successful dimerization. Central LysM domain present in *Os*CEBiP and is essential for the initiation of defense signaling.

Later on, another LysM protein, called Chitin Elicitor Receptor Kinase 1 (*At*CERK1) or LysM Receptor-Like Kinase 1 (LysM RLK1) which was similar with CEBiP for CHOS recognition, were reported (Miya et al., 2007; J. Wan et al., 2008) in *Arabidopsis thaliana*. In contrast to CEBiP, *At*CERK1 (AtLYK1) possess the transmembrane and intracellular domains, and several reports showed that CERK1 is the main component in CHOS recognition (Petutschnig et al., 2010) (Fig 1.6a). There are reports that longer chitin oligosaccharide such as (GlcNAc)8 crosslink two *At*CERK1 molecules like a horizontal bar (Liu et al., 2012) (Fig 1.6b). Two new LysM-RLKs, *At*LYK4 and *At*LYK5 in chitin signaling and that the *At*LYK5 serves as the primary chitin receptor and forms an active receptor complex with *At*CERK1 in *A. thaliana* (Cao et al., 2014) (Fig 1.6c). Eventually, CEBiP and CERK1 homologs have been reported from other plants such as tomato and maize (Fliegmann et al., 2011; Gust et al., 2012; Lee et al., 2014; Tanaka et al., 2010; Zeng et al., 2012) suggesting that the recognition of CHOS is universal in both monocotyledons (monocots) and dicotyledons (dicots). Along with signal recognition and transduction via a receptor mode, CHOS may also be capable of entering into the plant nucleus and act on chromatin which in turn leads to the changes in the genes expression without the requirement for specific transcription factors (Hadwiger, 2013).

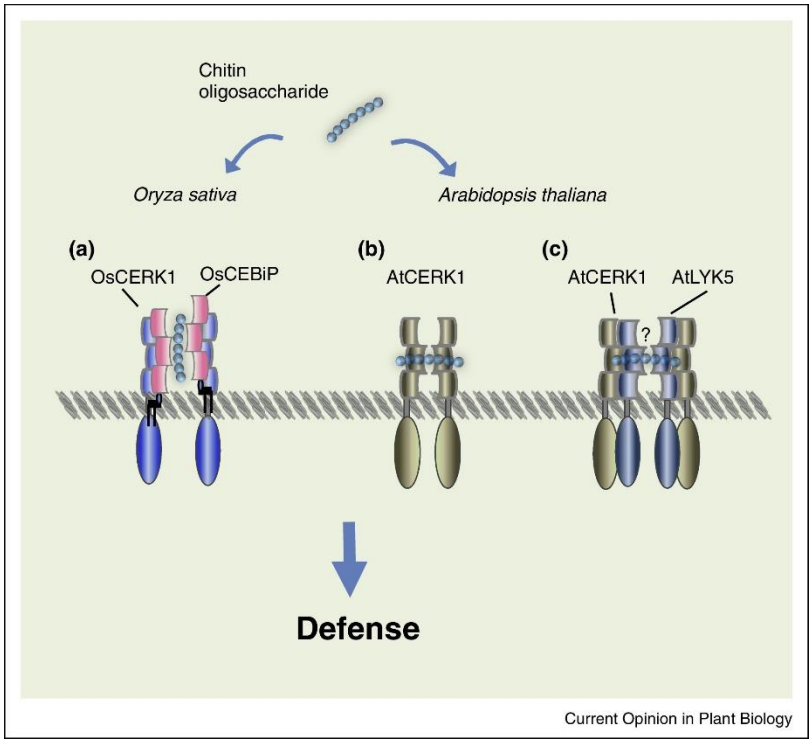


Fig 1.6: Models for ligand-induced activation of chitin receptors. (a) Receptor complex model in rice. (b) Hanging model for *At*CERK1 homodimerization in *Arabidopsis*. (c) Receptor complex model in *Arabidopsis* (Shinya et al., 2015).

Plant/crop	Disease/condition	Efficacy	Reference
Curcuma longa L	---	Increased activity of defense related enzymes	(Sathiyabama et al., 2016)
Zingiber officinale Roscoe	Rhizome rot	Inhibit ginger rhizome rot in storage.	(Liu et al., 2016)
Dendrobium sp	---	Protocorm Like Bodies (PLB) Induction	(Restanto et al., 2016)
Jute	Stem rot	Enhanced the activity of defense-related enzymes	(Chatterjee et al., 2014)
Rice	Leaf streak, leaf blight	Accumulated defense-related enzymes	(Li et al., 2013a)
Watermelon	Fruit blotch disease	Direct killing effect	(Li et al., 2013b)
Peach	Brown rot	Enhanced antioxidant and defense-related enzymes	(Ma et al., 2013)
Pine	Pitch canker	Upregulated the expression level of defense-related enzymes	(Fitza et al., 2013)
Camellia	Anthracnose	Accumulated H_2O_2, defense-related enzymes, and soluble protein	(Li and Zhu, 2012)
Broccoli	Native microflora	Antimicrobial coating served as carriers for bioactive compounds	(Alvarez et al., 2013)

Table 1.4: Some recent applications of chitin/chitosan as an elicitor of plant defense response.

1. 14 Significance of the present study

Flavobacterium johnsoniae is an aerobic gram-negative bacterium that degrades chitin and numerous other macromolecules (McBride et al., 2009). Analysis of the genome sequence revealed that ten glycohydrolases could be involved in chitin digestion. These enzymes comprise five chitinases that cut the long chitin polymers and five *N*- acetylglucosaminidases that release *N*-acetylglucosamine (GlcNAc) and/or chitobiose $(GlcNAc)_2$ from the oligomers. The predicted five chitinases are different. Three chitinases were close to bacterial chitinases. Among the three chitinases that were closer to bacterial chitinases, two of them (*Fj* ChiB and *Fj* ChiD), contain a single GH18 catalytic domain, while the third chitinase (*Fj* ChiA) had two GH18 domains. Fourth chitinase (*Fj* ChiC) was closer to animal chitinase with GH18 domain and the fifth chitinase (*Fj* GH19) was closer to plant chitinases containing the GH19 domain. Since, analysis of the *F. johnsoniae* genome sequence has given us an opportunity to dig the hidden messages of polysaccharide utilization (McBride et al., 2009), especially chitin, we further characterized the two chitinases namely *Fj* ChiB and *Fj* ChiC.

Serratia marcescens is one of the most studied soil bacteria for efficient degradation of chitin, biological control of plant diseases and plant growth promotion. Detailed knowledge of enzymology and the structures of these chitinases provided more insights into how a natural set of chitinolytic enzymes may find in several fields of biotechnology. The chitinolytic machinery of *Serratia marcescens* includes five chitin-active enzymes: *Sm* ChiA, *Sm* ChiB, *Sm* ChiC (C1 and C2), chitobiase (chb) and CBP21, a CBM33-type lytic polysaccharide monooxygenase. Chitin modifying enzymes reported in *S. marcescens* were listed in Table 1.5. Along with chitinases from *F. johnsoniae*, cloning and biochemical characterization of *Sm* ChiD, hitherto unknown, from a plant growth promoting *S. marcescens* GPS5 was also carried out.

Gene (protein)	Molecular weight (kDa)	Localization	Signal peptide	Reference
chiA (ChiA)	58	Extracellular	Yes	(Watanabe et al., 1997)
chiB (ChiB)	54	Periplasmic/ Extracellular	No	(Watanabe et al., 1997)
chiC (ChiC1)	52	Extracellular	No	(Watanabe et al., 1997)
chiC (ChiC2)	36	Extracellular	No	(Watanabe et al., 1997)
chb	95	Periplasmic	Yes	(Vaaje-Kolstad et al., 2013)
cbp (CBP21)	21	Extracellular	Yes	(Watanabe et al., 1997)
chiD (ChiD)	44	---	---	Present study

Table 1.5: Chitinases and CBP from *S. marcescens*.

1.15 Objectives of the work

In view of the importance of CHOS of specific compositions and specific length in mind, two bacterial isolates were selected for mining chitinases that could serve as source for synthesis of longer chain CHOS for a detailed study as defined below:

1) Biochemical characterization of chitinases present in *Flavobacterium johnsoniae* UW101 and a novel chitinase *Sm* ChiD from plant growth promoting *Serratia marcescens* GPS5
2) Study the role of chitinase-generated chitosan hydrolysates in plant immunity
   - ✓ What are the different types of CHOS produced by *Fj* ChiC and mutant G106W from chitosan polymers?
   - ✓ Do the chitosan hydrolysates from *Fj* ChiC and G106W mutant vary in inducing the plant immunity in plant cell suspensions?

Chapter-2

Materials and Methods

2.1 Bacterial cultures

Flavobacterium johnsoniae DSM 2064 was procured from **D**eutsche **S**ammlung von **M**ikroorganismen und **Z**ellkulturen (DSMZ), Germany. *Serratia marcescens GPS5* was selected from the bacterial library from laboratory. *Escherichia coli* strain BL21-pG-KJE8 (Takara, Japan) and *E. coli* Rosetta-gami 2 (DE3) (Novagen, USA) were from our previous collection.

2.2 Media used

CY medium (g L^{-1}): Casitone, 3g; $CaCl_2$. 2 H_2O, 1.36g; Yeast extract, 1g; Agar, 15g; added to 900 ml of water. The pH was adjusted to 7.2 and volume was made up to 1000 ml with double distilled water.

LB medium (g L^{-1}): Tryptone, 10g; NaCl, 10g; Yeast extract, 5g; Agar, 15g added to 900 ml of water. The pH was adjusted to 7.2 and volume was made up to 1000 ml with double distilled water.

Chitin agar medium (g L^{-1}): Colloidal chitin, 10; Na_2HPO_4, 0.065; KH_2PO_4,1.5; NaCl, 0.25; NH_4Cl, 0.5; $MgSO_4$, 0.12; $CaCl_2$, 0.005 and agar, 15. The pH was adjusted to 7.5 and the volume was made up to 1000 ml with distilled water.

2.3 Chemicals and kits

Isopropyl-β-D-thiogalactoside (IPTG), phenol solution Tris equilibrated, X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), agarose and *N*-Acetylglucosamine (NAG) were purchased from Sigma-Aldrich (USA). Antibiotics ampicillin, kanamycin, and chloramphenicol were purchased from Calbiochem. All routine media components for bacterial culture and all other chemicals were obtained from Merck, HiMedia laboratories (India) unless mentioned separately. The polymeric substrates α- chitin, β-chitin, and chitosan were kindly provided by Dr. Dominique Gillete, Mahtani Chitosan Pvt.,Ltd. (Veraval, India). Ni-NTA His-bind resin was purchased from Novagen (Madison, USA) for protein purification. Chito-oligosaccharides (CHOS) with different degrees of polymerization (DP) from DP2-DP6 and mixture of all these oligosaccharides were purchased from Seikagaku Corporation (Tokyo, Japan).

Genomic DNA (gDNA), Plasmid DNA isolation Miniprep kit and Gel extraction kit were purchased from MN (MACHEREY-NAGEL GmbH & Co. KG), Germany.

2.4 Enzymes

Restriction enzymes, T4 DNA ligase, and *Taq* polymerase were obtained from MBI Fermentas (Ontario, Canada). High fidelity Q5 DNA polymerase was procured from NEB (New England Biolabs), USA. All the enzymes were used as per the instructions given by respective manufacturers.

2.5 Primers Used for PCR

The primers used in this study (Table 2.1) were procured from Integrated DNA Technologies, Inc. (IDT), Coralville, IA, USA.

2.6 Identification and selection of *Flavobacterium johnsoniae* UW101 for chitinolysis

2.6.1 Molecular identification of *F. johnsoniae* UW101 based on 16S rDNA

Genomic DNA from *F. johnsoniae* was isolated by using genomic DNA purification kit. The 16S rRNA gene sequence was amplified from gDNA with the two bacterial universal primers (Table 2.1) using thermocycler (Eppendorf Mastercycler Gradient, Germany) at 50^{o}C annealing temperature. The amplified PCR product was examined on 1% agarose gel and eluted for sequencing at Scigenom Pvt. Ltd., Kerala, India. The partial sequences were matched with the nucleotide database available at GenBank, using BLAST tool in NCBI (National Centre for Biotechnology Information).

2.6.2 Chitinolysis by *F. johnsoniae* UW101 on the chitin-containing agar plate

A single bacterial colony of *F. johnsoniae* was grown on LB plate 3 days, was spot inoculated onto the 1% colloidal chitin (CC) containing minimal medium (MM) chitin-agar plate and incubated at 30^{o}C for 15-20 days to observe the zone of clearance around the bacterial colony.

S.No	GeneBank I.D	Gene Name	Primer name	Primer sequence 5'→ 3'	Restriction Site	Name of the vector cloned	Amplicon Size (kb)	Expected protein size (kDa)
1	NR 042496.1	16S rDNA	27F	GTTTGATCCTGGCTCAG	--	--	1.5	--
			1489R	TACCTTGTTACGACTTCA				
2	ABQ07183.1	*Fj*ChiB	*Fj*ChiA FP	ATATATCCATGGGGCAAAAAAAAG TAATAGC	*Nco* I	pET 28a(+)	1.54	57.8
			*Fj*ChiA RP	ATATACTCGAGCTTTTTAATAAAAC GTCTCAC	*Xho* I			
3	ABQ07183.1	*FjChiB*CBD	FP	AAT CCATGGCCAGCAACCCTCCAACAG GAACA	*Nco* I	pET 28a(+)	0.4	12
			RP	AGTCTCGAGAGCTGCTTTTGTGATT CTAAA	*Xho* I			
4	ABQ07554.1	*FjChiA*	*Fj*ChiBF/ ChiBGH1/ChiBGH1A FP	ATAAACGGATCCATGCAGCCAGCC CACGGG AAAAAAG	*Bam* HI	pET 28a(+)	4.73	168.9
			NGH2Rp	GCGTTCTCGAGGTTTAAAAAGTTCA TTAC	*Xho* I			
5	ABQ07554.1	*FjChi*AGHIA	*Fj*ChiBF/ ChiBGH1/ChiBGH1A FP	ATAAACGGATCCATGCAGCCAGCC CACGGG AAAAAAG	*Bam* HI	pET 28a(+)	3.42	136.4
			NGH1Rp	GCATGCTCGAGTCTATTAGTGCTTA CTGT	*Xho* I			
6	ABQ07554.1	*FjChi*AGHIIA	*Fj*ChiBGH2A FP	ATAAACGGATCCATGGTTACAGTT GAATTA CGAAATG	*Bam* HI	pET 28a(+)	3.45	126.7
			NGH2Rp	GCGTTCTCGAGGTTTAAAAAGTTCA TTAC	*Xho* I			
7	ABQ07554.1	*FjChi*AGH1	*Fj*ChiBGH1FP	ATAAACCATGGTACAGCCAGCCCA CGGGAA AAAAG	*Nco* I	pET 28a(+)	1.28	46.9
			*Fj*ChiBGH1RP	GCATGCTCGAGATCCTGAGATAAT TCCCAAATC	*Xho* I			
8	ABQ07554.1	*FjChi*AGH1I	*Fj*ChiBGH2FP	ATAAACCATGGTAAAAATTATTTTA GGATATGC AC	*Nco* I	pET 28a(+)	1.23	35.4
			*Fj*ChiBGH2RP	GCGTTCTCGAGGTTTAAAAAGTTCA TTAC	*Xho* I			
9	ABQ07559.1	*FjChiC*	*Fj*ChiCFP	ATCATGGATCCATGTGTACCAGTG AAAAAG	*Bam* HI	pET 28a(+)	0.95	38.1
			*Fj*ChiCRP	ATTATCTCGAGATTACCGCACATGC CTGAAG	*Xho* I			
10	ABQ07756.1	*FjChiD*	*Fj*ChiDFP	GTTATCCATGGGGCAGAAAAATAA AAAATTCG	*Nco* I	pET 28a(+)	1.02	41.2
			*Fj*ChiDRP	GTCATCTCGAGATTTAGCCGTTTTA ACTTTATAA	*Xho* I			
11	ABQ05635.1	*FjGH19*	*Fj*GH19FP	GTATACCATGGGGGCAAAACTAAA AATATTTGG	*Nco* I	pET 28a(+)	2.36	90.08
			*Fj*GH19RP	GGCGCCTCGAGTTTTATTTTTTTTA AATTAGG	*Xho* I			
12	KX579968	*SmChiD*	*Sm*ChiDFP	GCTTACCATGGATGCCTATCTCTCC GTCGGC	*Nco* I	pET 28a(+)	1.24	44.4
			*Sm*ChiDRP	GACTACTCGAGCCGTTTCTCGCCTT TTATTCC	*Xho* I			
13	ABQ07559.1	*Fj*CG106W	*Fj*CG106WFP	CTTGCCGGCTGGGTCATTTCAACC	--	pET 28a(+)	0.95	38.1
			*Fj*CG106WFP	TGAAATGACCCAGCCGGCAAGTGA				
14	ABQ07559.1	*Fj*CD148N	*Fj*CD148NFP	GTAGATGTTAATCTCGAATGGGAT	--	pET 28a(+)	0.95	38.1
			*Fj*CD148NRP	CCATTCGAGATTAACATCTACACC				
15	ABQ07559.1	*Fj*CW211G	*Fj*CW211GFP	ACTGGACCAGGGAGTCCGAATAAA	--	pET 28a(+)	0.95	38.1
			*Fj*CW211GRP	ATTCGGACTCCCTGGTCCAGTACT				

* Protein sequence was recently submitted to NCBI

Table 2.1: Details of primers used for cloning of *Fj* chitinolytic genes. Sequences underlined, colored in red represent restriction sites. Sequences underlined, colored in green represent mutated amino acid.

2.7 Cloning and characterization of chitinases from *F. johnsoniae* and *S. marcescens* GPS5

2.7.1 Genomic DNA isolation

Genomic DNA (gDNA) from *F. johnsoniae* and *S. marcescens* was isolated using gDNA isolation kit purchased from MN (MACHEREY-NAGEL GmbH & Co. KG), Germany.

2.7.2 Amplification and cloning of chitinase from *F. johnsoniae* and *S. marcescens*

Based on genome sequences available in NCBI genomic database (http://www.ncbi.nlm.nih.gov/nuccore/CP000685), primers were designed for the amplification of five chitinases and their truncations encoding genes from the gDNA of *F. johnsoniae*. The genes encoding for *Fj chiA* (and truncations), *Fj chiB, Fj chiC, Fj chiD* and *Fj GH19* were PCR amplified from *F. johnsoniae* genomic DNA by referring the annotated sequence (GenBank accession no. ABQ07554.1, ABQ07183.1, ABQ07559.1, ABQ07756.1, and ABQ05635.1). The respective genes were amplified with gene-specific forward and reverse primers (Table 2.1) using *Q5* DNA polymerase between 48^{o}C-55^{o}C annealing temperature with standard conditions mentioned in manual. Amplicons were gel extracted using MN Gel Cleanup kit. Bacterial expression vectors pET- 28a(+) and the amplicons were double digested with *Nco* I and *Xho* I (until unless mentioned separately), gel purified both the amplicons and vector were ligated into pET- 28a(+) using T4 DNA ligase at 22^{o}C for 1 h. The resultant plasmids were designated as pET 28a-*Fj chiA*, pET 28a-*Fj chiB*, pET 28a-*Fj chiC*, pET *28a-Fj chiD* and pET 28a-*Fj GH19* to express them respectively in expression host *E.coli* Rosetta-gami 2 (DE3) (Novagen, USA).

The genomic sequence of *S. marcescens* WW4 was downloaded from the public NCBI database (http://www.ncbi.nlm.nih.gov/nuccore/CP003959). Primers were designed based on the nucleotide sequence of *S. marcescens* WW4 for *Sm* ChiD. *Sm ChiD* gene was amplified from genome sequence of *S. marcescens* GPS5. Gene was cloned in expression vector pET- 28a(+) between *Nco* I and *Xho* I restriction sites. Recombinant plasmid pET 28a(+)-*Sm ChiD* was transformed into expression host *E. coli* Rosetta-gami 2 (DE3) (Novagen, USA).

2.8 Protein expression and purification of *Fj* chitinases

2.8.1 Protein expression

Expression and purification of pET 28a-*Fj chiA* (and truncations), pET 28a-*Fj chiB*, pET 28a-*Fj chiC*, pET *28a-Fj chiD*, pET 28a-*Fj GH19* and pET 28a(+)-*Sm chiD* were done as described by Neeraja et al., (2010). *E. coli* BL21 DE3 and Rosetta-gami 2 (DE3) competent cells were transformed with confirmed recombinant plasmids and plated on LB-kanamycin (50 µg/ml), chloramphenicol (25 µg/ml) plate. Confirmed single colony was inoculated onto 5 ml of Luria Bertani (LB) broth with appropriate antibiotics and incubated overnight with 180rpm at 37^{o}C. The overnight culture was diluted to 1% with fresh LB broth with antibiotics and incubated at appropriate conditions. After reaching OD_{600} to 0.5, cells were induced with 0.1-0.5 mM IPTG to express the chitinases and different truncated versions. Cells were harvested after 3 h of induction. The centriguged cell pellet was suspended in Ni-NTA equilibration buffer (50 mM NaH_2PO_4, 100 mM NaCl, and 10 mM imidazole at pH 8.0). The cells were lysed by sonication at 20% amplitude with 30×15 s pulses (with 15 s delay between pulses) on ice, with a Vibra cell Ultrasonic Processor, converter model CV33, equipped with a 3 mm probe (Sonics, Newtown, CT, USA). To seperate the insoluble cell debris, sonicate was centrifuged at 15000 g for 30 min at 4^{o}C. The expressed protein was purified using Ni-NTA column as the expressed protein was having C-terminal His-tag. Protein preparations were compared with uninduced controls to check the expression of proteins by 12% SDS-PAGE.

2.8.2 Ni-NTA Purification

Six ml of Ni-NTAgarose (CloneTech, Takara) was packed in a polypropylene column and equilibrated with 25 ml of Tris-NaCl buffer with 10 mM imidazole. Sonicated supernatant was applied to the column and flow through was stored at -20^{o}C. The column was washed with 20 ml of washing buffer (Tris-NaCl buffer with 20 mM imidazole). Finally, the protein was eluted with 15 ml of elution buffer (Tris-NaCl buffer with 50, 100, 150 and 200 mM imidazole) and fractions was collected. The purity of the protein was analyzed by SDS-PAGE.

2.8.3 SDS-PAGE analysis

The protein samples checked for their purity by SDS-PAGE on vertical gels according to Laemmli (Laemmli, 1970). The stacking gel containing 4.5 % polyacrylamide in 0.125 M Tris-HCl, pH 6.8 and the resolving gel composed of 12% polyacrylamide in 0.375 M Tris-HCl, pH 8.8. Electrode buffer contained 0.025 M Tris-HCl, 0.192 M glycine and 0.1% (w/v) of SDS at pH 8.5. The samples were boiled at 100^{o}C for 5 min in sample buffer containing 1% SDS (w/v) and 12% glycerol (v/v), in 0.063 M Tris-HCl, pH 6.8 and electrophoresis was carried out at 70V in stacking gel and at 120V in resolving gel. The gels were stained with a solution containing 0.5% (w⁄v) Coomassie brilliant blue G-250, 30% (v⁄v) methanol and 10% (v⁄v) glacial acetic acid, and kept on a rotary shaker. The stained SDS gel was destained in a solution containing 30% (v⁄v) methanol and 10% (v⁄v) glacial acetic acid till the protein bands clearly visible.

2.8.4 Preparation of the protein

Protein fractions with the highest purity were pooled and concentrated using Macrosep Centrifugal Devices (Pall Corporation, USA) of 10 kDa cut-off. The concentrated proteins were initially buffer-exchanged with 50 mM sodium acetate buffer pH 7.0 and stored at 4^{o}C until use. The purified protein concentration was quantified using Pierce BCA protein assay kit (Thermo Scientific, USA). The slope from the standard calibration curve constructed as per the instructions given in the kit for quantification of the protein amount.

2.8.5 Zymogram analysis for purified chitinases

A simple dot blot assay was carried out to detect the activity of purified recombinant chitinases. A poly acrylamide gel supplemented with 0.1% glycol chitin was prepared. Five µg of the purified enzyme was spotted onto the gel and placed in a humid chamber at 37^{o}C for overnight. After incubation, the gel was stained with 0.01% calcofluor white M2R in 0.5 M Tris-HCl pH 8.9 for 10 min at 4^{o}C. Finally, the brightener solution was removed, and the gel was washed with distilled water for 10 min at 4^{o}C for two times. Zone of clearance was visualized by placing the gels on a UV transilluminator.

2.9 Characterization of chitinases

2.9.1 Chitinase assay

Chitinase activity was checked by a modified Schales procedure using colloidal chitin as the substrate (Imoto and Yagishita, 1971). The reaction mixture (200 µL) consisting of recombinant chitinase (*Fj* ChiB: 200ng, *Fj* ChiC: 5 µg and *Sm* ChiD: 25 µg) and colloidal chitin (25 mg/ml) in 50 mM buffer (*Fj* ChiB: glycine-NaOH buffer pH 9.0, *Fj* ChiC: sodium citrate pH 6.0, and *Sm* ChiD: sodium citrate pH 5.0) at 40^{o}C for 1 h with constant shaking at 190 rpm. The reaction mixture was subjected to centrifuge at 150g for 20 min at 4^{o}C. 40 µl of reaction supernatant was mixed with 300 µl of color reagent (0.5 M sodium carbonate, 0.05% potassium ferricyanide) and boiled for 15 min in dark and centrifuged at 16100 g at 30^{o}C for 1 min. Then, 200 µl of supernatant containing reducing sugars was separated and triplicate samples were loaded in 96 well microtiter plates. OD was measured at 420 nm by microtitre plate reader (Multiscan, Labsystems, Finland). One unit was determined as the amount of enzyme that released 1 µmol of reducing sugar per minute.

2.9.2 Kinetics analysis

Activity of chitinases was measured by incubating the recombinant enzymes (*Fj* ChiB:200ng, *Fj* ChiC: 5 µg and *Sm* ChiD: 25 µg) with different concentration of colloidal chitin (0-50 mg/ml) in 50 mM buffer (*Fj* ChiB: Glycine-NaOH pH 9.0, *Fj* ChiC: sodium citrate pH 6.0, and *Sm* ChiD: sodium citrate pH 5.0) with respective controls in triplicates at 40^{o}C for 1 h at 180 rpm. Chitinase assay was done as described by Purushotham and Podile., (2012). Enzyme activity was defined as the release of one micromole of *N*-acetyl-glucosamine (NAG) per sec under standard experimental conditions. Specific activity in nanokat mg-1 of protein was calculated and kinetic values were obtained from three independent sets of data fitting to the Michaelis-Menten equation by nonlinear regression function available in GraphPad Prism software version 5.0 (GraphPad Software Inc., San Diego, CA).

2.9.3 Optimum temperature

The optimum temperature of *Sm* and *Fj* chitinases was ascertained by incubating the enzymes (*Fj* ChiB: 200ng, *Fj* ChiC: 5 µg and *Sm* ChiD: 25 µg) with colloidal chitin (30 mg/ml) in 50 mM buffers (*Fj* ChiB: 50 mM glycine-NaOH pH 9.0, *Fj* ChiC: 50 mM sodium citrate pH 6.0, and *Sm*

ChiD: 50 mM sodium citrate pH 5.0) for 1 h at different temperatures of 10, 20, 30, 40, 50, 60, 70 and 80^{0}C. Relative specific activity (%) was determined under standard assay condition as mentioned in 2.9.1.

2.9.4 Optimum pH

The optimum pH of *Sm* and *Fj* chitinases was determined by incubation of enzymes (*Fj* ChiB: 200ng, *Fj* ChiC: 5 µg and *Sm* ChiD: 25 µg) in a buffer at different pHs (2.0-10.0) for 1 h at 37^{o}C under standard assay conditions using colloidal chitin (30 mg/ml) as a substrate. The buffers used were 50 mM glycine- HCl buffer pH-2, 50 mM citrate buffer pH-3-6, 50 mM sodium acetate buffer pH-4-5.6, 50 mM sodium phosphate buffer pH-6-8, 50 mM tris-HCl buffer pH-8-9, 50 mM glycine-NaOH buffer pH-9-10. The relative specific activity (%) was ascertained under standard assay conditions as mentioned in 2.9.1.

2.9.5 Substrate specificity

The substrate specificity of purified *Sm* and *Fj* chitinases on chitin and non-chitin derived substrates were tested. The substrates used were α-chitin, β-chitin, colloidal chitin, chitosan with 75% DDA (sigma, Cat No: 419419), CM-cellulose (Himedia, Cat no: RM329) and Avicel (microcrystalline cellulose) (Fulka, Cat No: 11363). Reducing end assay was performed by incubating purified recombinant enzymes (*Fj* ChiB: 200ng, *Fj* ChiC: 5 µg and *Sm* ChiD: 25 µg) with 5.0 % of polymeric chitin and non-chitin substrates at 37^{o}C for 1 h in respective buffers (*Fj* ChiB: Glycine-NaOH pH 9.0, *Fj* ChiC: sodium citrate pH 6.0, and *Sm* ChiD: sodium citrate pH 5.0). Enzyme assay was done under standard chitinase assay conditions as mentioned in 2.9.1.

2.9.6 Soluble substrate binding

The binding of *Sm* ChiD to different soluble or insoluble polysaccharides was performed as described by Purushotham et al., (2012), with slight modifications. Native 8.0% polyacrylamide gels incorporated with different soluble polymeric substrates like glycol chitin, laminarin, and carboxymethyl cellulose (CM-cellulose), were used for the soluble substrate binding assay. One µM *Sm* ChiD or non-interacting BSA (as a control) were electrophoresed in the substrate impregnated gels under non-denaturing conditions at 4^{o}C and the protein bands were visualized by staining with Coomassie blue G-250. In a different reaction set up, the insoluble substrate

binding assay was performed with 1 mg ml^{-1} of α-chitin, β-chitin, colloidal chitin, or Avicel in 50 mM sodium acetate buffer, pH 6.0. The binding assay was performed by adding 2 µM *Sm* ChiD or non-interacting BSA to the reaction mixture and the binding assay mixture was incubated for 1 h at 4^{o}C (to minimize enzymatic hydrolysis) with continuous shaking at 1200 rpm. All the assays were performed in triplicate with appropriate controls and the amount of bound protein was quantified as described by Purushotham et al., (2012).

2.10 Analysis of products of purified chitinases on chitooligosaccharides (CHOS) and colloidal chitin by High-performance liquid chromatography (HPLC)

2.10.1 Reaction time-course of DP3-DP6 substrates hydrolysis/transglycosylation (TG) catalyzed by *Fj* ChiC and *Sm* ChiD

Analyses of the hydrolysis of chitin oligomers by *Sm* and *Fj* chitinases were conducted by incubating recombinant enzymes (*Fj* ChiB: 4 nM, *Fj* ChiC: 50 nM and *Sm* ChiD: 350 nM) in 50 mM buffer (*Fj* ChiB: glycine-NaOH pH 9.0, *Fj* ChiC: sodium citrate pH 6.0, and *Sm* ChiD: sodium citrate pH 5.0) with 1 mM (2 mM for *Sm* ChiD) of each individual substrate ranging from DP2-DP6. Reaction mixtures were incubated at 40^{o}C in a thermomixer, and reaction samples were collected at 0, 1, 3, 5, 10, 15, 20, 30, 45, 60, 90, 120, 160, 180, 360 and 720 min. The reaction was stopped by transferring 30 µL of the reaction mixture into an eppendorf tube containing 30 µL of 70% acetonitrile. Reaction mixtures were stored at -20^{o}C until further processed by isocratic HPLC at 25^{o}C using a Shimadzu 10ATvp UV/VIS HPLC system (Shimadzu corporation, Tokyo, Japan) equipped with a Shodex Asahipack NH2P-50 4E column (Showa Denko K.K,USA). Product profile was analyzed by injecting twenty microliters (20 µl) of the reaction mixture into the HPLC using Hamilton syringe (HAMILTON Bonaduz, Switzerland). The liquid phase consisted of 70% acetonitrile: 30% MilliQ H2O and the flow rate was set to 0.70 ml/min, eluted CHOS were monitored by at 210 nm. CHOS concentration was calculated using authentic oligosaccharide solution obtained from Seikagaku Corp., (Tokyo, Japan). CHOS mixture having DP1- DP6 was used for standard graph preparation. Standard calibration curves of CHOS moieties were constructed for each oligosaccharide separately. These data points yielded a linear curve for each standard sugar with the R^2 values of 0.997–1.0 allowing molar concentration of CHOS to be determined with confidence.

2.10.2 Time-course of colloidal chitin hydrolysis

All the three recombinant enzymes (*Fj* ChiB: 4 nM, *Fj* ChiC: 50 nM and *Sm* ChiD: 350 nM) were incubated with 1 mg/ml of colloidal chitin at 40^0C. Twenty microliters (20 µl) of reaction samples were collected at different time points and the reaction was stopped-up by adding an equal amount of 70% acetonitrile. Samples were centrifuged at 16,000 g for 10 min at 4ºC to remove the undigested chitin polymers. The supernatant was further concentrated (Eppendorf concentrator, Germany) for complete evaporation of the solvent without heating. The residue was dissolved in 40 µL of 35% acetonitrile and reaction mixtures were stored at -20ºC until analyzed by isocratic HPLC at 25^0C.

2.10.3 MALDI-TOF-MS

After HPLC analysis, the remaining reaction mixtures (60^{th} min sample for DP3, DP4, DP5 and DP6 of *Sm* ChiD) were analyzed by MALDI-TOF-MS. A portion of the reaction mixture (15 µL) was concentrated in a concentrator till the complete evaporation of buffer solvent and dissolved in 5 µL of HPLC grade MilliQ water. Two microliter of a 9 mg/ml mixture of 2,5- dihydroxybenzoic acid (DHB) in 30% acetonitrile (ACN) was applied to a MTP 384 target plate ground steel TF (Bruker Daltonics). Two microliters (2 µL) of the sample was mixed into the DHB droplet and dried under a stream of air. The samples were analyzed with an Ultraflex MALDI-TOF/TOF instrument (Bruker Daltonics GmbH, Germany) with an auto flex 123 smart beam. The instrument was operated in positive acquisition mode and controlled by the FlexControl 3.0 software. All spectra were obtained in the reflectron (single stage reflectron) mode with an acceleration voltage of 25kV, a reflector voltage of 26, and pulsed ion extraction of 40 ns in the positive ion mode. The acquisition range used was from m/z 50 to 2000. The data were collected from averaging 500 laser shots, with the lowest laser energy necessary to obtain sufficient signal to noise ratios. Peak lists were obtained from the MS spectra using Bruker FlexAnalysis software (Version 3.0).

2.10.4 Salt tolerance of *Fj* ChiC

Reducing end assay described in (2.9.1) was performed to determine the salt tolerance of *Fj* ChiC under various concentrations of salt. Five microgram (5µg) of purified *Fj* ChiC was incubated with 30 mg/ml of colloidal chitin (CC) for 1 h at 37^0C, with different concentrations of NaCl (0, 0.5, 1, 2, 3 and 3.5M) under standard assay conditions. Released reducing ends were calculated by

modified Schales procedure (Imoto and Yagishita, 1971). The average of triplicate data was used for analysis.

2.11 A structure-based sequence alignment of *Fj* chitinases and *Sm* chitinases and homology modeling of *Fj* ChiB and *Fj* ChiC

Catalytic domains of *Fj* ChiA GHI, *Fj* ChiA GHII, *Fj* ChiB, *Fj* ChiC and *Fj* ChiD were aligned using clustal omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). The models of *Fj* ChiB and *Fj* ChiC were generated by Modeller9v8 (http://www.salilab.org/modeller/) using templates of insect chitinase from the Asian corn borer, *Ostrinia furnacalis* (PDB ID: 3W4R) for *Fj* ChiB and chitinase from *Bacteroides thetaiotaomicron* (PDB ID: 3CO4) respectively as structural templates and the figures were generated by using PyMOL (http://www.pymol.org/).

2.12 Site-directed mutagenesis (SDM)

Single point mutations were generated by PCR using the wild-type plasmid DNA (pET 28a-*Fj chiC)* as a template. The primers used for mutation was listed in Table 2.1. Q5 high fidelity polymerase was used for SDM. Amplified PCR product was subjected to run in 10% agarose gel (3µl) to confirm the amplification. After PCR amplification, the reaction mixture was incubated with *Dpn*I restriction enzyme (10U) for overnight at 37^0C to get rid of methylated (parental) DNA. Three microliters (3µL) of digested PCR reaction was transformed into Rosetta-gami 2 (DE3) competent cells. Mutants were confirmed by colony PCR, double digestion, and sequencing.

2.13 Determination of subsite specificity of *Fj C*hiC and G106W mutant

A subsite specificity assay was performed to know the occupancy of sugar (whether acetylated or deacetylated) at particular enzyme subsites. Subsite mapping was done with DA35% and DA60% chitosan polymers. Both the polymers were completely hydrolyzed with *Fj C*hiC (10 nM) and G106W (500 nM) mutant by incubating at 37°C for 24h. Hydrolysate (30µg) was freeze-dried and resuspended in 25 µl of water (H_2O), 25 µl of 100 mM $NaHCO_3$ buffer pH8.5 and 50 µl of MeOH. Total 100 µl reaction mixture was re-acetylated by adding 1 µl of acetic anhydride at 37°C and 1200 rpm for every 15 min repeatedly 5 times. Total mixture was lyophilized and tested for re-acetylation by LC-MS. Five micrograms (5µg) of acetylated CHOS were incubated with 10 µl of $H_2{}^{18}O$ for overnight at 70^0C to label the reducing end for detection of fragmented ions by LC-

MS2. On the other hand, the acetylated CHOS were also mixed with the isotopic labeled internal standard to quantify the CHOS according to their DP and DA. The internal standard (R*) was produced by reacetylation of D-glucosamine oligomers with a double isotopic labeled ($^{13}C_4$-$^{2}H_6$) acetic anhydride. Subsites preference was identified after a series of computational analysis.

2.14 Elicitor and priming activity of CHOS produced by *Fj C*hiC & Mutant G106W

2.14.1 Preparation & Characterization of CHOS from Chitosan Substrates

We have generated CHOS by incubating different DA of chitosan polymer substrates (1 mg/ml of DA35%, DA50% and DA60%) with *Fj C*hiC (10 nM) and the mutant G106W (500 nM) in 40 mM ammonium acetate buffer (pH-5.5). Fractions collected at different time intervals *viz* 2 h, 6 h, 12 h, 24 h, and 48 h were analyzed through HPTLC. For further analysis, 48 h samples were collected for DA35% and DA60%. The generated crude hydrolysates were collected and stopped the reaction by keeping at 70^{0}C for 20 min. Reaction samples were lyophilized overnight and dissolved in sterile MilliQ water to make a final concentration of 1 mg/ml. Further, these samples were characterized by MALDI-TOF-MS and tested for their elicitor activity in rice cell suspension culture systems.

2.14.2 Estimation of reducing group by MBTH assay

Reducing ends of CHOS generated by both the chitinases *Fj C*hiC and mutant G106W were measured by 3-methyl-2- benzothiazolinone hydrazone (MBTH) method as described earlier (Horn and Eijsink, 2004). All the reactions were performed in triplicate in 200 µl reaction consisting of appropriate amount of enzyme (10 nM of *Fj C*hiC or 500 nM of G106W) and chitosan substrates of different DAs (DA1.6%, DA20%, DA35%, DA50% and DA60%) in 40 mM ammonium acetate buffer pH-5.5 at 40^{0}C for 20 min with constant shaking at 400 rpm. Forty microliter (40 µl) of reaction mixture containing reducing sugars collected at different time intervals (15, 180, 300, 600 and 1200 secs) and transferred to 40 µl 0.5 M NaOH containing Eppendorf (1.5ml) to inactivate the enzyme followed by addition of 20 µl (3 mg.ml^{-1}) MBTH and 20 µl (1 mg.ml^{-1}) DTT. The total 120 µl of reaction mixture was incubated at 80^{0}C for 15 min. Thereafter, 80 µl of colour reagent [0.5% (w/v) $FeNH_4$ (SO4)3+0.5% (w/v) H_3NSO_3+0.25 M HCl] was added and cooled to 28^{0}C to develop the color. Then the complete reaction mixture (200 µl)

was taken in 96 well microtiter plate, and the absorbance (OD) was measured at 620 nm by using microtiter plate reader.

2.15 High-performance thin layer chromatography (HPTLC)

Reaction fractions were collected at time intervals and separated by high-performance thin layer chromatography (HPTLC) based on their DP. The chitosan hydrolysate generated by the wild-type or mutant enzyme was applied in 20µL (40µg of hydrolysate) aliquots to HPTLC silica gel 60 F_{254} plates (Merck) using a Camag automatic TLC sampler 4 (Camag, Berlin, Germany). A solvent system consisting butanol/methanol/ammonia/water in the ratio of 5:4:2:1 was used as mobile phase. To detect the CHOS, the plate was sprayed with aniline diphenylamine reagent (400 µL aniline, 400 mg diphenylamine, 20 ml acetone, 3 ml 85% phosphoric acid) followed by baking for 8–10 min at high temperature (~180^{0}C -200 ^{0}C) using a hot air gun (Black & Decker, Germany) carefully. As standard, 4 µg each of chitosan oligomers $(GlcN)_{1–6}$ and chitin oligomers $(GlcNAc)_{1–6}$ (Seikagaku Corporation, Tokyo, Japan) were used.

2.16 Analysis of CHOS by UHPLC-ELSD-ESI-MS

The chemical composition of hydrolysates was analyzed by advanced UHPLC-ELSD-ESI-MS method described by Hamer et al., (2015). Aliquots of 2µl (1 mg/ml) were analyzed by UHPLC-ELSD-ESI-MS. In brief, CHOS were separated by hydrophilic interaction chromatography (HILIC) by using UPLC BEH Amide column. Samples were run at a flow rate of 0.4 $ml.min^{-1}$ in isocratic conditions with the following solvents: 80:20 acetonitrile: water, 10 mM NH_4HCO_2 and 0.1% (v/v) formic acid. Mass spectra were obtained over a scan range from m/z 50–2000 in positive scan mode. Internal standards [chitin oligomers (D1-D6) and chitosan oligomers (A1-A6)] of known concentration were injected for quantification of oligomers generated from the polymer. Data were analyzed by Data Analysis 4.1 software (Bruker, Germany).

2.17 Maintenance of *Medicago* and *Tobacco* cell suspensions

Medicago (*Medicago truncatula*), initial line was kindly provided by Dr. Karsten Niehaus, University of Bielefeld) and tobacco (*Nicotiana tabacum)* cells were kindly supplied by Dr. Burkhard Schmidt (Rheinisch-Westfälische Technische Hochschule Aachen University, Aachen, Germany). The cell suspensions were grown in 50 ml of modified Murashige & Skoog medium supplemented with 30 g/L sucrose and 1 mg/L 2,4-dichlorophenoxyacetic acid and were sub-

cultured every 7 days in 20 ml medium (Murashige and Skoog, 1962). The cells were maintained at 26^{o}C in the dark with constant agitation on a rotary shaker at 120 rpm.

2.18 Determination of oxidative burst

The oxidative burst was characterized by measuring H_2O_2 levels using a modified luminol-dependent chemiluminescence method (Warm and Laties, 1982). The cells were softly separated from the medium with a sintered glass filter on the third day after subculturing. Then 300 mg of cells were suspended in 5 ml of oxidative burst medium [10 mM MES with 5% (v/v) culture medium pH 5.8 and 3% (w/v) sucrose] and were resuspended on a rotary shaker. The cells were pre-incubated at 26^{o}C in the dark with constant agitation at 120 rpm for 4 h, before eliciting or priming with oligomers derived from chitosan (DA35% and DA 61%). 200µL aliquots of rice or medicago cell suspensions were transferred to sterile microtiter plates (Thermo Fisher Scientific, Waltham, MA) and 50 µL of 0.1 mg/ml luminol (Sigma; 1.21 mM in phosphate buffer) was added to each well before measuring the chemiluminescence by luminometer (Luminoskan Ascent, Thermo Fisher Scientific, Tokyo, Japan). The chemiluminescence value was presented as relative light units (RLU), which are proportional to the amount of H_2O_2 generated after stimulation. The concentration of H_2O_2 was determined using a standard calibration curve.

2.19 Elicitor activity

Five hours pre-incubated cell suspensions were treated with the CHOS mixture generated before measuring the oxidative burst (OB) by luminometer. HF chitosan (10 µg.ml^{-1}) was used as positive control for both medicago and rice cell lines. Graphical representation is given in (Fig 2.1).

2.20 Priming activity

To test priming activity, 3 h pre-incubated cell suspensions were treated with CHOS and incubated in the dark at 26^{0}C with shaking at 120 rpm. Pre-treated cells were again incubated for another 3 h at the same condition before elicitation. Priming was triggered by adding 10µg.ml-1 HF chitosan to tobacco and medicago cells. Immediately after adding elicitors, OB was measured by luminometer. Salicylic acid (SA) at a concentration of 5 µg.ml-1 and ulvan (10 µg.ml-1) were used as positive control for medicago and rice cells, respectively. Deionized water was used as negative control. Graphical representation can be seen in (Fig 2.1)

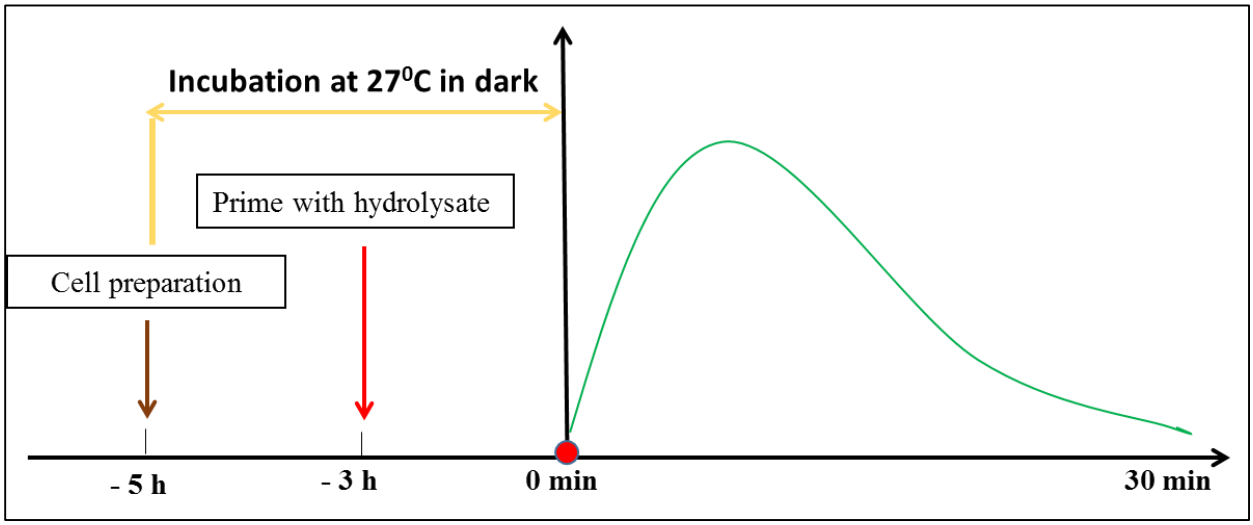


Fig 2.1: Graphical representation of oxidative burst measurements performed using *Nicotiana* and *Medicago* cell suspension cultures.

Chapter-3

Results and Discussion

3.1 Identity confirmation and screening for chitinolytic potential of *F. johnsoniae*

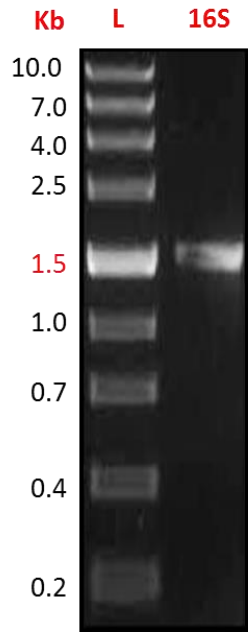
F. johnsoniae UW101 was procured from DSMZ (**D**eutsche **S**ammlung von **M**ikroorganismen und **Z**ellkulturen), Germany. The isolate was tested for its molecular confirmation by 16S rDNA gene sequencing. 16S rDNA was amplified with universal primers (Table 2.1) (Fig 3.1), sequenced and submitted for BLAST search in NCBI, showed 99% homology to *F. johnsoniae* UW101. Actively growing *F. johnsoniae* was spot inoculated on chitin agar plate containing 3% colloidal chitin as the sole carbon (C) and nitrogen (N) source. Culture plate was incubated at 30^0C and zone of clearance around the bacterial colony was regularly observed for 20 days (Fig 3.2). The zone of clearance of chitin increased with time. After 20 days entire plate was clear due to chitinolytic activity of *F. johnsoniae*

3.2 Cloning and characterization of *F. johnsoniae* chitinases

3.2.1 Amplification and cloning of chitinases

Fj chitinases (*Fj chiA, Fj chiA GHI, Fj chiA GHII, Fj chiA GH1A, Fj chiA GHIIA, Fj chiB, Fj chiC, Fj chiD* and *Fj GH19*) were amplified using gene specific primers with *F. johnsoniae* gDNA as template. Based on SMART database *Fj* ChiA was predicted to contain N-terminal leader peptide directing sec-dependent secretion (Letunic et al., 2015). Complete genes were amplified for rest of the chitinase genes. Since *Fj c*hiA had dual catalytic domains, we generated four truncations to know the importance of individual domain in chitin degradation. The amplicons, 4.73 kb of *Fj chiA*, 1.28 kb of *Fj chiA GHI*, 1.23 kb of *Fj chiA GHII*, 3.42 kb of *Fj chiA GH1A*, 3.45 kb of *Fj chiA GHIIA*, 1.54 kb of *Fj chiB*, 0.95 kb of *Fj chiC*, 1.02 kb of *Fj chiD* and 2.36 kb of *Fj GH19* were cloned in between respective restriction sites of expression vector pET- 28a (+) (Fig 3.3a). The clones were confirmed by double digestion (Fig 3.3b) and the insert gene sequence was confirmed by automated DNA sequencing to check for mutations (Scigenom, Kerala, India).

Similarly, *Sm chiD* was also amplified using gene specific primers. The gene encoding *Sm* ChiD without signal peptide was amplified using polymerase chain reaction with gene-specific forward (5'-GCTTACCATGGATGCCTATCTCTCCGTCGGC-3') and reverse (5'-GACTACTCGAGCCGTTTCTCGCCTTTTATTCC-3') primers. The amplicons corresponding to 1.2 kb were double-digested and ligated to *Nco* I & *Xho* I sites of pET- 28a (+) expression vector. All the ligation reactions were performed at 16^0C for 16 h, using T4 DNA ligase. Highly



Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Flavobacterium johnsoniae strain UW101 16S ribosomal RNA gene, partial sequence	1807	1807	100%	0.0	99%	NR_042496.1
Flavobacterium johnsoniae strain NBRC 14942 16S ribosomal RNA gene, partial sequence	1805	1805	100%	0.0	99%	NR_113704.1
Flavobacterium johnsoniae strain UW101 16S ribosomal RNA gene, complete sequence	1801	1801	100%	0.0	99%	NR_074455.1
Flavobacterium johnsoniae strain DSM 2064 16S ribosomal RNA gene, partial sequence	1753	1753	96%	0.0	99%	NR_118479.1
Flavobacterium johnsoniae strain ATCC 23107 16S ribosomal RNA gene, complete sequence	1731	1731	100%	0.0	98%	NR_044738.2
Flavobacterium defluvii strain EMB117 16S ribosomal RNA gene, partial sequence	1679	1679	100%	0.0	97%	NR_043772.1
Flavobacterium ginsengiterrae strain DCY55 16S ribosomal RNA, partial sequence	1653	1653	100%	0.0	97%	NR_132661.1
Flavobacterium chungbukense strain CS100 16S ribosomal RNA gene, partial sequence	1640	1640	100%	0.0	97%	NR_109016.1
Flavobacterium johnsoniae strain UW101 16S ribosomal RNA gene, partial sequence	1622	1622	95%	0.0	97%	NR_115473.1
Flavobacterium tilapiae strain Ruve-71 16S ribosomal RNA gene, partial sequence	1615	1615	99%	0.0	96%	NR_109052.1
Flavobacterium cutihirudinis strain E89 16S ribosomal RNA gene, partial sequence	1613	1613	99%	0.0	96%	NR_109728.1
Flavobacterium oncorhynchi strain 631-08 16S ribosomal RNA gene, partial sequence	1607	1607	100%	0.0	96%	NR_117031.1
Flavobacterium ginsenosidimutans strain THG 01 16S ribosomal RNA gene, partial sequence	1607	1607	100%	0.0	96%	NR_108688.1
Flavobacterium chungangense strain CJ7 16S ribosomal RNA gene, partial sequence	1602	1602	99%	0.0	96%	NR_044581.1
Flavobacterium glycines strain NBRC 105008 16S ribosomal RNA gene, partial sequence	1596	1596	100%	0.0	96%	NR_114280.1

Fig 3.1: Molecular confirmation of *F. johnsoniae*: *F. johnsoniae* 16S rDNA was amplified with Taq polymerase using bacterial universal primers (Table 2.1). 16S: Amplified 16S rDNA, and L: DNA ladder mix (200 bp - 10 kb).

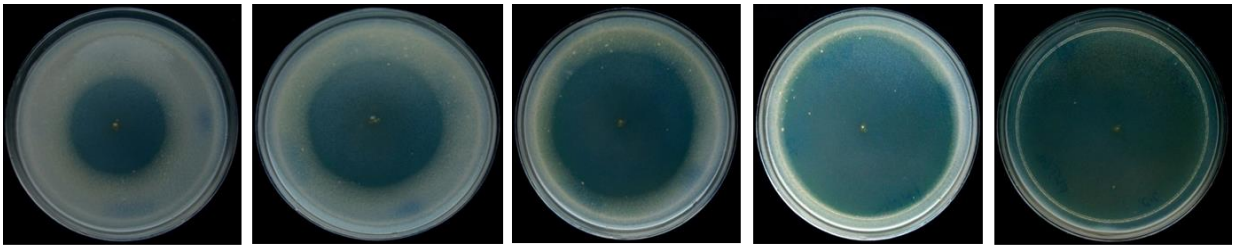


Fig 3.2: *F. johnsoniae* on chitin-containing agar plate: *F. johnsoniae* was spotted on colloidal chitin-containing CY minimal medium agar plate and incubated for 20 days at 30^0C.

efficient competent cells of *Escherichia coli* Rosetta-gami II (DE3) were used for transformation. Positive clones were selected on suitable antibiotic plates and confirmed by both double digestion and sequencing (Fig 3.4).

3.2.2 Expression and purification of *Fj* chitinases and *Sm* ChiD

All the nine *Fj* chitinase genes were overexpressed with C-terminal His-tag in expression host *Escherichia coli* Rosetta-gami II (DE3). The expressed chitinases are extracted from whole cell lysate (ChiC and *Sm* ChiD). *Fj* ChiB was extracted by 8M urea method. The extracted proteins were subjected to Ni-NTA agarose chromatography for purification. SDS-PAGE analysis (Fig 3.5) of purified chitinases revealed a molecular weight of 58, 38, 44 kDa which corresponds to *Fj* ChiB, *Fj* ChiC, and *Sm* ChiD, respectively (Fig 3.5).

3.2.3 Dot blot activity assay of purified chitinases

The purified recombinant chitinases were spotted on glycol chitin substrate containing polyacrylamide gel (PAGE) to detect chitinase activity. All the three recombinant chitinases showed activity zones on a substrate containing gel (Fig 3.6).

3.2.4 Characterization

3.2.4.1 Steady state kinetics analysis

Kinetic studies were carried out for three purified enzymes with various concentrations of CC (10-50 mg/ml) and enzyme in 50 mM optimized buffers and temperatures by reducing end assay. Specific activity [nanokat mg-1 protein] and substrate concentration (mg/ml) data were directly fitted to the Michaelis–Menten equation by nonlinear regression function available in (Fig 3.7 and Table 3.1) GraphPad Prism version 5.0 software. *Fj* ChiB (3.036×10^{2}) showed highest V_{max} [(nano kat mg-1 of protein)] value than *Fj* ChiC (11.2). The *K*m [(mg ml^{-1}] values for *Fj* ChiB (62.14) was little higher than *Fj* ChiC (49.38) indicating the substrate-binding affinity of *Fj* ChiC was highest than *Fj* ChiB. Catalytic activity *k*cat [sec^{-1}] of *Fj* ChiB (8.20×10^{7}) was more than *Fj* ChiC (7.94×10^{4}). Among the two chitinases, the overall catalytic activity, k_{cat}/K_m [mg-1 ml sec-1] of *Fj* ChiB has highest value (132.04×10^{4}) than *Fj* ChiC which was counted at 1.40×10^{3}. *Sm* ChiD exhibited 2.119, 52.82, 3.76×10^{2}, and 7.124 of V_{max}, K_m, k_{cat}, and k_{cat}/K_m, respectively.

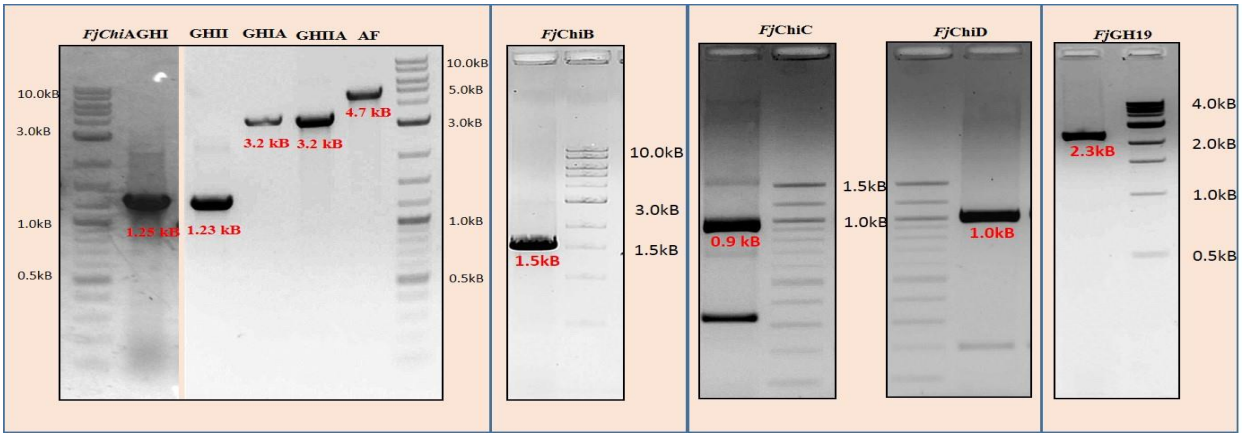


Fig 3.3.a: Amplification and cloning of chitinases from *F. johnsoniae*. All chitinases and their truncations of *F. johnsoniae* (*Fj chiA, Fj chiA GHI, Fj chiA GHII, Fj chiA GH1A, Fj chiA GHIIA, Fj chiB, Fj chiC, Fj chiD and Fj GH19*) were PCR-amplified with high fidelity Q5 DNA polymerase with gene specific primers using gDNA as a template and resolved on a 1.2% agarose gel.

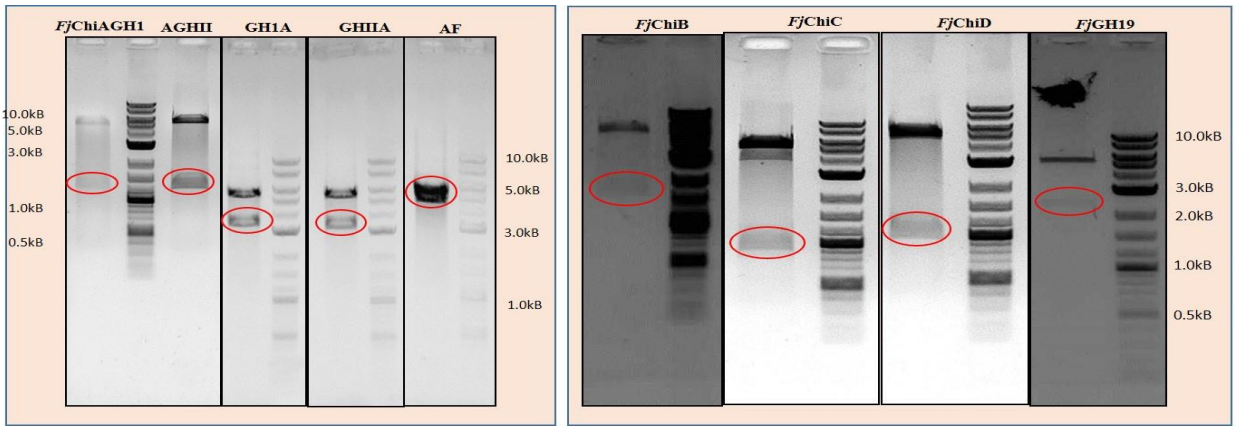


Fig 3.3.b: Confirmation of the insert by double digestion: The presence of insert was confirmed by double digestion with suitable restriction enzymes (*Nco* I and *Xho* I for *Fj chiB, Fj chiD, and Fj GH19* and *Bam* H1 and *Xho* I for *Fj chiA, Fj chiA GH1A, Fj chiA GHIIA and Fj chiC)* **(B)**. The molecular weight marker was DNA ladder mix (200 bp-10 kb) from NEB.

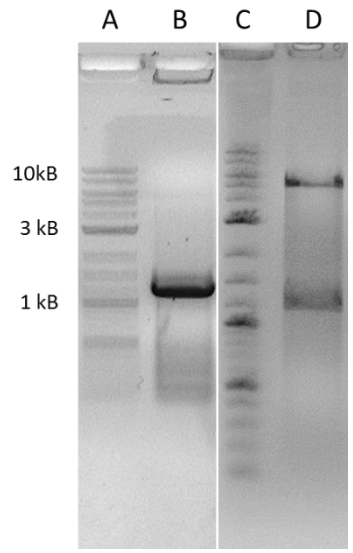


Fig 3.4: Amplification and cloning of chitinases-D from *Serratia marcescens* GPS5.
Sm chiD was PCR-amplified with gene specific primers and resolved on 1.2% agarose gel **(A, C)**. DNA molecular marker (100bp-10 kb marker, NEB), **(B)**. Amplied *Sm chiD* (1.2 kb) The molecular weight marker was DNA ladder mix from Fermentas (100 bp-10 kb) and **(D)**. Double digested with *Nco* I and *Xho* I restriction enzymes.

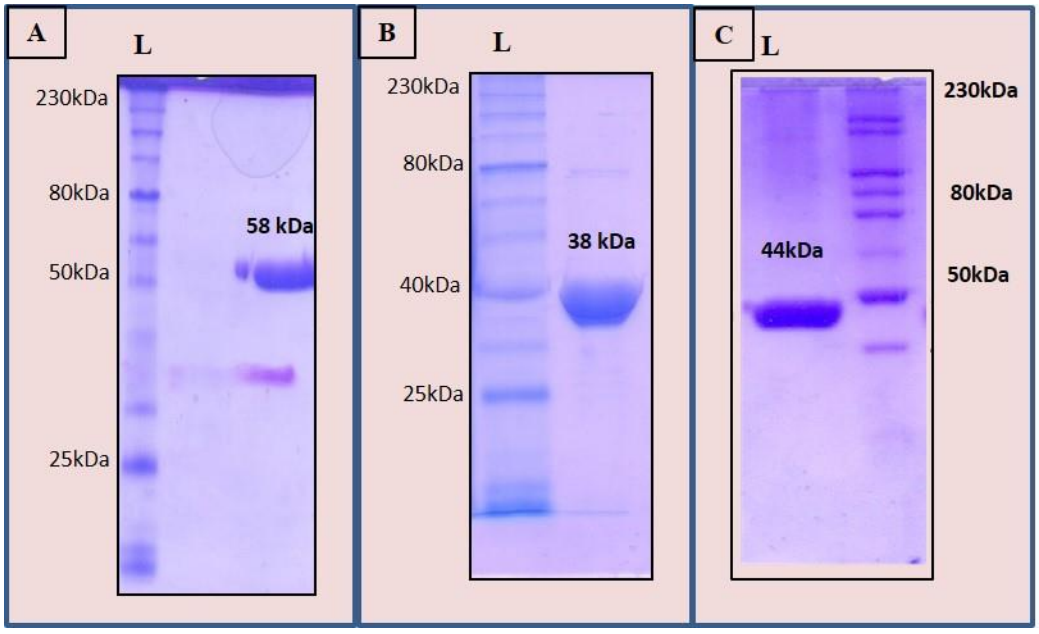


Fig 3.5: Purification of chitinases. Recombinant A) *Fj* ChiB, B) *Fj* ChiC, and C) *Sm* ChiD were purified using Ni-NTA agarose chromatography. Elution buffer containing 100, 150, 250 mM imidazole was used to elute chitinases from the column and loaded on 12% SDSPAGE followed by staining with Coomassie brilliant blue G-250. The molecular weight (Mw) of the standards is indicated in kDa. L: Protein standard.

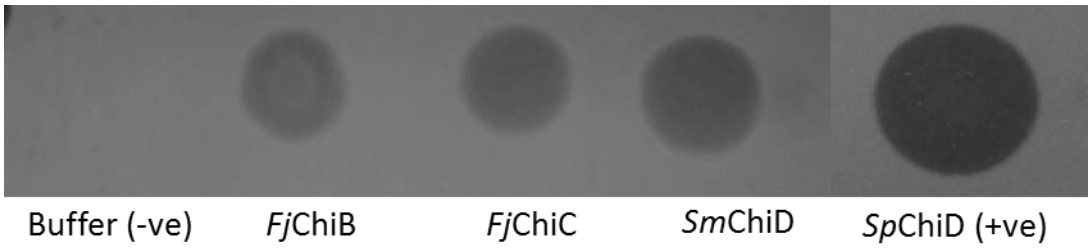


Fig 3.6: Chitinase activity of purified chitinases. Five micrograms (5 µg) of Purified chitinases were spotted on glycol chitin substrate containing polyacrylamide gel (PAGE) and incubated at 37^{0}C in a humid chamber for overnight. The gel was stained with 0.01% Calcofluor white M2R for 15 min at 4^{0}C. Eventually, the brightener solution was removed and the gel was washed two times with distilled water for 10 min at 4^{0}C. The gel was placed on UV transilluminator to visualize lytic zones which indicate the chitinase activity. Buffer (-ve): 50 mM Sodium citrate pH6.0. *Sp*ChiD: Chitinase-D from *Serratia proteamaculans* (5 µg).

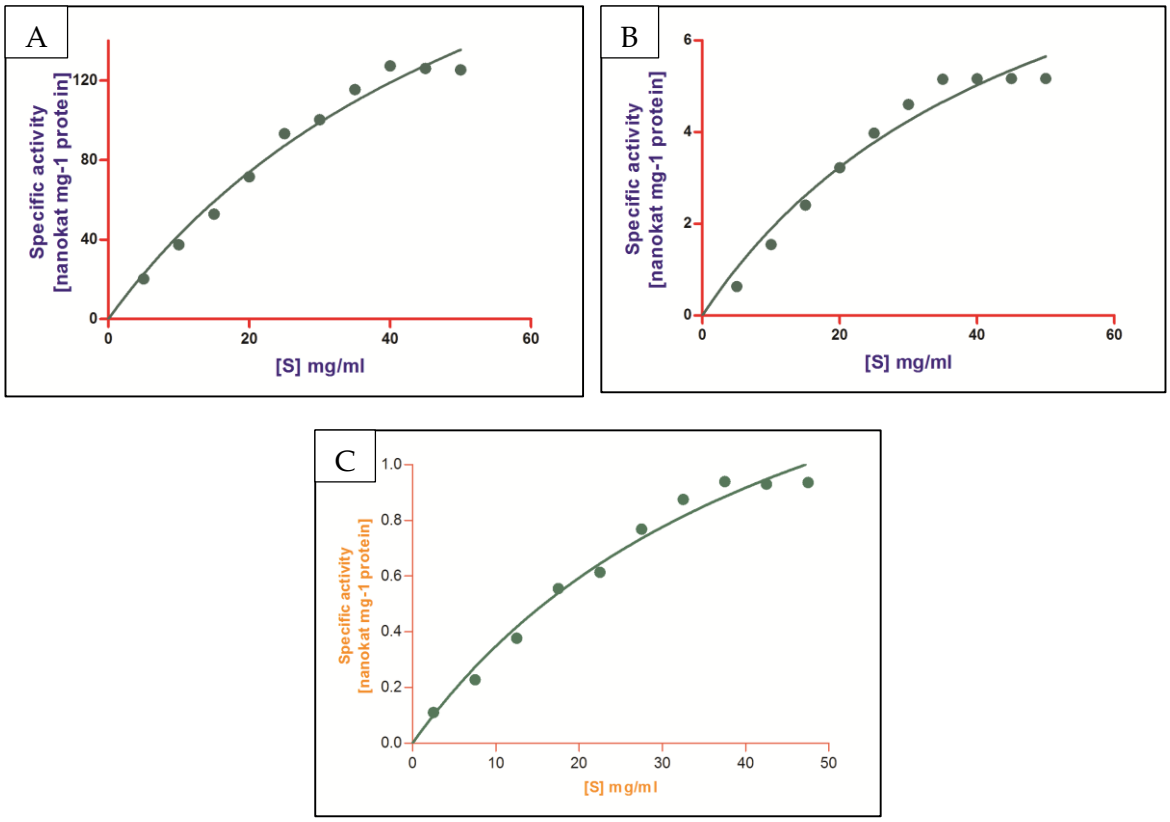


Fig 3.7: Kinetic analyses of purified chitinases. Kinetic studies of chitinases were calculated by estimating reducing sugars released from colloidal chitin substrate by enzymes. Varying concentration of substrate (10-50 mg.ml^{-1}) was incubated with chitinases (*Fj* ChiB: 5µg, *Fj* ChiC: 0.2µg, and *Sm* ChiD: 25 µg) in 50 mM buffer with at optimum pH and temperature for 1 h at 200 rpm. Specific activity [nanokat mg^{-1} protein] of protein was calculated and plotted the graph against substrate concentration. The data was fitted to the Michaelis-Menten equation by nonlinear regression function available in GraphPad Prism software version 5.0 to construct the kinetic graph and kinetic parameters. **A-C:** Curve fitting for *Fj* ChiB, *Fj* ChiC, and *Sm* ChiD respectively.

Enzyme	V_{max} (nano kat mg^{-1} of protein)	K_m (mg ml^{-1})	K_{cat} (sec^{-1})	K_{cat}/K_m (mg^{-1} ml sec^{-1})
*Fj*ChiB	3.036×10^{2}	62.14	8.20×10^{7}	132.04×10^{4}
*Fj*ChiC	11.2	49.38	7.94×10^{4}	1.40×10^{3}
SmChiD	2.119	52.82	3.76×10^{2}	7.124

Table 3.1: Kinetic parameters of colloidal chitin hydrolysis by chitinases.
GraphPad Prism software version 5.0 was used to calculate *V*max, *K*m, k_{cat} and *K*m/k_{cat}.

3.2.4.2 Effect of pH

The effect of pH on the hydrolytic activity of *Fj* ChiB and *Fj* ChiC was estimated on colloidal chitin substrate. Enzyme assay with different pH range buffers revealed that *Fj* ChiB exhibited broad range optimum pH ranging between 6.0-9.0 and optimum at 50 mM glycine-NaOH pH 9.0. *Fj* ChiC also showed broad range pH optimum between pH 5.0-9.0 and optimum activity in sodium citrate buffer pH 6.0. *Fj* ChiB maintained 80% of its maximum activity at pH 4.0 and 10.0 and very less activity (10%) at pH 2.0 and 3.0. *Fj* ChiC had negligible activity below pH 4.0 and 80% activity remained at pH 9.0-10.

Sm ChiD showed highest specific activity in 50 mM sodium citrate buffer pH.6.9 and completely lost its activity at pH 2.0 and remained 10% was at pH 3.0. Enzyme activity was 80% and 70% at pH 9.0 and 10.0 respectively (Fig 3.8).

3.2.4.3 Effect of temperature

The reaction was carried out with purified chitinases by incubating with colloidal chitin, at different temperatures (20-100^{0}C) to study the optimum temperature for the three chitinases. *Fj* ChiB showed maximum activity at 30^{0}C. *Fj* ChiC showed good activity between 20^{0}C-50^{0}C and optimum at 40^{0}C. Both the enzymes exhibited 50% activity at 10^{0}C. *Fj* ChiB maintained 80% of activity at 40^{0}C which decreased gradually till 70^{0}C and almost no activity at 80^{0}C. *Fj* ChiC had similar activity between 20^{0}C-50^{0}C. 80% activity was lost at 60^{0}C and no activity at 70^{0}C.

Sm ChiD showed ~80% activity at 30^{0}C and optimally active at 40^{0}C. The activity of *Sm* ChiD gradually decreased above 50^{0}C; only 10% activity was detectable at 70^{0}C and no activity 80^{0}C (Fig 3.9).

3.2.4.4 Hydrolytic activity on polymeric substrates

Substrate specificity of purified chitinases was determined with different chitinous and non-chitinous polymeric substrates. Both the chitinases *Fj* ChiB and *Fj* ChiC exhibited more activity towards chitosan (75% DDA) we tested. *Fj* ChiB and *Fj* ChiC preferred colloidal chitin next to cellulose. *Fj* ChiB showed similar activity on colloidal chitin. Both the enzymes preferred β-chitin next to colloidal chitin. α-chitin, which is more abundant and tightly packed

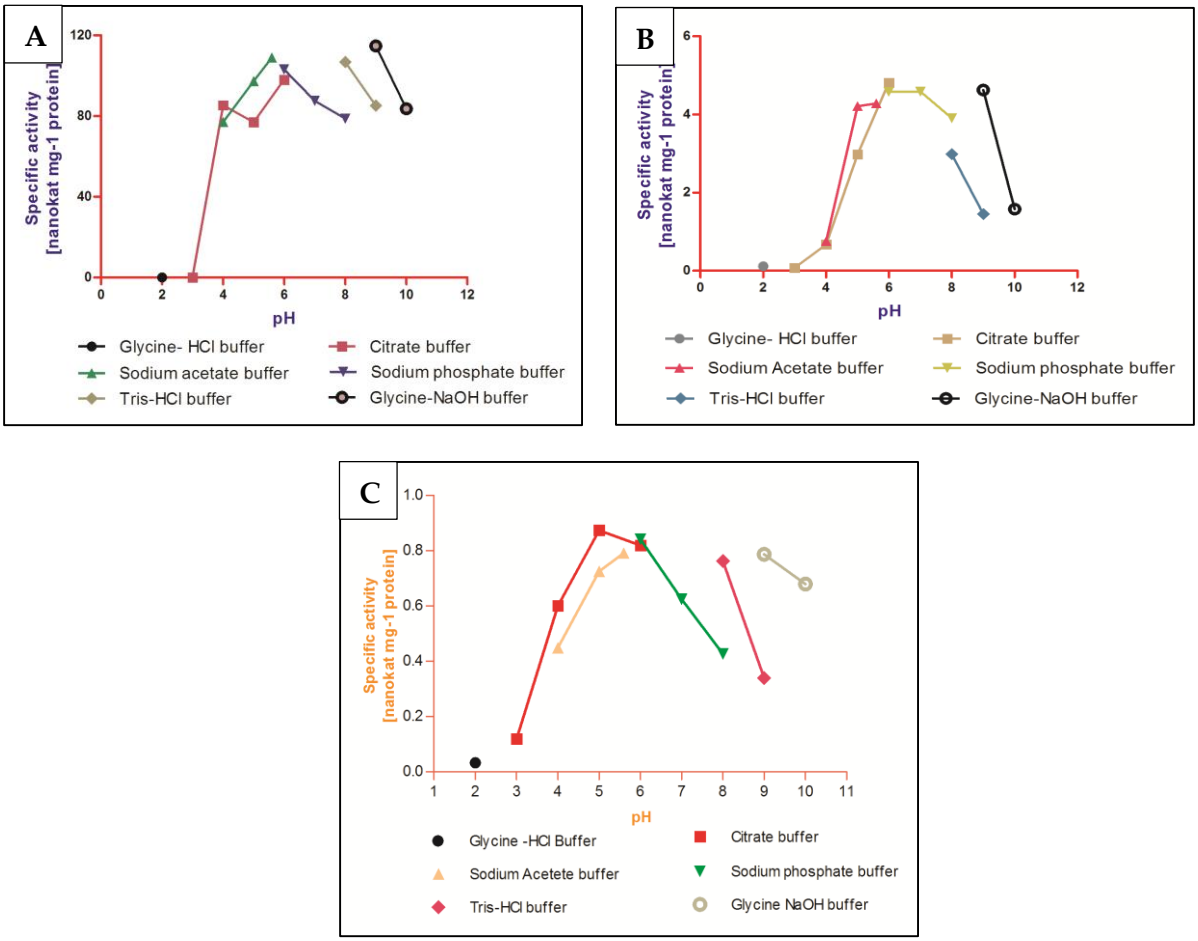


Fig 3.8: Optimum pH for activity of chitinases. The optimal pH of chitinases was determined by incubating the enzymes (*Fj* ChiB: 0.2 µg, *Fj* ChiC 5 µg, and *Sm* ChiD: 25 µg) with colloidal chitin (30 mg.ml^{-1}) at 40^{0}C for 1 h in 50 mM of different buffers pH ranging from 2-11 and the enzyme activity was estimated under standard assay condition. **A-C:** pH optima for *Fj* ChiB, *Fj* ChiC, *Sm* ChiD.

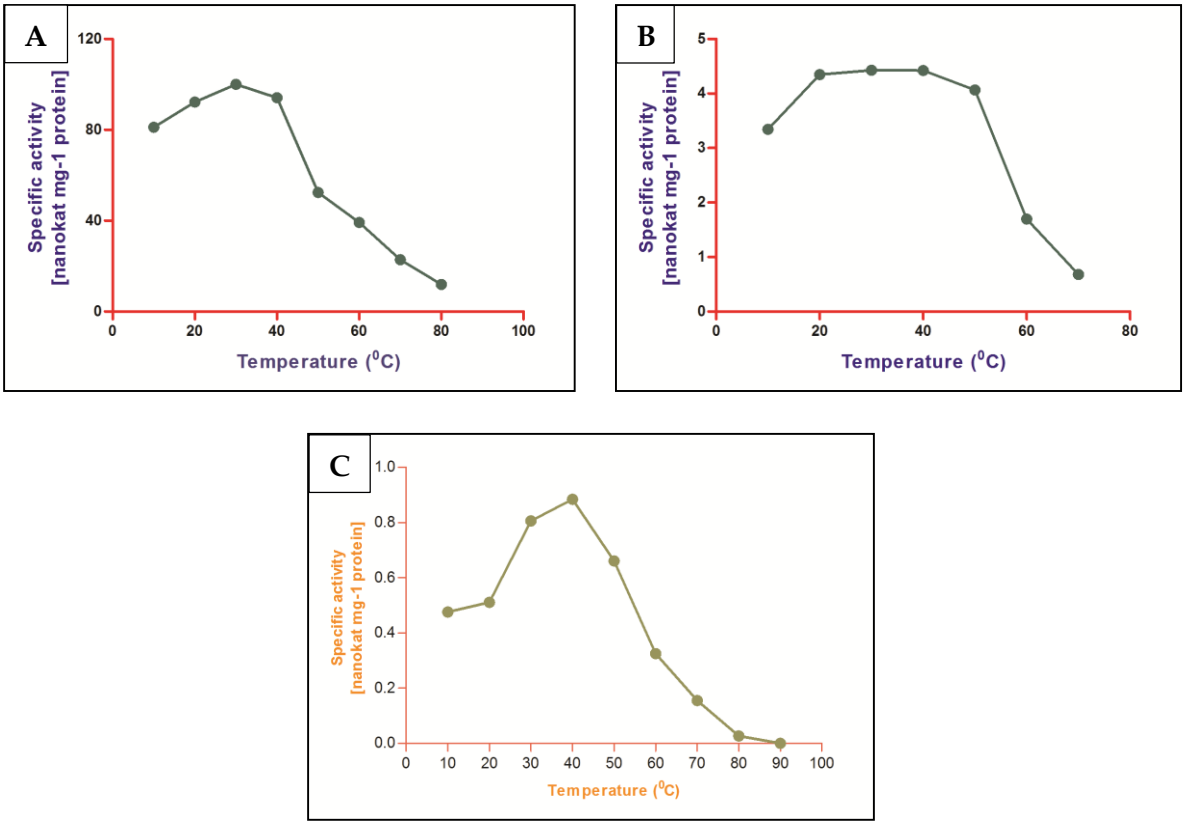


Fig 3.9: Optimum temperature for activity of chitinases. The optimal temperature of chitinases was determined by incubating the enzymes (*Fj* ChiB: 0.2 µg, *Fj* ChiC 5 µg and *Sm* ChiD: 25 µg) with colloidal chitin (30 mg.ml^{-1}) at 40^{0}C for 1 h in 50 mM buffer (*Fj* ChiB: glycine-NaOH buffer pH 9.0, *Fj* ChiC: sodium citrate pH 6.0, and *Sm* ChiD: sodium citrate pH 5.0) and the enzyme activity was estimated under standard assay condition. **A-C:** Temperature optima for *Fj* ChiB, *Fj* ChiC, *Sm* ChiD.

polymere was least preferred by *Fj* ChiC. *Fj* ChiB did not show activity on α-chitin. Both the enzymes were not active on non-chitinous polysaccharides avicel (Crystalline cellulose) and CM-cellulose (Carboxy Methyl cellulose) (Fig 3.10).

Sm ChiD exhibited more activity on colloidal chitin than chitosan. α-chitin and β-chitin were least preferred by *Sm* ChiD. *Sm* ChiD was not active on non-chitinous polysaccharides avicel (Crystalline cellulose) and CM-cellulose (Carboy Methyl cellulose) as expected (Fig 3.10).
Order of preference for the three chitinases follows: *Fj* ChiB: Chitosan≥Colloidal chitin> β-Chitin>α-Chitin. *Fj* ChiC: Chitosan > Colloidal chitin > β-Chitin>α-Chitin. *Sm* ChiD: Colloidal chitin> Chitosan> β-Chitin>α-Chitin.

3.2.4.5 Binding of *Sm* ChiD towards insoluble and soluble polymeric substrates

3.2.4.5.1 Soluble substrate binding

Binding preference of *Sm* ChiD towards soluble polysaccharide substrates was looked into by native polyacrylamide gel electrophoresis (Native-PAGE) with and without polysaccharides. Binding was attributed to the retardation in the mobility of the proteins in the gel. Comparison of electrophoretic pattern of *Sm* ChiD in presence or absence of the substrates revealed that *Sm* ChiD was not affected by the presence of non-chitinous substrates like CM-cellulose, laminarin or without substrate. The mobility was less in the presence of glycol chitin (Fig 3.11A).

3.2.4.5.2 Insoluble substrates binding

The binding ability of *Sm* ChiD towards the insoluble substrates was investigated at 4^{o}C, to minimize the enzyme-mediated hydrolysis of bound substrates. *Sm* ChiD (76.8%) showed maximum binding to colloidal chitin followed by 43% to α-chitin, 40% to avicel and 38% to β-chitin (Fig 3.11b).

3.2.4.6 Hydrolytic activities of chitinases on colloidal chitin

To gain more insights into their mode of action and characteristics, the three chitinases (*Fj* ChiB, *Fj* ChiB, and *Sm* ChiD) enzymes were incubated with colloidal chitin and analyzed those hydrolytic

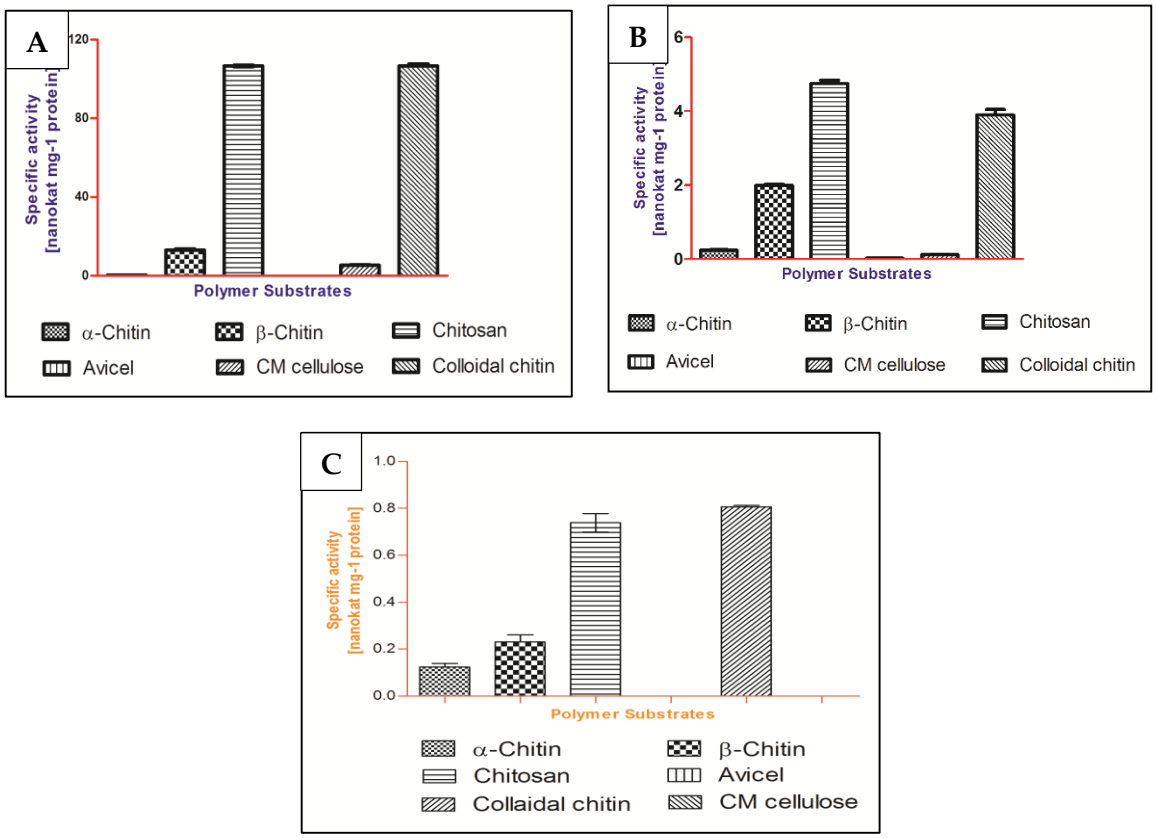


Fig 3.10: Substrate specificity for the activity of chitinases. The optimal temperature of chitinases was determined by incubating the enzymes (*Fj* ChiB: 0.2 µg, *Fj* ChiC 5 µg and *Sm* ChiD: 25 µg) with colloidal chitin (30 mg.ml^{-1}) at 40^{0}C for 1 h in 50 mM buffer (*Fj* ChiB: glycine-NaOH buffer pH 9.0, *Fj* ChiC: sodium citrate pH 6.0, and *Sm* ChiD: sodium citrate pH 5.0) and the enzyme activity was estimated under standard assay condition. **A-C:** Substrate preference for *Fj* ChiB, *Fj* ChiC, *Sm* ChiD**.**

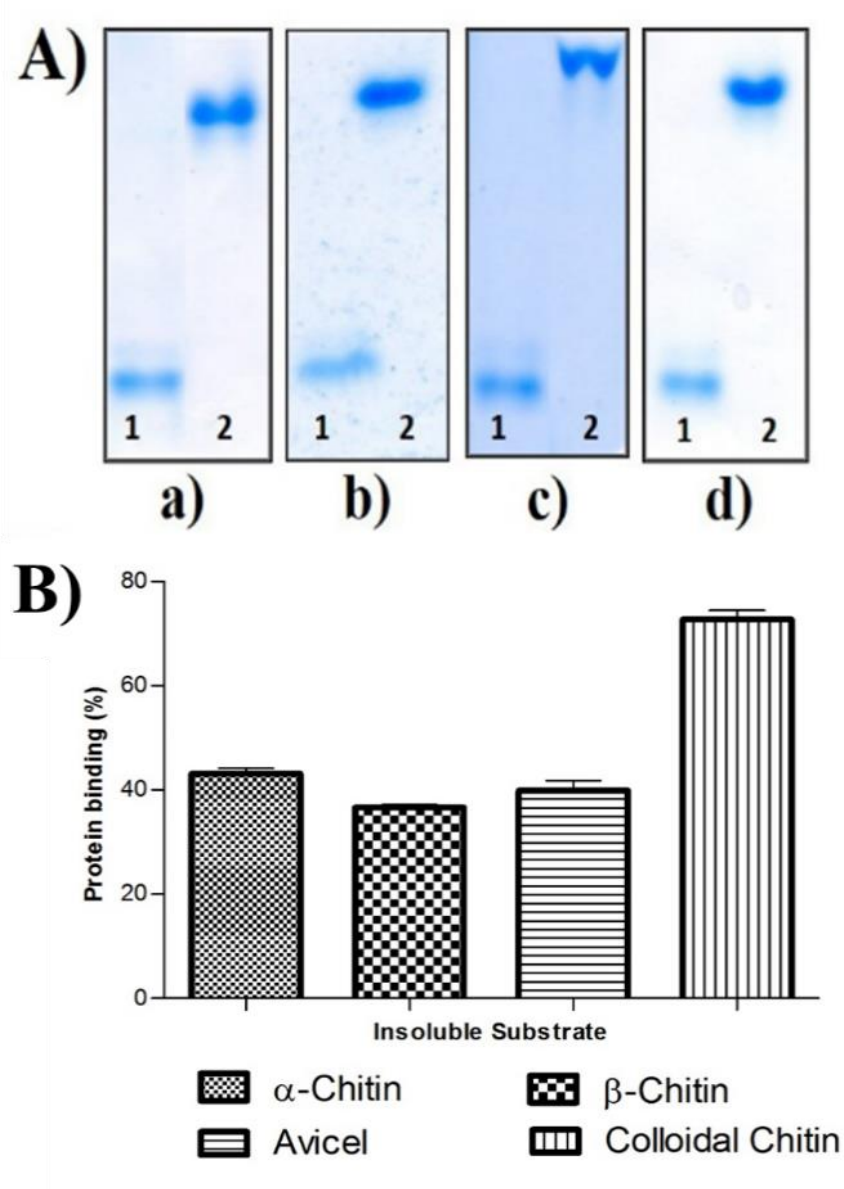


Fig 3.11: (A) Soluble substrate binding ability of *Sm* ChiD. Affinity non-denaturing gel electrophoresis was done at 4 ^{0}C by preparing 8% polyacrylamide gels. Six µg of *Sm* ChiD and BSA were electrophoresed without substrate (a) or with 0.1% (wt/vol) substrate CM-cellulose (b), glycol chitin (c), laminarin (d). Proteins were visualized by coomassie blue G-250 staining after electrophoresis. Lanes 1-2: BSA and *Sm* ChiD. **(B)** The insoluble substrate binding ability of *Sm* ChiD.

products by HPLC. Both the *Fj* chitinases were active on colloidal chitin and DP2 and DP3 are the major products. HPLC profile of the reaction product revealed that DP3 was the major end product produced by *Fj* ChiB followed by DP2, while DP2 was the major end product produced by *Fj* ChiC followed by DP3 at 720 min. At initial time points, DP4 was also detected for *Fj* ChiB at 10 min. Gradually DP4 was degraded to DP2. DP2 and DP3 concentration increased gradually from 0 min onwards for *Fj* ChiC. No DP1 formation was observed for both the *Fj* chitinases (Fig 3.12). Products were quantified from respective peak areas by using standard calibration curves of CHOS quantified ranging from DP1-DP6 (Fig 3.12).

The purified *Sm* ChiD exhibited more activity on colloidal chitin followed by chitosan. We further analyzed the activity by performing time-course degradation of colloidal chitin. The enzyme activity gradually increased till 9 h, where it reached to saturation. We could not see a change in enzyme activity from 9 - 11 h. It may be due to the saturation of reacted CHOS with Schales reagent. Simultaneous, TLC (thin layer chromatography) analysis of the fractions collected at different time intervals revealed that DP2 was the major hydrolytic product till 3 h. A prolonged incubation resulted in the accumulation of more of DP1 over DP2.

3.2.4.7 Hydrolytic activity of purified chitinases on oligomeric chitin substrates

The ability and efficiency of purified chitinases to degrade oligomeric chitin substrates were assessed by analysing the hydrolytic product profile formed in the course of their action. Both the chitinases (*Fj* ChiB and *Fj* ChiC) were not active on small chitin oligomers; DP2 and DP3. No hydrolytic products were observed with these two oligomers till 720 minutes.

DP2 was the major end product with DP4 substrate. DP2 formation was observed from 1 min onwards with both the enzymes. DP2 formation was gradually increased till 720 min. No DP1, DP3 formation observed with the DP4 substrate for *Fj* ChiB and *Fj* ChiC (Fig 3.13; Fig 3.14).

DP2 and DP3 were the major end products for *Fj* ChiB and *Fj* ChiC with DP5 substrate. Hydrolytic products were detected from 1 min onwards and complete hydrolysis occurred by 720 min. No DP4 and DP1 were detected throughout the reaction. *Fj* ChiC exhibited transglycosylation (TG) activity on DP5 substrate. DP6, DP7, and DP8 were detected from 10 min to 30 min.

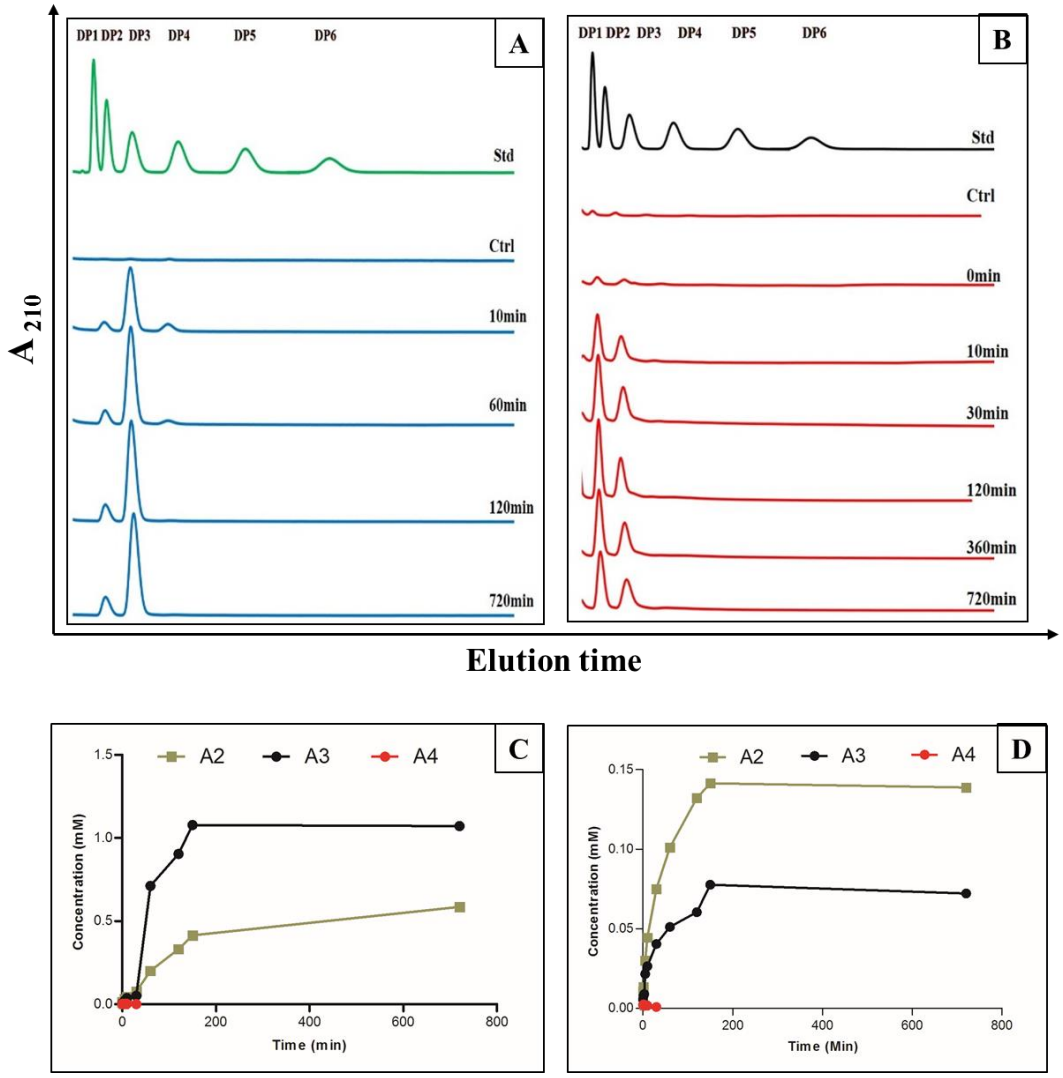


Fig 3.12: Time course of colloidal chitin hydrolysis by *Fj* ChiB and *Fj* ChiC . The reaction mixture (1 ml) containing colloidal chitin (30 mg/ml) in 50 mM buffer was incubated with *Fj* chitinases (*Fj* ChiB: 4 nM and *Fj* ChiC: 50 nM) for different time periods starting from 0-720 min at 40^0C. Each time point, 30 µL of reaction mixtures were withdrawn and an equal amount of 75% acetonitrile was added to stop the reaction. The reaction mixtures (60 µL each) were centrifuged, and the supernatants were concentrated and dissolved in 30 µL of 35 % acetonitrile. The reaction solution (20 µL) was analyzed by isocratic HPLC and eluted CHOS were monitored by recording absorption at 210 nm. The top most chromatogram shows a CHOS commercially available standard mixture of ranging from DP-DP6, while other chromatograms reflect the product profile after specified incubation time as indicated. Products were quantified from respective peak areas by using standard calibration curves of CHOS quantified ranging from DP1-DP6.

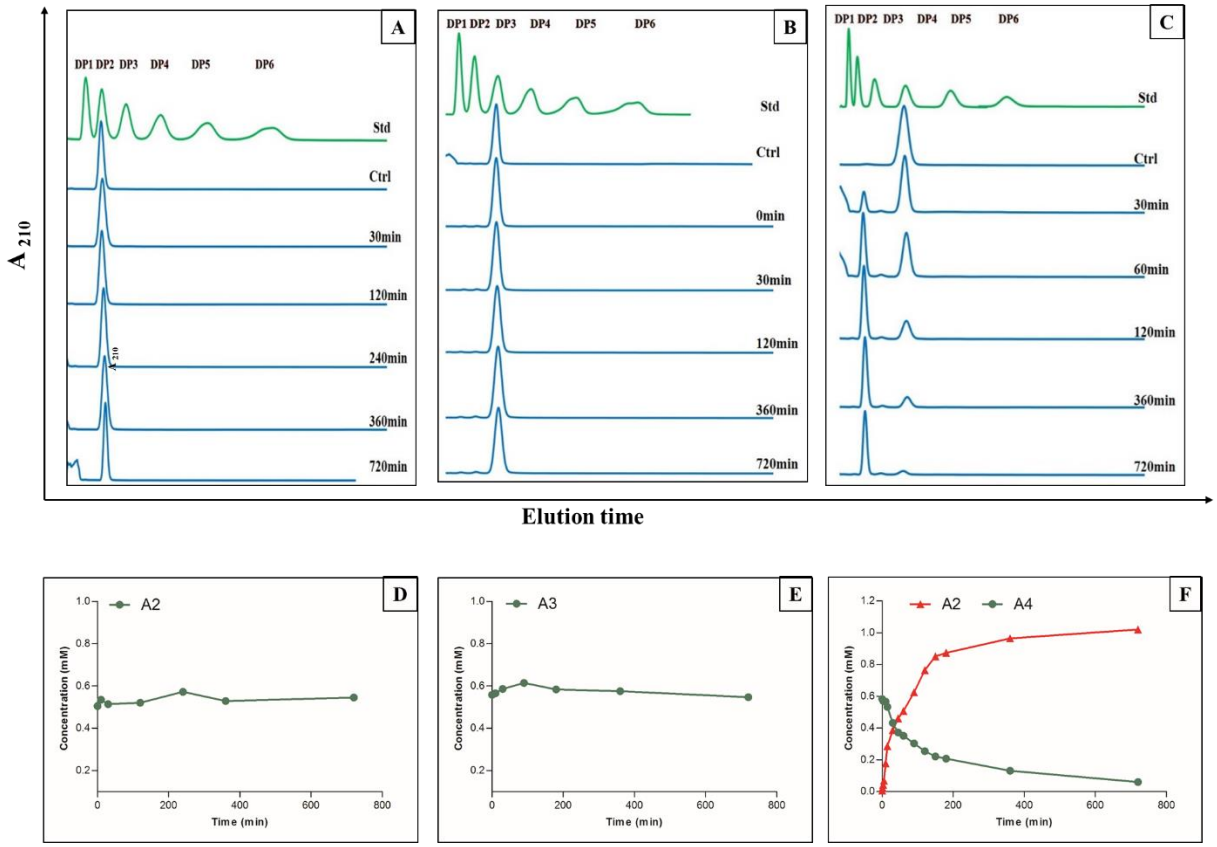


Fig 3.13: Time course of DP2, DP3, and DP4 hydrolysis by *Fj* ChiB. The reaction mixture (1 ml) containing substrate (1 mM) in 50 mM buffer was incubated with 4 nM of *Fj* ChiB: for different time periods starting from 0-720 min at 40^0C. Each time point, 30 µL of reaction mixtures were withdrawn and an equal amount of 75% acetonitrile was added to stop the reaction. The reaction mixtures (60 µL each) were centrifuged, and the supernatants were concentrated and dissolved in 30 µL of 35 % acetonitrile. The reaction solution (20 µL) was analyzed by isocratic HPLC and eluted CHOS were monitored by recording absorption at 210 nm. The top most chromatogram shows a CHOS commercially available standard mixture of ranging from DP-DP6, while other chromatograms reflect the product profile after specified incubation time as indicated. Products were quantified from respective peak areas by using standard calibration curves of CHOS quantified ranging from DP1-DP6.

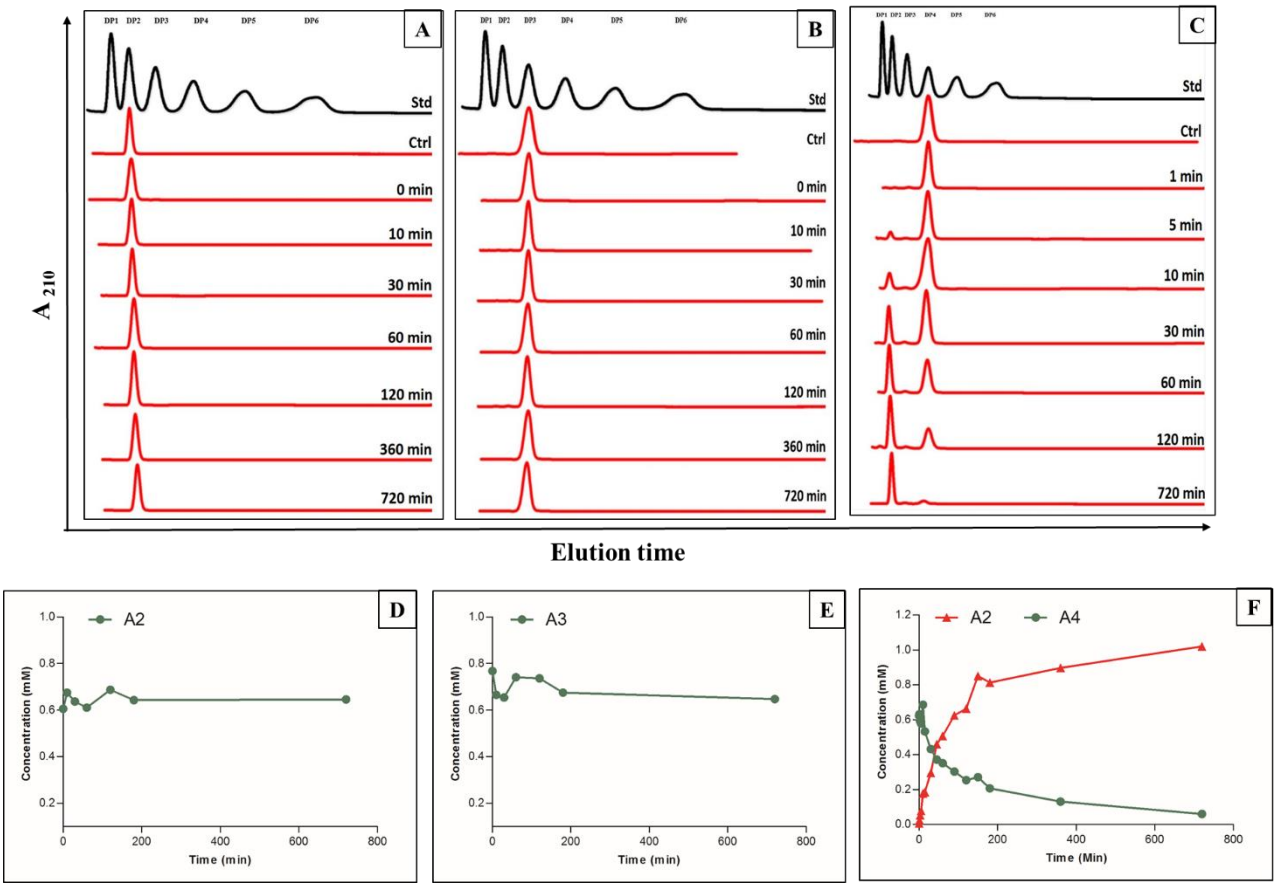


Fig 3.14: Time course of DP2, DP3, and DP4 hydrolysis by *Fj* ChiC. The reaction mixture (1 ml) containing substrate (1 mM) in 50 mM buffer was incubated with 50 nM of *Fj* ChiC for different time periods starting from 0-720 min at 40^0C. Each time point, 30 µL of reaction mixtures were withdrawn and an equal amount of 75% acetonitrile was added to stop the reaction. The reaction mixtures (60 µL each) were centrifuged, and the supernatants were concentrated and dissolved in 30 µL of 35 % acetonitrile. The reaction solution (20 µL) was analyzed by isocratic HPLC and eluted CHOS were monitored by recording absorption at 210 nm. The top most chromatogram shows a CHOS commercially available standard mixture of ranging from DP-DP6, while other chromatograms reflect the product profile after specified incubation time as indicated. Products were quantified from respective peak areas by using standard calibration curves of CHOS quantified ranging from DP1-DP6.

The TG products were gradually degraded after 30 min. No TG was observed for *Fj* ChiB (Fig 3.15; Fig 3.16).

Fj ChiB produced DP3 as a major product with DP6 substrate followed by DP2. DP5, DP4, DP3, and DP2 were detected at 10 min. All the given substrate was completely degraded by 180 min. No DP1 formation was detected till 720 min. DP3 is the major product at the end of the reaction followed by DP2 and DP4. *Fj* ChiC exhibited TG activity on DP6 substrate. DP7, DP8, and DP9 were observed from 10 min to 30 min. TG products were gradually degraded after 30 min. DP2 was the major end product followed by DP3 and DP4 at 720 min (Fig 3.15; Fig 3.16).

Sm ChiD was hardly active on DP2 substrate. It exhibited good TG activity on DP3, DP4, DP5, and DP6 substrate. DP2 was the major end product with DP3, DP4, DP5, and DP6. DP4 and DP5 are TG products with DP3 substrate. DP5 and DP6 were TG products formed from DP4 substrate. With DP5 substrate, *Sm* ChiD produced DP6 and DP7 as TG products. DP7, DP8, and DP9 were generated from DP6 substrate. All the TG products lasted long till 720 min. TG products started detectible from1 min onwards with all the tested CHOS, except DP2 (Fig 3.17).

The formation of long chain CHOS in the reaction mixtures was further confirmed by MALDI-TOF-MS analysis as described by Purushotham and Podile., (2012) (Fig 3.18).

3.3 Salt tolerance of *Fj* ChiC

The salinity tolerance of *Fj* ChiC was determined by incubating 30 mg/ml of colloidal chitin (CC) and 5µg of purified enzyme for 1 h at 37^0C, with different concentrations of NaCl (0-3.5M) under standard assay conditions. Enzyme activity decreased gradually with the increase of NaCl. At 3.5M NaCl, *Fj* ChiC retained 60% activity (Fig 3. 19).

3.4 Site-directed mutagenesis of *Fj* ChiC to improve TG

Among the two characterized chitinases from *F. johnsoniae*, *Fj C*hiC showed feeble TG activity by generating DP6-DP8 from DP5 substrate and DP7-DP9 products from DP6 substrate, in addition to hydrolytic activity. This finding encouraged us to improve the TG activity of *Fj* ChiC in terms of increasing the quantity and extending the duration of TG products by site-directed

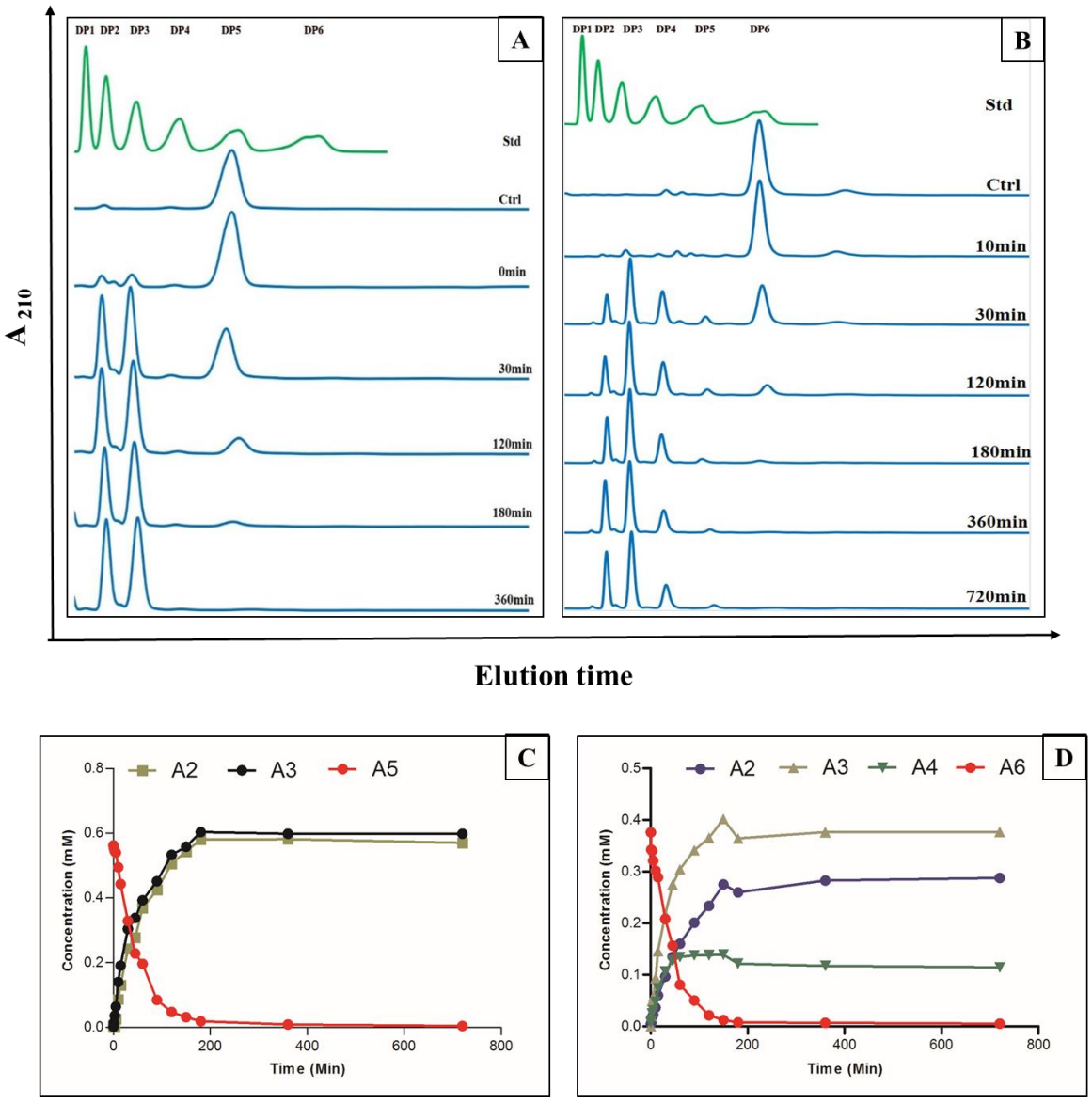


Fig 3.15: Time course of DP5 and DP6 hydrolysis by *Fj* ChiB . The reaction mixture (1 ml) containing substrate (1 mM) in 50 mM buffer was incubated with *Fj* chitinase (*Fj* ChiB: 4 nM) for different time periods starting from 0-720 min at 40^0C. Each time point, 30 µL of reaction mixtures were withdrawn and an equal amount of 75% acetonitrile was added to stop the reaction. The reaction mixtures (60 µL each) were centrifuged, and the supernatants were concentrated and dissolved in 30 µL of 35 % acetonitrile. The reaction solution (20 µL) was analyzed by isocratic HPLC and eluted CHOS were monitored by recording absorption at 210 nm. The top most chromatogram shows a CHOS commercially available standard mixture of ranging from DP-DP6, while other chromatograms reflect the product profile after specified incubation time as indicated. Products were quantified from respective peak areas by using standard calibration curves of CHOS quantified ranging from DP1-DP6.

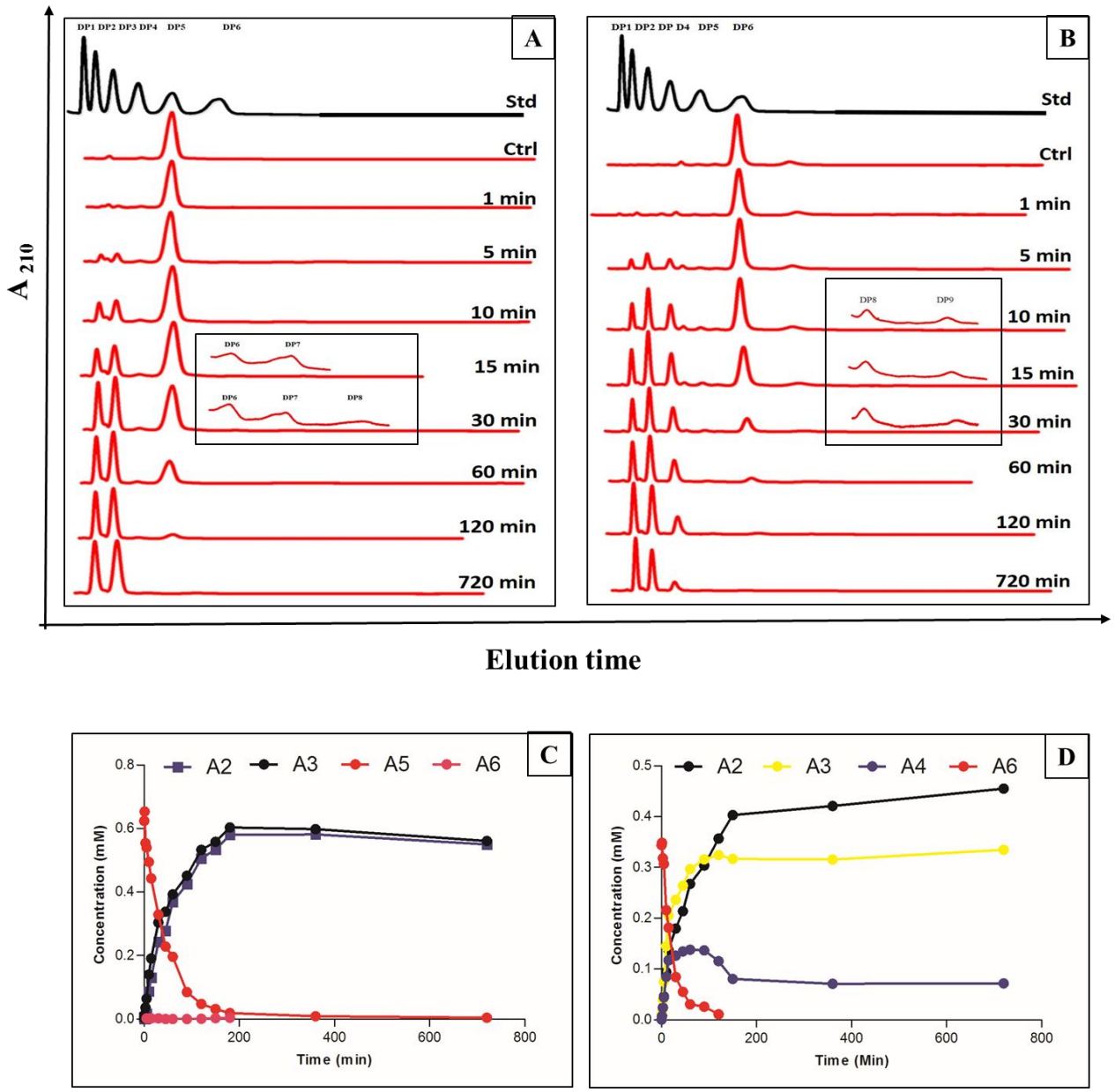


Fig 3.16: Time course of DP5 and DP6 hydrolysis by *Fj* ChiC. The reaction mixture (1 ml) containing oligomeric substrates (1 mM) in 50 mM buffer was incubated with *Fj* chitinase (*Fj* ChiC: 50 nM) for different time periods starting from 0-720 min at 40^{0}C. Each time point, 30 µL of reaction mixtures were withdrawn and an equal amount of 75% acetonitrile was added to stop the reaction. The reaction mixtures (60 µL each) were centrifuged, and the supernatants were concentrated and dissolved in 30 µL of 35 % acetonitrile. The reaction solution (20 µL) was analyzed by isocratic HPLC and eluted CHOS were monitored by recording absorption at 210 nm. Products were quantified from respective peak areas using standard calibration curves of CHOS quantified ranging from DP1-DP6.

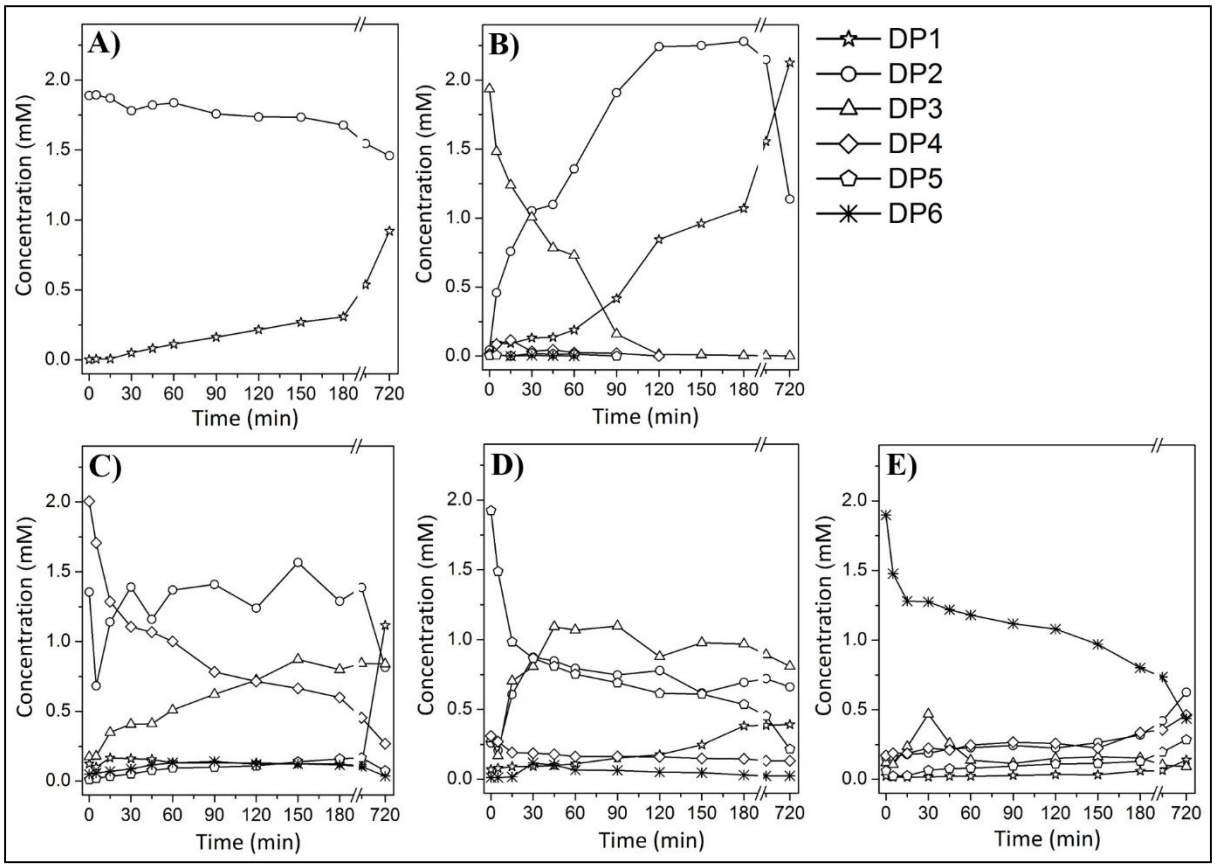


Fig 3.17: Hydrolytic and TG activities of *Sm* ChiD on CHOS. The reaction was carried out by incubating 2 mM of chitin oligomeric substrates DP2 (A), DP3 (B), DP4 (C), DP5 (D) and DP6 (E) with 350 nM of *Sm* ChiD in 50 mM sodium citrate buffer pH-5.0 at 40^{0}C. 40µl of samples was collected at different time intervals and the enzyme was inactivated by adding equal amounts of 70% acetonitrile. Products were analyzed by isocratic HPLC and quantified from respective peak areas by using standard calibration curves of CHOS ranging from DP1 to DP6.

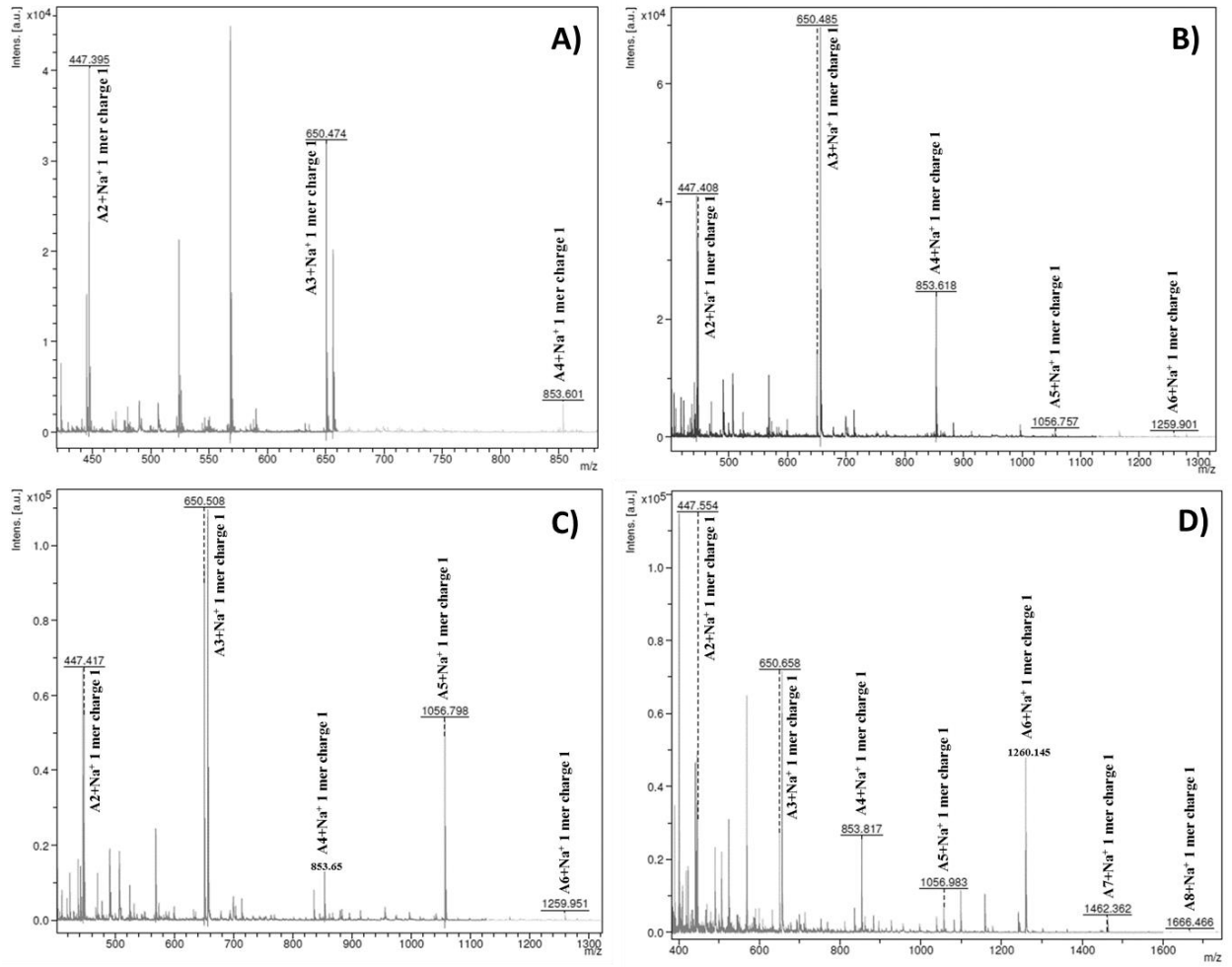


Fig 3.18: MALDI-TOF-MS analysis of hydrolytic or TG products generated by *Sm* ChiD. MALDI-TOF-MS analysis of hydrolysis or TG products generated by *Sm* ChiD with DP3 (A), DP4 (B), DP5 (C) and DP6 (D) substrates. After HPLC analysis of reaction products from DP3 to DP6 substrates (60 min samples), 4 µl of reaction sample was mixed with an equal volume of 2, 5-dihydroxybenzoic acid (2,5-DHB), and proceeded for mass measurements using an Ultraflex MALDI-TOF/TOF instrument. Peaks in all MALDI-TOF MS spectra were labeled based on their atomic mass and their degree of polymerization.

mutagenesis (SDM). The amino acid residues from the catalytic center, catalytic groove and within the catalytic triad (labeled in yellow color) were selected for mutagenesis (Fig 3.20). In the catalytic center, the residue G106 was closer to the catalytic triad D*X*D*X*E. D148 from the catalytic triad and W211 from the catalytic groove were selected for mutagenesis. Due to the conversion of G106W, enzyme activity decreased. Secondary hydrolysis of TG products decreased and thereby TG products remained for a long time due to D148N conversion. TG activity was completely lost due to W211G mutation. Chitobiase activity was observed due to W211G conversion (Fig 3.21).

3.5 Sequence analysis of *Fj* and *Sm* chitinases

The sequence alignment of catalytic domains of *F. johnsoniae* and *S. marcescens* GPS5 was done using clustal omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). All the GH18 catalytic domains (*Fj* ChiA GHI, *Fj* ChiA GHII, *Fj* ChiB, *Fj* ChiC and *Fj* ChiD) of *F. johnsoniae* was downloaded from NCBI. All the chitinases had conserved motif, DXXDXDXE in their catalytic center. Along with DXDXE motif, SXGG motif was also conserved in all the chitinases except *Fj* ChiC (Fig 3.22). Both *Fj* ChiB and *Fj* ChiC doesn't contain α+β insertion. *Sm* ChiD shared less similarity among the three chitinases already reported from *S. marcescens* (Fig 3.23) (Vaaje-Kolstad et al., 2013). Homology modeling revealed that both *Fj* ChiB and *Fj* ChiC doesn't contain α+β insertion. The α+β insertion was present in *Sm* ChiD (Fig 3.24).

3.6 Determination of subsite specificity of *Fj* ChiC and G106W mutant

Subsite preference studies revealed that *Fj* ChiC wild type chitinase was not specific at -2 and +2 subsites. It was able to accept both acetylated and deacetylated sugar at this position. All the chitinases prefer acetylated sugar at -1 subsite which was observed in *Fj* ChiC. *Fj* ChiC is accepting de-acetylated sugar at +1 subsite. But because of mutation, the preference was completely changed to acetylated sugar in G106W mutant (Fig 3.25).

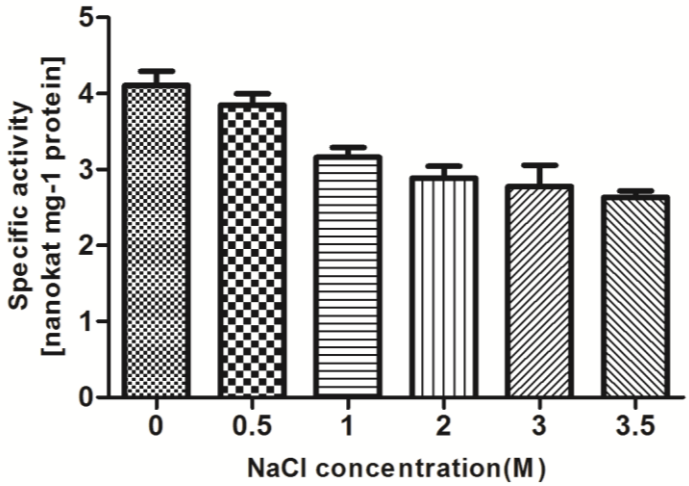


Fig 3.19: Salt tolerance of *Fj* ChiC. Salt tolerance of *Fj* ChiC was tested in the presence of NaCl. Five microgram of *Fj* ChiC was incubated with 30 mg/ml (w/v) colloidal chitin at 40^0C for 1h. Enzyme activity was measured by reducing end assay (Imoto and Yagishita, 1971). The average of triplicate data was used for analysis. Vertical bars represent the standard deviation of three experiments with three different individuals.

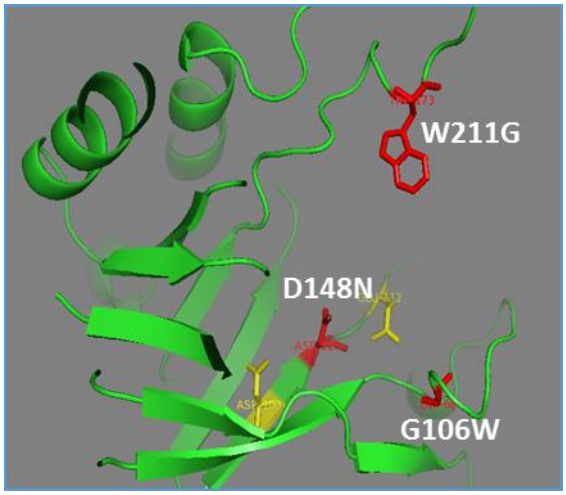


Fig 3.20: Amino acid residues selected for site-directed mutagenesis. The amino acid residues selected for site-directed mutagenesis were labeled in white color. G106 is located at the catalytic center, D148 located within catalytic triad (Labeled in yellow color) and W211 is located at the catalytic groove.

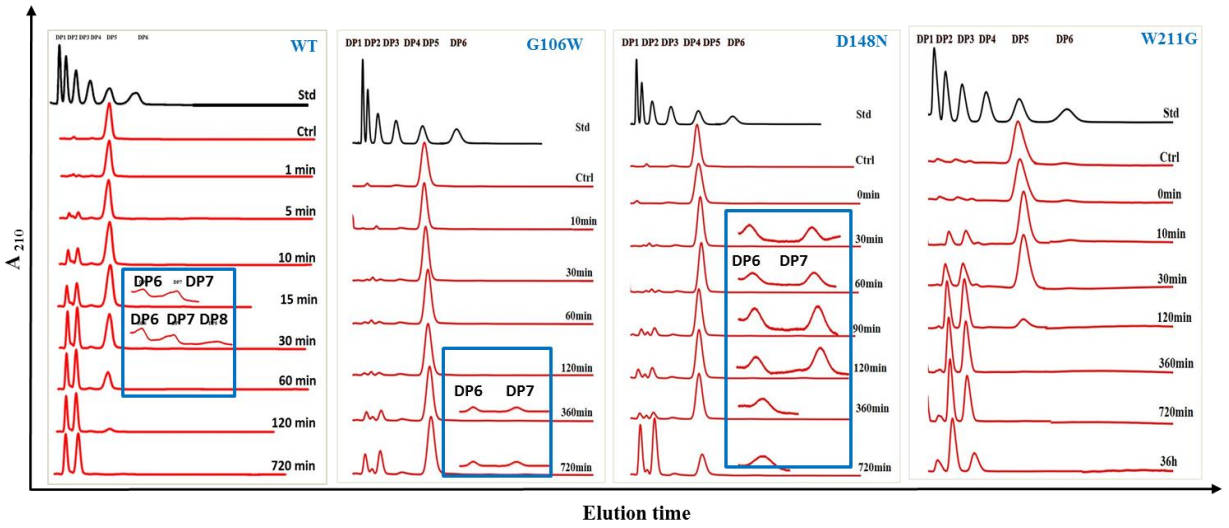


Fig 3.21: Profile of products generated by *Fj* ChiC mutants with DP5 substrate analysed by HPLC. The reaction mixture (1 ml) containing substrate (1 mM) in 50 mM buffer was incubated with *Fj* chitinases (*Fj* ChiC: 50 nM) for different time periods starting from 0-720 min at 40^0C. Each time point, 30 µL of reaction mixtures were withdrawn and an equal amount of 75% acetonitrile was added to stop the reaction. The reaction mixtures (60 µL each) were centrifuged, and the supernatants were concentrated and dissolved in 30 µL of 35 % acetonitrile. The reaction solution (20 µL) was analyzed by isocratic HPLC and eluted CHOS were monitored by recording absorption at 210 nm. The top most chromatogram shows a CHOS commercially available standard mixture of ranging from DP-DP6, while other chromatograms reflect the product profile after specified incubation time as indicated.

```
Fj_ChiB          ----------------------------------KKVIAYIPNWIDL-NAFS--STIQYS
Fj_ChiC          MIKNKALLGILFFMSSLFSVYSCTSEKENNPEEVKSKKARVVGYLSA-DSFDKITSIEFC
Fj_ChiA_GHI      -----------KKVVGYYAQWSIYARDFNVPKIDGSKLTHLNYSFYG-TTYDP-AHPENT
Fj_ChiD          ------------------------------------FDIIAYYTGD-SQLID--QYEVS
Fj_ChiA_GHII     ----------------------------------KIILGYAHSWENAGAPFLYFSQMVGS


Fj_ChiB          KLTHINIAFENPDANGYLSFNSGSN----AIINAAHAQN--IKVFVSLGGGSVSEGGAIR
Fj_ChiC          KLTHLNIAFANPDKNGNLVFDGDID----AVTKYVRSVNSNIVISISLAGGVISTEQAA-
Fj_ChiA_GHI      KLKCLDTYADFEHMEGGIPWDAPVKGN-FYDLMKLKQKYPHLKILISVGGWTKGQ-----
Fj_ChiD          KLNQIIFSFCHLK-DGKLSVDSAKDSLTIKHLVSLKAKNPQLKIIVSLGGWGGCEP----
Fj_ChiA_GHII     KTVNRDGYTPILTTNDTRYLTNGVFNKQLLKNDIKSLRDSGVPVIVSIGGKFNVVDYSFV
                 *:. :          .        .          .       :  * .*

Fj_ChiB          DNYFNLIT-PANRTAFIQKIYDYVVAHN-FDGVDVDLEGPAING-----------------
Fj_ChiC          -NWSLLIDKPENRPAFMQNISKFVTDHN-LDGVDVDLEWDAVTS-----------------
Fj_ChiA_GHI      -DLSPIAASPVARAALAADMANFIVTYPFIDGFDIDWEYPLSGGTDGTEIVNGMPVPPQK
Fj_ChiD          --CSDAFSTAEGRLKFAKSVKEVSDYFK-VDGLDLDWEYPSIEG-----------LPGHL
Fj_ChiA_GHII     QNGHVVLDNVTQKNIFVNGLKAIIDEYQ-FDGVDIDFEGGSMNFNAGG---LRDISYAGI
                             :  :   :      .  .**.*:* *

Fj_ChiB          ----DYGGFVIALANKLHAN--GKLISAALSEGYGGAN-------------VPSSTFAAY
Fj_ChiC          ----GYSGFVVELRKELTDR--KKLLTAALPNNTRFVN-------------INSEALNAF
Fj_ChiA_GHI      YSPDDNKNLVLLLKAMRQAMP-NKLVTIAAGNNVRNVSKQYLGPNNRAQYGMTEDISTYC
Fj_ChiD          YQAADKPNFTELVKILRSTLG-KKYELSFAAGGFQKCLDES---------IDWKAVAPFV
Fj_ChiA_GHII     SAYPRLKNVVDAFKELKAYYGPGFLLTAAPETQYVQGGYTTYTDTFGSFLPIIQNLRNEL
                           ...  .                                    .

Fj_ChiB          DWINIMAYDATGPWA--------------PGNPGQHSPYSMAVNQFN--YWTGRGLPASK
Fj_ChiC          DFINIMAYDSTGPWS--------------PNKIEQHSSFEFAKEGVE--FWKKQNVPSEK
Fj_ChiA_GHI      DYITYFGYDFGGNWYDKTCYNAPLYASGNPNDPLYGATQSESLDELTNQYLNVIGFPANK
Fj_ChiD          NRINIMSYDLVNGYSKVTG----------HHTPLYSTNPKEESTDRAVEFLLKQGVPAEK
Fj_ChiA_GHII     DLLAVQLYNTGGENG-------------LDGQYYGTAKKSNMVTALTDMVIKGYNIASTG
                 : :    *:  .                        :  .             ...:

Fj_ChiB          AIIG-LPFYGYGFGASANQGIS-----------------------------------------
Fj_ChiC          LTLG-VPFYGYNFTYPEVTSST-----------------------------------------
Fj_ChiA_GHI      LIMG-LPFYGKKFDNVAANSTNGLFVAAPRYIVPGCTNPQNPTGTWDGSGACEKSGSIEI
Fj_ChiD          LVIG-GAFYTRQWKNVENINNG-----------------------------------------
Fj_ChiA_GHII     MRFDGLPASKVLIALPACPSAAG-----------------------------------------
                        :.  .            .

Fj_ChiB          ---YANIVAQYPGAENL----------------------------DQVGNTIYYNGIPT
Fj_ChiC          ---FGEIIQAGTQFADQ----------------------------DEIG-KIYYNGRPT
Fj_ChiA_GHI      CDLVGNPVTNSHAYLDPNTMMVTPSAASAGWVRYFDNTTKVPYLYNSTLKQFISYEDKQS
Fj_ChiD          -LYQAGEHFQGVDFKNYATTYTEAN----GWKYFWDDKAKAPYWYNAQTKTFATSDDLKS
Fj_ChiA_GHII     ----SGYLTPTEGINAMH----------------------------YLRTGTTFSGRTYT
                           .                                              :

Fj_ChiB          IKQKTTFA-VQNAGGVMIWELSQD
Fj_ChiC          ILKKVEYA-SQNTGGIMIWELAQD
Fj_ChiA_GHI      MDLKVQYIKSRNLAGGMIWELSQD
Fj_ChiD          IKAKTEYVKEKKLGGIMFWELTLD
Fj_ChiA_GHII     MQPGGPYP---SLRGLMTWSVNWD
                 :     :    .  * * *.:  *
```

Fig 3.22: Sequence alignment of *Fj* chitinases. All the GH18 chitinase catalytic domains of *F. johnsoniae* were subjected to sequence alignment to check their conserving nature of amino acids. Conserved motif (DXXDXDXE) is labeled in yellow.

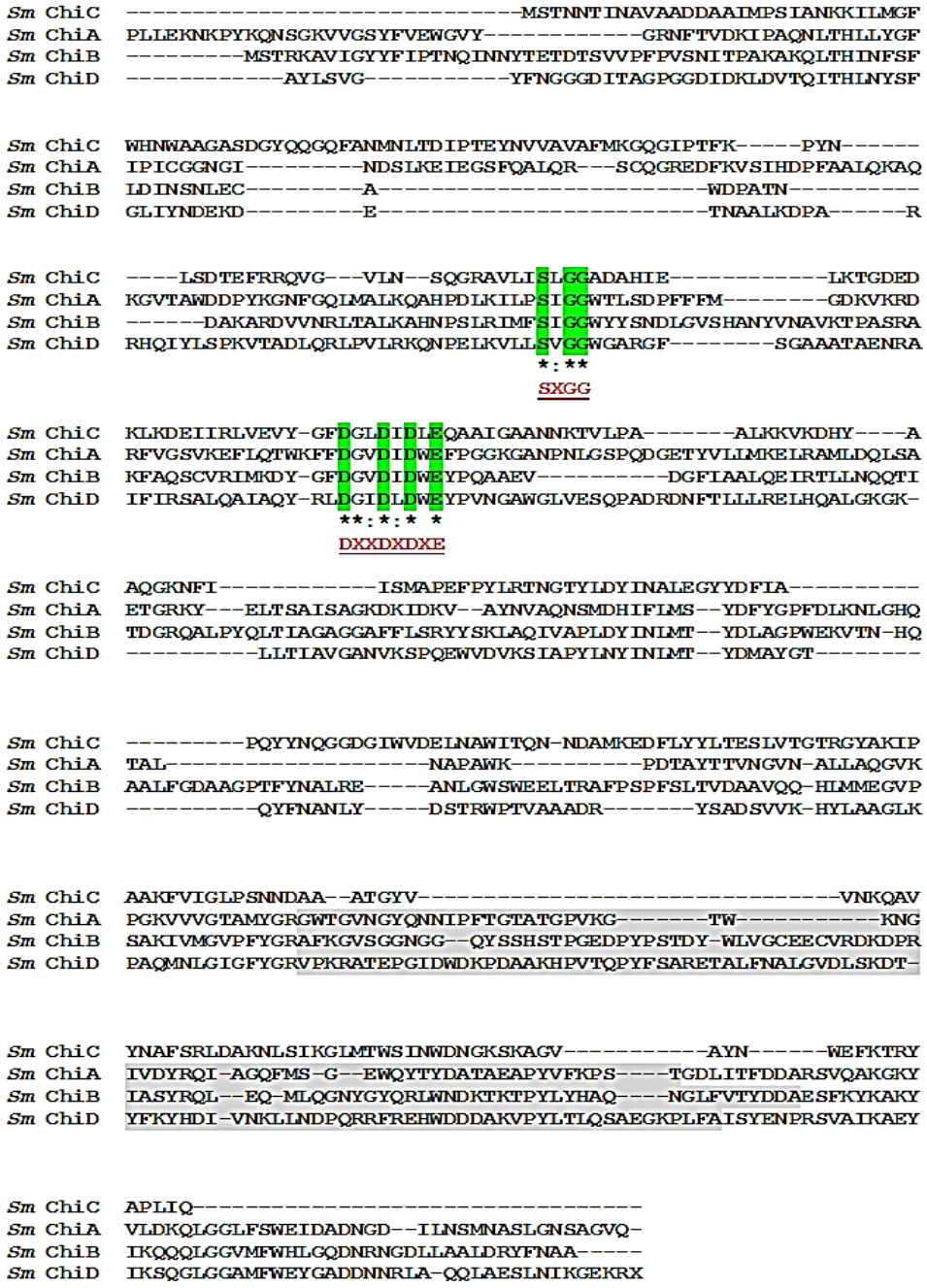


Fig 3.23: Sequence alignment for *Sm* ChiD with reported *S. marcescens* chitinases. Sequence alignment for *Sm* ChiD. Catalytic domains of *S. marcescens* chitinases (*Sm* ChiA, *Sm* ChiB, and *Sm* ChiC) and *Sm* ChiD were aligned using clustalw2. The segment of residues constituting for the α+β domains were present in *Sm* ChiA, *Sm* ChiB, and *Sm* ChiD, but absent in *Sm* ChiC, are shaded gray. Signature sequence motifs containing conserved residues that are crucial for catalysis ("SXGG" and "DXXDXDXE") are shaded green and shown at the bottom with red underline.

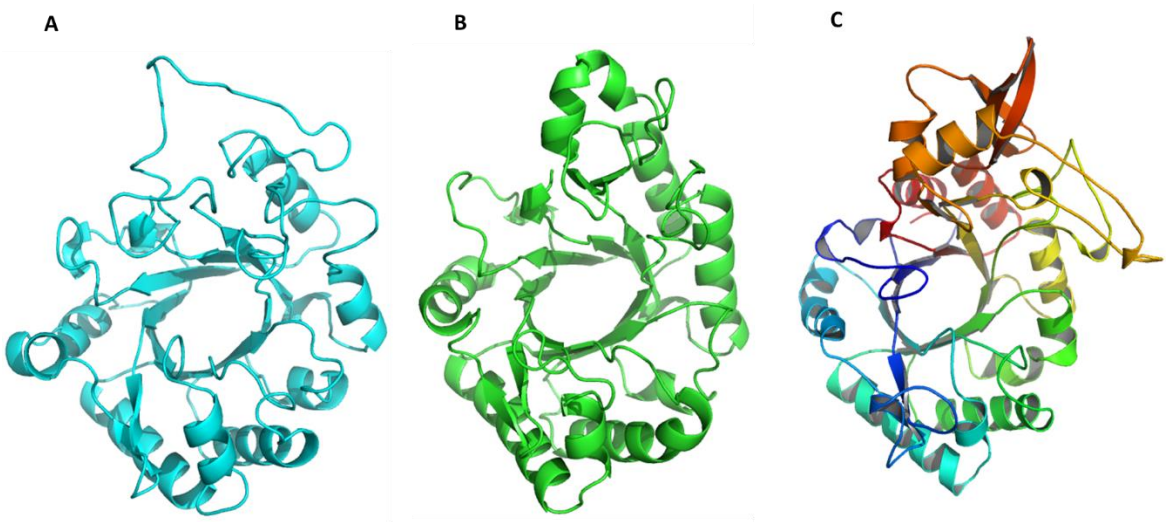


Fig 3.24: Homology modeling of chitinases. Homology modeling of *Fj* ChiB (A), *Fj* ChiC (B) and *Sm* ChiD (C). The sequence of chitinases (except for *Sm* ChiD) were retrieved from NCBI Database (http://www.ncbi.nlm.nih.gov/) for protein modeling. The BLAST program was used for sequence search against known 3D structure available in the Protein Databank (PDB) (http://www.rcsb.org/), to select appropriate templates for constructing 3D structure models of the *Fj* ChiB, *Fj* ChiC and *Sm* ChiD. The homology model program MODELLER v9.12 (Sali and Blundell, 1993) was employed to generate 3D models. The stereo-chemical quality of the modeled protein structure was checked in Ramachandran plot (Ramachandran et al., 1963) using PROCHECK (Laskowski et al., 1993). The compatibility of the model with its sequence was measured by Verify-3D graph (Lüthy et al., 1992).

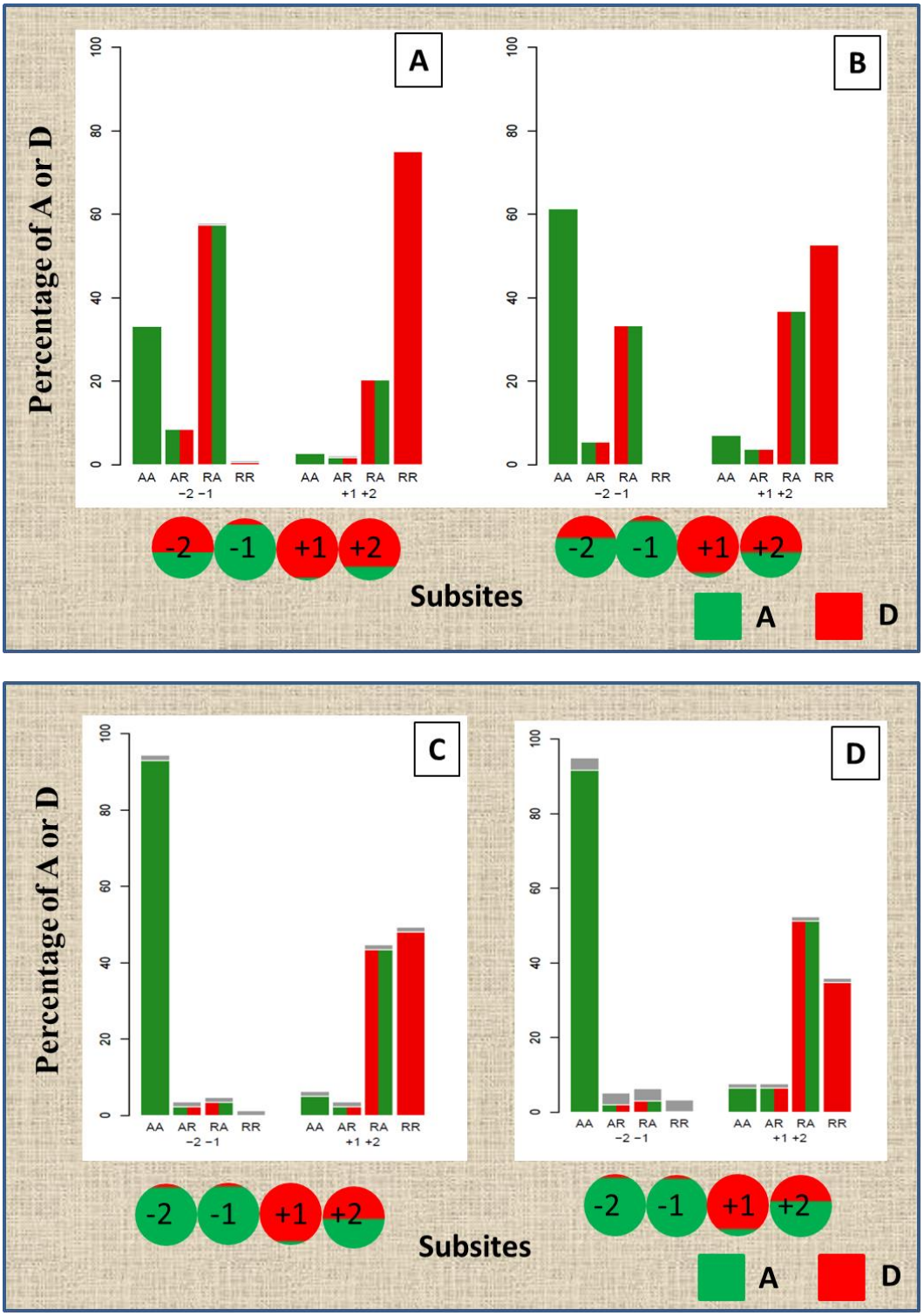


Fig 3. 25: Subsite preference of *Fj* ChiC and G106W mutant. Subsite preference of *Fj* ChiC (A & B) and G106W (C & D) mutant on DA35% (A & C) and DA60% (B & D) chitosan polymers respectively.

3.7 Production of CHOS from different DA chitosans

In search of selecting preferable chitosan substrate for production of longer chain CHOS by *Fj C*hiC and mutant G106W, we tested DA1.6%, DA20%, 35% DA, DA50% and DA60% chitosan polymers (Fig 3.26). The activity of both chitinases increased with increasing DA% of polymer. There was less or no activity on DA1.6% chitosan indicating the necessity of acetyl groups on C2 carbon of chitin polymer for the chitinase. *Fj C*hiC exhibited good activity on DA35%, DA50%, and DA60% chitosan polymers, whereas G106W exhibited good activity on DA50% and DA60% chitosan polymers and moderate activity on DA35% polymer. Therefore, DA35%, DA50%, and DA60% chitosan polymers were selected for further studies.

3.8 High-performance thin layer chromatography (HPTLC)

The *Fj C*hiC and mutant G106W were used to generate bioactive chitosan oligomers to test them as elicitors of plant defense. Chitosan polymers (1 mg/ml) with DA35%, DA50% and DA60% were hydrolyzed with either *Fj C*hiC (10 nM) or G106W (500 nM). Fractions of the reaction mixture were collected at 2 h, 6 h, 12 h, 24 h and 48 h, and analyzed by HPTLC (Fig 3.27). *Fj C*hiC was completely hydrolyzed all the substrates by 48 h except DA35% polymer where we observed a small amount of polymer was left. *Fj C*hiC generated a good number of CHOS by 48 h from all polymers by 48 h (Fig 3.27A). Whereas, G106W exhibited partial activity on all the tested polymers but generated longer chain polymers (Fig 3.27B). Both the enzymes showed increased activity with increasing DA%.

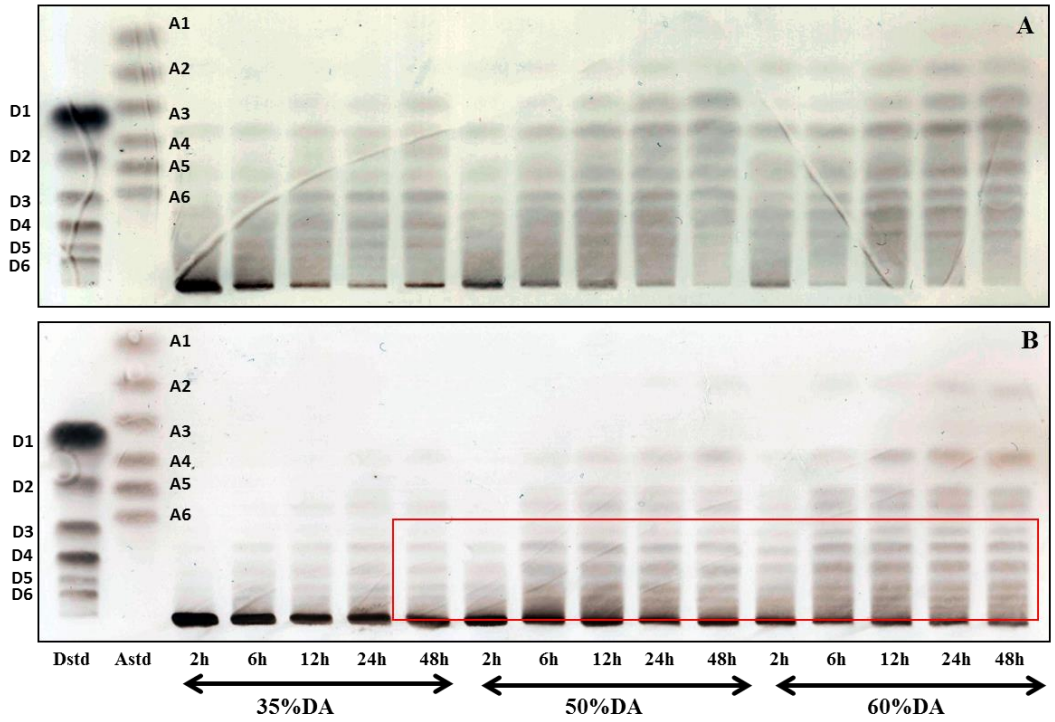


Fig 3.27: HPTLC analysis of crude chitosan hydrolysates obtained using *Fj* ChiC (A) and G106W (B). Fractions collected at different time intervals (arrows mark the selected sampling points) during the hydrolysis of chitosan (DA61% DA50% and DA35%) by *Fj* ChiC and G106W were analyzed by HPTLC. A_{std} was the standard mixture of $(GlcNAc)_1$–$(GlcNAc)_6$ and D_{std} represents the mixture of $(GlcN)_1$–$(GlcN)_6$. Red box showing the generated longer chain CHOS by G106W mutant.

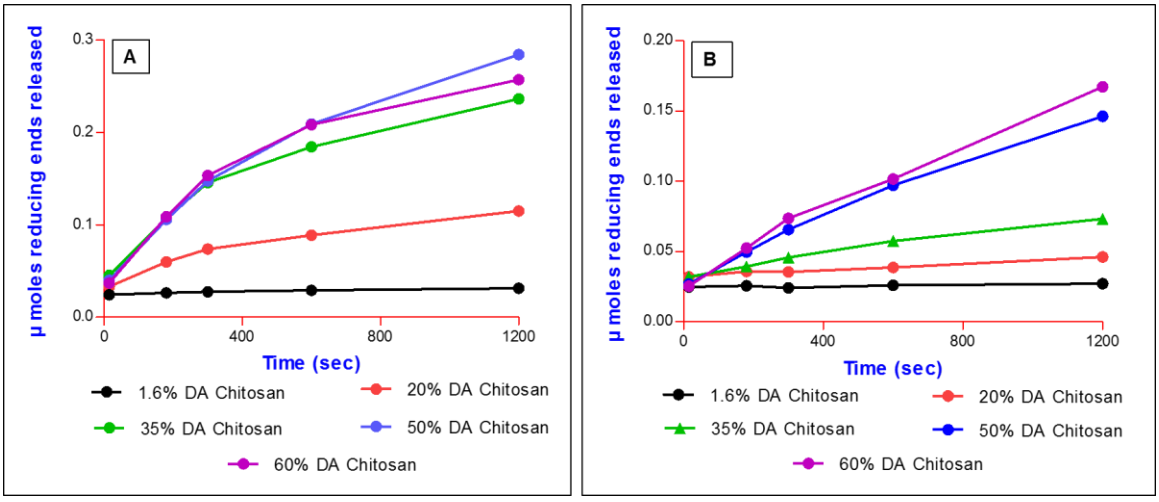


Fig 3.26: Activity of *Fj* ChiC and mutant G106W on various DA% of chitosan polymers. A) 1 mg/ml of polymer was incubated with 10 nM of *Fj* ChiC in 40 mM ammonium acetate buffer pH-5.5. MBTH assay was performed to estimate the reducing ends. B) 1 mg/ml of the polymer was incubated with 500 nM of G106W in 40 mM ammonium acetate buffer pH-5.5. MBTH assay was performed to estimate the reducing ends.

3.9 UHPLC-ELSD-ESI-MS analysis of purified COS

CHOS generated by the action of both the wild-type and mutant enzymes were analyzed by UHPLC-ELSD-ESI-MS. DA35% and DA60% chitosan polymers (1 mg/ml) were incubated with *Fj* ChiC and G106W mutant in 40 mM ammonium acetate buffer pH-5.5 at 40^0C. The reaction was carried out till 48 h. Samples were collected and stopped the reaction by adding an equal amount of 70% acetonitrile (ACN). The complete reaction mixture was lyophilized and resuspended in ultrapure MQ water to make 1 mg/ml concentration. Four microliters (4 µl) of the sample was analyzed for CHOS by UHPLC-ELSD-ESI-MS. Generated oligomers were quantified by using internal standards. No DP1 formation was observed in mutant (G106W) treated polymers. The quantity of longer chain CHOS are higher in mutant treated polymers (Fig 3.28). Qualitatively there was a considerable change in CHOS produced by *Fj* ChiC and G106W mutants respectively (Table 3.2).

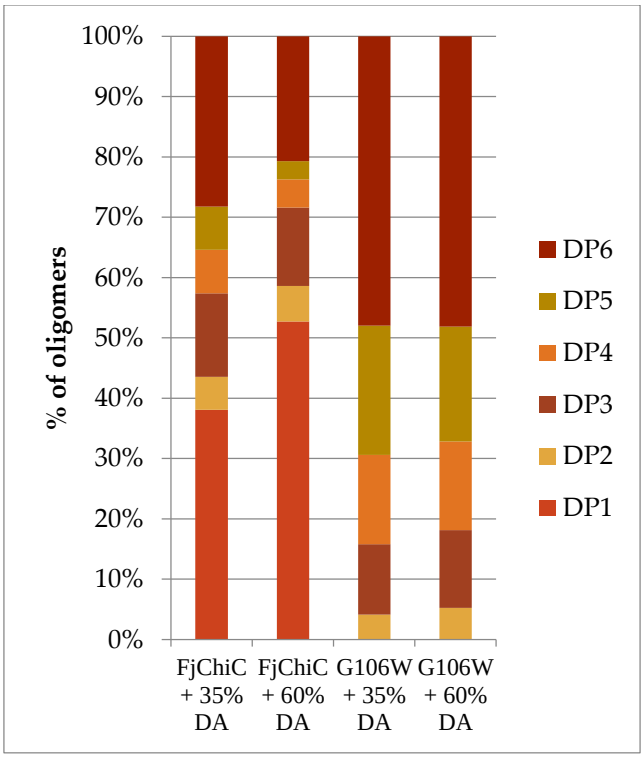


Fig 3.28: Quantification of generated oligomers from DA35% and DA60% chitosan polymers with *Fj* ChiC and G106W mutant.

Chitosan polymer	Degree of polymerization	*Fj* ChiC	G106W
35% DA	DP1	**A1**	
	DP2	A2, **A1D1**	A2
	DP3	A2D1, **A1D2**	**A3**, A2D1,
	DP4	A2D2, **A1D3**	**A3D1**, A2D2
	DP5	A2D3, **A1D4**	**A3D2**, A2D3
	DP6	**A2D4, A1D5**	**A3D3**
60% DA	DP1	**A1**	
	DP2	A2, **A1D1**	A2
	DP3	A2D1, **A1D2**	**A3**, A2D1
	DP4	A2D2, A1D3	**A3D1**, A2D2, A1D3
	DP5	A3D2, A2D3, **A1D4**	**A4D1**, A3D2, A2D3
	DP6	**A2D4, A1D5**	**A4D2, A3D3, A2D4**

Table 3.2: UHPLC-ELSD-ESI-MS analysis of purified CHOS showing different DA and DP oligomers. Unique products of each enzyme are shown in bold red.

3.10 Elicitor activity of purified CHOS in *Medicago truncatula* and *Nicotiana tabacum*

Elicitor activity was measured through cellular oxidative burst to crude CHOS mixture by measuring the release of H_2O_2. For elicitation, suspension-cultured cells of *Medicago* and *Nicotiana* were elicited with 20 $\mu g.ml^{-1}$ crude CHOS mixture right before measuring the production of H_2O_2 (Fig 2.1). The crude hydrolysates elicited a fast and sharp oxidative burst similar to HF chitosan (positive control). Maximum response was observed at 3 min after treatment with the hydrolysates generated by *Fj* ChiC and G106W than individual polymers in *Medicago*. Hydrolysates generated by G106W mutant treatment exhibited good elicitor activity than the *Fj* ChiC generated polymers. Hydrolysate from DA60% chitosan was superior in elicitor activity followed by DA50% and DA35% (Fig 3.29).

Polymers exhibited higher oxidative burst than enzyme treated samples in *Nicotiana*. *Fj* ChiC treated hydrolysates exhibited marginal elicitor activity than G106W treated hydrolysates. Hydrolysate of DA35% chitosan was superior elicitors followed by hydrolysates DA50% and DA60% chitosan. But the elicitation duration was longer (Fig 3.30).

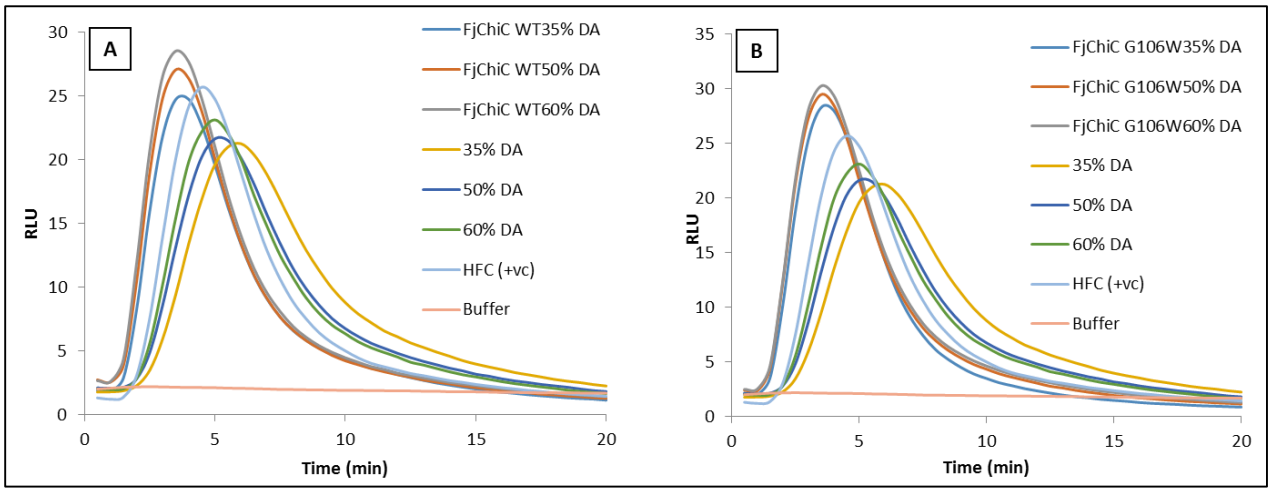


Fig 3.29: Elicitor response of the crude chitosan hydrolysate in *Medicago* cell suspensions. Crude mixture of *Fj* ChiC-generated (A) and mutant G106W generated (B) CHOS was used for elicitation in suspension cultured medicago cells. Released H_2O_2 was detected as relative light unit (RLU) by luminol-dependent chemiluminescence method. For elicitation, 20 µg.ml^{-1} of enzyme generated COS mixture was used. HF chitosan and sterilized buffer (40 mM ammonium acetate buffer) were used as a positive and negative control, respectively.

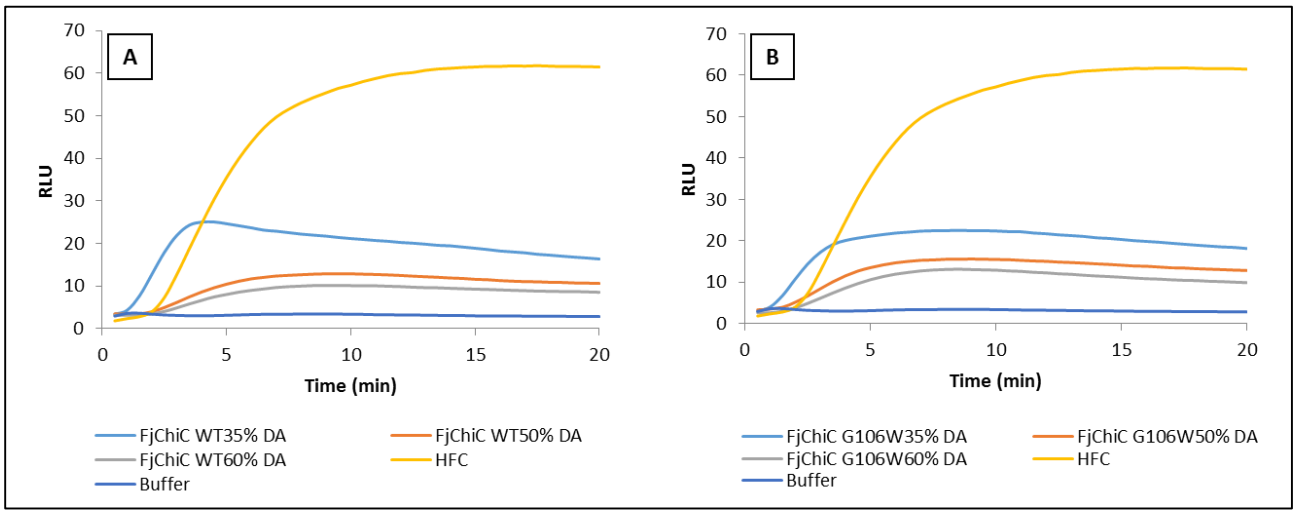


Fig 3.30: Elicitor response of the crude chitosan hydrolysate in *Nicotiana* cell suspensions. Crude mixture of *Fj* ChiC-generated (A) and mutant G106W generated (B) CHOS was used for elicitation in suspension cultured medicago cells. Released H_2O_2 was detected as relative light unit (RLU) by luminol-dependent chemiluminescence method. For elicitation, 20 µg.ml^{-1} of enzyme generated COS mixture was used. HF chitosan and sterilized buffer (40 mM ammonium acetate buffer) were used as a positive and negative control, respectively.

3.11: *Flavobacterium johnsoniae* has diverse chitinases

Chitinases are vital for the depolymerization of chitin-containing waste products from both aquatic and terrestrial ecosystems. In terms of biotechnological applications, chitinases are often used for the preparation of protoplasts from fungal cells, as a protective agent against phytopathogenic fungus and in the generation of biologically active compounds. Most chitinase producers taxonomically belong to a select population of *Bacillus* spp., *Aeromonas* spp., S*erratia* spp., *Pseudomonas* spp., *Vibrio* spp., as well as *Streptomyces* sp. Some of the chitinolytic species reported to date are listed in Table 3.3. *Flavobacterium johnsoniae* is the only species reported to be chitinolytic from genus *Flavobacterium*.

F. johnsoniae utilizes a variety of polysaccharides as nutrients. Genome analysis identified 138 predicted glycoside hydrolases and nine predicted polysaccharide lyases. *F. johnsoniae* was originally identified as a chitinolytic bacterium. Analysis of genome sequence of *F. johnsoniae* resulted in the identification of ten possible chitinolytic enzymes which could contribute to the chitinolytic activity. These enzymes include five candidate chitinases that cut the long chitin polymers and five β-*N*-acetylglucosaminidases that produce *N*-acetylglucosamine and/or chitobiose from the chitin oligomers (Fig 3.31). The predicted chitinases are diverse and include enzymes that are related to chitinases of bacteria (*Fj* ChiA, Fjoh_4555; *Fj* ChiB, Fjoh_4175; *Fj* ChiD, Fjoh_4757), animals (*Fj* ChiC, Fjoh_4560), and plants (*Fj* GH19, Fjoh_2608) (Kharade and McBride, 2014; McBride et al., 2009) (Table 3.4).

We confirmed the molecular identification of *F. johnsoniae* by 16S rDNA analysis. The BLAST search of the 16S rDNA sequence showed 99% homology with commercially procured strain *F. johnsoniae* UW101 (Fig 3.1). We confirmed the chitinase activity of *F. johnsoniae* on the basis of colloidal chitin degradation and zone of clearance on chitin agar plate. *F. johnsoniae* secretes chitinases extracellularly during growth in the presence of colloidal chitin. The diameter of the zone of clearance significantly increased with time indicative of the robust chitinolytic ability of *F. johnsoniae* (Fig 3.2).

Serratia marcescens was extensively studied for enzymatic hydrolysis of chitin. Chitin degradation by *S. marcescens* is a long-familiar enzyme system for the bioconversion of insoluble chitin polysaccharides (Suzuki *et al.*, 2002; Vaaje-Kolstad *et al.*, 2005, 2010; Gutierrez-Roman *et al.*, 2014), involving five chitin-degrading enzymes including ChiA, ChiB (processive chitinases),

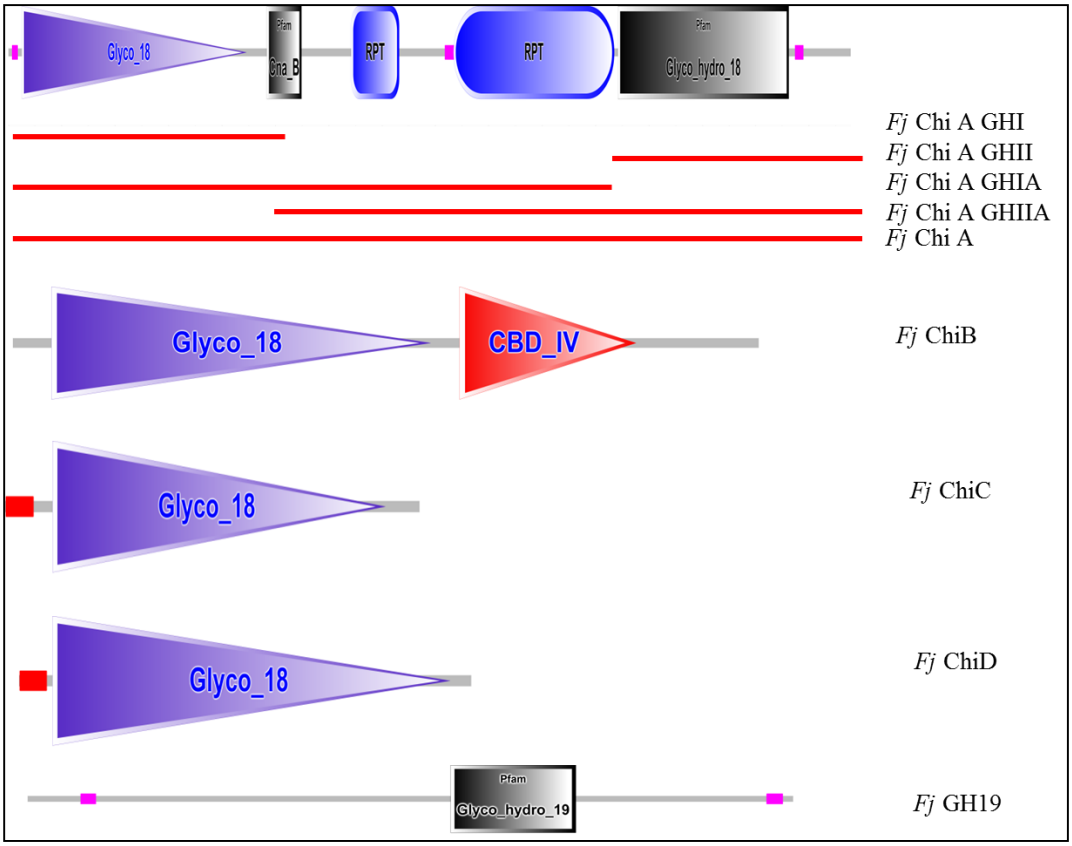


Fig 3.31: The chitinolytic machinery of *F. johnsoniae*. Domain architecture of chitinases present in *F. johnsoniae*. The protein sequence of enzymes was downloaded from NCBI and tested for its architecture in SMART database (Letunic et al., 2015). *Fj* ChiA is a dual GH18 catalytic domain chitinase, *Fj* ChiB is single GH18 catalytic domain chitinase associated with accessory domain CBD_IV (Cellulose binding domain_IV). *Fj* ChiC and *Fj* ChiD are single catalytic domain GH18 chitinase. Among all *Fj* GH19 is the only chitinase with the GH19 catalytic domain.

Bacterial source	Reference
Alteromonas sp. strain O-7.	(Tsujibo et al., 1993)
Pseudomonas aeruginosa K-187	(Wang et al., 1995)
Bacillus sp. WY22	(Woo et al., 1996)
Vibrio harveyi	(Svitil and Kirchman, 1998)
Bacillus sp.BG-11	(Bhushan, 2000)
Bacillus cereus CH	(Mabuchi et al., 2000)
Bacillus thuringiensis subsp. pakistani	(Thamthiankul et al., 2001)
Bacillus sp. NCTU2	(Wen et al., 2002)
Chitinimonas taiwanensis gen. nov. sp. nov	(Chang et al., 2004)
Clostridium sp. E-16,	(Konagaya et al., 2006)
Serratia sp. KCK	(Kim et al., 2007)
B. laterosporous. MML2270	(Shanmugaiah et al., 2008)
Bacillus subtilis NPU 001	(Chang et al., 2010)
Paenibacillus elgii B69	(Ding et al., 2011)
Serratia proteamaculans 568	(Purushotham et al., 2012)
Stenotrophomonas maltophilia	(Suma and Podile, 2013)
Serratia marcescens	(Vaaje-Kolstad et al., 2013)
Flavobacterium johnsoniae	(Chen et al., 2013)
Bacillus subtilis ATCC 11774	(Saber et al., 2015)
Paenicibacillus barengoltzii	(Fu et al., 2016)
Chitinolyticbacter meiyuanensis SYBC-H1	(Hao et al., 2016)
Streptomyces anulatus	(Mander et al., 2016)

Table 3.3: List of some reported chitinolytic bacteria.

Gene name	Gene length (bp)	Molecular weight (kDa)	Domains organization	Similarity
Fj ChiA	4737	168.9	GH18-GH18	Dual catalytic domain
Fj ChiAGHI	1281	46.9	GHI	*B. circulans* ChiA1 (33%)
Fj ChiAGHII	1232	35.4	GHII	*B. circulans* ChiD (37%)
Fj ChiA GHIA	3421	136.4	GHI +accessory	
Fj ChiA GHIIA	3456	126.7	GHII+ accessory	
Fj ChiB	1545	57.8	GH18-CBM6-D5	*B.circulans* ChiA1 (26%)
Fj ChiC	954	38.1	GH18	Bovine ChiA (25%)
Fj ChiD	1029	41.2	GH18	ChiA1 *B. circulans* (37%)
Fj GH19	2367	90.8	GH19	Potato CHTB2 endochitinase 2 (24%)

Table 3.4: Brief details of *F. johnsoniae* chitinases we cloned and their domain architectures.

a non-processive chitinase, ChiC, CBP21, a CBM33-type lytic polysaccharide monooxygenase which causes chain breaks by oxidative cleavage and a chitobiase which cleaves chitin oligomers to monomers (Vaaje-Kolstad et al., 2013).

3.12 Cloning and characterization of chitinases from *F. johnsoniae* and *Sm* ChiD

3.12.1 Cloning of chitinases

All the five chitinases and truncated sequences (*Fj* ChiA, *Fj* ChiA GHI, *Fj* ChiA GHII, *Fj* ChiA GH1A, *Fj C*hiA GHIIA, *Fj* ChiB, *Fj* ChiC, *Fj* ChiD and *Fj* GH19*)* were cloned in expression vector pET- 28a (+). All cloned chitinases were confirmed for proper insertion, transformed to protein expression host *Escherichia coli* strain BL21-pG-KJE8 (Takara, Japan) and *Escherichia coli* Rosetta-gami 2 (DE3) (Novagen, USA). Proteins were overexpressed in *E. coli*, and purified by Ni-NTA agarose column chromatography (Fig 3.3; 3.4 and 3.5).

Among all the chitinases we expressed, only *Fj* ChiB, *Fj* ChiC chitinases could be purified through soluble fractions. *Fj chiA GHI* was successfully expressed and failed to get into soluble form because of expression in inclusion bodies (IB) (Carrió et al., 2000). We could not express *Fj* ChiA, *Fj* ChiA GHII, *Fj* ChiA GH1A, *Fj* GH19 and *Fj C*hiA GHIIA. We could not detect the expression of these chitinases under different conditions. We tried by changing the expression vector (pET-22b (+), pETM-30, pETM-40; EMBL and pGEX-4T-1) by changing the expression host (*Escherichia coli* strain Lemo 21, ArcticExpress (DE3) and BL21 (DE3) pLysS E), and also by changing the temperature of the induction culture (14^0C, 24^0C, and 30^0C). We could observe the expression of *Fj* ChiA GHII in pETM-30, pETM-40 both the vectors, but resulted in inclusion bodies (IB).

3.12.1.1 *Fj* ChiB

Fj ChiB, Fjoh_4175 is single GH18 catalytic domain chitinase with a cellulose binding domain_IV (CBD_IV) (Fig 3.31). It has a calculated molecular weight of 57.8 kDa and pI/Mw as 6.23. *Fj* ChiB contains an N-terminal family 18 catalytic domain (Lys-28 through Asp-303) and C-terminal cellulose binding domain (CBD_IV) (Pro-324 through Lys-452), which belongs to carbohydrate binding module family 6 (CBM6). The CBDs were reported to enhance the cellulase activity by concentrating the cellulases on a cellulose polymeric surface (Gilkes et al., 1988). CBDs may

disrupt the noncovalent interactions between adjacent glucose units (Din et al., 1991). But the apparent role of these CBD_IV in chitinases was not reported yet. *Fj* ChiB exhibited 18.93%, 31.07 and 17.7% similarity with reported TG chitinase human chitotriosidase (HCHT) (Boot et al., 1998), ChiA1 of *Bacillus circulans* WL-12 (Chen et al., 2004) and ChiD of *Serratia proteamaculans* (Purushotham and Podile, 2012), respectively.

3.12.1.2 *Fj* ChiC

Fj ChiC (Fjoh_4560) is single catalytic domain chitinase (Fig 3.31). Analysis by LipoP 1.0 Server further suggested that the secreted protein may be a lipoprotein (Rahman et al., 2008). The closest homolog is ChiA from *Bos taurus* (bovine) with 25% identity over 244 amino acids. Hence, it was as animal type bacterial chitinase. It encodes for a signal peptide which spanned between 1-22 amino acids. *Fj* ChiC exhibited 18.28, 26.16 and 17.3% similarity with reported TG chitinases like human chitotriosidase (HCHT) (Boot et al., 1998), ChiA1 of *Bacillus circulans* WL-12 (Chen et al., 2004) and ChiD of *S. proteamaculans* (Purushotham and Podile, 2012), respectively.

3.12.1.3 *Sm* ChiD

Sm ChiD is a single GH18 catalytic domain chitinase-like *Fj* ChiC. *Sm* ChiD had 79% homology with chitinase II from *Klebsiella pneumoniae* (ACZ01996), and 43% to chitinase from *B. subtilis* (ACU33923). *Sm*ChiD that showed 85% identity with the crystal structure of chitinase-D (PDB ID: 4NZC) from *S. proteamaculans* (*Sp*ChiD) (Purushotham and Podile, 2012) 35% to ChiA from *S. maltophilia* (EEK46733) (Suma and Podile, 2013), and 32% to ChiB from *S. marcescens* (ACX42072).

3.12.2 Effect of pH and temperature on the activity

Among the two *Fj* chitinases, *Fj* ChiB had a broad range optimal activity over pH 4-10, similar to *S. proteamaculans* chitinases (Purushotham et al. 2012), ChiA from *S. marcescens* 2170 (Suzuki et al., 2002), *Serratia* sp.KCK (Kim et al., 2007), and *S. proteamaculans* 18A1 (Mehmood et al., 2009). *Fj* ChiC was optimally active in 50 mM citrate buffer pH 6.0 on colloidal chitin substrate similar to *Sp* ChiD (Purushotham and Podile, 2012; Purushotham et al., 2012). *Fj* ChiB exhibited optimum activity at 30^0C. All the four *Sp* chitinases displayed optimum activity at 40^0C, Similar

to *Serratia sp*.KCK (Kim et al., 2007). *Fj* ChiC exhibited activity over 20^0C- 50^0C and optimum at 40^0C similar to ChiA, ChiB and ChiC1 from *S. marcescens* (Suzuki et al., 2002). *Fj* ChiB showed 50% decrease in activity at 50^0C. Even though it exhibits broad optimal for *Fj* ChiC, 80% loss of activity was recorded at 60^0C.

Chitinase assay using 3% colloidal chitin as a substrate in different pH range buffers and different temperatures revealed that *Sm* ChiD was optimally active at pH-5.0 and 40^0C, respectively (Fig 3.8 & 3.9). Whereas, ChiA, ChiB and ChiC1 from *S. marcescens* showed a nearly similar optimum pH, but, very high optimum temperatures $\geq 60^0$C (Suzuki et al., 2002) compared to *Sm* ChiD. The optimal reaction conditions of *Sm* ChiD are similar to *Sp* ChiD (pH-6.0 and 40^0C) (Purushotham and Podile, 2012).

3.12.3 Catalytic activity

We observed a significant difference in overall catalytic efficiency between the two selected chitinases. *Fj* ChiB exhibited more catalytic efficiency k_{cat}/Km at 132.04×10^4 (mg^{-1} ml sec^{-1}), whereas for *Fj* ChiC had at 1.40×10^3(mg^{-1} ml sec^{-1}). The difference may be due to the presence of extra accessory domain CBM6 for *Fj C*hiB. Similar single catalytic domain chitinase *Sp* ChiD exhibited significantly lower catalytic efficiency (4.7 h $mg^{-1}ml^{-1}$) and 2- fold higher Km (83 mg ml^{-1}) than *Fj* ChiC towards colloidal chitin substrate (Purushotham and Podile 2012). *Sp* ChiA and *Sp* ChiB from *S. proteamaculans* exhibited similar catalytic efficacy towards colloidal chitin (Purushotham et al. 2012) like *Fj* ChiB might be because of the presence of accessory domains (CBM's, FN3 and PKD) in both the chitinases.

The kinetic parameters obtained with increasing concentrations of colloidal chitin revealed that *Sm* ChiD has higher *K*m and *V*max values (47.92 mg/ml and 2.02 nkat/mg of protein) (Fig 3.7) compared to *Sp* ChiD (35.12 mg/ml and 0.89 nkat/mg of protein) under similar assay conditions. *Sm* ChiD also showed a 2.5-fold higher overall catalytic efficiency (74.8 s^{-1} mg^{-1} ml) than *Sp* ChiD (29.3 s^{-1} mg^{-1} ml) (Madhuprakash et al., 2015b). Inspite of the high percentage identity between *Sm* ChiD and *Sp* ChiD, the differences in the kinetic parameters of these two enzymes gives a clue on the evolutionary divergence of the chitinolytic machinery of *S. marcescens* and *S. proteamaculans*.

3.13 Activity on polymeric carbohydrate substrates

Both the *Fj* chitinases exhibited good activity towards 75% DA of chitosan polymer (Sigma, CNo: 419419). The order of preference for *Fj* ChiB was Chitosan=Colloidal chitin> β-Chitin, whereas it was Chitosan > Colloidal chitin > β-Chitin for *Fj* ChiC. Similar kind of substrate specificity was observed for *Sp* ChiD from *S. proteamaculans* (Purushotham and Podile, 2012). The activity of both the enzymes was not detectable on non-chitinous substrates like avicel and CM-cellulose indicating the high substrate specificity of *Fj* ChiB and *Fj* ChiC (Fig 3.10).

Sm ChiD showed highest specific activity on colloidal chitin followed by chitosan, β–chitin and α –chitin. There was no detectable activity on non-chitinous substrates like Avicel and CM-cellulose (Fig 3.10). The hydrolyzing activity of *Sm* ChiD on different chitinous substrates indicated that higher the degree of crystallinity of substrate lower was the activity. This could be due to the lack of accessory binding domains for *Sm* ChiD. For efficient degradation, in addition to the catalytic domains, catalytic enzymes often evolved to have one or more carbohydrate binding modules (CBMs). It was shown that the fusion of accessory domains to *Sp* ChiD improved the catalytic efficiency on insoluble chitinous substrates (Madhuprakash et al., 2015b). Another key feature of the enzymes acting on crystalline substrates is the presence of a path of solvent exposed aromatic residues leading from a CBM to the substrate binding cleft (Hamre et al., 2015; Horn et al., 2006; Katouno et al., 2004; Uchiyama et al., 2001; Watanabe et al., 2003).

3.14 Substrate binding properties of *Sm* ChiD

Native PAGE in presence or absence of different soluble polymeric substrates showed that the movement of *Sm* ChiD was retarded in the gel incorporated with glycol chitin (Fig 3.11A). There was no difference in the mobility of *Sm* ChiD in the gels impregnated with laminarin, CM-cellulose or with no substrate (Fig 3.11A). The results indicated preferential binding of *Sm* ChiD to soluble chitinous substrates over non-chitinous substrates. This property of *Sm* ChiD was in line with *Sp* ChiD, which had the similar preference for glycol chitin (Purushotham and Podile, 2012). In contrast to *Sp* ChiD, *Sm* ChiD bound to both α- or β-chitin with nearly equal preference followed by colloidal chitin (Fig 3.11B). *Sm* ChiD exhibited moderate affinity towards all insoluble β(1→4)polysaccharides examined in this experiment, and thus suggesting the affinity was not

limited to chitin, even though the binding activity to colloidal chitin was the highest among the assayed insoluble polysaccharides. Hence, the moderate binding activity of the intact *Sm* ChiD towards Avicel may be due to the affinity of catalytic domain for this polysaccharide. Similarly, the binding affinity of Chitinase A1 from *Bacillus circulans* WL-12 was not restricted to chitinous substrates (Hashimoto et al., 2000).

3.15 Activities on colloidal chitin

Hydrolytic profile of *Fj* ChiB and *Fj* ChiC was tested on polymeric substrates like colloidal chitin and different DP of CHOS i.e DP2-DP6 by HPLC. DP3 was the major end product with colloidal chitin for *Fj* ChiB whereas DP2 was the major hydrolytic product with *Fj* ChiC (Fig 3.12).

The purified *Sm* ChiD exhibited more activity on colloidal chitin followed by chitosan. We further analyzed the activity by performing time-course degradation of colloidal chitin. The enzyme activity gradually increased till 9 h, where it reached to saturation. We could not see a change in enzyme activity from 9 - 11 h. It may be due to the saturation of reacted CHOS with Schales reagent. Simultaneous, TLC (thin layer chromatography) analysis of the fractions collected at different time intervals revealed that DP2 was the major hydrolytic product till 3 h. A prolonged incubation resulted in the accumulation of more of DP1 over DP2. The latter observation hints the possibility of chitobiase activity of *Sm* ChiD and the chances of synergistic effect of *Sm* ChiD with the other three chitinases. The time-course degradation of colloidal chitin indicated that *Sm* ChiD could be an endo-acting enzyme.

3.16 Activities on chitin oligomeric substrates & Transglycosylation (TG)

3.16.1: *Fj* ChiB and *Fj* ChiC

Both the chitinases were not active on DP2 and DP3 oligomeric substrates indicating the endo-active nature of both the chitinases (Fig 3.13 and 3.14). Product profile with DP4 substrate for both the enzymes indicated the formation of DP2 (chitobiose) suggesting the minimum substrate length for both the enzymes was DP4. DP3 and DP2 were the major end products, respectively for *Fj* ChiB and *Fj* ChiC with DP5 and DP6 oligomeric substrates (Fig 3.15 and 3.16).

We also observed a low level of TG activity for *Fj* ChiC on DP5 and DP6 substrates. DP6, DP7, and DP8 formed at 15 min. Secondary hydrolysis of TG products was observed at 30 min. DP7, DP8, and DP9 formed with DP6 substrate because of TG activity of *Fj* ChiC. TG products were completely degraded by the same wild-type enzyme within 30 min. Indeed, bioengineering of chitinases has been successfully applied to enhance the TG activity of GH18 chitinases. SDM was performed to significantly enhance the TG/hydrolysis (T/H) activity ratio of several chitinases (Madhuprakash et al., 2012; Zakariassen et al., 2011). Even though the mechanism behind this effect is not fully understood, conserved amino acid residues were targeted to produce efficient TG chitinases. Here, we generated three mutants G106W, D148N and W211G spanning substrate exit site, catalytic center, and catalytic groove, respectively (Fig 3.20). Mutant G106W had decreased hydrolytic activity and TG products were observed late at 720 min (Fig 3.21). The mutation D148N made the enzyme to retain TG products from 30 min to 720 min (Fig 3.21). Secondary hydrolysis decreased due to D148N conversion similar to *Sm*ChiA-D313N (Zakariassen et al., 2011). W211G mutant produced DP1 at 720 min, which was not observed in wild-type *Fj* ChiC. The TG activity of W211G mutant was completely lost. Interestingly, chitobiase activity was gained because of this mutation (Fig 3.21).

The amino acid motif, 'DXDXE' was the most conserved among all GH18 chitinase. Aspartic acid situated in the middle of the motif plays a crucial role in catalysis. Initially, it acts a general acid and later same amino acid acts as a general base to complete the catalysis. Mutating this amino acid residue to asparagine (D→N) increased the TG activity in *S. marcescens* (Zakariassen et al., 2011). D148 is the corresponding amino acid in *Fj* ChiC. We mutated this particular amino acid to asparagine (D→N). Due to this particular mutation, secondary hydrolysis of TG products decreased significantly. TG products remained for long, till 720 min.

Another highly conserved motif in GH18 chitinases, was SXGG motif. Amino acid residue present immediate to this motif plays a crucial role in processivity (Payne et al., 2012). Along with processivity, the same amino acid is crucial for TG, revealed by mutational studies on *Sp* ChiD and *Af* ChiB1. Mutations at corresponding residues in *Sp* ChiD and *Af* ChiB1 (W120A, W137E, respectively) resulted in the loss of complete TG activity (Lu *et al.*, 2009; Madhuprakash *et al.*, 2012). SXGG motif was extended with tryptophan in other well-known TG GH18 chitinases, viz Human chitotriosidase (Aguilera et al., 2003), *Bacillus circulans* ChiA (Watanabe et al., 2003),

Coccidioides immitis chitinase 1(Hollis et al., 2000), ChiA and ChiB from *S. marcescens* (Horn et al., 2006). In contrast to above-mentioned chitinases, G106 was present at positions in *Fj* ChiC. But, in *Fj* ChiC, the mutation at G106W greatly decreased the enzyme activity, with no improvement in TG.

3.16.2: *Sm* ChiD

The activity of *Sm* ChiD on different CHOS (DP2-6) was monitored by HPLC. Reaction with DP2 substrate revealed that the enzyme *Sm* ChiD hydrolyzed DP2 to monomeric GlcNAc. After 60 min, most of the DP2 remained unaltered (94.2%) and decreased to 84.5% by 180 min (Fig 3.17A). The reaction time-course was followed until 720 min, where the proportion of DP2 and DP1 was 61.3% and 38.7%, respectively (Fig 3.17A). The present data of time course degradation of DP2, clearly indicated that the reaction progressed at a very low rate but, gave a clue on the enigmatic behavior of *Sm* ChiD compared to other chitinases of *S. marcescens*, which generated DP2 as the major end product of chitin hydrolysis (Suzuki et al., 2002).

The reaction of *Sm* ChiD with DP3 substrate generated DP1-6 products, wherein, DP1-2 were the hydrolytic products, while DP4-6 formed due to the TG activity. Of the three TG products, only DP4 and DP5 were detected at the early time of reaction *i.e*. at 5 min (Fig 3.17B). DP6 formation was noticed from 30 min. The proportion of quantifiable TG products (DP4-6) was only 2.6% compared to the proportion of hydrolytic products (52.6%). Reaction mixture collected at the end of 90 min showed a further decrease in the proportion 0.86% of TG products. A complete loss of TG was noticed at 180 min (Fig 3.17B), wherein, only DP2 and DP1 were observed as the major products with 68% and 32%, respectively. This relative proportion of hydrolytic products was reversed at the end of 720 min with more of DP1 (65.2%) than DP2 (34.8%) (Fig 3.17B). These data indicated that *Sm* ChiD exhibited chitobiase activity after prolonged incubation.

Sm ChiD produced DP1-6 products from DP4 substrate, among which DP1-3 are hydrolytic products and DP5-6 formed due to the TG activity. Both the hydrolytic and TG products were observed from the 0 min (Fig 3.17C). Though the TG products were detectable from 0 min, a considerable quantity of TG products was only observed from 5 min, with DP5 and DP6 at 1.6% and 2.75%, respectively. Among the quantifiable TG products formed with DP4 as a substrate,

DP6 (4.1%) was more than DP5 (2.8%) product until 60 min. This pattern was overturned from 180 min, where a clear decrease in the quantity of DP6 (3.7%) with a concomitant increase in the quantity of DP5 (5.2%) was observed that remained until the end of 360 min, with DP5 and DP6 at 5.4% and 3.4%, respectively. The reaction with DP4 was followed until 720 min, where a clear decrease in the quantity of TG products, DP5 (2.4%) and DP6 (1.2%), was noticed. A substantial increase in the hydrolytic products was observed at 720 min, wherein, the relative proportion of DP1-3 was 35.5%, 25.8% and 26.6%, respectively (Fig 3.17C).

CHOS with a range of DP1-6 were produced by *Sm* ChiD with DP5 as a substrate, among which DP1-4 were produced as a result of hydrolytic activity and DP6 was the only quantifiable TG product (Fig 3.17D). Formation of DP6 was observed right from the 0 min. The proportion of DP6 increased to a maximum of 3.9% by 30 min that remained until 45 min (3.3%). A clear decrease in the amount of DP6 was noticed from 60 min (2.2%), but, these low levels of DP6 were maintained until the end of 720 min (1.1%). Most of the DP5 substrate was converted to products by 720 min with more of hydrolytic products (DP1-4 = 89.2%) than the only quantifiable TG product DP6 (1.1%) (Fig 3.17D).

Sm ChiD produced both hydrolytic and TG products with DP6 substrate, but, the present study has a limitation that the quantification of CHOS was done up to DP6 only, due to the lack of CHOS standards larger than DP6. However, the presence of long-chain TG products, DP7 and DP8 was also confirmed using MALDI-TOF-MS analysis (Fig 3.18). Nearly 25% of the DP6 substrate was converted to products within 5 min, wherein, the reaction was dominated by hydrolytic activity over TG (Fig 3.17E). By the end of 180 min, 56% of the DP6 substrate was converted to products and the proportion of even chain length hydrolytic products *i.e.* DP4 (18.6%) and DP2 (17.6%) was predominant. A similar pattern was observed even at 720 min with more of DP4 (22.6%) and DP2 (30.7%) than DP5 (14%), DP3 (4.5%) and DP1 (6.8%) (Fig 5E). Activity studies with DP5 and DP6 substrates indicated that *Sm* ChiD required a reaction time beyond 720 min to produce DP1 as the major end product, suggesting a preference for CHOS with DP > 2 substrates, in spite of its chitobiase activity. Indeed, it should be noted that there was no temporal difference between the hydrolytic and TG activities of *Sm* ChiD similar to *Sp* ChiD (Madhuprakash et al., 2012; Purushotham and Podile, 2012). The TG activity of *Sm* ChiD with CHOS can be exploited further

to produce longer chain CHOS in higher amounts, in view of their potential applications especially to elicit immune responses in plants (Das et al., 2015). It is equally important to tailor *Sm* ChiD to show TG activity on fully/partially deacetylated chitosan oligomers for synthesizing oligomers with defined degree or pattern of acetylation, which are known to act as elicitors of plant immune response (Madhuprakash et al., 2015a).

On the other hand, chitobiase activity was also important for the efficient production of monomeric GlcNAc, which has industrial value in the medicinal and biotechnological fields (Aam et al., 2010; Chen et al., 2010). Extreme insolubility of polymeric chitinous substrates is the major limitation for the large scale production of GlcNAc and thus requires harsh chemical hydrolysis methods (Chen et al., 2010). These processes are not eco-friendly and moreover, generate low yields of GlcNAc. Enzymatic processes can be used to supplement the chemical methods of chitin degradation, wherein a concerted action of chitinolytic enzymes is necessary. Suzuki et al. (2002) reported that the chitinases (ChiA, ChiB, and ChiC) of *S. marcescens* act synergistically for efficient hydrolysis of powdered chitin. Later, it was proved that the chitin binding protein 21(Vaaje-Kolstad et al., 2005) and the chitobiase (Gutierrez-Roman *et al.*, 2014) of *S. marcescens* can further potentiate the activities of ChiA, ChiB, and ChiC for improved degradation of polymeric chitinous substrates. Also, by using a combination of chitinase-C and *N*-acetyl hexosaminidase from *Streptomyces coelicolor* A3(2), 90% pure GlcNAc yields were obtained after 8 h incubation from crab shell chitin (Nguyen-Thi and Doucet, 2016). In line with these reports, *Sm*ChiD may also act synergistically with the other three chitinases or chitobiase for efficient production of GlcNAc from polymeric chitin.

3.17 Salt tolerance of *Fj* ChiC

Microorganisms can be found over an array of extreme conditions like salinity, pH, temperature and oxygen. Halophilic enzymes were found to have a high proportion of aspartic and glutamic acids. The percentage of aspartic and glutamic acids was 13.34% in *Fj* ChiC. Activity was tested at different concentrations of NaCl with colloidal chitin. Enzyme activity decreased gradually in the presence of NaCl from 0.5M to 3.5M. But, 60% of activity was retained even at 3.5M of NaCl (Fig 3.19). A salt tolerant chitinase from *Halobacterium salinarum* NRC-1 has been reported to have major activity in the NaCl concentration range 1–5 mol L^{-1} (Hatori et al., 2006) .

Halophilic inhabitants such as *Salinivibrio costicola* 5SM-1 (Aunpad and Panbangred, 2003), *Vibrio harveyi* and *Alteromonas* sp. strain O-7 (Chang et al., 2003), and *Planococcus rifitoensis* (Essghaier et al., 2010) were capable of producing chitinase, but their enzymatic activity decreases with increasing the concentration greater than 15% of NaCl. Salinity tolerant chitinase has great biotechnological potential applications. Understanding more details about *Fj* ChiC would be useful.

3.18 Sequence analysis of *Fj* and *Sm* chitinases

We aligned all the GH18 chitinases present within the genome of *F. johnsoniae* to know the identity of the each chitinase. These chitinases shared less sequence similarity with other chitinases present within this organism. *Fj* ChiAGHII shared 22.73%, 18.22%, 20.53%, 19.69% with *Fj* ChiB, *Fj* ChiC, *Fj* ChiAGHI and *Fj* ChiD respectively. While *Fj* ChiGHI showed 20.53%, 27.14%, 25.09% and 28.53% with *Fj* ChiAGHII, *Fj* ChiB, *Fj* ChiC and *Fj* ChiD, respectively. Among them, *Fj* ChiB shared 39.12% similarity towards *Fj* ChiC. The highly conserved motif "DXXDXDXE" was conserved in all the chitinases. Another conserved motif "SXGG" was conserved in all the chitinases except *Fj* ChiC in which this conserved motif replaced with "SXAG". The amino acid situated next to "SXGG" motif known to play a role in enzyme processivity. The chitinases with bulky aromatic amino acids at that particular position are known to act in a processive manner, while enzymes with small amino acid are known to act in a non-processive manner (Vaaje-Kolstad et al., 2013). Amino acid glycine (Gly) was present for *Fj* ChiB and *Fj* ChiC, so these enzymes might act in a non-processive manner. Tryptophan (Trp) was present for *Fj* ChiAGHI and *Fj* ChiD, so these two chitinases might act in a processive manner. In *Fj* ChiAGHII, amino acid Lysine (K) was present after "SXGG" motif (Fig 3.22).

Sm ChiD exhibited very less similarity with remaining chitinases present in *S. marcescens***.** It exhibited 20.65%, 25% and 28.08% with *Sm* ChiC, *Sm* ChiA, and *Sm* ChiB respectively. Both "DXXDXDXE" and "SXGG" of GH18 chitinases were conserved in *Sm* ChiD. The presence of Tryptophan (Trp) after "SXGG" motif indicated its processive nature (Fig 3.23).

3.19 Determination of subsite specificity of *Fj* ChiC and G106W mutant

The catalytic activity of enzymes greatly affected by substrate-binding sites distant from the cleavage bond actually undergoing hydrolysis. Subsite mapping of glycosyl hydrolases (GHs) has begun in the late 1960's with primitive studies on amylolytic GHs. The -n to +n subsite nomenclature was widely accepted by molecular enzymologists. Subsites were labeled from -n to +n, where +n represents the non-reducing end and -n the reducing end of carbohydrate. Cleavage of sugar bond takes place between the -1 and +1 subsites (Sunna et al., 1997). Finding out subsite preference of chitinase will be useful because there will be a great chance to engineering the enzyme to generate a specific pattern of CHOS. Here first time we performed these experiments by comparing wild-type *Fj* ChiC and G106W mutant. G106W mutant substrate preference was completely changed to acetylated sugar at -2 subsite. Hence, there will be a great chance of generating a different pattern of CHOS by mutating the amino acid residues present at enzyme catalytic groove.

3.20 Production and elicitor activities of bioactive chitosan oligosaccharides generated by *Fj* ChiC and G106W mutant

Unlike animals, plants don't have an adaptive immune system, which could recognize and respond to an immense number of microbial pathogens. So, plants have evolved multiple ways of defense to protect themselves from microbial attacks. Plants possess an amazing immune system that allows guarding themselves versus a wide range of microbes including bacteria, oomycetes and fungi (Boller and Felix, 2009; Zipfel and Felix, 2005). Activation of defense response is the essential step of the microorganism detection by conserved molecular patterns called PAMPs (Pathogen Associated Molecular Patterns) or MAMPs (Microbe-Associated Molecular Patterns) (Nürnberger et al., 2004). Their perception during the infection triggers defense in plants, so they were named as general elicitors i.e., compounds that are able to induce plant defenses (Eder and Cosio, 1994).

In the present study, we investigated the ability of *Fj C*hiC (an endo-acting non-processive enzyme) and G106W mutant to generate different bioactive CHOS from chitosan polymers of different DA and tested their plant strengthening activity using plant cell suspensions.

Chitosan polymers of various DA% were selected to generate diverse CHOS by *Fj C*hiC and G106W mutant. Hydrolytic activity was tested on DA1.6%, DA20%, DA35%, DA50%, and DA60%. As expected both the chitinases exhibited less or no activity on DA1.6% chitosan polymer. The activity increased with the increasing DA% of chitosan polymers. Chitinases cannot act on DA1.6% chitosan polymer because of no acetyl groups present on C2 of sugar residues in the polymer. The subsites present in chitinase play a crucial role in catalysis. Chitinases cleave the glycosidic bond present between sugar units bound to the -1 and +1 subsites. Hydrolysis by the family GH18 chitinases needed the presence of *N*-acetyl group of the sugar located in the -1 subsite (Sikorski et al., 2006). By increasing the DA% of polymer, there are great chances of acetyl groups to be occupied at -1 subsite of chitinase. This is the probable reason to observe the increasing hydrolysis with increasing DA% of polymers.

HPTLC and UHPLC-ELSD-ESI-MS analysis revealed the generation of various DA, DP of CHOS by *Fj* ChiC and G106W mutant. The crude chitosan hydrolysates obtained by *Fj* ChiC and G106W were tested for both elicitor and priming activities in suspension cells of *Medicago* and tobacco. Hydrogen peroxide (H_2O_2), a sensitive indicator of plant defense response, was measured during an oxidative burst of the cell suspensions with selected crude chitosan hydrolysates.

When applied as an elicitor, the crude hydrolysate of the chitosan DA35%, DA50%, and DA 60% triggered a considerable oxidative burst in *Medicago* (Fig 3.29). Hydrolysates produced by G106W mutant exhibited good elicitor activity than *Fj* ChiC generated hydrolysates. Even the polymers alone also exhibited good elicitor activity but low when compared to enzyme hydrolysates. Hydrolysates of DA60% exhibited good elicitor activity followed by DA50% and DA35% indicative of role of CHOS in elicitor activity than polymers (Shibuya and Minami, 2001). Since we observed almost double the longer chain CHOS in G106W generated hydrolysates, the role of longer CHOS in elicitor activity (Wan et al., 2008). For instance, previous reports showed that the functional mechanisms of CHOS primarily depend on DA of given oligomer. Hydrolysates generated from chitosan polymers (CH-63, CH-88) showed the highest antipathogenic activity against *Phytophthora parasitica* (strain 227) than chitosan native polymer (Falcón et al., 2008).

The length of oligosaccharides generally must have a DP>4 for successful induction of biological response in plants (Côté and Hahn, 1994). The HR, which is activated by several other elicitors, is also characterized by the plant cell death and deposition of chemical barriers, production of phytoalexins that prevent pathogen spreading into healthy neighboring tissues. Application of CHOS caused the cell death in a dose-dependent manner in tobacco (Wang et al., 2008). Similarly, studies on *Arabidopsis thaliana* cell suspensions demonstrated the dependency of CHOS elicitor activity on their physicochemical features and concentration (Cabrera et al., 2006).

When to compare with *Medicago*, *Fj* ChiC generated hydrolysates exhibited good elicitor activity than G106W generated hydrolysates in *Nicotiana*. Even the native polymers showed good elicitor activity than hydrolysates. Hydrolysates of DA35% are better elicitors in *Nicotiana* followed by DA50% and DA60% (Fig 3.30). These results on *Nicotiana* were different from *Medicago.* Both the cell suspensions responded differently to native chitosan polymers and hydrolysates. It might be possible that CHOS with different DA or different DP can also show different activities on a different plant. However, it still remains unclear how this activity varies from plant to plant. The hydrolysates generated by *Fj* ChiC and G106W mutant did not show priming activity on both the tested plant cell suspensions (*Medicago* and *Nicotiana*).

Chapter-4

Summary and conclusions

Chitin occupied the second place after cellulose in biological turnover of the ecosystem and it is a structural component of many organisms from different taxonomic groups. In soil, chitin is mainly found in fungal cell walls and exoskeleton of arthropods (e.g. crustaceans such as crabs and shrimps). The main degradation pathway of chitin depolymerization in nature is by chitinases (EC 3.2.1.14) resulting in the hydrolysis of the 1, 4-β- glycosidic bonds present in between the *N*-acetyl- D-glucosamine units (GlcNAc). The main objective of this present study is to get a better understanding of bacterial chitinases and, in particular, for the synthesis of bioactive chitooligosaccharides (CHOS) from polymer chitosan substrates. It was assumed that the diverse chitinase produced by fresh water habitat *Flavobacterium johnsoniae* UW101 and one new chitinase from *Serratia marcescens* GPS5 could be biochemically different to those reported so far.

F. johnsoniae was originally identified as a fresh water habitat chitinolytic bacterium. Genome sequence analysis of *F. johnsoniae* resulted in the identification of ten diverse chitinolytic enzymes which could responsible to chitinolytic activity. Among these, five are candidate chitinases and five are β-*N*-acetylglucosaminidases. Those five chitinases are diverse in their sequence and include enzymes that are similar to chitinases of bacteria (*Fj* ChiA, Fjoh_4555; *Fj* ChiB, Fjoh_4175; *Fj* ChiD, Fjoh_4757), animals (bovine) (*Fj* ChiC, Fjoh_4560), and plants (tobacco) (*Fj* GH19, Fjoh_2608). *S. marcescens* is well-studied bacterium for enzymatic bioconversion of insoluble chitin polysaccharides. Chitin degradation by *S. marcescens* involving five chitinases, *Sm* ChiA, *Sm* ChiB, *Sm* ChiC (C1 & C2), CBP21, a CBM33-type lytic polysaccharide monooxygenase and a chitobiase.

4.1 Biochemical characterization of *Fj* ChiB, *Fj* ChiC, and *Sm* ChiD

Among the two *Fj* chitinases, *Fj* ChiB had optimum activity in 50 mM glycine-NaOH buffer pH-10.0, whereas, *Fj* ChiC in 50 mM citrate buffer pH-6.0. *Fj* ChiB was optimally active at 30^0C, whereas, *Fj* ChiC was active at 40^0C. *Sm* ChiD was optimally active in 50 mM sodium citrate buffer pH-5.0 and 40^0C. A significant difference was observed in catalytic efficiency of *Fj* chitinases. *Fj* ChiB exhibited more catalytic efficiency *k*cat/Km at 132.04×10^4 (mg^{-1} ml sec^{-1}) than *Fj* ChiC which had at 1.40×10^3(mg^{-1} ml sec^{-1}). The difference could be due to the presence of additional accessory domain CBM6 in *Fj* ChiB. *Sm* ChiD exhibited a 2.5-fold higher overall

catalytic efficiency (74.8 s^{-1} mg^{-1} ml) than *Sp* ChiD (29.3 s^{-1} mg^{-1} ml) and which is a lower that other reported chitinases of *S. marcescens*.

4.2 Hydrolytic and transglycosylation (TG) activities on oligomeric substrates

Hydrolytic profile with colloidal chitin revealed that DP3 was the major end product with colloidal chitin for *Fj* ChiB whereas DP2 for *Fj* ChiC. *Sm* ChiD produced both DP1 and DP2 as major end products. HPLC analysis revealed that there were significant changes in hydrolytic products of all there chitinases (*Fj* chiB, *Fj* ChiC, and *Sm* ChiD) with oligomeric substrates. DP4 was the minimum oligomeric substrate for both *Fj* ChiB and *Fj* ChiC. *Fj* ChiC exhibited transient transglycosylation (TG) activity on DP5 and DP6, which was not observed with *Fj* ChiB. *Fj* ChiC synthesized DP6, DP7, and DP8 as TG products from DP5 substrate, whereas, DP7, DP8 and DP9 were the TG products with DP6 substrate. But, the TG products remained till 30 min, and were degraded later. Site-directed mutants were generated for *Fj* ChiC to improve the TG activity. The activity of mutant G106W decreased and TG products were observed at 720 min. TG products lasted long from 15 min to 720 min because of mutation of D148N. The mutant W211G resulted in complete loss of TG activity and gaining of chitobiase activity at 720 min.

Sm ChiD exhibited good TG activity on DP3, DP5, DP5 and DP6 oligomeric substrates. DP6 is longest TG product observed with DP4 and DP5 substrate. DP7 and DP8 were observed as TG products with DP6 substrate. DP1 and DP2 were the major end products with all the oligomeric substrates. *Sm* ChiD did not exhibit TG activity on DP2 substrate. TG products lasted long for 720 min.

Homology modeling and protein sequence alignment revealed that *Sm* ChiD shared less similarity with chitinases present in *S. marcescens*. The α+β insertion which plays a crucial role in processivity was present in *Sm* ChiD and absent in both the *Fj* chitinases.

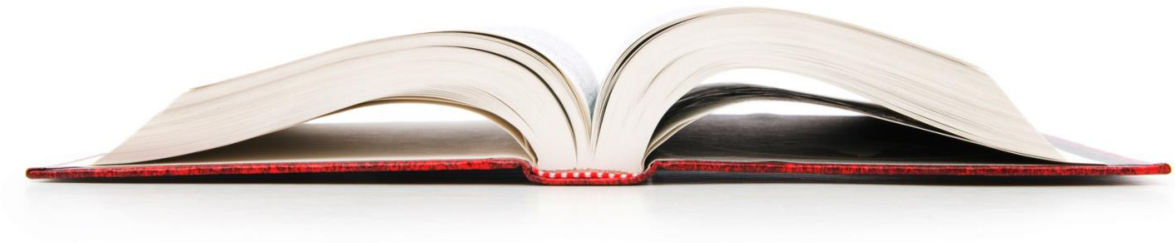
4.3 Plant-strengthening activities of chitosan oligosaccharides generated by *Fj* ChiC and G106W mutant

The ability of chitooligosaccharides (CHOS) to induce the plant immunity was studied in *Medicago truncatula* and *Nicotiana tabacum* cell suspension cultures. Three types of chitosan polymers (DA35%, DA50%, and DA60%) were selected to generate bioactive CHOS. The

hydrolytic products were analyzed by HPTLC and UHPLC-ELSD-ESI-MS. The CHOS produced by both the chitinases showed significant quantitative difference in their DP and DA. Crude hydrosylates showed good elicitor activity than untreated polymers in *M. truncatula*. G106W mutant treated hydrolysates exhibited marginal elicitor activity than *Fj* ChiC treated. Hydrolysates of DA60% is good at activity followed by DA50% and DA35%. *N. tabacum* cells were responded differently than *M. truncatula*. Polymers exhibited higher oxidative burst than enzyme treated samples in *N. tabacum* cell suspensions. *Fj* ChiC generated hydrolysates exhibited marge elicitor activity than G106W generated hydrolysates. Hydrolysates of DA35% had better elicitor activity followed by hydrolysatef from DA50% and DA60%.

4.4 Conclusions

- A new chitinase was characterized from *S. marcescens* and named it as *Sm* ChiD
- *Sm* ChiD exhibited high TG on DP3, DP4 and DP5 substrates
- *Sm* ChiD exhibits less sequence similarity with *S. marcescens* chitinases
- *Fj* ChiB showed more catalytic efficiency likely due to the presence of additional binding domain CBM6
- *Fj* ChiC exhibited very low TG activity on longer chain length oligomers DP5 and DP6
- D148N conversion retained TG products longer duration and this residue may regulate the secondary hydrolysis
- Due to conversion of G106W, enzyme sub-site preference was completely changed at -2 position to “Acetylated sugar” in *Fj* ChiC
- There was a significant qualitative and quantitative change in oligomeric pattern between *Fj* ChiC and G106W mutant treated polymers
- Chitosan hydrolysates produced by mutant G106W exhibited marginal elicitor activity in *M. truncatula*
- Polymers without hydrolysis exhibited good elicitor activity in *N. tabacum*



References

Aam, B.B., Heggset, E.B., Norberg, A.L., Sorlie, M., Varum, K.M., Eijsink, V.G.H., 2010. Production of chitooligosaccharides and their potential applications in medicine. Mar. Drugs. doi:10.3390/md8051482

Aguilera, B., Ghauharali-van der Vlugt, K., Helmond, M.T.J., Out, J.M.M., Donker-Koopman, W.E., Groener, J.E.M., Boot, R.G., Renkema, G.H., van der Marel, G.A., van Boom, J.H., Overkleeft, H.S., Aerts, J.M.F.G., 2003. Transglycosidase activity of chitotriosidase: improved enzymatic assay for the human macrophage chitinase. J. Biol. Chem. 278, 40911–6. doi:10.1074/jbc.M301804200

Alvarez, M. V., Ponce, A.G., Moreira, M. del R., 2013. Antimicrobial efficiency of chitosan coating enriched with bioactive compounds to improve the safety of fresh cut broccoli. LWT - Food Sci. Technol. 50, 78–87. doi:10.1016/j.lwt.2012.06.021

Aronson, N.N., Halloran, B.A., Alexeyev, M.F., Zhou, X.E., Wang, Y., Meehan, E.J., Chen, L., 2006. Mutation of a conserved tryptophan in the chitin-binding cleft of *Serratia marcescens* chitinase A enhances transglycosylation. Biosci. Biotechnol. Biochem. 70, 243–51.

Aunpad, R., Panbangred, W., 2003. Cloning and characterization of the constitutively expressed chitinase C gene from a marine bacterium, *Salinivibrio costicola* strain 5SM-1. J. Biosci. Bioeng. 96, 529–36. doi:10.1016/S1389-1723(04)70145-0

Bellich, B., D'Agostino, I., Semeraro, S., Gamini, A., Cesàro, A., 2016. "The Good, the Bad and the Ugly" of Chitosans. Mar. Drugs 14, 99. doi:10.3390/md14050099

Bhushan, B., 2000. Production and characterization of a thermostable chitinase from a new alkalophilic *Bacillus* sp. BG-11. J. Appl. Microbiol. 88, 800–8.

Boer, H., Munck, N., Natunen, J., Wohlfahrt, G., Söderlund, H., Renkonen, O., Koivula, A., 2004. Differential recognition of animal type beta-4-galactosylated and alpha-3-fucosylated chito-oligosaccharides by two family 18 chitinases from *Trichoderma harzianum*. Glycobiology 14, 1303–13. doi:10.1093/glycob/cwh121

Boller, T., Felix, G., 2009. A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. Annu. Rev. Plant Biol. 60, 379–406. doi:10.1146/annurev.arplant.57.032905.105346

Boot, R.G., Renkema, G.H., Verhoek, M., Strijland, A., Bliek, J., de Meulemeester, T.M.A.M.O., Mannens, M.M.A.M., Aerts, J.M.F.G., 1998. The human chitotriosidase gene: nature of inherited enzyme deficiency. J. Biol. Chem. 273, 25680–25685. doi:10.1074/jbc.273.40.25680

Cabrera, J.C., Messiaen, J., Cambier, P., Van Cutsem, P., 2006. Size, acetylation and concentration of chitooligosaccharide elicitors determine the switch from defense involving PAL activation to cell death and water peroxide production in *Arabidopsis* cell suspensions. Physiol. Plant. 127, 44–56. doi:10.1111/j.1399-3054.2006.00677.x

Cao, Y., Liang, Y., Tanaka, K., Nguyen, C.T., Jedrzejczak, R.P., Joachimiak, A., Stacey, G., 2014. The kinase LYK5 is a major chitin receptor in *Arabidopsis* and forms a chitin-induced complex with related kinase CERK1. Elife 3. doi:10.7554/eLife.03766

Carrió, M.M., Cubarsi, R., Villaverde, A., 2000. Fine architecture of bacterial inclusion bodies. FEBS Lett. 471, 7–11.

Chang, S.C., Wang, J.T., Vandamme, P., Hwang, J.H., Chang, P.S., Chen, W.M., 2004. *Chitinimonas taiwanensis* gen. nov., sp. nov., a novel chitinolytic bacterium isolated from a freshwater pond for shrimp culture. Syst. Appl. Microbiol. 27, 43–9. doi:10.1078/0723-2020-00252

Chang, W.T., Chen, C.S., Wang, S.L., 2003. An antifungal chitinase produced by *Bacillus cereus* with shrimp and crab shell powder as a carbon source. Curr. Microbiol. 47, 102–8.

Chang, W.T., Chen, M.L., Wang, S.L., 2010. An antifungal chitinase produced by *Bacillus subtilis* using chitin waste as a carbon source. World J. Microbiol. Biotechnol. 26, 945–950. doi:10.1007/s11274-009-0244-7

Chatterjee, S., Chatterjee, B.P., Guha, A.K., 2014. A study on antifungal activity of water-soluble chitosan against *Macrophomina phaseolina*. Int. J. Biol. Macromol. 67, 452–457. doi:10.1016/j.ijbiomac.2014.04.008

Chen, C.-T., Huang, C.-J., Wang, Y.-H., Chen, C.-Y., 2004. Two-step purification of Bacillus circulans chitinase A1 expressed in *Escherichia coli* periplasm. Protein Expr. Purif. 37, 27–31. doi:10.1016/j.pep.2004.03.017

Chen, J.K., Shen, C.R., Liu, C.L., 2010. N-acetylglucosamine: Production and applications. Mar. Drugs. doi:10.3390/md8092493

Chen, S., Kaufman, M.G., Miazgowicz, K.L., Bagdasarian, M., Walker, E.D., 2013. Molecular characterization of a cold-active recombinant xylanase from *Flavobacterium johnsoniae* and its applicability in xylan hydrolysis. Bioresour. Technol. 128, 145–155. doi:10.1016/j.biortech.2012.10.087

Collinge, D.B., Kragh, K.M., Mikkelsen, J.D., Nielsen, K.K., Rasmussen, U., Vad, K., 1993. Plant chitinases. Plant J. 3, 31–40.

Côté, F., Hahn, M.G., 1994. Oligosaccharins: structures and signal transduction. Plant Mol. Biol. 26, 1379–411.

Dahiya, N., Tewari, R., Hoondal, G.S., 2006. Biotechnological aspects of chitinolytic enzymes: A review. Appl. Microbiol. Biotechnol. doi:10.1007/s00253-005-0183-7

Das, S.N., Dutta, S., Kondreddy, A., Chilukoti, N., Pullabhotla, S.V.S.R.N., Vadlamudi, S., Podile, A.R., 2010. Plant growth-promoting chitinolytic *Paenibacillus elgii* responds positively to tobacco root exudates. J. Plant Growth Regul. 29, 409–418. doi:10.1007/s00344-010-9152-1

Das, S.N., Madhuprakash, J., Sarma, P.V.S.R.N., Purushotham, P., Suma, K., Manjeet, K., Rambabu, S., Gueddari, N.E. El, Moerschbacher, B.M., Podile, A.R., 2015. Biotechnological approaches for field applications of chitooligosaccharides (COS) to induce innate immunity in plants. Crit. Rev. Biotechnol. 35, 29–43. doi:10.3109/07388551.2013.798255

Dash, M., Chiellini, F., Ottenbrite, R.M., Chiellini, E., 2011. Chitosan - A versatile semi-synthetic polymer in biomedical applications. Prog. Polym. Sci. 36, 981–1014. doi:10.1016/j.progpolymsci.2011.02.001

Day, R.B., 2001. Binding site for chitin oligosaccharides in the soybean plasma membrane. Plant Physiol. 126, 1162–1173. doi:10.1104/pp.126.3.1162

Di Rosa, M., Malaguarnera, L., 2016. Chitotriosidase: A new inflammatory marker in diabetic complications. Pathobiology 83, 211–9. doi:10.1159/000443932

Din, N., Gilkes, N.R., Tekant, B., Miller, R.C., Warren, R.A.J., Kilburn, D.G., 1991. Non–hydrolytic disruption of cellulose fibres by the binding domain of a bacterial cellulase. Bio/Technology 9, 1096–1099. doi:10.1038/nbt1191-1096

Ding, R., Li, Y., Qian, C., Wu, X., 2011. Draft genome sequence of *Paenibacillus elgii* B69, a strain with broad antimicrobial activity. J. Bacteriol. 193, 4537. doi:10.1128/JB.00406-11

Duo-Chuan, L., 2006. Review of Fungal Chitinases. Mycopathologia 161, 345–360. doi:10.1007/s11046-006-0024-y

Dutta, P.K., Ravikumar, M.N. V., Dutta, J., 2002. Chitin and chitosan for versatile applications. J. Macromol. Sci. Part C Polym. Rev. 42, 307–354. doi:10.1081/MC-120006451

Eder, J., Cosio, E.G., 1994. Elicitors of Plant Defense Responses. pp. 1–36. doi:10.1016/S0074-7696(08)62404-3

Einbu, A., Vårum, K.M., 2007. Depolymerization and de-N-acetylation of chitin oligomers in hydrochloric acid. Biomacromolecules 8, 309–314. doi:10.1021/bm0608535

Essghaier, B., Rouaissi, M., Boudabous, A., Jijakli, H., Sadfi-Zouaoui, N., 2010. Production and partial characterization of chitinase from a halotolerant *Planococcus rifitoensis* strain M2-26. World J. Microbiol. Biotechnol. 26, 977–984. doi:10.1007/s11274-009-0259-0

Falcón, A.B., Cabrera, J.C., Costales, D., Ramírez, M.A., Cabrera, G., Toledo, V., Martínez-Téllez, M.A., 2008. The effect of size and acetylation degree of chitosan derivatives on tobacco plant protection against *Phytophthora parasitica* nicotianae. World J. Microbiol. Biotechnol. 24, 103–112. doi:10.1007/s11274-007-9445-0

Fernández-de Castro, L., Mengíbar, M., Sánchez, Á., Arroyo, L., Villarán, M.C., Díaz de Apodaca, E., Heras, Á., 2016. Films of chitosan and chitosan-oligosaccharide neutralized and thermally treated: Effects on its antibacterial and other activities 73, 368–374. doi:10.1016/j.lwt.2016.06.038

Fitza, K.N.E., Payn, K.G., Steenkamp, E.T., Myburg, A.A., Naidoo, S., 2013. Chitosan application improves resistance to *Fusarium circinatum* in Pinus patula. South African J. Bot. 85, 70–78. doi:10.1016/j.sajb.2012.12.006

Flach, J., Pilet, P.E., Jollès, P., 1992. What's new in chitinase research? Experientia 48, 701–16.

Fliegmann, J., Uhlenbroich, S., Shinya, T., Martinez, Y., Lefebvre, B., Shibuya, N., Bono, J.-J., 2011. Biochemical and phylogenetic analysis of CEBiP-like LysM domain-containing extracellular proteins in higher plants. Plant Physiol. Biochem. 49, 709–720. doi:10.1016/j.plaphy.2011.04.004

Fu, X., Yan, Q., Wang, J., Yang, S., Jianga, Z., 2016. Purification and biochemical characterization of novel acidic chitinase from *Paenicibacillus barengoltzii*. Int. J. Biol. Macromol. doi:10.1016/j.ijbiomac.2016.06.050

Fukamizo, T., Goto, S., Torikata, T., Araki, T., 1989. Enhancement of transglycosylation activity of lysozyme by chemical modification. Agric. Biol. Chem. 53, 2641–2651. doi:10.1271/bbb1961.53.2641

Fukamizo, T., Sasaki, C., Schelp, E., Bortone, K., Robertus, J.D., 2001. Kinetic properties of chitinase-1 from the fungal pathogen *Coccidioides immitis*. Biochemistry 40, 2448–54.

Gautier, S., Xhauflaire-Uhoda, E., Gonry, P., Piarard, G.E., 2008. Chitin-glucan, a natural cell scaffold for skin moisturization and rejuvenation. Int. J. Cosmet. Sci. 30, 459–469. doi:10.1111/j.1468-2494.2008.00470.x

Gilkes, N.R., Warren, R.A., Miller, R.C., Kilburn, D.G., 1988. Precise excision of the cellulose binding domains from two *Cellulomonas fimi* cellulases by a homologous protease and the effect on catalysis. J. Biol. Chem. 263, 10401–10407.

Gooday, G.W., 1990. The Ecology of Chitin Degradation. pp. 387–430. doi:10.1007/978-1-4684-7612-5_10

Gust, A.A., Willmann, R., Desaki, Y., Grabherr, H.M., Nürnberger, T., 2012. Plant LysM proteins: modules mediating symbiosis and immunity. Trends Plant Sci. 17, 495–502. doi:10.1016/j.tplants.2012.04.003

Gutierrez-Roman, M.I., Dunn, M.F., Tinoco-Valencia, R., Holguin-Melendez, F., Huerta-Palacios, G., Guillen-Navarro, K., 2014. Potentiation of the synergistic activities of chitinases ChiA, ChiB, and ChiC from *Serratia marcescens* CFFSUR-B2 by chitobiase (Chb) and chitin binding protein (CBP). World J. Microbiol. Biotechnol. 30, 33–42. doi:10.1007/s11274-013-1421-2

H.P.S, A.K., Saurabh, C.K., A.S., A., Nurul Fazita, M.R., Syakir, M.I., Davoudpour, Y., Rafatullah, M., Abdullah, C.K., M. Haafiz, M.K., Dungani, R., 2016. A review on chitosan-cellulose blends and nanocellulose reinforced chitosan biocomposites: Properties and their applications. Carbohydr. Polym. 150, 216–226. doi:10.1016/j.carbpol.2016.05.028

Hadwiger, L.A., 2013. Multiple effects of chitosan on plant systems: Solid science or hype. Plant Sci. 208, 42–49. doi:10.1016/j.plantsci.2013.03.007

Hamed, I., Özogul, F., Regenstein, J.M., 2016. Industrial applications of crustacean by-products (chitin, chitosan, and chitooligosaccharides): A review. Trends Food Sci. Technol. 48, 40–50. doi:10.1016/j.tifs.2015.11.007

Hamel, L.P., Beaudoin, N., 2010. Chitooligosaccharide sensing and downstream signaling: Contrasted outcomes in pathogenic and beneficial plant-microbe interactions. Planta. doi:10.1007/s00425-010-1215-9

Hamer, S.N., Cord-Landwehr, S., Biarnés, X., Planas, A., Waegeman, H., Moerschbacher, B.M., Kolkenbrock, S., 2015. Enzymatic production of defined chitosan oligomers with a specific pattern of acetylation using a combination of chitin oligosaccharide deacetylases. Sci. Rep. 5, 8716. doi:10.1038/srep08716

Hamre, A.G., Schaupp, D., Eijsink, V.G.H., Sorlie, M., 2015. The directionality of processive enzymes acting on recalcitrant polysaccharides is reflected in the kinetic signatures of oligomer degradation. FEBS Lett. 589, 1807–1812. doi:10.1016/j.febslet.2015.05.034

Hao, Z., Wu, H., Yang, M., Chen, J., Xi, L., Zhao, W., Yu, J., Liu, J., Liao, X., Huang, Q., 2016. Cloning, expression and 3D structure prediction of chitinase from *Chitinolyticbacter meiyuanensis* SYBC-H1. Int. J. Mol. Sci. 17. doi:10.3390/ijms17060825

Hashimoto, M., Ikegami, T., Seino, S., Ohuchi, N., Fukada, H., Sugiyama, J., Shirakawa, M., Watanabe, T., 2000. Expression and characterization of the chitin-binding domain of chitinase A1 from *Bacillus circulans* WL-12. J. Bacteriol. 182, 3045–3054. doi:10.1128/JB.182.11.3045-3054.2000

Hatori, Y., Sato, M., Orishimo, K., Yatsunami, R., Endo, K., Fukui, T., Nakamura, S., 2006. Characterization of Recombinant Family 18 Chitinase from Extremely Halophilic Archaeon *Halobacterium salinarum* Strain NRC-1. Chitin Chitosan Res. 12, 201.

Hayafune, M., Berisio, R., Marchetti, R., Silipo, A., Kayama, M., Desaki, Y., Arima, S., Squeglia, F., Ruggiero, A., Tokuyasu, K., Molinaro, A., Kaku, H., Shibuya, N., 2014. Chitin-induced activation of immune signaling by the rice receptor CEBiP relies on a unique sandwich-type dimerization. Proc. Natl. Acad. Sci. 111, E404–E413. doi:10.1073/pnas.1312099111

Henrissat, B., Bairoch, A., 1993. New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem. J 293, 781–788.

Henrissat, B., Davies, G., 1997. Structural and sequence-based classification of glycoside hydrolases. Curr. Opin. Struct. Biol. doi:10.1016/S0959-440X(97)80072-3

Hollis, T., Monzingo, a F., Bortone, K., Ernst, S., Cox, R., Robertus, J.D., 2000. The X-ray structure of a chitinase from the pathogenic fungus *Coccidioides immitis*. Protein Sci. 9, \544–551. doi:10.1110/ps.9.3.544

Horn, S.J., Eijsink, V.G.H., 2004. A reliable reducing end assay for chito-oligosaccharides. Carbohydr. Polym. 56, 35–39. doi:10.1016/j.carbpol.2003.11.011

Horn, S.J., Sikorski, P., Cederkvist, J.B., Vaaje-Kolstad, G., Sørlie, M., Synstad, B., Vriend, G., Vårum, K.M., Eijsink, V.G.H., 2006. Costs and benefits of processivity in enzymatic degradation of recalcitrant polysaccharides. Proc. Natl. Acad. Sci. U. S. A. 103, 18089–94. doi:10.1073/pnas.0608909103

Imoto, T., Yagishita, K., 1971. A simple activity measurement of lysozyme. Agric. Biol. Chem. 35, 1154–1156. doi:10.1271/bbb1961.35.1154

Iseli, B., Armand, S., Boller, T., Neuhaus, J.M., Henrissat, B., 1996. Plant chitinases use two different hydrolytic mechanisms. FEBS Lett. 382, 186–188. doi:10.1016/0014-5793(96)00174-3

Jung, W.J., Park, R.D., 2014. Bioproduction of chitooligosaccharides: Present and perspectives. Mar. Drugs. doi:10.3390/md12105328

Kadowaki, S., Saskiawan, I., Watanabe, J., Yamamoto, K., Bunno, M., Ichihara, Y., Kumagai, H., 1997. Transglycosylation activity of β-*N*-acetylhexosaminidase from *Penicillium oxalicum* and its application to synthesis of a drug carrier. J. Ferment. Bioeng. 83, 341–345. doi:10.1016/S0922-338X(97)80139-0

Kaku, H., Nishizawa, Y., Ishii-Minami, N., Akimoto-Tomiyama, C., Dohmae, N., Takio, K., Minami, E., Shibuya, N., 2006. Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor. Proc. Natl. Acad. Sci. 103, 11086–11091. doi:10.1073/pnas.0508882103

Kaku, H., Shibuya, N., Xu, P., Aryan, A.P., Fincher, G.B., 1997. *N*-acetylchitooligosaccharides elicit expression of a single (13)-beta-glucanase gene in suspension-cultured cells from barley (*Hordeum vulgare*). Physiol. Plant. 100, 111–118. doi:10.1111/j.1399-3054.1997.tb03460.x

Katouno, F., Taguchi, M., Sakurai, K., Uchiyama, T., Nikaidou, N., Nonaka, T., Sugiyama, J., Watanabe, T., 2004. Importance of exposed aromatic residues in chitinase B from *Serratia marcescens* 2170 for crystalline chitin hydrolysis. J. Biochem. 136, 163–168. doi:10.1093/jb/mvh105

Kharade, S.S., McBride, M.J., 2014. *Flavobacterium johnsoniae* chitinase ChiA is required for chitin utilization and is secreted by the type IX secretion system. J. Bacteriol. 196, 961–970. doi:10.1128/JB.01170-13

Khor, E., Lim, L.Y., 2003. Implantable applications of chitin and chitosan. Biomaterials 24, 2339–49.

Khoushab, F., Yamabhai, M., 2010. Chitin Research Revisited. Mar. Drugs 8, 1988–2012. doi:10.3390/md8071988

Kim, H.-S., Golyshin, P.N., Timmis, K.N., 2007. Characterization and role of a metalloprotease induced by chitin in *Serratia* sp. KCK. J. Ind. Microbiol. Biotechnol. 34, 715–721. doi:10.1007/s10295-007-0245-1

Kim, S., Rajapakse, N., 2005. Enzymatic production and biological activities of chitosan oligosaccharides (COS): A review. Carbohydr. Polym. 62, 357–368. doi:10.1016/j.carbpol.2005.08.012

Konagaya, Y., Tsuchiya, C., Sugita, H., 2006. Purification and characterization of chitinases from *Clostridium* sp. E-16 isolated from the intestinal tract of the South American sea lion (*Otaria flavescens*). Lett. Appl. Microbiol. 43, 187–193. doi:10.1111/j.1472-765X.2006.01926.x

Koshland, D.E., Stein, S.S., 1954. Correlation of bond breaking with enzyme specificity; cleavage point of. J. Biol. Chem. 208, 139–148.

Krajewska, B., 2004. Application of chitin- and chitosan-based materials for enzyme immobilizations: A review. Enzyme Microb. Technol. doi:10.1016/j.enzmictec.2003.12.013

Kumar, A.B.V., Gowda, L.R., Tharanathan, R.N., 2004. Non-specific depolymerization of chitosan by pronase and characterization of the resultant products. Eur. J. Biochem. 271, 713–23.

Kuranda, M.J., Robbins, P.W., 1991. Chitinase is required for cell separation during growth of *Saccharomyces cerevisiae*. J. Biol. Chem. 266, 19758–67.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of *bacteriophage* T4. Nature 227, 680–685. doi:10.1038/227680a0

Langer, R.C., 2002. Monoclonal antibody against the *Plasmodium falciparum* Chitinase, PfCHT1, recognizes a malaria transmission-blocking epitope in *Plasmodium gallinaceum* ookinetes unrelated to the chitinase *Pg*CHT1. Infect. Immun. 70, 1581–1590. doi:10.1128/IAI.70.3.1581-1590.2002

Laskowski, R.A., MacArthur, M.A., Moss, D.S., Thornton, J.M., 1993. 20.19. (1993). PROCHECK - a program to check the stereochemical quality of protein structures. J. App. Cryst., 26, . J. App. Cryst 26, 283–291. doi:10.1107/S0021889892009944

Lavall, R.L., Assis, O.B.G., Campana-Filho, S.P., 2007. Beta-chitin from the pens of Loligo sp.: extraction and characterization. Bioresour. Technol. 98, 2465–72. doi:10.1016/j.biortech.2006.09.002

Lee, W.-S., Rudd, J.J., Hammond-Kosack, K.E., Kanyuka, K., 2014. *Mycosphaerella graminicola* LysM effector-mediated stealth pathogenesis subverts recognition through both CERK1 and CEBiP homologues in wheat. Mol. Plant-Microbe Interact. 27, 236–243. doi:10.1094/MPMI-07-13-0201-R

Lemieux, M.J., Mark, B.L., Cherney, M.M., Withers, S.G., Mahuran, D.J., James, M.N.G., 2006. Crystallographic structure of human β-hexosaminidase A: Interpretation of tay-sachs mutations and loss of GM2 ganglioside hydrolysis. J. Mol. Biol. 359, 913–929. doi:10.1016/j.jmb.2006.04.004

Letunic, I., Doerks, T., Bork, P., 2015. SMART: recent updates, new developments and status in 2015. Nucleic Acids Res. 43, D257–D260. doi:10.1093/nar/gku949

Li, B., Liu, B., Shan, C., Ibrahim, M., Lou, Y., Wang, Y., Xie, G., Li, H.Y., Sun, G., 2013a. Antibacterial activity of two chitosan solutions and their effect on rice bacterial leaf blight and leaf streak. Pest Manag. Sci. 69, 312–320. doi:10.1002/ps.3399

Li, B., Shi, Y., Shan, C., Zhou, Q., Ibrahim, M., Wang, Y., Wu, G., Li, H., Xie, G., Sun, G., 2013b. Effect of chitosan solution on the inhibition of *Acidovorax citrulli* causing bacterial fruit blotch of watermelon. J. Sci. Food Agric. 93, 1010–1015. doi:10.1002/jsfa.5812

Li, S. j., Zhu, T. -h., 2012. Biochemical response and induced resistance against anthracnose (*Colletotrichum camelliae*) of camellia (*Camellia pitardii*) by chitosan oligosaccharide application. For. Pathol. n/a–n/a. doi:10.1111/j.1439-0329.2012.00797.x

Lin, F., Jia, X.-G., Lei, W.-X., Li, Z.-J., Zhang, T.-Y., 2009. [Spectra analyses of chitosans degraded by hydrogen peroxide under optimal conditions]. Guang Pu Xue Yu Guang Pu Fen Xi 29, 43–7.

Liu, T., Liu, Z., Song, C., Hu, Y., Han, Z., She, J., Fan, F., Wang, J., Jin, C., Chang, J., Zhou, J.-M., Chai, J., 2012. Chitin-induced dimerization activates a plant immune receptor. Science 336, 1160–4. doi:10.1126/science.1218867

Liu, Y., Wisniewski, M., Kennedy, J.F., Jiang, Y., Tang, J., Liu, J., 2016. Chitosan and oligochitosan enhance ginger (Zingiber officinale Roscoe) resistance to rhizome rot caused by Fusarium oxysporum in storage. Carbohydr. Polym. 151, 474–479. doi:10.1016/j.carbpol.2016.05.103

Lodhi, G., Kim, Y.-S., Hwang, J.-W., Kim, S.-K., Jeon, Y.-J., Je, J.-Y., Ahn, C.-B., Moon, S.-H., Jeon, B.-T., Park, P.-J., 2014. Chitooligosaccharide and it's derivatives: preparation and biological applications. Biomed Res. Int. 2014, 1–13. doi:10.1155/2014/654913

Lu, Y., Yang, H., Hu, H., Wang, Y., Rao, Z., Jin, C., 2009. Mutation of Trp137 to glutamate completely removes transglycosyl activity associated with the *Aspergillus fumigatus* AfChiB1. Glycoconj. J. 26, 525–534. doi:10.1007/s10719-008-9203-z

Lüthy, R., Bowie, J.U., Eisenberg, D., 1992. Assessment of protein models with three-dimensional profiles. Nature 356, 83–85. doi:10.1038/356083a0

Ma, Z., Yang, L., Yan, H., Kennedy, J.F., Meng, X., 2013. Chitosan and oligochitosan enhance the resistance of peach fruit to brown rot. Carbohydr. Polym. 94, 272–277. doi:10.1016/j.carbpol.2013.01.012

Mabuchi, N., Hashizume, I., Araki, Y., 2000. Characterization of chitinases excreted by *Bacillus cereus* CH. Can. J. Microbiol. 46, 370–5.

Madhuprakash, J., El Gueddari, N.E., Moerschbacher, B.M., Podile, A.R., 2015a. Production of bioactive chitosan oligosaccharides using the hypertransglycosylating chitinase-D from *Serratia proteamaculans*. Bioresour. Technol. 198, 503–509. doi:10.1016/j.biortech.2015.09.052

Madhuprakash, J., El Gueddari, N.E., Moerschbacher, B.M., Podile, A.R., 2015b. Catalytic efficiency of chitinase-D on insoluble chitinous substrates was improved by fusing auxiliary domains. PLoS One 10. doi:10.1371/journal.pone.0116823

Madhuprakash, J., Tanneeru, K., Purushotham, P., Guruprasad, L., Podile, A.R., 2012. Transglycosylation by Chitinase D from *Serratia proteamaculans* improved through altered substrate interactions. J. Biol. Chem. 287, 44619–44627. doi:10.1074/jbc.M112.400879

Maksimov, I. V, Valeev, a S., Safin, R.F., 2011. Acetylation degree of chitin in the protective response of wheat plants. Biochem. Biokhimiia 76, 1342–6. doi:10.1134/S0006297911120078

Malerba, M., Cerana, R., 2016. Chitosan Effects on Plant Systems. Int. J. Mol. Sci. 17, 996. doi:10.3390/ijms17070996

Mander, P., Cho, S.S., Choi, Y.H., Panthi, S., Choi, Y.S., Kim, H.M., Yoo, J.C., 2016. Purification and characterization of chitinase showing antifungal and biodegradation properties obtained from *Streptomyces anulatus* CS242. Arch. Pharm. Res. doi:10.1007/s12272-016-0747-3

Martinez, E.A., Boer, H., Koivula, A., Samain, E., Driguez, H., Armand, S., Cottaz, S., 2012. Engineering chitinases for the synthesis of chitin oligosaccharides: Catalytic amino acid mutations convert the GH-18 family glycoside hydrolases into transglycosylases. J. Mol. Catal. B Enzym. 74, 89–96. doi:10.1016/j.molcatb.2011.09.003

McBride, M.J., Xie, G., Martens, E.C., Lapidus, A., Henrissat, B., Rhodes, R.G., Goltsman, E., Wang, W., Xu, J., Hunnicutt, D.W., Staroscik, A.M., Hoover, T.R., Cheng, Y.-Q., Stein, J.L., 2009. Novel features of the polysaccharide-digesting gliding bacterium *Flavobacterium johnsoniae* as revealed by genome sequence analysis. Appl. Environ. Microbiol. 75, 6864–6875. doi:10.1128/AEM.01495-09

Mehmood, M.A., Xiao, X., Hafeez, F.Y., Gai, Y., Wang, F., 2009. Purification and characterization of a chitinase from *Serratia proteamaculans*. World J. Microbiol. Biotechnol. 25, 1955–1961. doi:10.1007/s11274-009-0094-3

Meins, F., Sperisen, C., Neuhaus, J.-M., Ryals, J., 1992. The primary structure of plant pathogenesis-related glucanohydrolases and their genes. pp. 245–282. doi:10.1007/978-3-7091-6684-0_10

Mellor, K.J., Nicholas, R.O., Adams, D.J., 1994. Purification and characterization of chitinase from *Candida albicans*. FEMS Microbiol. Lett. 119, 111–117.

Merzendorfer, H., Zimoch, L., 2003. Chitin metabolism in insects: structure, function and regulation of chitin synthases and chitinases. J. Exp. Biol. 206, 4393–412.

Miya, A., Albert, P., Shinya, T., Desaki, Y., Ichimura, K., Shirasu, K., Narusaka, Y., Kawakami, N., Kaku, H., Shibuya, N., 2007. CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in *Arabidopsis*. Proc. Natl. Acad. Sci. 104, 19613–19618. doi:10.1073/pnas.0705147104

Morganti, P., Fabrizi, G., Palombo, P., Palombo, M., Ruocco, E., Cardillo, A., Morganti, G., 2008. Chitin-nanofibrils: A new active cosmetic carrier. J. Appl. Cosmetol. 26, 113–128.

Morris, V.B., Neethu, S., Abraham, T.E., Pillai, C.K.S., Sharma, C.P., 2009. Studies on the condensation of depolymerized chitosans with DNA for preparing chitosan-DNA nanoparticles for gene delivery applications. J. Biomed. Mater. Res. B. Appl. Biomater. 89, 282–92. doi:10.1002/jbm.b.31214

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. doi:10.1021/jf9040386

Nanjo, F., Sakai, K., Ishikawa, M., Isobe, K., Usui, T., 1989. Properties and transglycosylation reaction of a chitinase from *Nocardia orientalis*. Agric. Biol. Chem. 53, 2189–2195. doi:10.1271/bbb1961.53.2189

Neeraja, C., Moerschbacher, B., Podile, A.R., 2010. Fusion of cellulose binding domain to the catalytic domain improves the activity and conformational stability of chitinase in *Bacillus licheniformis* DSM13. Bioresour. Technol. 101, 3635–3641. doi:10.1016/j.biortech.2009.12.118

Nguyen-Thi, N., Doucet, N., 2016. Combining chitinase C and *N*-acetylhexosaminidase from *Streptomyces coelicolor* A3(2) provides an efficient way to synthesize *N*-acetylglucosamine from crystalline chitin. J. Biotechnol. 220, 25–32. doi:10.1016/j.jbiotec.2015.12.038

Nürnberger, T., Brunner, F., Kemmerling, B., Piater, L., 2004. Innate immunity in plants and animals: striking similarities and obvious differences. Immunol. Rev. 198, 249–66.

Ochiai, H., Ohmae, M., Kobayashi, S., 2004. Enzymatic glycosidation of sugar oxazolines having a carboxylate group catalyzed by chitinase. Carbohydr. Res. 339, 2769–2788. doi:10.1016/j.carres.2004.08.016

Park, J., Morita, K., Fukumoto, I., Yamasaki, Y., Nakagawa, T., Kawamukai, M., Matsuda, H., 1997. Purification and characterization of the chitinase (ChiA) from *Enterobacter* sp. G-1. Biosci Biotechnol Biochem 61, 684–689.

Parvez, S., Rahman, M.M., Khan, M.A., Khan, M.A.H., Islam, J.M.M., Ahmed, M., Rahman, M.F., Ahmed, B., 2012. Preparation and characterization of artificial skin using chitosan and gelatin composites for potential biomedical application 69, 715–731. doi:10.1007/s00289-012-0761-7

Patil, R.S., Ghormade, V., Deshpande, M. V., 2000. Chitinolytic enzymes: An exploration. Enzyme Microb. Technol. doi:10.1016/S0141-0229(00)00134-4

Payne, C.M., Babans, J., Horn, S.J., Backe, P.H., Arvai, A.S., Dalhus, B., Bjaras, M., Eijsink, V.G.H., Sorlie, M., Beckham, G.T., Vaaje-Kolstad, G., 2012. Hallmarks of processivity in glycoside hydrolases from crystallographic and computational studies of the *Serratia marcescens* chitinases. J. Biol. Chem. 287, 36322–36330. doi:10.1074/jbc.M112.402149

Petutschnig, E.K., Jones, A.M.E., Serazetdinova, L., Lipka, U., Lipka, V., 2010. The Lysin Motif Receptor-like Kinase (LysM-RLK) CERK1 is a major chitin-binding protein in *Arabidopsis thaliana* and subject to chitin-induced phosphorylation. J. Biol. Chem. 285, 28902–28911. doi:10.1074/jbc.M110.116657

Pillai, C.K.S., Paul, W., Sharma, C.P., 2009. Chitin and chitosan polymers: Chemistry, solubility and fiber formation. Prog. Polym. Sci. doi:10.1016/j.progpolymsci.2009.04.001

Purushotham, P., Podile, A.R., 2012. Synthesis of long-chain chitooligosaccharides by a hypertransglycosylating processive endochitinase of *Serratia proteamaculans* 568. J. Bacteriol. 194, 4260–4271. doi:10.1128/JB.06473-11

Purushotham, P., Sarma, P.V.S.R.N., Podile, A.R., 2012. Multiple chitinases of an endophytic *Serratia proteamaculans* 568 generate chitin oligomers. Bioresour. Technol. 112, 261–269. doi:10.1016/j.biortech.2012.02.062

Rahman, O., Cummings, S.P., Harrington, D.J., Sutcliffe, I.C., 2008. Methods for the bioinformatic identification of bacterial lipoproteins encoded in the genomes of Gram-positive bacteria. World J. Microbiol. Biotechnol. 24, 2377–2382. doi:10.1007/s11274-008-9795-2

Rajitha, P., Gopinath, D., Biswas, R., Sabitha, M., Jayakumar, R., 2016. Chitosan nanoparticles in drug therapy of infectious and inflammatory diseases. Expert Opin. Drug Deliv. 13, 1177–1194. doi:10.1080/17425247.2016.1178232

Ramachandran, G.N., Ramakrishnan, C., Sasisekharan, V., 1963. Stereochemistry of polypeptide chain configurations. J. Mol. Biol. 7, 95–99. doi:10.1016/S0022-2836(63)80023-6

Ramírez, M.Á., Rodríguez, A.T., Alfonso, L., Peniche, C., 2010. Chitin and its derivatives as biopolymers with potential agricultural applications. Biotecnol. Apl. 27, 270–276.

Restanto, D.P., Santoso, B., Kriswanto, B., Supardjono, S., 2016. The application of chitosan for protocorm like bodies (PLB) induction of orchid (*Dendrobium* sp) *in vitro*. Agric. Agric. Sci. Procedia 9, 462–468. doi:10.1016/j.aaspro.2016.02.164

Rinaudo, M., 2006. Chitin and chitosan: Properties and applications. Prog. Polym. Sci. 31, 603–632. doi:10.1016/j.progpolymsci.2006.06.001

Riva, R., Ragelle, H., Des Rieux, A., Duhem, N., Jorame, C., Prvat, V., 2011. Chitosan and chitosan derivatives in drug delivery and tissue engineering. Adv. Polym. Sci. doi:10.1007/12_2011_137

Saber, W.I.A., Ghoneem, K.M., Al-Askar, A.A., Rashad, Y.M., Ali, A.A., Rashad, E.M., 2015. Chitinase production by *Bacillus subtilis* ATCC 11774 and its effect on biocontrol of Rhizoctonia diseases of potato. Acta Biol. Hung. 66, 436–48. doi:10.1556/018.66.2015.4.8

Salah, R., Michaud, P., Mati, F., Harrat, Z., Lounici, H., Abdi, N., Drouiche, N., Mameri, N., 2013. Anticancer activity of chemically prepared shrimp low molecular weight chitin evaluation with the human monocyte leukaemia cell line, THP-1 52, 333–339. doi:10.1016/j.ijbiomac.2012.10.009

Sali, A., Blundell, T.L., 1993. Comparative protein modelling by satisfaction of spatial restraints. J. Mol. Biol. 234, 779–815. doi:10.1006/jmbi.1993.1626

Sathiyabama, M., Bernstein, N., Anusuya, S., 2016. Chitosan elicitation for increased curcumin production and stimulation of defence response in turmeric (*Curcuma longa* L.). Ind. Crops Prod. 89, 87–94. doi:10.1016/j.indcrop.2016.05.007

Sato, K., Saimoto, H., Morimoto, M., Shigemasa, Y., 2003. Depolymerization of chitin and chitosan under hydrothermal conditions. Sen'i Gakkaishi. doi:10.2115/fiber.59.104

Shanmugaiah, V., Mathivanan, N., Balasubramanian, N., Manoharan, P.T., 2008. Optimization of cultural conditions for production of chitinase by *Bacillus laterosporous* MML2270 isolated from rice rhizosphere soil. African J. Biotechnol. 7, 2562–2568.

Shibuya, N., Ebisu, N., Kamada, Y., Kaku, H., Cohn, J., Ito, Y., 1996. Localization and binding characteristics of a high-affinity binding site for *N*-Acetylchitooligosaccharide elicitor in the plasma membrane from suspension-cultured rice cells suggest a role as a receptor for the elicitor signal at the cell surface. Plant Cell Physiol. 37, 894–898. doi:10.1093/oxfordjournals.pcp.a029030

Shibuya, N., Kaku, H., Kuchitsu, K., Maliarik, M.J., 1993. Identification of a novel high-affinity binding site for *N*-acetylchitooligosaccharide elicitor in the membrane fraction from suspension-cultured rice cells. FEBS Lett. 329, 75–78. doi:10.1016/0014-5793(93)80197-3

Shibuya, N., Minami, E., 2001. Oligosaccharide signalling for defence responses in plant. Physiol. Mol. Plant Pathol. 59, 223–233. doi:10.1006/pmpp.2001.0364

Shinya, T., Nakagawa, T., Kaku, H., Shibuya, N., 2015. Chitin-mediated plant-fungal interactions: catching, hiding and handshaking. Curr. Opin. Plant Biol. 26, 64–71. doi:10.1016/j.pbi.2015.05.032

Sikorski, P., Sorbotten, A., Horn, S.J., Eijsink, V.G.H., Varum, K.M., 2006. *Serratia marcescens* chitinases with tunnel-shaped substrate-binding grooves show endo activity and different degrees of processivity during enzymatic hydrolysis of chitosan. Biochemistry 45, 9566–9574. doi:10.1021/bi060370l

Sinha, S., Chand, S., Tripathi, P., 2016. Enzymatic production of glucosamine and chitooligosaccharides using newly isolated exo-β-D-glucosaminidase having transglycosylation activity. 3 Biotech 6, 13. doi:10.1007/s13205-015-0330-5

Sirimontree, P., Suginta, W., Sritho, N., Kanda, Y., Shinya, S., Ohnuma, T., Fukamizo, T., 2014. Mutation strategies for obtaining chitooligosaccharides with longer chains by transglycosylation reaction of family GH18 chitinase. Biosci. Biotechnol. Biochem. 78, 2014–2021. doi:10.1080/09168451.2014.948373

Song, J., Remmers, S.J.A., Shao, J., Kolwijck, E., Walboomers, X.F., Jansen, J.A., Leeuwenburgh, S.C.G., Yang, F., 2016. Antibacterial effects of electrospun chitosan/poly(ethylene oxide) nanofibrous membranes loaded with chlorhexidine and silver 12, 1357–1364. doi:10.1016/j.nano.2016.02.005

Songsiriritthigul, C., Pantoom, S., Aguda, A.H., Robinson, R.C., Suginta, W., 2008. Crystal structures of *Vibrio harveyi* chitinase A complexed with chitooligosaccharides: implications for the catalytic mechanism. J. Struct. Biol. 162, 491–9. doi:10.1016/j.jsb.2008.03.008

Souza, C.P., Almeida, B.C., Colwell, R.R., Rivera, I.N.G., 2011. The importance of chitin in the marine environment. Mar. Biotechnol. (NY). 13, 823–30. doi:10.1007/s10126-011-9388-1

Suginta, W., Songsiriritthigul, C., Kobdaj, A., Opassiri, R., Svasti, J., 2007. Mutations of Trp275 and Trp397 altered the binding selectivity of *Vibrio carchariae* chitinase A. Biochim. Biophys. Acta 1770, 1151–60. doi:10.1016/j.bbagen.2007.03.012

Suma, K., Podile, A.R., 2013. Chitinase A from *Stenotrophomonas maltophilia* shows transglycosylation and antifungal activities. Bioresour. Technol. 133, 213–220. doi:10.1016/j.biortech.2013.01.103

Sunna, A, Moracci, M., Rossi, M., Antranikian, G., 1997. Glycosyl hydrolases from hyperthermophiles. Extremophiles 1, 2–13. doi:10.1007/s007920050009

Suzuki, K., Sugawara, N., Suzuki, M., Uchiyama, T., Katouno, F., Nikaidou, N., Watanabe, T., 2002. Chitinases A, B, and C1 of *Serratia marcescens* 2170 produced by recombinant *Escherichia coli*: enzymatic properties and synergism on chitin degradation. Biosci. Biotechnol. Biochem. 66, 1075–1083. doi:10.1271/bbb.66.1075

Suzuki, K., Taiyoji, M., Sugawara, N., Nikaidou, N., Henrissat, B., Watanabe, T., 1999. The third chitinase gene (chiC) of *Serratia marcescens* 2170 and the relationship of its product to other bacterial chitinases. Biochem. J. 343, 587. doi:10.1042/0264-6021:3430587

Svitil, A.L., Kirchman, D.L., 1998. A chitin-binding domain in a marine bacterial chitinase and other microbial chitinases: implications for the ecology and evolution of 1,4-beta-glycanases. Microbiology 144 (Pt 5, 1299–308. doi:10.1099/00221287-144-5-1299

Szymańska, E., Winnicka, K., 2015. Stability of chitosan - A challenge for pharmaceutical and biomedical applications. doi:10.3390/md13041819

Taira, T., Fujiwara, M., Dennhart, N., Hayashi, H., Onaga, S., Ohnuma, T., Letzel, T., Sakuda, S., Fukamizo, T., 2010. Transglycosylation reaction catalyzed by a class V chitinase from cycad, *Cycas revoluta*: a study involving site-directed mutagenesis, HPLC, and real-time ESI-MS. Biochim. Biophys. Acta 1804, 668–75. doi:10.1016/j.bbapap.2009.10.015

Tanaka, S., Ichikawa, A., Yamada, K., Tsuji, G., Nishiuchi, T., Mori, M., Koga, H., Nishizawa, Y., O'Connell, R., Kubo, Y., 2010. HvCEBiP, a gene homologous to rice chitin receptor CEBiP, contributes to basal resistance of barley to *Magnaporthe oryzae*. BMC Plant Biol. 10, 288. doi:10.1186/1471-2229-10-288

Thamthiankul, S., Suan-Ngay, S., Tantimavanich, S., Panbangred, W., 2001. Chitinase from *Bacillus thuringiensis* subsp. *pakistani*. Appl. Microbiol. Biotechnol. 56, 395–401. doi:10.1007/s002530100630

Trouvelot, S., HÃ©loir, M.-C., Poinssot, B., Gauthier, A., Paris, F., Guillier, C., Combier, M., TrdÃ¡, L., Daire, X., Adrian, M., 2014. Carbohydrates in plant immunity and plant protection: roles and potential application as foliar sprays. Front. Plant Sci. 5. doi:10.3389/fpls.2014.00592

Tsujibo, H., Orikoshi, H., Tanno, H., Fujimoto, K., Miyamoto, K., Imada, C., Okami, Y., Inamori, Y., 1993. Cloning, sequence, and expression of a chitinase gene from a marine bacterium, *Altermonas* sp. strain O-7. J. Bacteriol. 175, 176–81.

Uchiyama, T., Katouno, F., Nikaidou, N., Nonaka, T., Sugiyama, J., Watanabe, T., 2001. Roles of the exposed aromatic residues in crystalline chitin hydrolysis by chitinase A from *Serratia marcescens* 2170. J. Biol. Chem. 276, 41343–41349. doi:10.1074/jbc.M103610200

Umemoto, N., Ohnuma, T., Mizuhara, M., Sato, H., Skriver, K., Fukamizo, T., 2013. Introduction of a tryptophan side chain into subsite +1 enhances transglycosylation activity of a GH-18 chitinase from *Arabidopsis thaliana*, AtChiC. Glycobiology 23, 81–90. doi:10.1093/glycob/cws125

Umemoto, N., Ohnuma, T., Osawa, T., Numata, T., Fukamizo, T., 2015. Modulation of the transglycosylation activity of plant family GH18 chitinase by removing or introducing a tryptophan side chain. FEBS Lett. 589, 2327–2333. doi:10.1016/j.febslet.2015.07.018

Usui, T., Hayashi, Y., Nanjo, F., Sakai, K., Ishido, Y., 1987. Transglycosylation reaction of a chitinase purified from *Nocardia orientalis*. Biochim. Biophys. Acta 923, 302–9.

Vaaje-Kolstad, G., Horn, S.J., Sørlie, M., Eijsink, V.G.H., 2013. The chitinolytic machinery of *Serratia marcescens* - A model system for enzymatic degradation of recalcitrant polysaccharides. FEBS J. 280, 3028–3049. doi:10.1111/febs.12181

Vaaje-Kolstad, G., Horn, S.J., Van Aalten, D.M.F., Synstad, B., Eijsink, V.G.H., 2005. The non-catalytic chitin-binding protein CBP21 from *Serratia marcescens* is essential for chitin degradation. J. Biol. Chem. 280, 28492–28497. doi:10.1074/jbc.M504468200

Vaaje-Kolstad, G., Westereng, B., Horn, S.J., Liu, Z., Zhai, H., Sørlie, M., Eijsink, V.G.H., 2010. An oxidative enzyme boosting the enzymatic conversion of recalcitrant polysaccharides. Science. 330, 219–222. doi:10.1126/science.1192231

Van Aubel, G., Cambier, P., Dieu, M., Van Cutsem, P., 2016. Plant immunity induced by COS-OGA elicitor is a cumulative process that involves salicylic acid. Plant Sci. 247, 60–70. doi:10.1016/j.plantsci.2016.03.005

Varum, K., Smidsrød, O., 2006. Chitosans, in: food polysaccharides and their applications. CRC Press, pp. 497–520. doi:10.1201/9781420015164.ch14

Venkatesan, J., Kim, S.-K., 2010. Chitosan composites for bone tissue engineering--an overview. Mar. Drugs 8, 2252–66. doi:10.3390/md8082252

Wan, J., Zhang, X.-C., Neece, D., Ramonell, K.M., Clough, S., Kim, S. -y., Stacey, M.G., Stacey, G., 2008. A LysM Receptor-Like Kinase Plays a Critical Role in Chitin Signaling and Fungal Resistance in *Arabidopsis*. Plant Cell Online 20, 471–481. doi:10.1105/tpc.107.056754

Wan, J., Zhang, X.-C., Stacey, G., 2008. Chitin signaling and plant disease resistance. Plant Signal. Behav. 3, 831–3. doi:10.4161/psb.3.10.5916

Wang, M., Chen, Y., Zhang, R., Wang, W., Zhao, X., Du, Y., Yin, H., 2015. Effects of chitosan oligosaccharides on the yield components and production quality of different wheat cultivars (*Triticum aestivum* L.) in Northwest China. F. Crop. Res. 172, 11–20. doi:10.1016/j.fcr.2014.12.007

Wang, S.L., Chang, W.T., Lu, M.C., 1995. Production of chitinase by *Pseudomonas aeruginosa* K-187 using shrimp and crab shell powder as a carbon source. Proc. Natl. Sci. Counc. Repub. China. B. 19, 105–12.

Wang, W., Li, S., Zhao, X., Du, Y., Lin, B., 2008. Oligochitosan induces cell death and hydrogen peroxide accumulation in tobacco suspension cells. Pestic. Biochem. Physiol. 90, 106–113. doi:10.1016/j.pestbp.2007.10.003

Warm, E., Laties, G.G., 1982. Quantification of hydrogen peroxide in plant extracts by the chemiluminescence reaction with luminol. Phytochemistry 21, 827–831. doi:10.1016/0031-9422(82)80073-3

Watanabe, T., Ariga, Y., Sato, U., Toratani, T., Hashimoto, M., Nikaidou, N., Kezuka, Y., Nonaka, T., Sugiyama, J., 2003. Aromatic residues within the substrate-binding cleft of *Bacillus circulans* chitinase A1 are essential for hydrolysis of crystalline chitin. Biochem. J. 376, 237–244. doi:10.1042/BJ20030419

Watanabe, T., Kimura, K., Sumiya, T., Nikaidou, N., Suzuki, K., Suzuki, M., Taiyoji, M., Ferrer, S., Regue, M., 1997. Genetic analysis of the chitinase system of *Serratia marcescens* 2170. J. Bacteriol. 179, 7111–7117.

Wen, C.-M., Tseng, C.-S., Cheng, C.-Y., Li, Y.-K., 2002. Purification, characterization and cloning of a chitinase from *Bacillus* sp. NCTU2. Biotechnol. Appl. Biochem. 35, 213–9. doi:10.1042/

Woo, C.J., Yun, U.J., Park, H.D., 1996. Isolation of chitin-utilizing bacterium and production of its extracellular chitinase. J. Microbiol. Biotechnol. 6, 439–444.

Wu, C.-T., 2003. Class I chitinase and -1,3-glucanase are differentially regulated by wounding, methyl jasmonate, ethylene, and gibberellin in tomato seeds and leaves. Plant Physiol. 133, 263–273. doi:10.1104/pp.103.024687

Wu, T., Zivanovic, S., Hayes, D.G., Weiss, J., 2008. Efficient reduction of chitosan molecular weight by high-intensity ultrasound: Underlying mechanism and effect of process parameters. J. Agric. Food Chem. 56, 5112–5119. doi:10.1021/jf073136q

Xing, R., Liu, S., Yu, H., Guo, Z., Wang, P., Li, C., Li, Z., Li, P., 2005. Salt-assisted acid hydrolysis of chitosan to oligomers under microwave irradiation. Carbohydr. Res. 340, 2150–2153. doi:10.1016/j.carres.2005.06.028

Yang, T.L., 2011. Chitin-based materials in tissue engineering: Applications in soft tissue and epithelial organ. Int. J. Mol. Sci. doi:10.3390/ijms12031936

Yin, H., Du, Y., Dong, Z., 2016. Chitin oligosaccharide and chitosan oligosaccharide: Two similar but different plant elicitors. Front. Plant Sci. 7, 522. doi:10.3389/fpls.2016.00522

Yin, H., Li, Y., Zhang, H.-Y., Wang, W.-X., Lu, H., Grevsen, K., Zhao, X., Du, Y., 2013. Chitosan oligosaccharides–triggered innate immunity contributes to oilseed rape resistance against *Sclerotinia sclerotiorum*. Int. J. Plant Sci. 174, 722–732. doi:10.1086/669721

Yin, H., Zhao, X., Du, Y., 2010. Oligochitosan: A plant diseases vaccine—A review. Carbohydr. Polym. 82, 1–8. doi:10.1016/j.carbpol.2010.03.066

Yoksan, R., Akashi, M., Miyata, M., Chirachanchai, S., 2004. Optimal gamma-ray dose and irradiation conditions for producing low-molecular-weight chitosan that retains its chemical structure. Radiat. Res. 161, 471–80.

Zakariassen, H., Hansen, M.C., Joranli, M., Eijsink, V.G.H., Soorlie, M., 2011. Mutational effects on transglycosylating activity of family 18 chitinases and construction of a hypertransglycosylating mutant. Biochemistry 50, 5693–5703. doi:10.1021/bi2002532

Zeng, L., Velásquez, A.C., Munkvold, K.R., Zhang, J., Martin, G.B., 2012. A tomato LysM receptor-like kinase promotes immunity and its kinase activity is inhibited by AvrPtoB. Plant J. 69, 92–103. doi:10.1111/j.1365-313X.2011.04773.x

Zipfel, C., Felix, G., 2005. Plants and animals: a different taste for microbes? Curr. Opin. Plant Biol. 8, 353–60. doi:10.1016/j.pbi.2005.05.004