Production of chitooligosaccharides by different approaches and testing their plant strengthening activity in tomato

Thesis submitted for the degree of
DOCTOR OF PHILOSOPHY

by

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CERTIFICATE

This is to certify that **Mr. Rajesh Rao Maddu** 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 **"Production of chitooligosaccharides by different approaches and testing their plant strengthening activity in tomato"** for submission for the degree of Doctor of Philosophy of the University.

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This is to declare that the work embodied in this thesis entitled **"Production of chitooligosaccharides by different approaches and testing their plant strengthening activity in tomato"** 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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3.	PL 801	Lab Work	4	Pass
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Rajesh Rao M

Contents

Abbreviations and Symbols

ABA	Abscisic acid
BSA	Bovine Serum Albumin
CC	Colloidal chitin
CCR	Cinnamoyl-CoA reductase
COS	Chitin/Chitosan Oligosaccharides
DA or FA	Degree of Acetylation or Fraction of Acetylation
DDA	Degree of DeAcetylation
DP	Degree of Polymerization
ETRII	Electron transport rate
FESEM	Field emission scanning electron microscopy
F_m	Maximum florescence levels measured in dark
F_m'	Maximum florescence levels measured in light
Fs	Steady state florescence
G	Gram
GHs	Glycosyl hydrolases
GlcN	Glucosamine
GlcNAc	N-acetyl-D-glucosamine
HPLC	High Performance Liquid Chromatography
HPTLC	High Performance Thin Layer Chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
JA	Jasmonic acid
kb	kilobase
kDa	kilo Dalton
L	Litre
LB	Luria-Bertani
LOX	Lineolate lipoxygenase
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar
MW	Molecular Weight

OD	Optical Density
PA	Pattern of N-acetylation
PAL	Phenylalanine Ammonia-lyase
PCR	Polymerase Chain Reaction
PAGE	Polyacrylamide Gel Electrophoresis
POD	Peroxidase
PPO	Polyphenol Oxidase
qL	Redox state of PSI
qN	Coefficient of non-photochemical quenching
qP	Coefficient of photochemical quenching
Rpm	Revolutions per minute
S	Second (s)
SDS	Sodium Dodecyl Sulphate
SSF	Solid state fermentation
TG	Transglycosylation
TLC	Thin layer chromatography
*T*m	Transition temperature/ Melting temperature
Y (II)	Efficient quantum yield
Y(NO)	Non-regulated energy dissipation
Y(NPQ)	Non regulated non-photochemical quenching
µM	Micromolar
°C	Degree Centigrade/ Degree Celsius
µg	Microgram
µL	Microlitre

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Dedicated to my beloved parents

and sister

Chapter I

Introduction

Introduction

1.1 Chitin, chitosans and chito-oligosaccharides (COS)

Chitin is a β (1→4) homopolymer linked to N-acetyl-D-glucosamine (NAG) units, and it is the second most abundant polymer in nature, next to cellulose. Chitin is a principal structural component in the fungal cell wall, the exoskeleton of insects and nematodes, and the outer shell of crustaceans. Chitin is highly insoluble in water because of its hydrophobic nature due to acetyl groups and exists in three different forms. Among the three types of chitin, namely α-chitin, β-chitin, and γ-chitin, the most stable and crystalline form is α-chitin. It exhibits a well-organized structure with polymer chains oriented in an antiparallel manner with strong hydrogen bonding that mainly exists in shrimps and crabs. In the case of β-chitin, chitin fibers are arranged in parallel form (Gardner and Blackwell, 1975) and exclusively exist in squid pens. The β-chitin is smooth and less rigid because of its weak hydrogen bonds; hence, β-chitin reacts with the chemical solvents (Kurita et al. 1994). The γ-chitin contains both α and β structures (Rinaudo. 2006) and is present mainly in the cell walls of some fungi. Chitosans are the deacetylated forms of chitin and are cationic in nature. The enzymes that convert chitin to chitosan are chitin deacetylases (CDA). In nature, fungi belonging to Zygomycetes family and other fungi which express CDAs are a rich source of chitosan. The fungal cell wall of *Mucor rouxii* possesses high amount of chitosan (Synowiecki and Al- khateeb, 2003). Chitosan is water soluble and is classified based on the degree of polymerization (DP), degree of acetylation (DA), and pattern of acetylation (PA) (Aam et al. 2010; Basa et al. 2020). Some pathogenic fungi can convert chitin in their cell wall to chitosan with the help of CDA for protection against plant defence response (Gueddari et al. 2002).

Chitinolytic microbes play a pivotal role in chitin recycling (Kurita et al. 2000), and they possess a great diversity of chitin-degrading enzymes. Chitinases and chitosanases act on chitin and chitosan, and produce chitooligosaccharides (COS). COS possess special properties like biocompatibility, biodegradability and are also bioactive (Synowiecki and Al-Khateeb 2003; Jayakumar et al. 2010). Because of these special properties, COS have many applications in different fields that include metal uptake, tissue engineering, wound dressing, water purification, drug delivery, food and environment, chemical modification, pharmaceuticals, nano-technology, medicine and agriculture (Das et al. 2015). COS can be synthesized using various methods, including physical, chemical, and enzymatic approaches. The physical techniques include (Mourya et al. 2011) irradiation, ultra-sonication, microwave-assisted hydrolysis, and high-

pressure homogenization, etc. Chemical methods include use of acids like sulphuric acid and hydrochloric acid to dissolve the calcium carbonate in the chitin shells. Acid-treated chitin shells are later treated with alkali to remove the protein and finally treated with sodium hypochlorite to make it odorless and colorless chitin, useful for COS production. The enzymatic method includes using chitinases or chitosanases produced by recombinant DNA technology and overexpressed in yeast or bacteria (Zeng et al. 2002).

The enzymatic method includes the use of chitinases or chitosanases that are produced by recombinant DNA technology and overexpressed in yeast or bacteria (Zeng et al. 2002). Chitin degradation by enzymatic method is advantageous over harsh chemicals method as it is eco-friendly. In our lab, a library of recombinant chitinases and a range of mutants was generated from different chitinolytic bacteria including *Bacillus thuringiensis*, *B. licheniformis*, *Paenibacillus elgii*, *Enterobacter cloacae*, *Flavobacterium johnsoniae*, *Serratia marcescens*, *S. proteamaculans* and *Chitiniphilus shinanonensis* etc, analyzed their mode of action on chitinous substrates and COS production (Neeraja et al. 2010; Purushotham et al. 2012; Madhuprakash et al. 2012; Das et al. 2016; Mallakuntla et al. 2017; Vaikuntapu et al. 2018; Bhuvanachandra et al. 2020). Most of the chitinases are either endo- or exo-acting enzymes. Chitobiose (DP2) stands out as the primary outcome when processive chitinases act upon chitinous substrates. To generate longer DP COS, suitable for biological uses, it becomes necessary to employ a non-processive endo-chitinase with a defined specificity or a chitinase exhibiting transglycosylation (TG) activity (Fukamizo et al. 2011; Ohnuma et al. 2011; Purushotham and Podile, 2012; Mallakuntla et al. 2017; Vaikuntapu et al. 2018). Certain chitinases exhibit TG along with hydrolytic function, facilitating the formation of fresh glycosidic bonds between donor and acceptor sugar molecules. This characteristic of chitinases can be harnessed for the synthesis of longer chain COS. In agriculture, long chain COS appear to be useful molecules that have the tendency to elicit immune response in plants.

1.2 Solid state fermentation (SSF)

Solid state fermentation (SSF) is a process in which solid substrate is used. In SSF, less amount of water is used without affecting the moisture levels required for the optimum growth and metabolism of the microbes. The substrate used in SSF can be a source of energy or a supportive material added with required nutrients that are necessary for proper growth of microorganisms. SSF is highly compatible for microbes, as it mimics the natural habitat for microbes (Rahardjo et

al. 2006). The main advantages with the use of SSF are the low water requirement, thin surface area, easy circulation of air, small fermenter size, perfect support for the microorganisms, use of mixed cultures, easy downstream processes and high yield of products (Holker et al. 2005; Nigam et al. 2009).

Utilization of low cost agricultural waste and shrimp waste in SSF can produce high value economical products, and indirectly overcomes the problem of disposal of chitin waste and agro-industrial waste that could cause environmental pollution. There is a rapid out flow of shellfish waste from the industries during the processing of the seafood meat. This shellfish waste is of low value and it faces problem of collection and proper disposal, leading to water pollution. Attempts to utilize the shrimp waste by using chemical methods led to environment pollution (Mao et al. 2017). So, there is a need for more efficient method(s) in the utilisation of shrimp waste. The most common biological method that could be implemented for low cost waste such as shrimp waste could be SSF (Abu et al. 2017). In the SSF, microbes can be used on shrimp waste to produce high value products without damaging the environment. This method is quite helpful as an alternative to chemical process to obtain COS products, secondary metabolites and enzymes from shrimp waste. SSF was successfully used for the production of industrially important secondary metabolites, enzymes, hormones and antibiotics (Hernandez et al. 1992). Till now, limited studies were done on use of shrimp shell waste as substrate in SSF (Setiawan et al. 2021).

1.3 Agro-industrial residues and SSF

Most of the agricultural residues are used as animal feed due to their nutritive value with good amount of proteins, carbohydrates and trace elements. At the same time these are also an excellent nutrient sources for microorganisms. When used in fermentation process microbes have developed the ability to recycle the wastes (Nguyen et al. 2010). Common agro-industrial wastes used in SSF include leaves, stem shell, husks, bagasse, stalk etc. Various agro-industrial residues namely, ground nut shells, wheat bran, paddy straw, sugarcane bagasse, sorghum straw and rice husk, corn cobs etc., are the most inexpensive and high energy rich substrates for fermentation industry. These agricultural wastes are often converted to value added products as an outcome of the waste management (Brzezinska et al. 2008). The selected substrate must support the growth of the organisms and has a cruitial role in the production of economically important products of interest.

1.4 Fungal biomass as source of chitin

Fungal biomass serves as a significant reservoir of chitin. This biomass can be obtained through the process of SSF. In certain fungi, such as *Aspergillus niger* and *Mucor rouxii*, chitin comprises up to 45% of their cell wall composition, while in *Penicillium notatum*, it accounts for about 20% of the cell wall (Kaur and Dhillon, 2014). *Fusarium oxysporum* and *Alternaria alternata* are saprophytic fungi that are abundant in the soil and contribute to decomposition of organic matter. Some of them are known to be pathogenic to plants. The fungal cell wall consists of 65% carbohydrate, 7.2% protein, 5.4% lipid and major component being chitin (40%). The cell walls of *Fusarium oxysporum,* when digested with chitinases, revealed that they contain acetylated chitosan, NAG disaccharide and deacetylated COS (Fukamizo et al. 1992) as the products, indicating that the NAG residues along with deacetylated glucosamine residues are present in the chitinous polysaccharide. The primary cell wall of *Alternaria* mainly contains chitin, β-1,3 mannoproteins and phosphomannan (De-Hoog and Guarro, 1995). Recent studies have been focussing on use of bio-wastes as an inexpensive carbon source for fungi culturing for COS production (Kannan et al. 2010).

1.5 Shrimp waste as chitin source

The demand for sea food consumption has increased several folds in recent times. This high demand led to the growth of many seafood processing units (Yadav et al. 2019). New advanced technologies in aquaculture have increased the shell fish production which includes crab, shrimp and krill (Hamed et al. 2016). A million tons of fish shell waste is being produced annually by seafood processing units. The main waste from the shellfish industry includes the head, shell and tail portion of shrimp which constitutes 45-60% of the discarded waste (Mao et al. 2017). The occurrence of high amounts of shrimp waste has generated interest in chitin, most abundant polysaccharide in the ocean ecosystem (Shahidi and Synowiecki, 1991; Biswas and Gargi, 2013; Kaur and Dhillon, 2015).

Chitin is extracted from shrimp waste by a two-step process that includes the removal of minerals (calcium carbonate) and proteins (Wahyuntari and Setyahadi, 2011). Shrimp waste is preferred over shellfish (like lobsters and crabs) because of the thinner shell wall (Bautista et al. 2001; Thirunavukkarasu and Shanmugam, 2009), which allows high efficiency of chitin extraction (Percot et al. 2003). Many enzymatic and chemical methods are used to obtain chitin from shrimp waste (Beaney et al. 2005). The use of sodium hydroxide (NaOH) for deproteinization resulted

in partial deacetylation, hydrolysis and also a decrease in the molecular weight of chitin (Younes and Rinaudo, 2015). Similarly, treatment with harsh acids on chitin has led to high ash content and also degradation of polymer. However, chemical treatment has certain problems due to high temperatures and use of concentrated acids which would lead to chitin deacetylation and depolymerisation (Charoenvuttitham et al. 2006).

Shrimp shell waste has been extensively studied for its potential to produce bioactive monosaccharides and oligosaccharides for long (Barran et al. 1975). Compounds, such as NAG, found applications in the cosmetics industry and also as an important nutritional supplement. Moreover, shrimp shell waste is a favourable substrate for synthesizing chitinases by Actinomycetes and various bacterial species, surpassing colloidal chitin in its effectiveness. Rattanakit et al. (2007) suggested that shrimp shell waste demonstrates excellent suitability as a culture medium for *Aspergillus* sp., resulting in the synthesis of comparable or higher levels of chitinolytic enzymes compared to the fungus grown on chitin-supplemented media. Wang et al. (1995) conducted a study highlighting the chitinolytic activity of bacteria from the genus *Pseudomonas*, utilizing shrimp and crab shell waste as substrates for chitinase production.

1.6. Microbes used for SSF

1.6.1 Serratia proteamaculans

S. proteamaculans 568 belong to family of Enterobacteriaceae, a root endophyte isolated from *Populus* (Taghavi et al. 2009). CAZY database (Henrissat and Davies, 1997) showed that *S. proteamaculans* has four chitinases (*Sp*ChiA, *Sp*ChiB, *Sp*ChiC and *Sp*ChiD), three chitin binding proteins (*Sp*CBP21, *Sp*CBP28 and *Sp*CBP50) and one N-acetylhexosaminidase (Purushotham et al. 2012). Out of four chitinases, three of them (*Sp*ChiA, *Sp*ChiB and *Sp*ChiC) showed good activity on β-chitin whereas, *Sp*ChiD had better activity on chitin/chitosan insoluble substrates. Enzymatic treatment of oligomeric and polymeric substrates by *Sp*ChiA, *Sp*ChiB and *Sp*ChiC has yielded DP2 as major end product whereas, *Sp*ChiD generated DP1 as major end product. Interestingly, *Sp*ChiD possessed high TG activity and was able to produce higher chain length COS from lower DP substrates (Purushotham and Podile, 2012). Moreover, partially purified *Sp*ChiD produced COS showed elicitor activity in plants (Madhuprakash et al. 2015; Ramakrishna et al. 2021). Chitin binding proteins of *S. proteamaculans* (*Sp*CBP21 and *Sp*CBP50) can bind effectively to β-chitin and synergistically work together with *Sp*ChiB in

chitin degradation (Manjeet et al. 2013). Since this organism possesses efficient chitinases and chitin binding proteins, *S. proteamaculans* was selected for the generation of COS through SSF.

1.6.2 Streptomyces sps.

Many soil bacteria mainly actinobacteria are known to degrade the chitin and contribute in the recycling of carbon and nitrogen in the soil. A good number of *Streptomyces* species are known for their chitinolytic activity and are predominantly found in the chitin-rich soils. Chitin is a precious source of nutrient in soil environment and *Streptomyces* are known to effectively utilise chitin to combat competition with other living microbes in their soil niche. *Streptomyces* are mycelial bacteria that live in soil and secrete a lot of proteins and secondary metabolites, including vital antibiotics. Actinomycetes, mainly *Streptomyces* sps. are used extensively in secondary metabolites production and commercial important enzymes that have a wide application in medicine and agriculture (Kumar and Gupta, 2006). Several chitinolytic enzymes have been identified in several *Streptomyces* spp. are known to produce many chitinases (Joo, 2005). Earlier reports on soil isolates of *S. coelicolor* A3 showed that chitin amendment to soil has drastically induced the chitin-related catabolic genes along with genes for primary and secondary metabolism (Hunger et al. 2006). Chitin amendment has increased the number count of actinobacteria mainly *Streptomyces* and was used as a biocontrol agent (Kong et al. 2001). Certain actinobacteria are even known to penetrate and degrade the chitin hyphal walls of pathogenic fungi by producing chitinase and antifungal compounds (Gomes et al. 2000). Antifungal activities of chitinases from *Streptomyces* against phytopathogenic fungi have been well documented. Majority of chitinases found in soils are thought to be produced by *Streptomyces* strains. For many *Streptomycetes*, chitin is a significant source of nutrients, and these microbes have evolved sophisticated extracellular systems for chitin utilisation (Chater et al. 2010).

1.7 Chitinase and transglycosylation (TG)

Chitinases that act on chitin are present in bacteria, actinomycetes, fungi, animals and higher plants (Brzezinska et al. 2008). Chitinases break the glycoside bonds between the sugar moieties hence known as glycoside hydrolases (GH) (Lombard et al. 2014). Chitinases belong to two families, GH18 and GH19 based on their sequence of amino acids and the mode of action. GH19 chitinases are exclusively present in plants and GH18 are restricted to bacteria, fungi and animals. Family 18 chitinases are known to act by a double displacement mechanism. The carbonyl

oxygen atom in the carbonyl group of the N-acetyl molecules occupying at '-1' sub-site act as the nucleophile that leads to the intermediate of an oxazolinium ion. Since the N-acetyl group is involved in catalysis, binding substrate requires GlcNAc in '-1' sub-site (Zakariassen et al. 2009). Higher chain length COS are synthesized by chitinases with TG properties.

TG is the transfer of a sugar residue from one glycoside to another, catalyzed by GH18 chitinases, and is usually completed in two steps. The first step is the formation of oxazolinium ions. In the second step, a water molecule which ultimately leads to hydrolysis attacks the oxazolinium ion intermediate. If a water molecule is outcompeted by another sugar molecule then TG takes place (Zakariassen et al. 2009). Chitinases from a variety of bacterial sources have been reported to have TG activity. For example, *Nocardia orientalis* chitinase was used to convert $GlcNAc_4$ substrate to $GlcNAc_6$ by saturated ammonium sulphate (Nanjo et al. 1989). *Sp*ChiD from *S. proteamaculans* showed higher TG activity with $GlcNAc_{3–6}$ substrates producing higher $GlcNAc_{7–13}$ products, later got degraded to smaller GlcNAc after 90 min of incubation. (Purushotham and Podile, 2012). Our lab has performed extensive research on the TG activity possessing enzymes from different bacteria. TG activity of *Stm*ChiA from *Stenotrophomonas maltophilia was* reported by Suma and Podile (2013). Mallakuntla et al. (2017), reported TG activity of *Ec*Chi1 from *Enterobacter cloacae* subsp. *cloacae.* TG activity of *Sm*ChiD from *S. marcescens* and *Cs*ChiL from *Chitiniphilus shinanonensis* were also reported generating products were DP6, DP7 and DP8 (Vaikuntapu et al. 2018; Bhuvanachandra et al. 2020). Among all the reported TG chitinases, *Sp*ChiD was the most potential enzyme to produce long chain COS.

1.8 Improvement of TG, use of ionic liquids, osmolytes and humectants

Higher chain length COS are of importance because of their ability to elicit immune response in plants. COS of higher chain length can be obtained by TG activity and by the action of specific or nonspecific enzymes on chitosan substrates of different DA. The acetyl group plays a key role in the elicitation of immune responses in plants (Petutschnig et al. 2010). At least five acetyl groups are strictly needed for binding COS and eliciting the immune response (Cord et al. 2016). DA in chitosan substrate plays an important role in plant defence response. Completely deacetylated COS molecules could not bind to plant receptors and elicit immune responses (Li et al. 2020). The immune system in rice gets activated by reactive oxygen species (ROS) generation when COS has at least five acetyl groups (Cord et al. 2016). In addition, Gubaeva et al. (2018) showed the impact of DA and DP of COS in triggering defence response in *Arabidopsis thaliana.*

They showed that DA 0% and DP 3-8 were not able to elicit any elicitor response. However, COS with partial acetylation of 1 to 60% DA were able to generate a response. DP of minimum 9 and more (DP14) plays a key role on the activity and COS absorption (Tanaka et al. 1997; Chae et al. 2005; Zeng et al. 2008).

A few GH18 chitinases exhibit both the primary hydrolytic activity and the intrinsic TG activity *via* the creation of a fresh glycosidic bond. TG involves the transfer of a sugar unit to another sugar. Retaining GHs with TG activity can be employed to selectively and regionally synthesise a glycosidic bond (Ly and Withers, 1999). Glycosylation and deglycosylation are two processes that can be used to explain the two-fold displacement mechanism used by GH18 chitinases to catalyse reactions. The creation of the enzyme-substrate complex during the glycosylation stage will eventually result in the synthesis of the intermediate oxazolinium ion, thereafter, during the deglycosylation phase the reaction depends on the nature of the acceptor. When sugar molecules are used as the acceptor instead of water, TG will take place resulting in the creation of a larger chain length COS (Bissaro et al. 2015). Inherent TG was reported in few chitinases like *At*ChiC in *A. thaliana* (Ohnuma et al. 2011), *Ac*MNPV in *Autographa california* (Fukamizo et al. 2011), *Bc*ChiA1 in *Bacillus circulans* WL-12 (Sasaki et al. 2002), chitotriosidase in human macrophages (Aguilera et al. 2003), *Ci*X1 in *Coccidioides immitis* (Fukamizo et al. 2001), *Cr*ChiA in *Cycas revoluta* (Taira et al. 2009), *Ec*Chi1 in *Enterobacter cloacae* subsp. *Cloacae* (Mallakuntla et al. 2017), *Nt*ChiV from *Nicotiana tabacum* (Ohnuma et al. 2011), *Sp*ChiD in *S. proteamaculans (*Purushotham and Podile, 2012), *Sm*ChiD in *S. marcescens* (Vaikuntapu et al. 2018), and *Vc*ChiA in *Vibrio carchariae* (Suginta et al. 2005). The chitinases with TG are extremely helpful in the synthesis of longer chain COS.

Characterization of chitinases is important to identify the optimal conditions for activity more importantly the TG activity. Indeed, enzyme engineering has been used to enhance the TG activity of chitinases and simultaneously decrease hydrolysis, mainly by site-directed mutagenesis (SDM). Using SDM, mutant enzymes with significantly enhanced TG/hydrolysis (T/H) ratio of chitinases were developed (Zakariassen et al. 2009; Madhuprakash et al. 2012). Amino acid mutations at the active site, substrate-binding cleft, and also in solvent accessible regions of *Sp*ChiD significantly enhanced the TG activity. These mutations have increased the amount of TG products and also increased the duration of TG activity (Madhuprakash et al. 2012; 2014; 2015). The TG activity was also improved by varying the substrate concentration and water

availability and changing the reaction conditions with different solvents like ionic liquids, osmolytes and humectants (Badola et al. 2017).

Salts that remain in liquid form and do not crystallize at room temperature are known as ionic liquids. These are polar, non-volatile and are non-flammable in nature. Ionic liquids tend to decrease hydrolysis in TG acting enzymes (Lang et al. 2006). Ionic liquids have low melting point and are thermally stable and are known as green solvents as they are not flammable. They are also called designer solvents because the properties of the ionic solvent like polarity, hydrophobic nature, relative density and viscosity can be altered by cation and anions present in it. They can be recycled and reused many times. Earlier reports suggest that enzyme activity can be altered by the properties of ionic liquid solvents (Yang and Pan, 2005). Immiscible forms of ionic liquid in water are known to act as best medium for enzyme-catalyzed reactions, because the bulkier hydrophobic alkyl chain has the capacity to pull of the water molecules from the catalytic site of the enzyme. High viscous ionic liquid tends to decrease the reaction rate mainly due to the limits in mass-transfer. A key factor *i.e.*, the water in the reaction medium, affects the glycosidase-catalyzed synthesis. In this context, ionic liquids could be used to alter the water activity and thereby secondary hydrolysis of the glycosidase-catalyzed reactions to enhance the yield of TG products.

Osmolytes are biological molecules that help in the protection of enzymes under stress condition. Common osmolytes include proline, glycerol, trehalose, glycine betaine urea, sucrose etc. Osmolytes are known to remove water molecules from interacting surfaces, catalytic sites and binding clefts with in the enzymes. Osmolytes, water-binding humectants such as glycerol lower water activity and are widely used in several industrial applications. Water activity plays a key role in steering hydrolysis and TG of GH18 chitinases. So, considering osmolytes and humectants as ingredients of reaction might open up new directions to improve the TG of chitinases.

1.9 Pre-treatment of chitinous substrates to increase COS production

The method of pre-treatment to dissolve chitin may vary based on the intended end product, and also the start material including chitin flakes, chitin powder, chitosan, or chitin nanofibers. Chitin can be dissolved through a process known as acidic hydrolysis. This method involves the treatment of chitin with strong acids like hydrochloric acid (HCl) or sulphuric acid, at elevated temperatures. The purpose of this treatment is to break down chitin into soluble components, including individual N-acetyl glucosamine monomers. One important application of acidic

hydrolysis is the production of chitosan. To achieve optimal results, a concentrated HCl solution with a concentration of 38% (w/w) is typically employed to dissolve the chitin. The resulting solution is then refluxed at approximately 100 °C for a minimum of 90 min to ensure a high yield of glucosamine hydrochloride (Gandhi and Laidler, 2002; Novikov, 2004; Mojarrad et al. 2007).

Alkaline hydrolysis is a method that can be used to dissolve chitin. In this process, chitin is subjected to high temperatures and treated with a strong base like potassium hydroxide (KOH) or sodium hydroxide (NaOH). Under these alkaline conditions, chitin undergoes deacetylation, resulting in the conversion of chitin into chitosan. The removal of acetyl groups from the chitin is known as deactylation reaction, allowing chitosan to become soluble in the alkaline solution (Synowiecki and Al-khateeb, 2003). Enzymatic hydrolysis is a technique utilized for chitin dissolution, involving the use of chitinases, enzymes responsible for breaking down chitin into degradation products like COS and NAG (Nakagawa et al. 2011). Tailored enzymes, such as chitinase or protease, are employed to achieve a controlled and gentle dissolution of chitin, making this method desirable (Villa-Lerma et al. 2016).

Ionic liquids, which are molten salts with low melting points, present a promising solution for the environmentally friendly and sustainable dissolution of chitin. The ionic liquid selection depends upon the dissolved chitin application (Wu et al. 2008). It was found that 1-butyl-3-methylimidazolium acetate ([BMIM]Ac) was an excellent solvent for native chitins from various sources. Jaworska et al. (2016) attempted to dissolve chitins in 1-butyl-3-methylimidazolium chloride ([BMIM]Cl) unsuccessfully. Whereas, Xie et al. (2006) reported successful dissolution of chitin and chitosan in [BMIM] Cl at concentration up to 10 %. These variations underscore the significance of carefully considering the specific ionic liquid and its compatibility with the targeted chitin dissolution process.

Many innovative pre-treatment methods have been identified to decrease the crystalline nature and improve the chitinases accessibility on chitin substrate. Pre-treatment methods are of three types and they are physical, chemical and microbial pre-treatment. Physical pre-treatment methods include gamma irradiation, mechanical milling, steam explosion and sub-critical liquids (Osada et al. 2012; Villa-Lerma et al. 2013). The chemical methods include ionic liquid, co-solvents and temperature cycling with alkaline solution (Fang et al. 2015; Husson et al. 2017). Microbial pre-treatment includes microbial fermentation to reduce the chitin crystallinity (Zhang et al. 2018). The main aim of different physical, chemical, and biotechnological approaches of various pre-treatment methods is to decrease the crystallinity and increase the solubility of chitin

(Zhou et al. 2019). Pre-treatment with ionic liquids and chemicals like acids has decreased the crystalline nature of chitin. Physical and biological methods were also more effective (Zhang et al. 2018). Ionic liquids are also used to dissolve chitin (Shimo et al. 2016). Ionic liquids can be used but are relatively expensive. Concentrated acids and ionic liquids cannot be used for industrial applications because of their hazardous nature, threat to the environment and high cost. In search of new methods that are more affordable and environmentally friendly, alkali and alkali in combination with urea are used for chitin pre-treatment.

1.10 Plant strengthening by COS

COS have attracted attention in recent years as an important molecule for agriculture as safer alternative to the expensive and toxic agrochemicals used for crop protection. COS can be applied as foliar spray (Lei et al. 2011) and can impart benefits to plant *via* enhancing plant growth and innate immunity. Application of COS has increased plant growth, germination of seeds, chloroplast and chlorophyll content, increase intake of nutrients and also helps in nitrogen fixation (Katiyar et al. 2014). Chemically synthesized plant growth regulators and chemical pesticides are very expensive and also harmful to the environment. Hence, there has been a desperate search for naturally occurring molecules that could induce plant growth and proliferation (Falcón-Rodríguez et al. 2012) along with plant defence response. COS elicit different biological responses in plants (Hadwiger et al. 2013). Chitosan is known to be used as plant growth bio-stimulant (Pichyangkura et al. 2015) and is also known to act as an elicitor in response to pathogen (Pospieszny et al. 1991). COS help in growth of plants (Nge et al. 2006), increase biomass in plants (Wang et al. 2017), increase the contents of photosynthesis machinery (Zou et al. 2015), and also increase the nutrient content in plants (Dzung et al. 2011).

Like many organisms, plants do have innate immunity that responds to the microbial attack (Jones and Dangl, 2006). Unlike animals, which have mobile defence response cells, plants have an autonomous cellular event that gets activated on pathogen attack. The defence response remains in dormant state until the inducing agents come in contact (Mandal et al. 2010). The inducing agent can be a pathogen or externally applied elicitor. The defence response is much stronger when compared to the plant that have not got any contact with the inducing agent. The defence responses in plants include activation of octadecanoid pathway that leads to the expression of many transcription factors and jasmonic acid (Shah et al. 2014). The activation of transcription factors ultimately leads to the expression of defence responsive genes that include pathogeneses-related (PR) proteins and antioxidant enzymes (Davar et al. 2013). Foliar

application of chitin and chitosan for different purposes has been reported in plants. Foliar application of COS showed positive impact on the rate of photosynthesis in maize and soybean (Khan et al. 2002). Decrease in transpiration rate is due to increase in ABA levels in bean plants after COS treatment (Iriti et al. 2009). COS foliar application has led to decrease in diseases caused by bacteria, virus, fungi and also the damage due to the pests (Rabea et al. 2005). Foliar application of COS has controlled the powdery mildew pathogen in barley (Faoro et al. 2008).

Scope of the work

In view of the importance of COS, it is prudent to have methods for production of COS from the abundant chitinous waste in a less expensive and eco-friendly manner. Since higher chain length COS are the useful molecules that bind to the plant immune receptors and elicit plant defence response, exploring the TG activity of chitinases, solvents that may increase the TG activity and decreasing the hydrolytic activity could be attractive approaches. Methods that decrease the crystallinity of chitin may also improve ways to produce COS enzymatically. Against this background, the following objectives were framed to prepare COS and test them for plant strengthening activity:

1. solid state fermentation with chitinous substrates,
2. reaction engineering to enhance COS production using trans-glycosylating chitinase,
3. pre-treatment of crystalline chitin with KOH and KOH-Urea for enhanced hydrolysis/release of COS, and
4. testing the plant strengthening activity of the COS on tomato.

Chapter II

Materials and methods

2.1 Chemicals

Regular laboratory chemicals were procured from HiMedia labs, Sigma, Merck and SRL. Chemicals like potassium hydroxide. Urea, sodium chloride, imidazole, sodium citrate and citric acid, Luria-Bertani (LB) broth, *Streptomyces* agar, potato dextrose agar (PDA) were from HiMedia, and the solvents like sulphuric acid (H_2SO_4), hydrochloric acid (HCl) were from SRL while HPLC grade acetonitrile was obtained from Merck. Invitrogen supplied the Ni-NTA agarose. Mahtani Chitosan Pvt. Ltd. India provided the α-chitin and β-chitin. Isopropyl-β-D-thiogalactopyranoside (IPTG), antibiotics and chitosan of DA 37% and 61% were obtained from Sigma. Chitin and chitosan oligosaccharides with DP1-6 were procured from Seikagaku Company (Tokyo, Japan).

2.2 Kits

Total RNA isolation kit was obtained from Sigma (USA), and the cDNA synthesis kit was procured from Takara (Japan).

2.3 Enzymes

*Sp*ChiD-Y28A enzyme from *Serratia proteamaculans* (Madhuprakash, 2014)

*Pe*Chi3 enzyme from *Paenibacillus elgii* (Das, 2016)

*Cs*ChiG enzyme from *Chitiniphilus shinanonensis* (Sivaramakrishna et al. 2020a)

2.4 Microbial strains

Fungal strains: *Fusarium oxysporum* and *Alternaria alternata* were from our lab culture collection.

Bacterial strains: *S. proteamaculans* was extensively studied in our lab (Purushotham, 2011; Madhuprakash et al. 2012, Manjeet et al. 2014), and *Streptomyces* sp**.** was from the collection of strains received from Dr. Gopalakrishnan, International Crops Research Institute for Semi-Arid Tropics (ICRISAT), Patancheru, Telagnana, India.

2.5 Primers used

S. No	Gene	Forward primer (5'→3')	Reverse primer (3'→5')
1	β-1,3-glucanase	TGCCCCATTTCAAGTTCCTG	CGTGTATCCCTCAAAAACCCAAC
2	Phenyl alanine-ammonia lyase (PAL)	CGACTTGAGGCATTTGGAGGA	CAGGGGTCATCAGCATAGGT
3	Peroxidase	GCTGACATTCTTGCTCTTGCT	CGCTTGAAACTCGTCCATCT
4	Actin	TGATGGTGGGTATGGGTCAA	AGGGGCTTCAGTTAGGAGGAC
5	Tubulin	AGGTTTGTCACTCACTTGGAGG	ACATTCATCAGCGTTCTCTACT
6	Chitinase	TCACACAACTACAACTATGGGC	GCTTTGGGGATTGAGGAGTCA
7	Polyphenol oxidase (PPO)	ATTGGCGGGAAAAGAAGGGA	GTGGGCATTGGCGGGTAA
8	Catalase	TCGCAGAGAATGAACAACTCG	TGATGACCACACTTGGGAGC
9	Transcription factor R2 R3-MYB	AGATGGAGGAGTTAGCAATGATG	ATGTGAATGGAGGAGGACTTG
10	WRKY transcription factor 4	CAAGAATCCAGCCAAGCAAATG	CCCGTTCGTCAATGTCCCTTC
11	Cinnamoyl-CoA reductase	GCAGCAGAAACCAAAGTTCG	ATCACTCCAGCAAGTCTCGT
12	Linoleate 13S-lipoxygenase 3-1, chloroplastic	ATAGGGGAAAGACAGCAACCG	TAATACACCAGCACCACACCT

Table 2.1: Details of primers used for real-time PCR

2.6 Substrates

Paddy straw, groundnut shells, sorghum straw, sugarcane bagasse (procured locally), chitin flakes and β-chitin (Chitin India Pvt Ltd, Gujarat)

2.7. Media used

2.7.1 Luria-Bertani (LB) media

LB media consists of tryptone-10g, NaCl-10g, yeast extract-5g added to distilled water of 900ml and pH (7.2) was adjusted and final it was make up to 1.0 L.

2.7.2 Streptomyces agar media

Streptomyces agar media consists of malt extract-10g, yeast extract-4g, dextrose-4g, $CaCO_3$-2g and agar-12g and were added to 1.0 L of distilled water and autoclaved.

2.7.3 Potato dextrose agar (PDA)

Potato dextrose agar consists of potato infusion-200g, dextrose-20g, and agar-20g were added to 900ml of water and pH (5.6) was adjusted and final it was make up to 1.0 L.

2.7.4 Minimal medium

Minimal media consists of the following components per 1.0 L of media: Na_2HPO_4-0.065g, KH_2PO_4-1.5g, NaCl-0.25g, NH_4Cl-0.5g, $MgSO_4$-0.12g, $CaCl_2$-0.005g and pH adjusted to 7.0.

2.8. Methods

2.8.1 Phylogenetic analysis of bacterial isolates

Colony PCR was done for the amplification of 16s rRNA gene of ANU-34 and CAI-21 bacterial isolate. Single isolated colony was picked carefully and dissolved in autoclaved MilliQ water and then kept for boiling at 120°C for 10 min. Samples were centrifuged for 10 min at 13115 g and supernatant was used as template for PCR. EzBioCloud database was used to determine the phylogenetic neighbours and pairwise 16S rRNA gene sequence similarity was calculated (Yoon et al. 2017). From the database, the FASTA sequence with high pairwise sequence identity was taken, and CLUSTALX2 v. 2.0.12 was used in the multiple sequence alignment. Using DAMBE 6.4.42, paired deletion was used to handle every gap (Xia, 2017). Using neighbour joining of MEGA 7.0 (Kumar et al. 2016) with bootstrap replicates of 1000 and the Kimura 2-parameter model the trimmed sequences were used to construct a phylogenetic tree.

2.8.2. Detection of COS

2.8.2.1 Thin layer chromatography (TLC)

COS samples, each of (20 µl), were loaded on a to a silica gel plate (TLC silica gel 60 Merck Germany). The TLC chamber was earlier equilibrated using a solvent system in the ratio of (5:4:2:1) of n-butanol, methanol, 25% ammonia solution and water. The samples were loaded on TLC plate carefully and placed in a TLC chamber for separation. When the solvent front reached $3/4^{th}$ of the plate, the plate was removed from the chamber and left for air drying. The chromatogram was developed using a spraying solution of (400 µl of aniline, 400 mg of diphenylamine, 20 ml of acetone and 3ml of 85% phosphoric acid) and later heated with a hot air gun (Bosch Germany) at 180°C for 3 min to visualize the samples.

2.8.2.2 High performance liquid chromatography (HPLC)

COS from the reaction mixture were subjected to HPLC analysis (Shimadzu, Tokyo, Japan). For identification of COS, samples of 20 µl were injected into the Shodex amino P-50 4E column using a Hamilton syringe (Hamilton, Switzerland). The solvent system for separation consisted of 70:30 of acetonitrile and Millipore water. The COS was detected at 210 nm with a flow rate of 0.7 ml min^{-1} at 25°C. The detected COS were quantified using known COS DP1-6 mix as standard.

2.9 FE-SEM of chitin and tomato leaves

Structural changes of chitin (α-chitin and β-chitin) upon treatment with *Streptomyces* strains ANU-34 and CAI-21 were visualized *via* field emission scanning electron microscopy (FE-SEM). Chitinous substrates, after 96h of incubation with *Streptomyces* strains, were prefixed using 2.5% glutaraldehyde solution and kept for overnight incubation at 4°C. Next day, the glutaraldehyde solution was removed and samples were subjected to dehydration in a gradient of ethanol solution (10-100%). The samples were air-dried and later mounted on to the stub with coverslip fixed using a double-sided magnetic tape. The stubs were gold coated for 90s using Hitachi E 1010 ion sputter using ultra 55 model of Carl Zeiss, Germany. The gold-coated samples were subjected to microscopy with a working range of image set up from 100-2µm of distance having a voltage of 5Kv with a magnification of 1500-12000x. Stomata of COS-treated tomato leaves were also observed through FE-SEM. Finely sectioned leaf slices were fixed in 2.5% glutaraldehyde solution followed by dehydration as indicated above and FE-SEM images were taken.

2.10 Design of experiment and SSF

For production of COS in SSF, fungus was grown in a suitable substrate (paddy straw, ground nut shells and sugar cane bagasse) added with minimal media and the fungal mycelium was carefully harvested after 7 days. This fungal mycelium was autoclaved for use as inducer for SSF. Chitin flakes were ground to a fine powder in a blender and autoclaved to use in fermentation process. SSF plates were prepared by taking sterile plastic Petri plates and making perforations on top and bottom. Solid media for COS production were prepared by blending chitin flakes and β-chitin with 1% of fungal grown chitin. SSF was carried out at 30ºC by inoculating chitinolytic *S. proteamaculans* on to solid media and kept for incubation. For every 3 h, the samples were collected and concentrated by vacuum evaporator, later filtered using 0.2 µm disc filters and loaded onto TLC and HPLC for analyses.

2.11. Time course reactions of *Pe*Chi3 and products analyses

2.11.1 High Performance Thin Layer Chromatography (HPTLC)

HPTLC (CAMAG, Muttenz, Switzerland) was employed to visualise the products formed from polymeric chitosan substrates - DA 37% and 61% (2 mg each) *Pe*Chi3 enzyme - 300 nM dissolved in a buffer of 50 mM sodium acetate buffer pH 5.6 at 50°C. Approximately 20 µg chitosan hydrolysate in a total volume of 10 µl was spotted onto silica gel 60 F_{254} TLC plates, 10 × 20 cm, thickness of 200 µm (Merck, Germany). A controlled software applicator was used to apply the samples in the band form sprayed on to the plates using compressed air. Samples system consisted of n-butanol, methanol, 25% liquor ammonia, and water in 5:4:2:1 ratio. To visualize the spots TLC plate was stained by using ammonium bisulphate and heated using a hot air gun at 140°C and spots are noted.

2.11.2 Characterization of PeChi3-products by MALDI-TOF MS

To visualise the products that are formed longer than DP 6 MALDI TOF was used, as described previously by Purushotham et al. (2012). A sample amount of 1.0 µl was mixed with equal ratio of (1:1) with DHB (2,5-dihydroxy benzoic acid) at 10 mg/ml present in 50% acetonitrile solution and applied on to target plate. The sample was air dried and detected using Ultraflex MALDI-TOF (Bruker Daltonics, Germany). The obtained MS spectra were analysed with mMass 5.0 software.

2.12 Chitin pre-treatment:

For the pre-treatment process, shrimp α-chitin was chosen as the starting material. It underwent treatment with KOH, along with the addition of acid and KOH-urea in the process. As elucidated previously, the chitin treated with acid was referred to as the colloidal form of chitin (CC), based on the work of Ramirez et al. (2004). Two concentrations of KOH, namely 11% and 20% aqueous solutions, were utilized for the chitin pre-treatment. The selection of these concentrations was guided by considerations of solubility issues below 11% and challenges with solubility and freezing at concentrations exceeding 20% KOH in aqueous solution, as reported by Gong et al. (2016). Therefore, 11% and 20% KOH solutions were chosen as the lower and higher concentrations for the KOH pre-treatment. In addition to KOH, urea was introduced at a 4% concentration in conjunction with the 11% and 20% KOH aqueous solutions. This inclusion aimed to assess the effect of combining urea with KOH in the pre-treatment process. To prepare the chitin solutions, 1% and 2% were used with both the 11% KOH and 20% KOH-urea aqueous solutions. These solutions were stored at a temperature of~ 30°C for a duration of 3 days. Subsequently, the

defrosting process was carried out at 27°C ± 2°C, and the resulting solution was stirred twice daily. Once the thawing process was complete, the pH of the chitin solutions was gradually reduced to 2 using concentrated H_2SO_4 added drop-wise. The solutions were then stirred to facilitate the dissolution of chitin, based on the methodology described by Hu et al. (2007). The resulting mixture was further agitated for one hour at 27°C and subsequently centrifuged for 20 min at 7000 g. This process led to forming a chitin pellet, carefully washed with double distilled water until a neutral pH of 7 was achieved. The thoroughly washed chitin residue was combined with a small amount of Milli-Q water and stored at a temperature of 4°C for preservation. To determine the chitin concentration in the treated substrates, 1.0 mL of chitin solution was taken in triplicate and placed in pre-measured empty vials. The samples were then lyophilized for 14-16 h to completely dry them. The average substrate concentration was subsequently measured. The treated substrates were assigned specific names as follows: 11K1, 11KU1, 11K2, 11KU2, 20K1, and 20KU1. In these names, "K" represents the treatment with KOH alone, "KU" denotes the treatment with KOH-urea, the first number indicates the percentage of KOH used (either 11 or 20), and the last number represents the proportion of chitin used in the treatment (either 1 or 2).

2.13. Expression and purification of *Cs*ChiG, *Sp*ChiD-Y28A and *Pe*Chi3

The pure enzymes used in the study were prepared according to Sivaramakrishna et al. (2020) for *Cs*ChiG, and Madhuprakash (2014) for *Sp*ChiD-Y28A, and Das (2016) for *Pe*Chi3. The recombinant chitinases were overexpressed in their respective host. Pure form of heterologously expressed proteins was obtained through nickle-nitrilotriacetic acid (Ni-NTA) affinity chromatography. Briefly, *Sp*ChiD (Y28A), *Pe*Chi3, and *Cs*ChiG clones were borrowed from the chitinase library of the lab. The culture was inoculated in LB broth with ampicillin (100 µg/mL) and kanamycin (50 µg/mL) antibiotic and grown at 37 °C overnight. A starter culture of 0.5-0.6 OD_{600} was added to the main culture and cells were grown at 37 °C. At log phase 0.5 mM of IPTG was added to media to induce protein expression, and cultures were grown at 28°C for 24 h. Later, cells were harvested at 10,000g for 15 min. The culture pellet was washed thoroughly to remove any remnants of LB. The cell pellet was resuspended in lysis buffer (10 mM imidazole, 100 mM NaCl, and pH 8.0) and subjected to sonication by using Vibra-cell ultrasonic processor at 35% amplitude for 15-20s with an on and off cycle equipped with a 3 mm probe (Sonics, USA). The cell lysate was centrifuged at 26000g for 45 min and supernatant was passed through a Ni-NTA resin packed in a 10 ml of syringe column. His-tagged protein bound to the resin, and the unbound protein was eluted out and collected as flow through. Wash buffer (20 mm imidazole, 300 mm NaCl, and 50 mm NaH_2PO_4, pH 8.0) double the volume of sonicated supernatant is passed through

the column to remove unbound proteins. To elute the recombinant protein bound to Ni-NTA, 50, 150, and 250 mM imidazole was used. Eluted protein was run on 12% SDS-PAGE.

2.13.1 Protein measurement

The eluted pure recombinant protein was buffer exchanged with sodium phosphate buffer, pH 8.0, and concentrated using 30 kDa cut-off centricons (Vivaspin Sartorious, Germany). Protein estimation is done using a BCA kit (Themofischer Scientific, USA). Protein concentration was estimated at 545 nm.

2.14 Time-course degradation of substrates

For calculation of enzyme activity 5 mg/mL of substrate concentration was used and 5 µM of pure enzyme (*Cs*ChiG in 50 mM sodium citrate buffer, pH 4.0) was used for 1, 12, or 24 h by incubating the reaction mixture at 50°C. Extra 5 µM of *Cs*ChiG was added at 12 h to see if hydrolysis of chitin was affected by incubating the combination for 48 h and examining the results at 24 h (24 h sp), or 48 h (48 h sp). From the reaction mixture, 100 µL sample was collected at each time point, and centrifuged for 15 min at 4°C. To the 40 µL of supernatant an equal volume 70% acetonitrile was added to stop the reaction and these samples were analysed through HPLC.

2.15 Preparation of COS from SSF

Chitin flakes were powdered in a grinder and passed through a 1 mm sieve. Two bacterial isolates of Actinomycetes family ANU-34 and CAI-21 isolated from soil samples were used for SSF. Chitin flakes (0.5% w/v) were used and inoculated with bacterial culture (ANU-34 and CAI-21). The moisture content was maintained to 70% with M9 buffer and grown for 10 days. Every two days, they were mixed with a sterile glass rod in a sterile environment. After 10 days of incubation, the contents in the SSF were added with 5X volume of 50 mM sodium citrate buffer agitated in an incubator for 30 min at 120 rpm to extract the formed COS into buffer. The bacterial-digested chitin flakes were filtered using cheese cloth and the COS solution was concentrated using lyophiliser and was stored at -20°C until use.

2.16 Plant material, growth conditions, and COS treatment

Tomato (*Arka vikas* variety) seedlings were grown in green house with a photoperiod of 14 h at 28°C and 70% humidity. Tomato seedlings of 5 to 6 leaf stage were selected for foliar application. COS from SSF at a concentration of 10 µg/mL was sprayed. Foliar application was done thrice with 5 min gap and tween-20 (0.01%) was added to the COS solution for proper adherence to the leaves. Ten days after foliar application, plant growth parameters like plant height, chlorophyll

content, relative water content, amino acid content, and photosystem-II efficiency were measured. Control and treated plants were harvested at different time points and were stored at -80°C for further processing. Every experiment was repeated twice with 3 replicates for each treatment.

2.17 Relative water content measurement

Relative water content of the freshly harvested leaves of both control and treated seedlings was determined. Fresh weight of both treated and control seedlings was noted and named as fresh mass (FM) and then the leaves were immersed in MilliQ water and stored at 4 °C for 24 h to get turgid mass. Turgid mass of the leaves was noted and denoted as (TM). To measure dry mass (DM) leaves were kept in hot air oven for 3 days at 70°C for drying and weirght was noted. Relative water content was calculated using the formula RWC [%] = [(FM) − (DM)/(TM) − (DM)] ×100.

2.18 Chlorophyll content estimation

Leaf chlorophyll content was measured according to Arnon (1949). Both control and treated leaves each of 1.0 cm length were collected. The collected leaves were cut into fine pieces and are crushed in ice-cold 80% acetone on a prechilled motor and pestle. The leaves lysate was centrifuged at 3214 g for 20 min 4°C and the supernatant absorbance was measured at 645nm and 663nm with acetone as a blank.

Calculation of chlorophyll a and b are as follows:

Total chlorophyll content - 20.2(A645) +8.02(A663)
Chlorophyll a content - 12.7 (*A*663) −2.69 (*A*645)
Chlorophyll b content - 22.9 (*A*645) −4.68 (*A*663)

2.19 Determination of PSII and PSI photochemical efficiencies

Photosynthetic parameters of PSII were measured by dual-PAM 100 (Walz, Effeltrich, Germany). Fully expanded second and third leaves from the tip of the tomato seedlings were selected and kept in the dark for 30 min before analysis. $(F_m' - F_s)/F_m'$ was used to determine the quantum yield Y(II). Parameters, Fs represent the steady state florescence, F_m and F_m' − represent the maximum flourescence levels measured in dark and light. Electron transport rate (ETR(II)) was calculated using the formula ETR(II)= 0.84×0.5×Y(II)×light intensity (µmol photon m^{-2} s^{-1}). Non-regulated quantum yield of heat dissipation Y(NO) was calculated by formula Y(NO)=Fs/Fm. Y(NPQ) regulated quantum yield of heat dissipation was calculated as follows

Y(NPQ) = 1-Y(II)-Y(NO). qP = $(F_m' - F_s)/(F_m'\text{-}Fo)$ where Fo represents minimum florescence and qN=1- $(F_m' - F_o)/(F_m\text{-}Fo)$ puddle model equations which were used to calculate the photochemical quenching parameters.

2.20 Isolation of RNA, cDNA synthesis and quantitative real-time PCR (qRT-PCR)

Real-time PCR was done according to Nadendla et al. (2018). In brief, total RNA was isolated with Qiagen's RNA plant kit, and the purity and concentration of RNA samples were evaluated with the help of nanodrop and ethidium bromide-stained agarose gel electrophoresis. A total of 12 defence-related genes were chosen to examine the expression pattern in tomato leaves exposed to treatments. cDNA synthesis kit from Takara Bio (Japan) was used to conduct reverse transcription (RT) PCR by the recommended procedure. In a nutshell, nuclease-free water was used to combine 4 g of total RNA, one µl of 50 µM Oligo-dT primer, and1 µL of 10 mM dNTP into a full volume of 10 µL. The combination was kept for 5 min at 65°C and was quickly cooled by being kept in an ice bath. Adding the combination of Blue Print RTase, recombinant RNase inhibitor, and 4 µL of 5X RT buffer was combined with 1 µL of nuclease-free water to make up 20 µL. The reaction combination was kept for 10 min at 37°C and later for 60 min at 42 °C. Through thermal denaturation at 95°C for 5 min, the enzyme was rendered inactive. Using gene-specific primers, one microliter of the RT reaction combination served as the template for the PCR (Table 2.1). The following ingredients were used for the PCR: 1X PCR buffer, 1 unit of *Taq* polymerase, 200 µM dNTP mix dNTP combination, two mM $MgCl_2$, 0.8 pmol each of the forward and reverse primers. In an Eppendorf master cycler gradient thermal cycler (Germany), PCR was carried out with varying annealing temperatures using cycling parameters of 94°C for initial denaturation, for 3 min followed by 38 cycles at 94°C for 2 min (denaturation), 2 min with gene-specific temperature for annealing with a gene-specific temperature for 2 min and 1 min at 72°C (extension), and 10 min at 72°C for final extension. The PCR products were compared to the loading control actin on a 1.2% TAE agarose gel.

2.21 Amino acid content

Treated and control leaf samples of 0.5 g each were ground in liquid nitrogen and extracted in ice cold 2.0 ml of 5% acetic acid for 1.0 h with mild agitation on a shaker at 37°C. Homogenate was later centrifuged at 4000 rpm for 15 min and the supernatant was subjected to free amino acids analysis (Sreeharsha et al. 2019).

Chapter III

Solid state fermentation with chitinous substrates

3.1 Introduction:

In search of economically viable processes as an alternate to the available chemical processes for COS production, bioprocess technology has a few advantages. It is relatively less expensive, less time consuming, eco-friendly, and also recycles agricultural and shrimp wastes to generate value-added products. Solid-state fermentation (SSF) is a fermentation process that consumes less water for the substrates. The substrate should have just enough water for proper growth of the microorganism (Pandey, 1992; Pandey et al. 2000; Pandey, 2003). SSF was used for the fermentation of chitin in the production of COS. SSF has advantages, compared to submerged fermentation process, with low water requirement, easy aeration, low sterility demands, small size of fermenter and ease of downstream process (Holker et al. 2005; Swiontek et al. 2008; Nigam et al. 2009). The methodology followed for design of experiment for SSF, microbial strains used, phylogenetic analysis of bacterial isolates, FE-SEM of chitin, TLC and HPLC analyses is described in the materials and methods chapter (Sections 2.10, 2.4, 2.8.1, 2.9, 2.8.2.1, 2.8.2.2).

In this work, shrimp waste was used as a source of chitin (substrate) for chitinolytic organisms like *S. proteamaculans* and *Streptomyces* sp. in SSF to generate value-added COS, with the following objectives:

3.2 Objectives:

1. **Collection and preparation of different substrates for SSF to grow fungi**
    - Collecting different agricultural wastes from different sources that can be used as substrate in SSF to generate fungal biomass
    - Procured agricultural wastes are ground into fine powder, autoclaved, mixed with mineral solution and used as substrate
2. **Selection of suitable substrate for fungal growth**
    - Growing different strains of fungus on different agricultural wastes
    - Checking for fungal growth on different agricultural wastes
    - Selection of the fungal strain growing optimally on a particular agricultural waste
3. **Design of SSF to grow chitinoytic bacteria on fungal biomass (chitin)/chitin flakes**
    - Designing the SSF that could allow proper aeration
    - Maintaining the SSF in incubation chamber at particular temperatures to support the optimum growth of bacterial strain for the production of chitinases

4. **Optimizing the growth conditions of *S. proteomaculans* and *Streptomyces* sp. on chitin flakes**
    - Standardizing the growth conditions like pH, temp, minerals, inoculum, moisture control, inducers etc. for the chitinolytic bacteria
5. **Detection and characterization of COS using chromatography techniques**
    - Detection of COS generated by SSF using TLC and HPLC

3.3. Results and discussion

3.3.1 Substrates used in SSF

To obtain mycelial biomass, the selected fungal cultures (*F. oxysporum* and *A. alternata*) were grown on different substrates (*viz.* ground nut shells, paddy straw, sorghum straw and sugar cane bagasse) to obtain fungal biomass rich in chitin. This was subsequently used as substrate in SSF to produce COS by *S. proteomaculans*. All the four substrates were procured from local fields/markets. The substrates were ground to powder using a blender and autoclaved (Fig. 3.1). All the substrates were converted into a dry powder and the particle size of the powdered substrate was not uniform. The selected substrates were separately inoculated with *F. oxysporium* or *A. alternata* and 1% minimal medium and grown at 28°C for 7 days. It was observed that growth of *F. oxysporum* was more on the groundnut shells (Fig. 3.2A), followed by sugarcane bagasse, sorghum straw and paddy straw. Paddy straw (Fig. 3.2B) supported less growth of *F. oxysporum,* while groundnut shells (Fig. 3.2A) served as good substrate, after 7 days of incubation.

In the case of *A. alternata,* more growth was observed on sorghum straw (Fig. 3.3C) followed by sugarcane bagasse, ground nut shells and paddy straw. On paddy straw (Fig. 3.3A) *A. alternata* had less growth, while on sorghum straw (Fig. 3.3C), there was much better growth after 7 days of incubation. *F. oxysporum* showed better growth on ground nut shells (Fig. 3.2A), while *A. alternata* showed better growth on sorghum straw (Fig. 3.3C). Therefore, these substrates were used for the SSF.

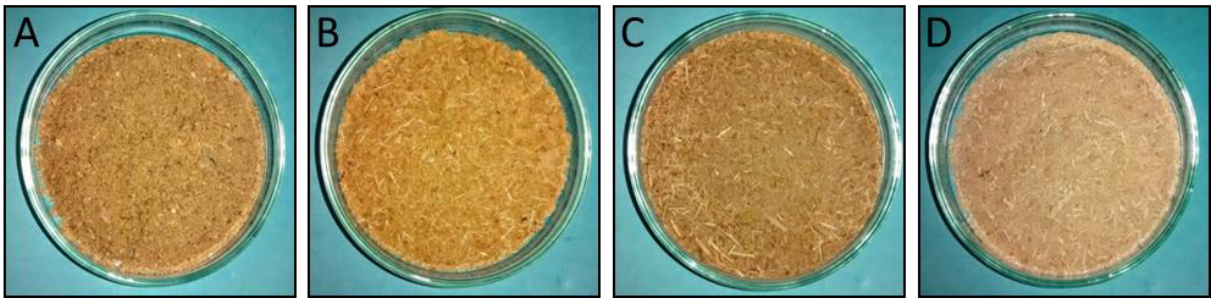

Fig. 3.1: Finely ground substrates used in solid state fermentation: (A) Groundnut shells, (B) paddy straw, (C) sorghum straw, and (D) sugarcane bagasse.

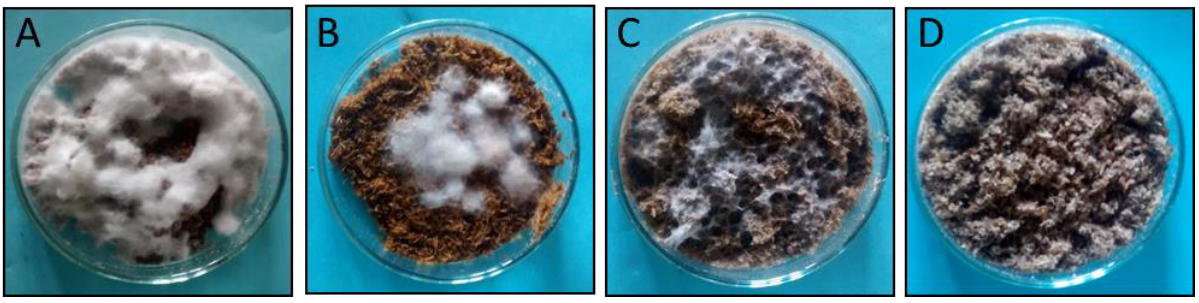

Fig. 3.2: Growth of *Fusarium oxysporum* on different substrates: Growth of *F. oxysporum* on (A) groundnut shells, (B) paddy straw, (C) sorghum straw, and (D) sugarcane bagasse. Selected substrates were inoculated with *F. oxysporum* along with 1% minimal medium and grown at 28°C for 7 days.

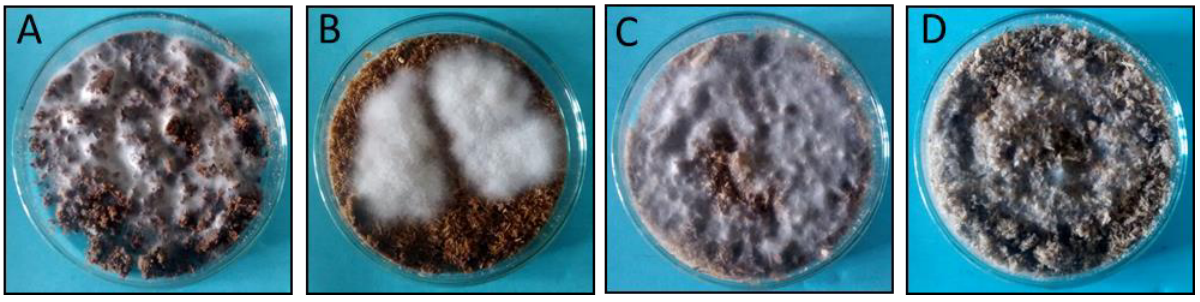

Fig. 3.3: Growth of *Alternaria alternata* on different substrates: Growth of *A. alternata* on (A) ground nut shells, (B) paddy straw, (C) sorghum straw, and (D) sugarcane bagasse. The selected substrates were inoculated with *A. laternata* along with 1% minimal medium and grown at 28°C for 7 days.

3.3.2 Selection of chitinolytic microbes for SSF

S. proteamaculans was originally isolated as a root endophyte of *Populus* tree (Taghavi et al. 2009). The extracellular chitinolytic system of *S. proteamaculans* has been well characterized by cloning, expression and characterization of chitinolytic enzymes and chitin binding proteins (Madhuprakash et al. 2012; Purushotham et al. 2012; Manjeet et al. 2013). *S. proteamaculans* consists of four chitinases (*Sp*ChiA, *Sp*ChiB, *Sp*ChiC and *Sp*ChiD), three chitin-binding proteins and one N-acetylhexosaminidase (Purushotham et al. 2012). Chitinases of *S. proteamaculans* can act on both α-chitin and β-chitin. Chitin-binding proteins of *S. proteamaculans* can bind effectively to β-chitin and synergistically work together with *Sp*ChiB in chitin degradation (Manjeet et al. 2013). *Sp*ChiD possessed exceptional TG activity and produced higher chain

length COS from lower DP substrates (Purushotham and Podile, 2012; Madhuprakash et al. 2012). Therefore, *S. proteamaculans* was selected as one of the bacterial strains to test its efficiency in converting chitinous substrates into COS in an SSF.

A collection of 19 *Streptomyces* strains isolated from the soil were obtained from Dr. Gopalkrishnan, ICRISAT. All the isolates were screened for their chitinolytic activity. Two strains of *Streptomyces* sp. (ANU-34 and CAI-21) that showed higher chitinolytic potential on chitin agar plates were selected for the SSF to generate COS from fungal biomass or chitin flakes. An attempt was made to tentatively identify these two chitinolytic strains by amplifying and sequencing the 16S rRNA gene. A phylogenetic tree was also constructed for the selected strains.

3.3.3. Phylogenetic tree analysis

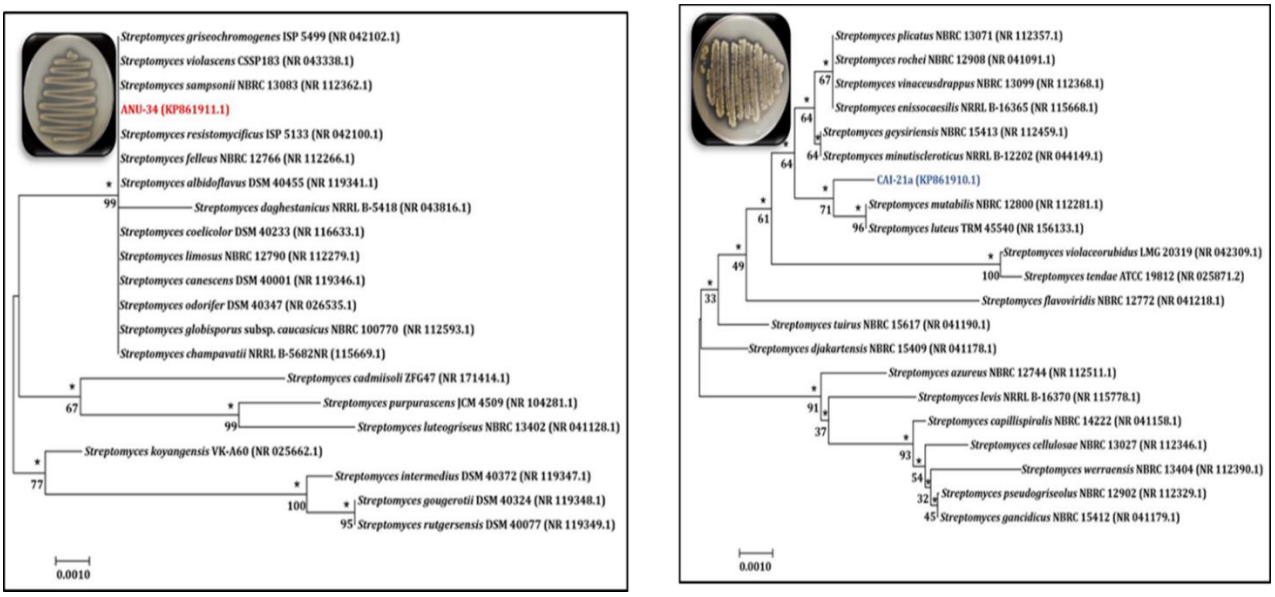

Fig. 3.4: Phylogenetic tree was constructed using MEGA 7.0 by employing neighbour joining and Kimura2-parameter model. The numerical values displayed at each node is the bootstrap percentage (based on 1000 replicates) and the scale bar indicates 0.001 substitutions per nucleotide position. The accession nos. for the sequences are shown in parenthesis. Asterisks indicate branches that were recovered using maximum likelihood method of phylogeny. Hydrolysis of chitin by the isolate *Streptomyces* sp. CAI-21 can be observed by the formation of zone of clearance around the bacteria in the colloidal chitin agar plate of top left corner.

The 16s rRNA-based sequence identity revealed that both the isolates belong to *Streptomyces*. *Streptomyces* sp. *(*ANU-34) strain showed closer identity (100%) towards a group of *Streptomyces* that include *S. griseochromogens, S. violasens, S. sampsonii, S. resistomycificus, S. albidoflavs* etc. The phylogenetic tree revealed that the *Streptomyces* sp.CAI-21 strain showed more sequence similarity with *S. mutabils* and *S. leuteus*. *S. mutabilis* is a known chitinolytic

strain (Rajendran et al. 2023). Whereas, *S. leuteus* has not yet been reported as a chitinolytic organism.

3.3.4 Scanning electron microscopy analysis of α-chitin treated with *Streptomyces* sp. (ANU-34) and *Streptomyces* sp. (CAI-21)

Crystalline α-chitin was treated with *Streptomyces* sp. (ANU-34) and *Streptomyces* sp. (CAI-21). After 96 h of incubation, crystalline chitin showed clear perforations and the substrate was more amorphous with both the strains when compared to control. Results clearly showed strong chitinolytic activity on crystalline α-chitin by both *Streptomyces* sp. (ANU-34) and *Streptomyces* sp. (CAI-21) after 96 h of incubation.

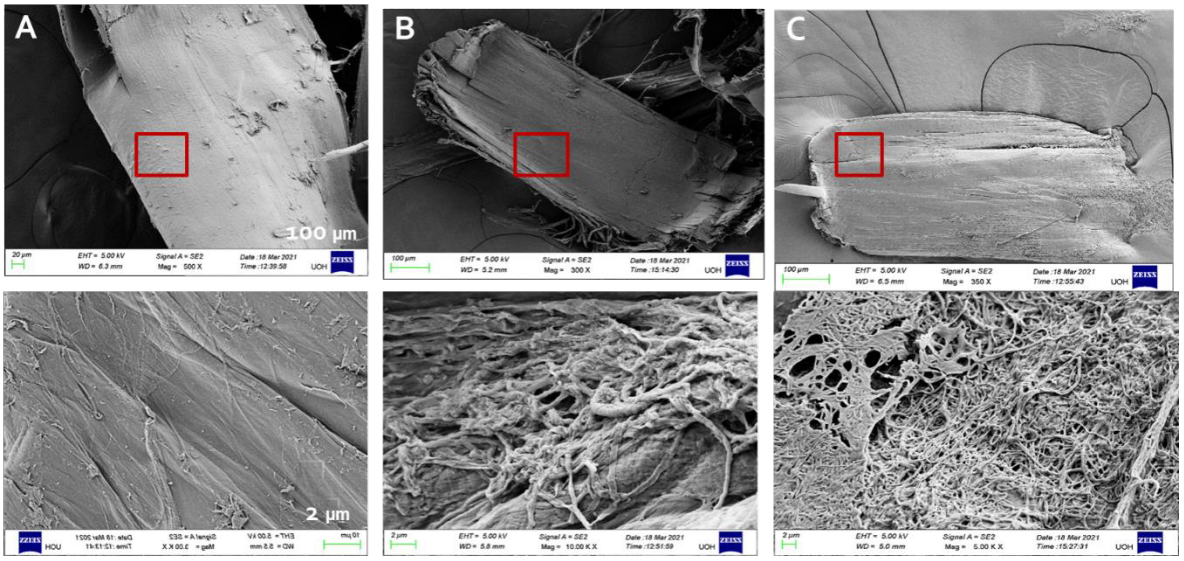

Fig. 3.5: FE-SEM analysis of α–chitin treated with *Streptomyces* sp. (ANU-34) and *Streptomyces* sp. (CAI-21): The figure shows structures of α–chitin particle alone (A) and incubated with *Streptomyces sp.* (ANU-34) after 96 h (B) and *Streptomyces* sp. CAI-21 after 96 h (C). The upper panel represents the untreated and treated individual α-chitin particles while the lower panel represents the close-up images of the target area (shown in B frame) of the respective particle. The scale bars of the images in the upper panel is 100 µm and those in the lower panel is 2 µm, respectively.

The FE-SEM images of α-chitin treated with chitinolytic *Streptomyces* sp. (CAI-21) and *Streptomyces* sp. (ANU-34) strains are presented in Fig 3.5. The untreated α-chitin was intact, smooth and retained rigid surface (Fig. 3.5A). Incubation of α-chitin with *Streptomyces* sp. (ANU-34) and *Streptomyces* sp. (CAI-21) for 96 h (Fig. 3.5B and C) resulted in perforations on the chitin surface. The perforations may be due to the chitinases secreted as reported earlier by Mukherjee et al. (2020). It was also observed that the chitin was less crystalline and was more amorphous with intense perforations with *Streptomyces* sp. (ANU-34) when compared to 96 h on α-chitin by *Streptomyces* sp. (CAI-21).

3.3.5 SEM analysis of β-chitin

Crystalline β-chitin was treated with both *Streptomyces* sp. (ANU-34) and *Streptomyces* sp. (CAI-21). After 96h of incubation β-chitin was also converted from crystalline to amorphous form and clear perforations were also seen on both strains treated substrates when compared to control. The results further confirmed the chitinolytic ability on crystalline β-chitin, by both *Streptomyces* sp. (ANU-34) and *Streptomyces* sp. (CAI-21) after 96h of incubation (Fig. 3.6). The untreated β-chitin surface was intact, soft and plain (Fig. 3.6A). Incubation of β-chitin with *Streptomyces* sp. (ANU-34) for 96h (Fig. 3.6B) resulted in perforations on β-chitin surface and clear chitin fibrils can be seen. The perforations could be due to the array of chitinases secreted by the *Streptomyces* strains.

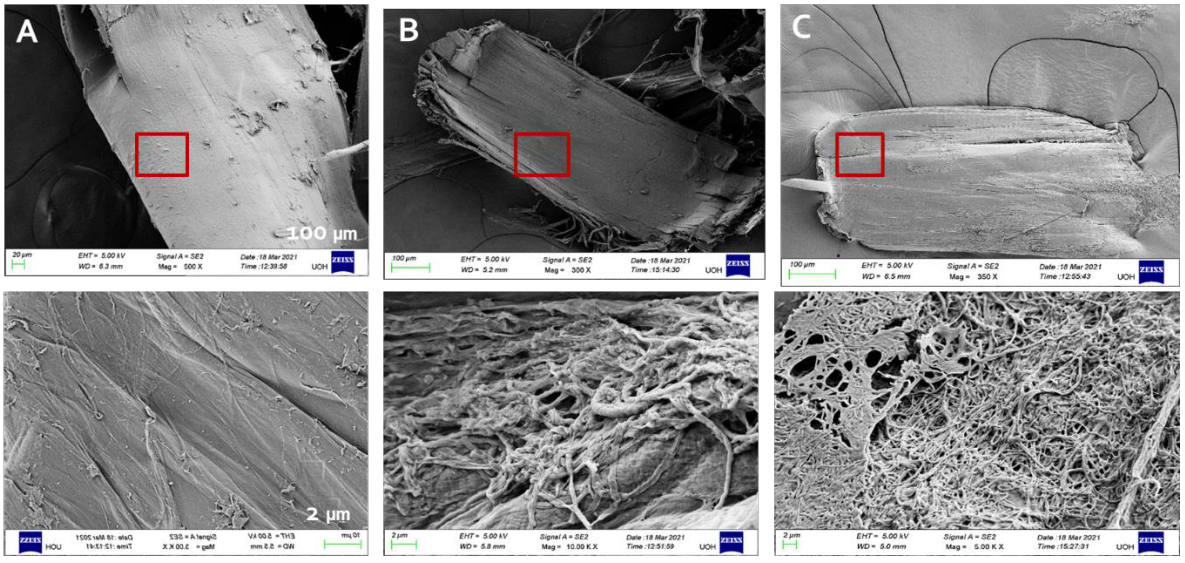

Fig. 3.6: FE-SEM analysis of β–chitin treated with *Streptomyces* sp. (ANU-34) and *Streptomyces* sp. (CAI-21): The figure shows structures of β-chitin particle alone (A) and incubated with *Streptomyces sp.* (ANU-34) after 96 h (B) and *Streptomyces* sp. CAI-21 (C) after 96 h. The upper panel represents the untreated and treated individual β-chitin particles while the lower panel represents the close-up images of the target area (shown in frame B) of the respective particle. The scale bars of the images in the upper panel is 100 µm and those in the lower panel is 2 µm, respectively.

3.3.6 HPLC profile of COS produced by *S. proteamaculans*

Crystalline chitin subjected to SSF by *S. proteamaculans* was analysed in a time-dependent manner using HPLC and quantification of obtained products was done using HPLC chromatogram. *S. proteamaculans* produced 0.5 mM DP6 as minor product; DP1 at a 7.5 mM and DP4 at 7.0 mM as major end product on α-chitin. *S. proteamaculans* when grown on α-chitin in combination with 1% fungus biomass has produced DP5 at a concentration of 0.5mM as minor products; DP3 and DP2 as major end products at 4.0 mM and 10.0 mM concentration

respectively. Purushotham et al. (2012) reported DP4 as the major end product from *S. proteamaculans* by *Sp*ChiC.

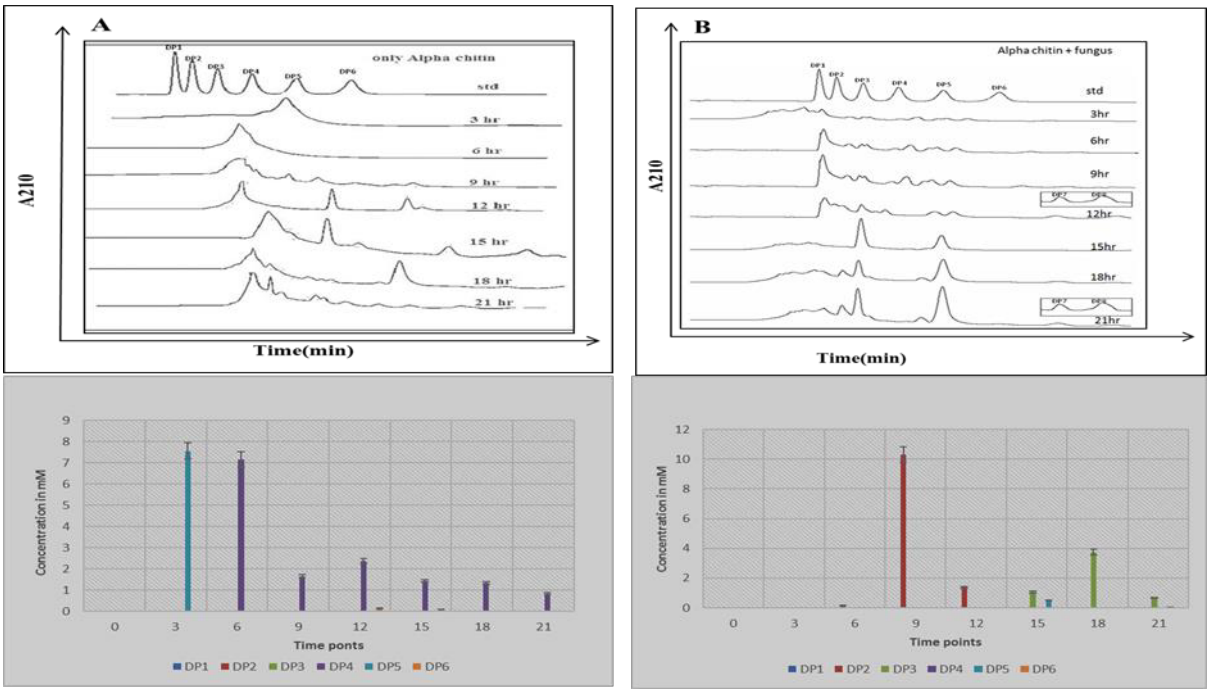

Fig. 3.7: HPLC profile of products formed from SSF with *S. proteamaculans* on α-chitin: Time course of COS formed at different time points, fractions collected from each time point were collected resuspended in MilliQ water filtered using 0.22 µm disc filters and concentrated through vacuum evaporator and detected by isocratic HPLC with a ratio of 70:30 of ACN:H_2O at 210nm. The upper most profile shows COS standard mix ranging from DP1 to DP6. The other profiles show the products formed from DP4, DP5 & DP6 in SSF at different time points from 3 to 21 h. The profile also shows the smaller -peak-area representing products of COS>6.

3.3.7 HPLC profile of *Streptomyces* sp. (ANU-34) and *Streptomyces* sp. (CAI-21) on α-chitin

The SSF of crystalline chitin by *Streptomyces* sps. (ANU-34 and CAI-21) was analyzed through HPLC. *Streptomyces* sp. ANU-34 produced 5.0 mM DP1 and 2.5mM DP4 as minor products and 25.0 mM DP3 and 30.0 mM DP5 were the major end products. Whereas, the SSF of crystalline chitin by *Streptomyces* sp. CAI-21 strain produced 15.0 mM DP4 and 35.0 mM DP5 as the major end products. Thus, *Streptomyces* sp. CAI-21 produced COS mixture in SSF consists of DP1, DP3, DP4 and DP5; *Streptomyces* sp. ANU-34 produced COS has DP4 and DP5. Anh et al. (2021) reported formation of COS (DP 2 to 10) by *S. macrosporeus* VTCC 940003.

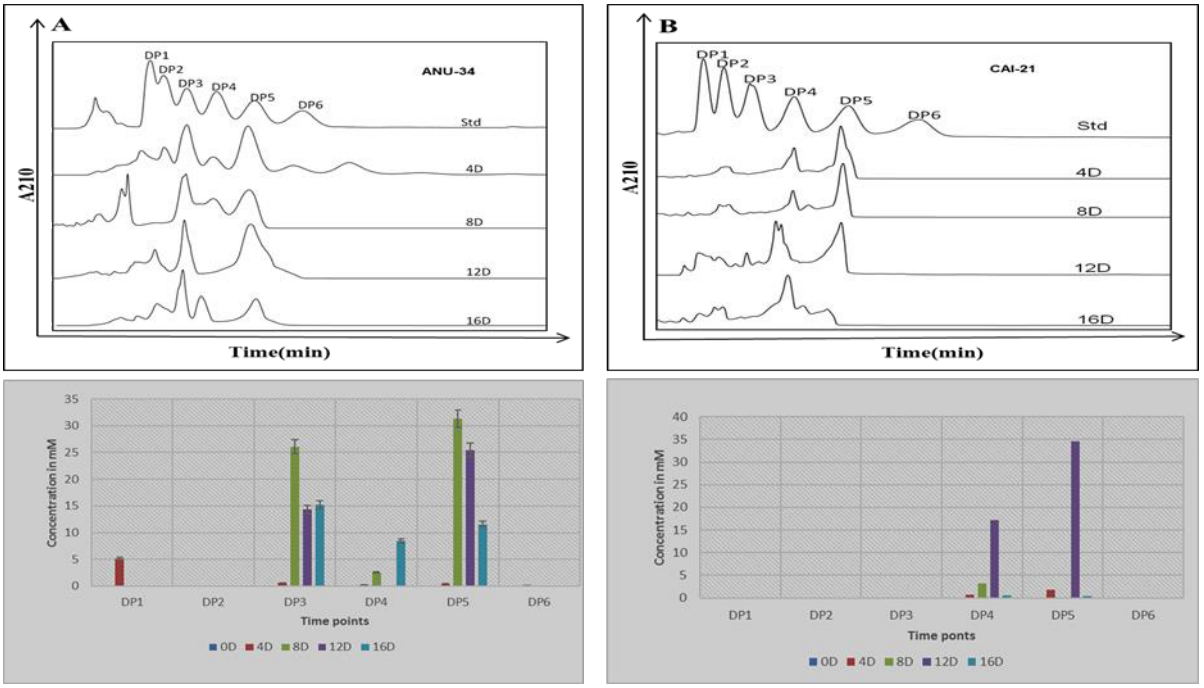

Fig. 3.8: HPLC profile of products formed from SSF of *Streptomyces sp.* (ANU-34) and *Streptomyces* sp. (CAI-21) on α-chitin. Time course of COS formed at different time points, fractions collected from each time point were collected resuspended in MilliQ water filtered using 0.22 µm disc filters and concentrated through vacuum evaporator and detected by isocratic HPLC with a ratio of 70:30 of ACN:H_2O at 210nm. The upper most profile shows COS standard mix ranging from DP1 to DP6. The other profiles show the products formed from DP4, DP5 & DP6 in SSF at different time points from 3 h to 21 h. The profile also shows the smaller -peak-area representing products of COS>6.

3.4. Effect of inducer concentration in SSF

The SSF of crystalline chitin by *Streptomyces* sp. (ANU-34) and *Streptomyces* sp. (CAI-21) was analyzed and quantification of obtained products was done using an HPLC chromatogram. *Streptomyces* sp. (ANU-34) produced 5.0 mM DP1 and 2.5 mM DP4 as minor products; 25.0 mM DP3 and 30.0 mM DP5 were the major end products. Similarly, the SSF of crystalline chitin by *Streptomyces* sp. (CAI-21) strain produced 15.0 mM DP4 and 35.0 mM DP5 as the major end products. Thus, the COS produced by *Streptomyces* sp. (ANU-34) in SSF consists of a mixture of DP1, DP3, DP4, and DP5. Whereas, *Streptomyces* sp. (CAI-21) produced, COS comprises DP4 and DP5. Anh et al. (2021) reported the presence of a mixture of DP2 to DP10 in COS produced by *S. macrosporeus* VTCC 940003.

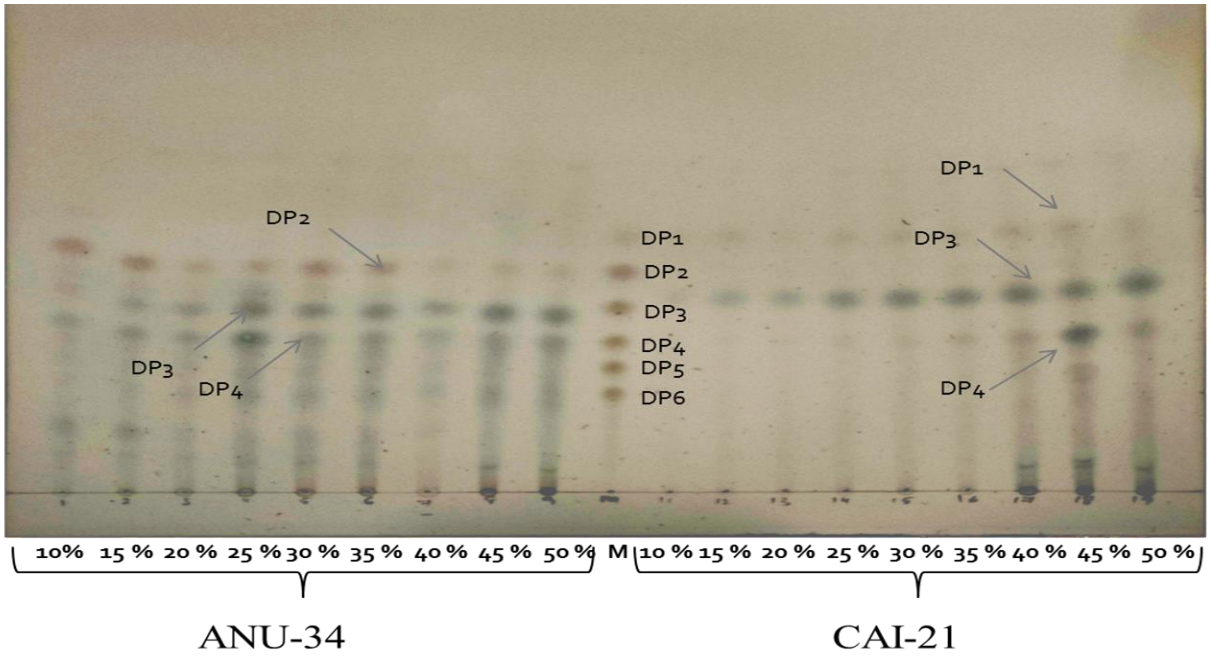

Fig. 3.9: Profiling of COS fractions collected from SSF flasks containing different percentages of wheat bran as an inducer: Chitin flakes were incubated with either *Streptomyces sp.* (ANU-34) or *Streptomyces* sp. (CAI-21) in SSF with different percentage (10% to 50%) of wheat bran as inducer. After six days of incubation COS fractions were collected resuspended in filtered (0.22µm filters) MilliQ water and concentrated in vacuum evaporator and loaded on TLC. DP1-DP6: COS mix.

To study the effect of wheat bran as an inducer on COS production in SSF, both *Streptomyces* sp. (ANU-34) and *Streptomyces* sp. (CAI-21) were grown in SSF flasks containing chitin flakes and different percentages of wheat bran (ranging from 10% to 50%) for six days. *Streptomyces* sp. (ANU-34) generated DP2, DP3, and DP4 as the major end products when the wheat bran concentration was 10% to 35%. But with the increase in wheat bran concentration from 35% to 40%, DP3 and DP4 were formed as major products. In the case of *Streptomyces* sp. (CAI-21), DP3 was the major end product at 10% to 35% of wheat bran concentration, and at the later concentrations (*i.e*., from 40% to 50%), DP3 and DP4 were formed. A higher inducer concentration may affect the product formation in lignocellulosic SSF (Singhania et al. 2006).

3.5. Effect of salt concentration in SSF

Different salt concentrations ranging from 0.1%-0.9% were used to optimize culture conditions for *Streptomyces* sp. in SSF to produce COS. Samples from different SSF flasks containing *Streptomyces* were grown in different salt concentrations was profiled on TLC to detect COS. *Streptomyces* sp. (ANU-34) strain produced DP2 and DP4 as the major end products in all the salt concentrations (*i.e.,* 0.1% to 0.9%). Whereas, in the case of *Streptomyces* sp. (CAI-21), DP1 was the major product formed along with DP3 and DP4. At later time points, only DP3 and DP4 were the major products, and the optimum percentage of salt was from 0.4% to 0.7%

for the *Streptomyces* sp. (CAI-21) strain. A lower concentration of NaCl is preferred in SSF. The primary role of NaCl is maintaining the ionic strength in SSF. NaCl plays a key role in the activity of enzymes (Falk, 1918) and increases the reaction rate to get more amount of products (Weinberg, 1967). Similar reports also suggest the effect of lower salt concentration is highly beneficial in SSF (Kamini et al.1998; Mahadik et al. 2002).

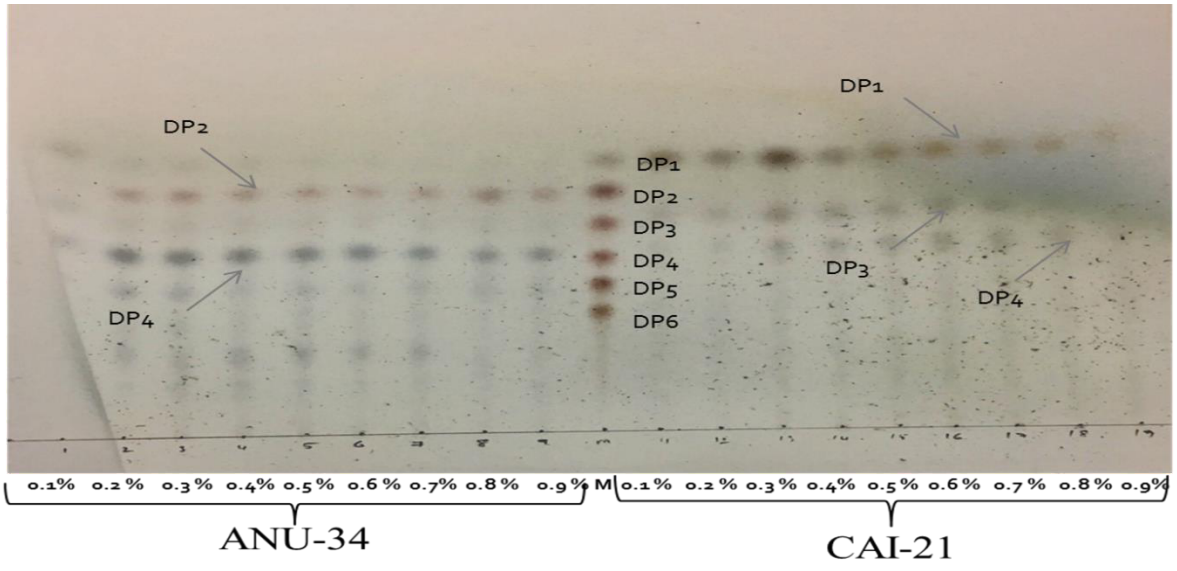

Fig. 3.10: Profiling of COS fractions collected from SSF flasks containing different concentrations of salt: Chitin flakes were incubated with either *Streptomyces sp.* (ANU-34) or *Streptomyces* sp. (CAI-21) in SSF with different concentrations (0.1-0.9%) of salt. After six days of incubation COS fractions were collected resuspended in filtered (0.22µm filters) MilliQ water and concentrated in vacuum evaporator and loaded on TLC. DP1-DP6: COS mix.

3.6. Effect of moisture content on SSF

Different moisture content ranging from 10 to 90% was used to optimize culture conditions for COS production by *Streptomyces* sp. ANU-34 and CAI-21 strains. *Streptomyces* sp. (ANU-34) strain yielded DP2 and DP4 as the major end products at the optimum moisture of 50 to 90%. In the case of *Streptomyces* sp. (CAI-21), DP3 was the major end product when the moisture content was between 10 to 50%; at moisture content beyond 50%, the major products were DP1, DP3, and DP4. The optimum moisture content for COS production was from 60 to 90% for the *Streptomyces* sp. (CAI-21) strain, but earlier studies suggest preference for a lower moisture concentration in SSF (Nigam et al. 1994). Microorganisms prefer low moisture levels for optimum growth in SSF. Higher moisture concentration could interfere with oxygen transfer, reducing product yield (Mitchell et al. 2002).

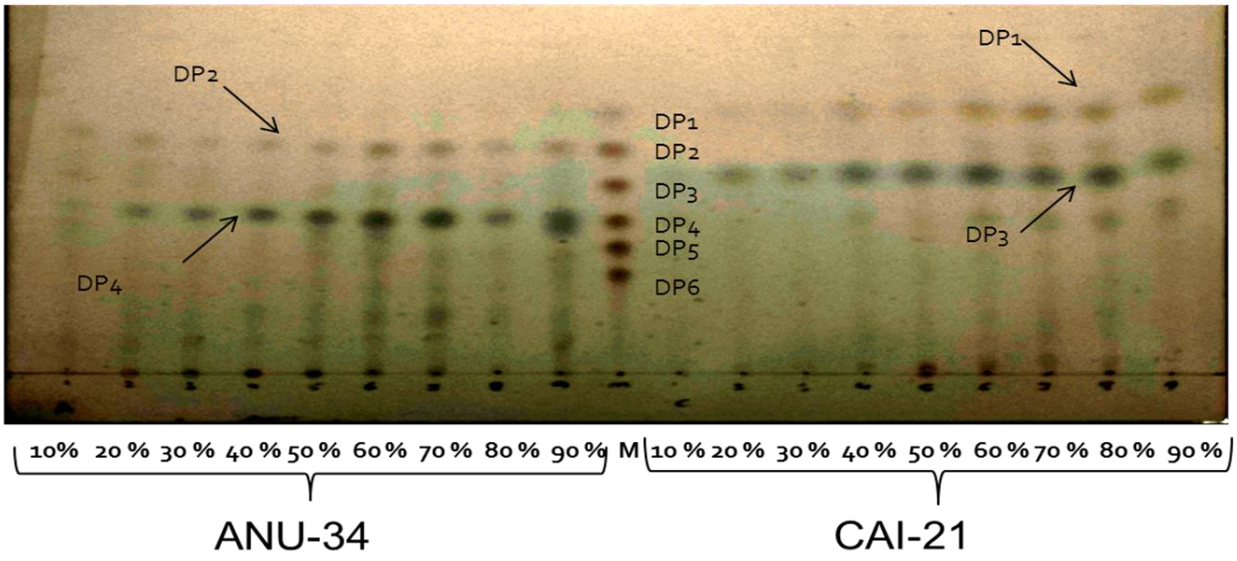

Fig. 3.11: Profiling of COS fractions collected from SSF flasks containing different amount of moisture content: Chitin flakes were incubated with either *Streptomyces sp.* (ANU-34) or *Streptomyces* sp. (CAI-21) in SSF with different moisture content (10-90%). After six days of incubation COS fractions were collected resuspended in filtered (0.22µm filters) MilliQ water and concentrated in vacuum evaporator and loaded on TLC. DP1-DP6: COS mix.

3.7. Effect of sugar concentration in SSF

The optimum percentage of sugar for the COS production for the *Streptomyces* sp. (ANU-34) strain was from 0.2 to 0.8%. During this optimum sugar concentration, the *Streptomyces* sp. (ANU-34) strain produced DP4 as the major end product at initial time points, and at later time points, DP4 and DP5 were the major products. Whereas, in the case of *Streptomyces* sp. (CAI-21) strain optimum percentage of sugar for the COS production was from 0.3 to 0.8%. *Streptomyces* sp. (CAI-21) strain has produced DP1 as the major end product at sugar concentrations from 0.1 to 0.3%. At later sugar concentrations, DP1 and DP3 were detected. Sucrose is a better carbon source when compared to other sugars (Walaszczyk et al. 2014). Sugar is known to provide an immediate energy source for the microbes in SSF. Earlier reports suggest initial sugar concentrations are beneficial for the optimum growth of microbes in SSF (Strasser et al. 1994).

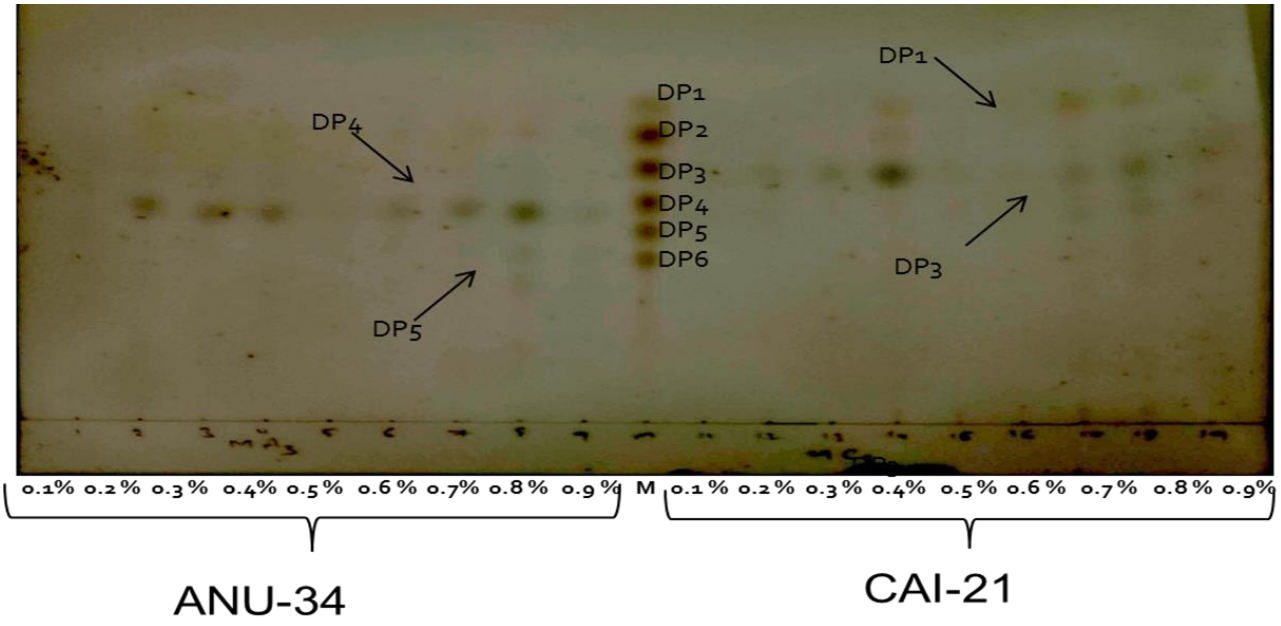

Fig. 3.12: Profiling of COS fractions collected from SSF flasks containing different concentration of sugar: Chitin flakes were incubated with either *Streptomyces sp.* (ANU-34) or *Streptomyces* sp. (CAI-21) in SSF with different concentration of sugar (0.1-0.9%). After six days of incubation COS fractions were collected resuspended in filtered (0.22µm filters) MilliQ water and concentrated in vacuum evaporator and loaded on TLC. DP1-DP6: COS mix.

Chapter IV

Reaction engineering to enhance COS production using trans-glycosylating chitinase

4.1 Introduction

Few chitinases have the capability to form long chain COS by using short length COS *via* transglycosylation (TG). Extensive studies on exploration of TG property of chitinases were done by Madhuprakash (2014) using chitinase D from *S. proteamaculans* (*Sp*ChiD) and Das (2016) using *P. elgii* (*Pe*Chi3). Madhuprakash et al. (2012) developed several mutants of *Sp*ChiD including *Sp*ChiD (Y28A) which had improved TG to produce long chain COS. Out of the four previously characterised chitinases (Das, 2016) (Table 4.1), *Pe*Chi3 possesses the highest hydrolytic activity on 90% DDA chitosan polymer apart from chitin hydrolysis. Based on this observation, *Pe*Chi3 was used for synthesis of COS of various DA using chitosan polymers (DA of 37% and 61%). Similarly, Y28A mutant showing improved TG was tested for further improvement in COS synthesis through reaction engineering using ionic liquids.

Enzyme	V_{max} (µmol s^{-1} mg^{-1})	K_m (mg ml^{-1})	k_{cat} (s^{-1})	Substrate-binding cleft	Activity on chitosan polymer (90%DDA)	Transglycosylation property
*Pe*Chi1	0.4301 x 10^{-2}	84.58	9.247	Deep tunnel	Not detected	Yes
*Pe*Chi3	3.377x 10^{-2}	29.26	439.01	Deep tunnel	High	No
*Pe*Chi4	2.142 x 10^{-2}	0.7263	299.88	Deep tunnel with closed roof	Moderate	No
*Pe*Chi5	0.2358x 10^{-2}	45.14	2.063	Open and shallow	Low	No

Table 4.1. Kinetic parameters of colloidal chitin hydrolysis by *P. elgi* chitinases. (Das, 2016)

TG reactions, like other glycosynthases, introduce new glycosidic bonds between shorter chain COS to form a longer-chain COS product. However, such chitinases yield very low quantity of desired COS due to their inherent hydrolytic activity. Therefore, to favor TG over hydrolytic activity, we need to decrease the water availability in the catalytic site of these enzymes. We hypothesized that performing catalysis in presence of ionic liquids may favor TG over hydrolysis. Ionic liquids are salts that do not crystallize at room temperature and could be used to alter the water activity, and secondary hydrolysis of the glycosidase catalyzed reactions to enhance yield of TG products. Mixture of dialkyl imidazolium cations and anions such as methyl sulfate or hexaflurophosphate can be used to influence the catalysis of glycoside hydrolases. Ionic liquids commonly used are mentioned in the table 4.2.

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Ionic liquid (%v/v)	Formate dehydrogenase (FDH)			β-galactosidase		
	25	50	75	25	50	75
[MIMIM] [$MeSO_4$]	65	73	98	74	14	-
[Et_3NH] [$MeSO_4$]	-	-	-	-	-	-
[Et3NMe] [$MeSO_4$]	82	55	-	-	-	-
[$PrNH_3$] [NO_3]	-	-	-	-	-	-
[BMIM] [BF_4]	-	-	-	31	-	6
[EMIM] [$PhCO_2$]	-	-	-	-	-	-
[BMIM] [$F3CSO_3$]	38	3	-	-	-	-
[BMIM [$OctSO_4$]	-	-	-	35	10	-

Table 4.2. Enzyme activity in the presence of ionic liquid, % of residual activity in comparison to the activity in pure buffer solution. (Kaftzik et al. 2002)

Osmolytes are noteworthy biological compounds which protect and enhance the stability of the enzymes *in vivo* by altering local water structure. Osmolytes are known to replace water from surface interaction, binding clefts, catalytic sites and other cavities present inside the enzymes and thereby influence the activity of enzyme. Water-binding humectants such as glycerol, lower water activity, and are widely used in several industrial applications (Badola et al. 2017). Water activity plays a vital role in steering hydrolysis and TG of GH18 chitinases. So, considering osmolytes and humectants as ingredients, catalytic reaction of *Sp*ChiD might open up new methods to improve TG. The methodology followed for expression and purification of *Sp*ChiD-Y28A and *Pe*Chi3, protein measurement, HPTLC, MALDI-TOF MS and HPLC analyses is described in the materials and methods (Sections 2.13, 2.13.1, 2.11.1, 211.2, 2.8.2.2).

4.2. Results and discussion

4.2.1 Generation of COS by PeChi3

Higher activity of the recombinant *Pe*Chi3 on chitosan polymer of DA ≤ 25% than glycol chitin and β-chitin (Table 4.1) encouraged to further evaluate the potential of this enzyme for COS synthesis using higher DA chitosan polymers. We tested both DA 37% and DA 61% chitosan polymers for COS production by *Pe*Chi3. The hydrolysates of 37% and 61% DA chitosans generated different shorter chain COS of DP<5 as observed in TLC (Fig 4.1). Appearance of smear below chitosan hexamer (D_6) indicated the generation of longer-chain COS.

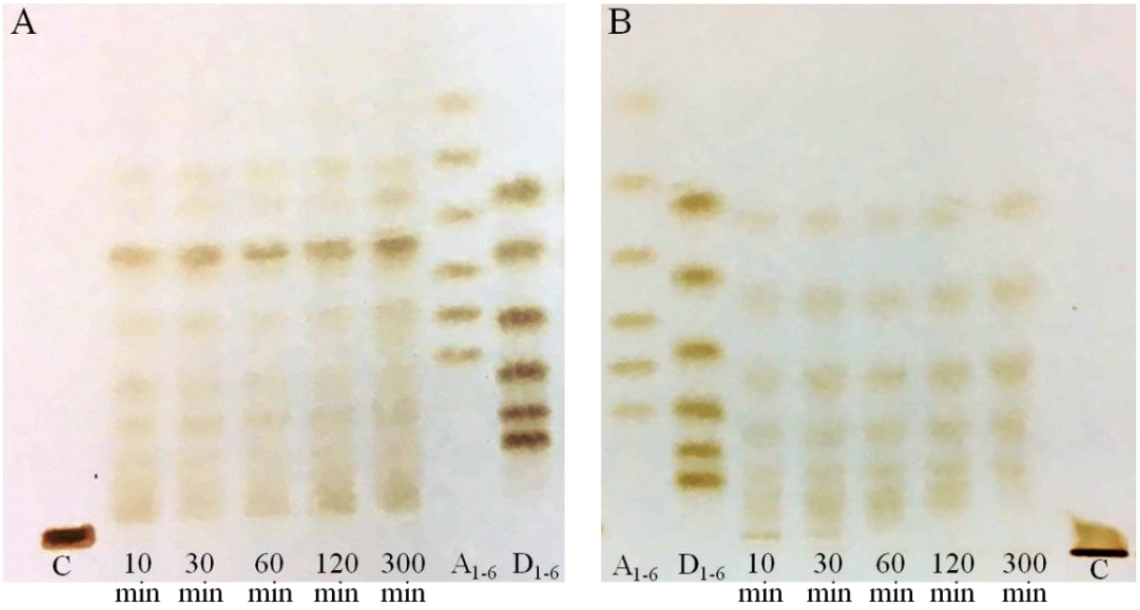

Fig. 4.1: TLC of hydrolysates generated by *Pe*Chi3: Profiling of hydrolysate generated by *Pe*Chi3 from chitosan polymers of DA 37% (A) and 61% (B). Fractions collected at different time intervals (shown in minutes) were loaded on to TLC plates and stained with ammonium bisulphate. A_{1-6} and D_{1-6} represent standard mixture of $(GlcNAc)_{1-6}$ and $(GlcN)_{1-6}$, respectively. Lane 'C' indicates the substrate control.

MALDI-ToF-MS analysis of hydrolysates from both the chitosan polymers revealed COS of various molecular weights representing chain lengths ranging from DP3-DP16. As expected, higher DA chitosan i.e. DA 61% yielded COS with higher percentage of '**A**' units (Fig 4.2). Hydrolysis of 37% DA chitosan showed the presence of various COS, including D_3, A_2D_1, A_1D_3, A_2D_2, A_1D_4, A_2D_3, and mono-acetylated COS ranging from DP6 to DP16. While 61% DA chitosan polymer resulted in COS of relatively higher percentages of '**A**' viz. D_3, A_2D_1, A_2D_2, A_4D_4, D_8, A_3D_6, A_4D_5, A_3D_7, A_4D_6, A_5D_5, A_4D_7, A_5D_6, A_4D_8, A_5D_7, A_4D_9, A_5D_8, A_6D_7, A_5D_9, A_6D_8, A_6D_9, and A_7D_8 (Fig 4.2). In addition to degradation of chitosans by chitosanases (Heggset et al. 2010; Pechsrichuang et al. 2018), several chitinases of GH18 and 19 have been assessed for their ability to cleave partially acetylated chitosan polymers into oligosaccharides (Heggset et al. 2009; Eide et al. 2012; Li et al. 2021). The substrate binding cleft of most of the GH18 chitinases requires a GlcNAc (A) at -1 subsite for catalysis. The +1 subsite is less absolute, and the presence of either A or D can lead to successful catalytic event (Van Aalten et al. 2001). Accumulation of long-chain mono-acetylated COS (Fig 4.2) in the hydrolysed products without subsequent cleavage into short-chain COS by *Pe*Chi3 substantiates the absolute preference of 'A' at -1 subsite and no preference for 'A' at -2 and -3 subsites. Li et al. (2021), detected similar mono-acetylated short-chain COS of DP3 and DP4 as major hydrolysed products by a chitinase (*Pp*Chi) from *Paenibacillus pabuli*.

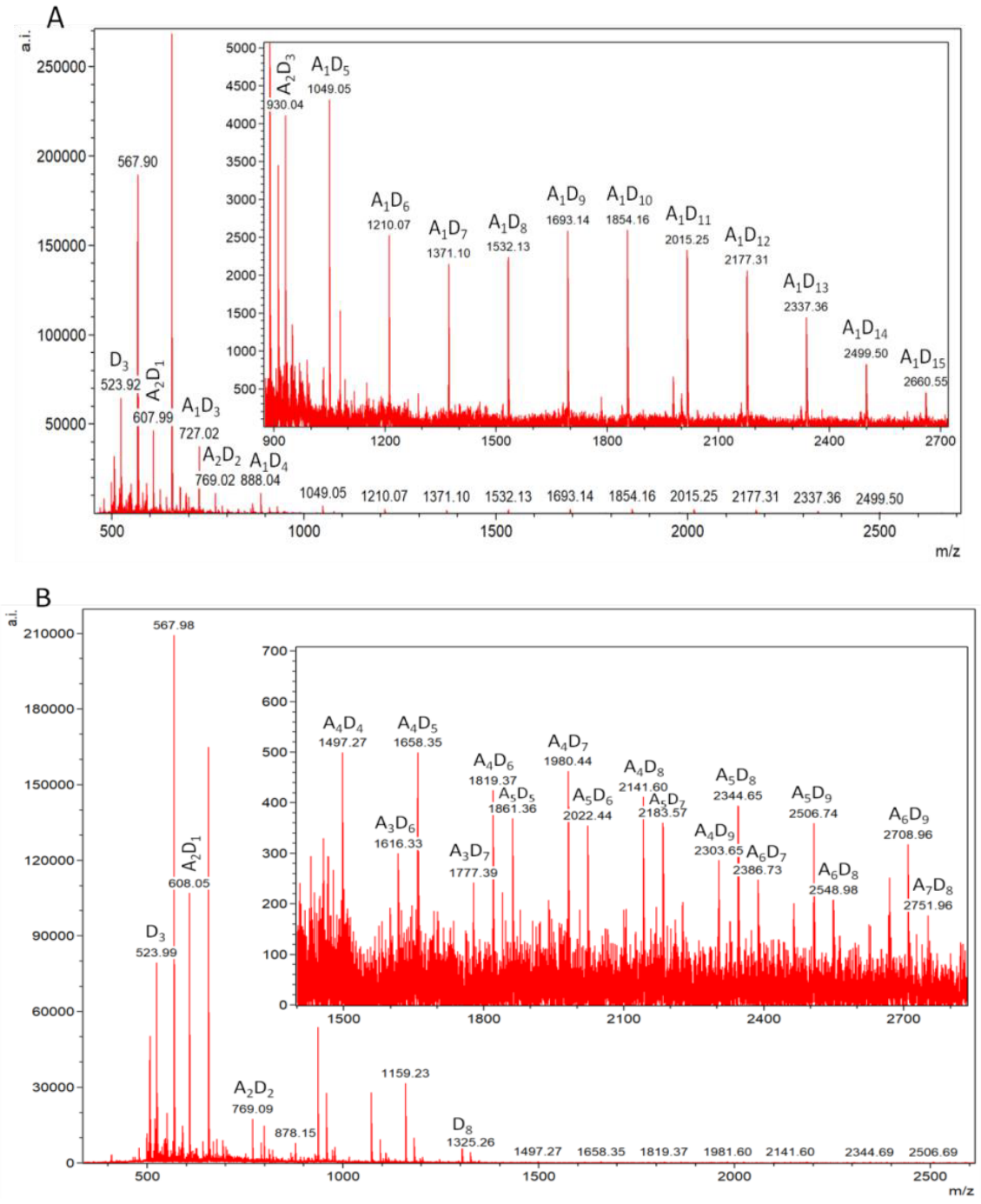

Fig. 4.2: MS analysis of COS generated by *Pe*Chi3: Chromatogram representing the COS generated by *Pe*Chi3 using 37% DA (A) and 61% DA (B) chitosan polymers. MALDI-ToF MS analysis of chitosan hydrolysates collected at 300 min showing different COS species. Masses shown are with Na adduct.

4.2.2 Effect of ionic liquids on TG of SpChiD-Y28A

To study the effect of ionic liquids on TG reaction catalysed by *Sp*ChiD-Y28A, we have tested four ionic liquids *viz*. 1-Butyl-3-methylimidazolium tetrafluroborate (I1), 1-Ethyl-3-methylimidazolium trifluoromethane sulfonate (I2), 1-Ethyl-3-methylimidazolium ethyl sulfate (I3) and 1-Butyl-3-methylimidazolium trifluoromethane sulfonate (I4). 200nM of *Sp*ChiD-Y28A was incubated with A5 (1 mg/ml) and samples were collected at various time points to monitor

the formation of longer chain CHOS (>5) as TG products. In presence of 10% 1-Butyl-3-methylimidazolium tetrafluroborate (I1), TG products were decreased slightly (Fig 4.3 A and B), whereas at 15%, both TG and hydrolytic activity were decreased drastically. The TG activity of *Sp*ChiD-Y28A decreased in presence of 10% (v/v) 1-Ethyl-3-methylimidazolium trifluoromethane sulfonate (I2), and TG products were not detected at 15% (v/v) (Fig 4.4 A and B). Hydrolysis was not affected at 10% (v/v) but drastically decreased in presence of 15% (v/v) I2 (Fig 4.4 B). In presence of 1-Ethyl-3-methylimidazolium ethyl sulphate (I3), both at 10% and 15% (v/v), TG products were reduced and hydrolysis by *Sp*ChiD-Y28A was drastically reduced at 15% (Fig 4.5 A and B). Similarly, addition of 1-Butyl-3-methylimidazolium trifluoromethane sulfonate (I4) into the reaction reduced TG activity of *Sp*ChiD-Y28A at both 10 and 15% (v/v) with reduction in hydrolysis only at 15% (v/v) (Fig 4.6 A and B).

1,3-dimethyl imidazolium methyl sulfate [MMIM][$MeSO_4$] has been shown to reduce secondary hydrolysis of β-galactosidase, and thereby increased the TG by 60% (Kaftzik et al. 2002). However, the four ionic liquids had similar negative effect on TG of *Sp*ChiD-Y28A possibly due to the similar type of cationic (imidazolium) components. The reduction of TG in presence of these ionic liquids, although hydrolysis retained at lower %, is not clearly understood. The reduction in hydrolysis at 15% (v/v) of ionic liquids may be due to the destabilizing effect of both imidazolium cations and anions on *Sp*ChiD-Y28A.

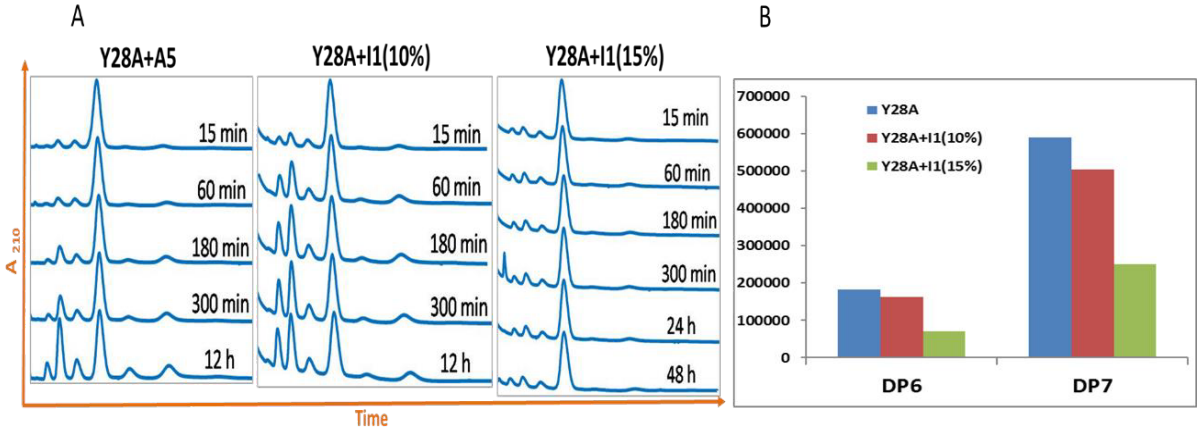

Fig. 4.3: Effect of I1 *i.e.*, 1-Butyl-3-methylimidazolium tetrafluroborate [BMIM][BF_4] on the TG activity of *Sp*ChiD-Y28A. A) HPLC chromatogram showing the peaks of hydrolysed and TG products in presence of 10% and 15% (v/v) I1. B) Comparison of peak areas of DP6 and DP7 TG products in presence of 10% and 15% (v/v) of I1. A5 (1mg/ml) was incubated with 200nM of *Sp*ChiD-Y28A and samples were collected at various time points as mentioned above each chromatogram.

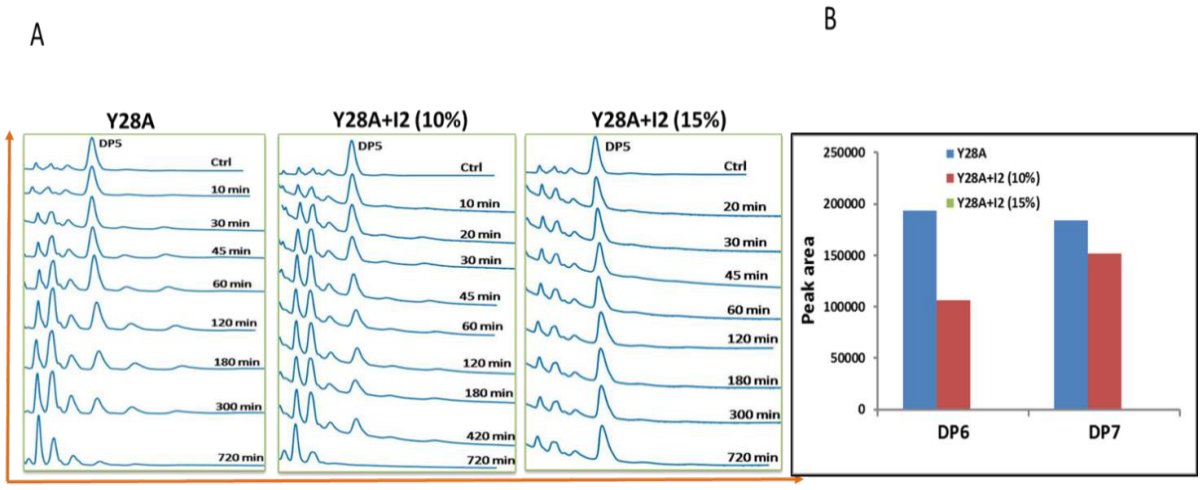

Fig. 4.4: Effect of I2 *i.e.*, 1-Ethyl-3-methylimidazolium trifluoromethane sulfonate on the TG activity of *Sp*ChiD-Y28A. A) HPLC chromatogram showing the peaks of hydrolysed and TG products in presence of 10% and 15% (v/v) I2. B) Comparison of peak areas of DP6 and DP7 TG products in presence of 10% and 15% (v/v) of I2.

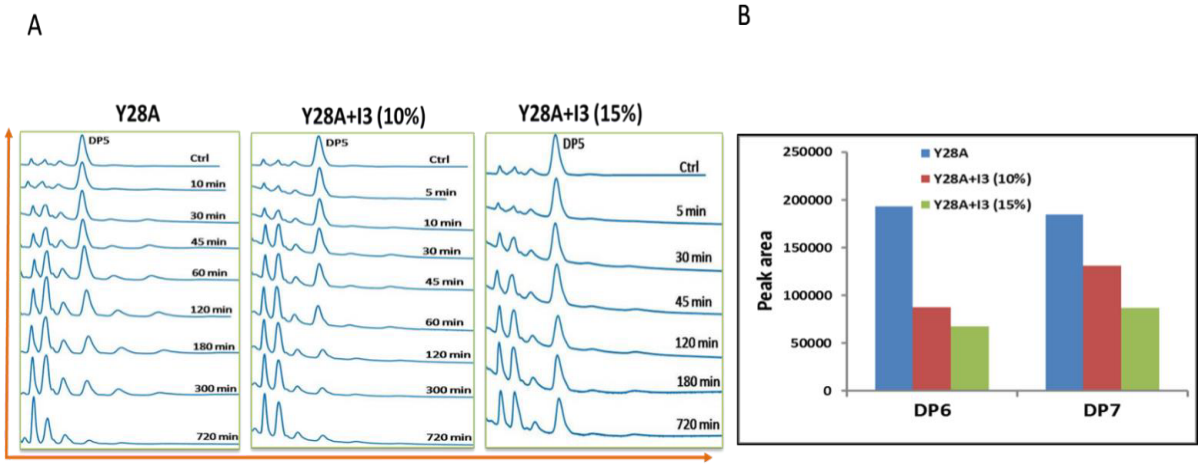

Fig. 4.5: Effect of I3 i.e. 1-Ethyl-3-methylimidazolium ethyl sulfate on the TG activity of *Sp*ChiD-Y28A. A) HPLC chromatogram showing the peaks of hydrolyzed and TG products in presence of 10% and 15% (v/v) I3. B) Comparison of peak areas of DP6 and DP7 TG products in presence of 10% and 15% (v/v) of I3.

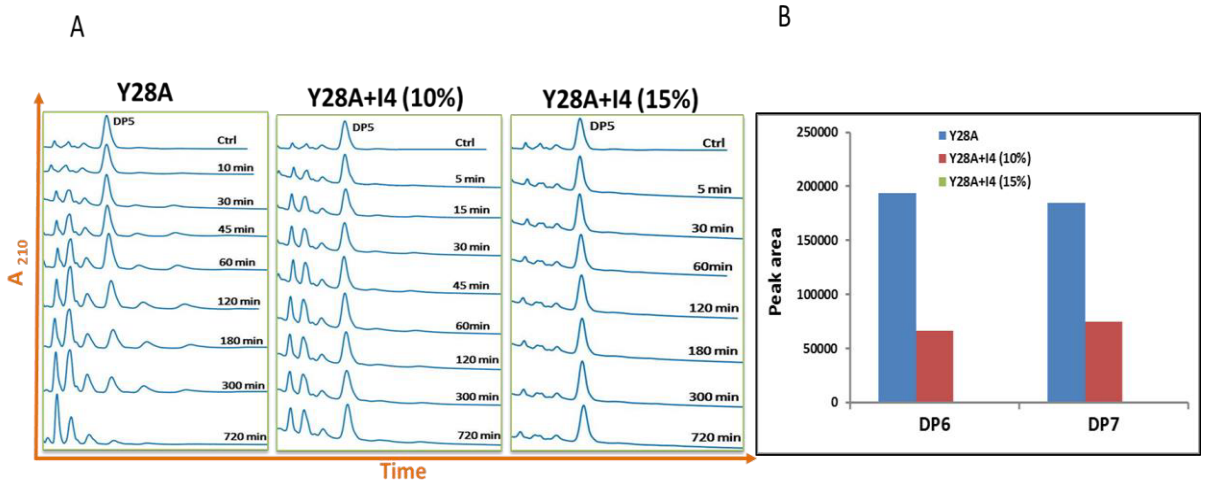

Fig. 4.6: Effect of I4 *i.e.*, 1-Butyl-3-methylimidazolium trifluoromethane sulfonate on the TG activity of *Sp*ChiD-Y28A. A) HPLC chromatogram showing the peaks of hydrolyzed and TG products in presence of 10% and 15% (v/v) I4. B) Comparison of peak areas of DP6 and DP7 TG products in presence of 10% and 15% (v/v) of I4.

4.2.3 Effect of osmolytes and humectants on TG of SpChiD

Hydrolytic and TG activity of *Sp*ChiD was tested in presence of different concentrations of osmolytes (5% of proline and *myo*-inositol) and humectants (15% of glycerol) as one of the components in the reaction mixture. The reaction was carried out at 40°C in 25mM of sodium acetate pH 5.6. We could not observe any improvement in TG with the tested osmolytes (proline, *myo*-inositol) and humectants (glycerol). However, enzyme hydrolysis was increased in presence of proline and glycerol (Fig 4.7).

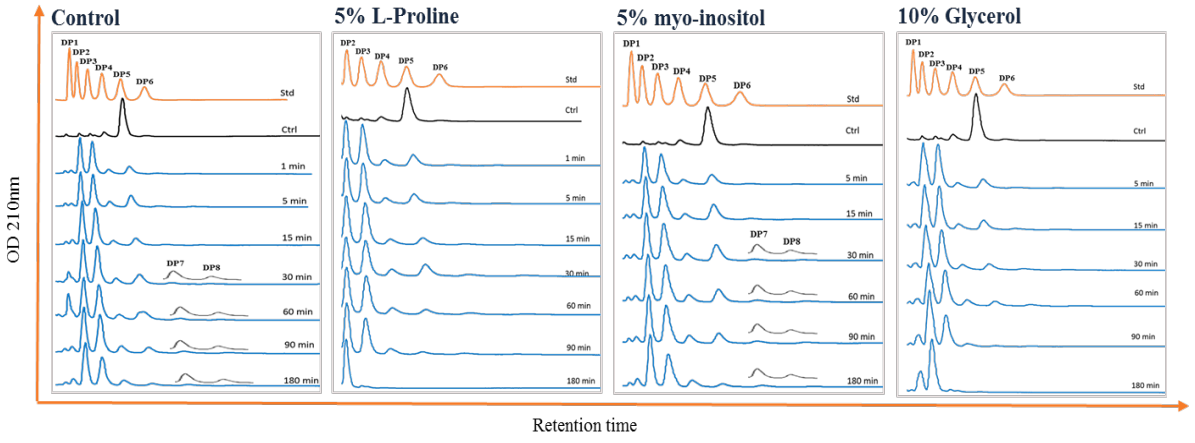

Fig. 4.7: Effect of osmolytes and humectants on the TG activity of *Sp*ChiD-Y28A: HPLC chromatogram showing the peaks of hydrolyses and TG products in presence of 5% proline and myo-inositol, and 10% glycerol.

In the present study, TG activity decreased in case of osmolytes and humectants. An increase in TG activity by 3.1-fold was reported for CelB when glycerol was incorporated in the enzyme reaction (Lang et al. 2006). However, enzyme hydrolysis was increased in presence of proline and glycerol (Fig 4.7). Similar result was observed when proline has improved the catalytic efficiency of PersiXyn2 (Norouzi et al. 2020). Similarly, glycerol was also known to increase the activity of human phenylamine mutant enzymes. (Leandro et al. 2001). Other similar osmolytes have also increased hydrolytic activity in case of *Pseudomonas cepacia* lipase enzyme (Azizi et al. 2011).

Chapter V

Pre-treatment of crystalline chitin with KOH and KOH–Urea for enhanced hydrolysis/release of COS

5.1. Introduction

Chitin is a hydrophobic polymer not soluble in water and many other solvents in crystalline form. Chitin is obtained from the exoskeletons of shrimps and crabs. The crystalline and water-immiscible nature of chitin makes it less amenable to hydrolysis by chitinases. The crystalline nature of chitin is the main hurdle for hydrolysis and product formation (Berton et al. 2018). The colloidal chitin obtained from concentrated acid treatment is used for enzymatic hydrolysis (Gao et al. 2018). Thus, there is a scope for a cost-effective green method that is environmentally friendly for the pre-treatment of chitin to make chitin amenable to hydrolysis by chitinases.

To dissolve chitin, less expensive laboratory bases like NaOH, LiOH, and KOH in combination with urea solutions have been used recently (Hu et al. 2007; Li et al. 2010). Chitin treated with ionic liquids and alkali/alkali-urea displayed broad applications (Shamshina et al. 2019). Chitin degradation by enzymatic methods is more advantageous than harsh chemicals because the enzymes work at lower temperatures in aqueous buffers. Pre-treatment of chitin can significantly enhance enzymatic hydrolysis. Pre-treatment of α-chitin with ionic liquid yielded significant enhancement in COS production on subsequent enzymatic hydrolysis with chitinases (Xu et al. 2019). The primary reason behind the enhanced yield was the substantial decrease in the crystalline nature of chitin after pre-treatment with ionic liquids (Hall et al. 2010).

Similarly, alkali-urea pre-treatment has significantly decreased the chitin crystallinity (Ding et al. 2019). Freeze-thaw method for alkali-urea solvent has not affected the molecular mass of chitin and the DA (Li et al. 2010). Different chitinases can be used on the KOH-urea pre-treated chitin substrates to get a good amount of COS products. Pre-treatment with KOH-urea can reduce the crystallinity of cellulose and chitin in an eco-friendly and cost-effective manner allowing the polymer to act upon enzymes more efficiently. Against this background, recently, chitin was subjected to KOH and KOH-urea pre-treatment followed by hydrolysis by *Ec*Chi1 and *Cs*ChiG chitinase enzymes (Sivaramakrishna et al. 2020a, 2020b). Here, six different pre-treated substrates were prepared using KOH and KOH-urea methods to check the efficiency of those substrates on subsequent hydrolysis with *Cs*ChiG to obtain COS.

The methodology followed for preparing *Cs*ChiG, substrate pre-treatment and HPLC analyses is described in the materials and methods (Sections 2.14, 2.8.2.2, 2.12).

5.2. Results and discussion

5.2.1 Time-course degradation of wet and dry substrates

Figures 5.1-5.6 show the HPLC chromatograms of *Cs*ChiG (5 µM) treated on wet and dry substrates (5 mg/mL) at different time points (1 h to 48 h sp) on six different pretreated substrates 11K1, 11KU1, 11K2, 11KU2, 20K1 and 20KU1, respectively. The reaction products were analyzed by binary gradient HPLC. The uppermost chromatogram profile corresponds to a standard COS mixture from DP1 to DP6. The other HPLC chromatogram profiles are for the reactions at different time points, (A) for wet substrate and (B) for dry substrate. Tables 5.1 and 5.2 represent mass values (individual and total) of DP1-DP3 product values of COS obtained by the hydrolysis of the *Cs*ChiG on wet and dry substrates, respectively. Fig. 5.7 indicates the overall formed products by wet and dry substrates of pre-treated substrates 11K1, 11KU1, 11K2, 11KU2, 20K1 and 20KU1 by *Cs*ChiG enzyme at different time points.

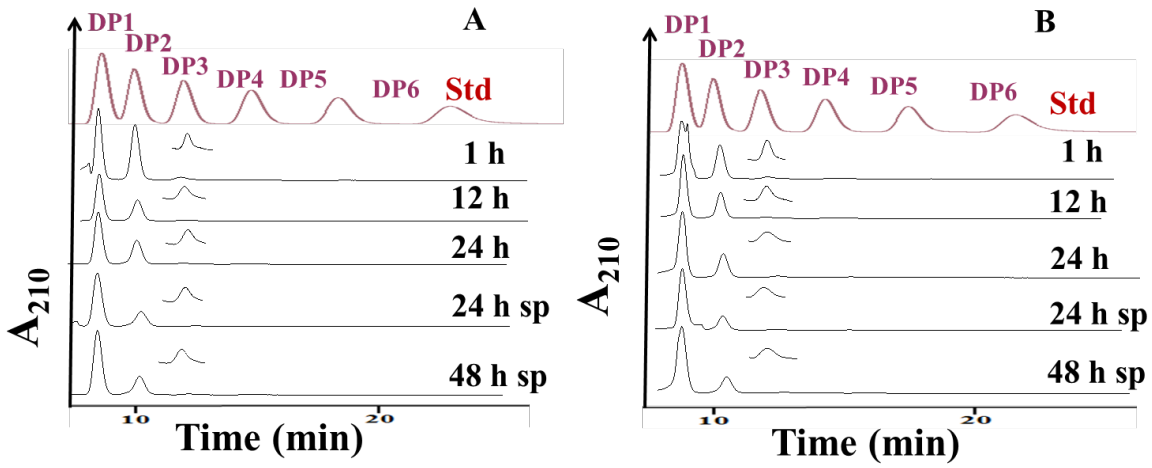

Fig. 5.1: Hydrolysis of 11K1 by *Cs*ChiG: The *Cs*ChiG (5 µM) was incubated with 5 mg/mL of the substrate in the reaction mixture for different time periods at 50 °C. The reaction products were analyzed by binary gradient HPLC. The topmost profile shows a standard mixture of COS ranging from DP1 to DP6. The remaining profiles are the reactions at different incubation times. (A) Wet substrate (B) Dry substrate.

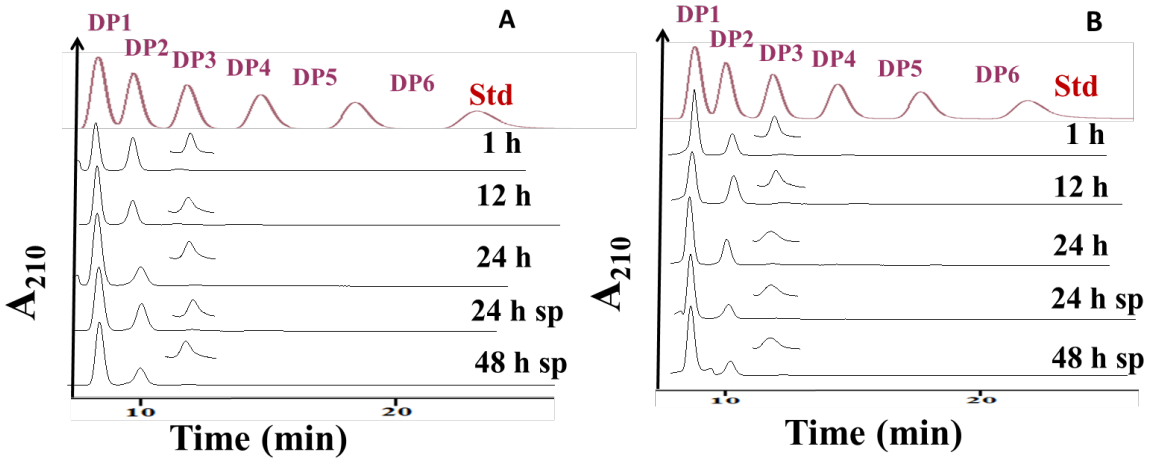

Fig. 5.2: Hydrolysis of 11KU1 by *Cs*ChiG: The *Cs*ChiG (5 µM) was incubated with 5 mg/mL of the substrate in the reaction mixture for different time periods at 50 °C. The reaction products were analyzed by binary gradient HPLC. The topmost profile shows a standard mixture of COS ranging from DP1 to DP6. The remaining profiles are the reactions at different incubation times. (A) Wet substrate (B) Dry substrate.

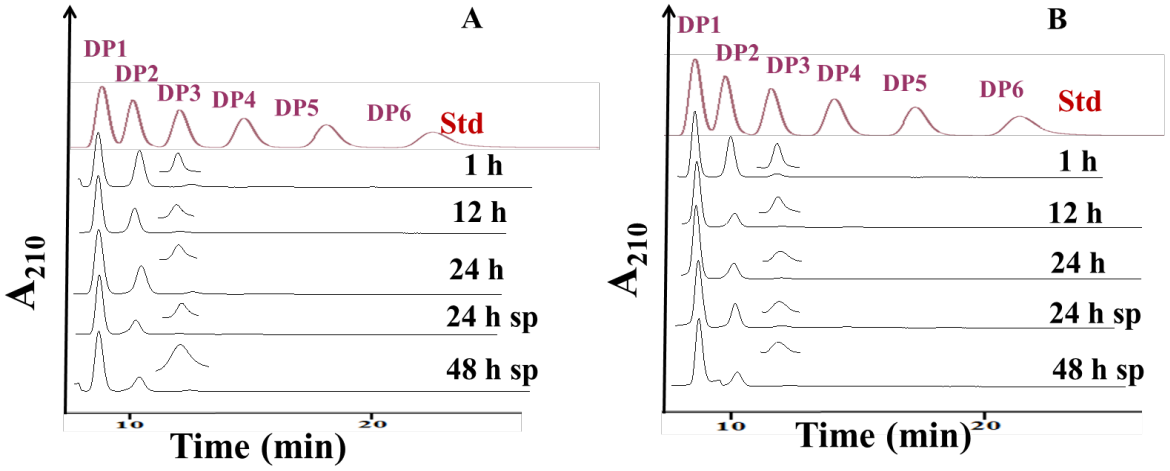

Fig. 5.3: Hydrolysis of 11K2 by *Cs*ChiG: The *Cs*ChiG (5 µM) was incubated with 5 mg/mL of the substrate in the reaction mixture for different time periods at 50 °C. The reaction products were analyzed by binary gradient HPLC. The topmost profile shows a standard mixture of COS ranging from DP1 to DP6. The remaining profiles are the reactions at different incubation times. (A) Wet substrate (B) Dry substrate.

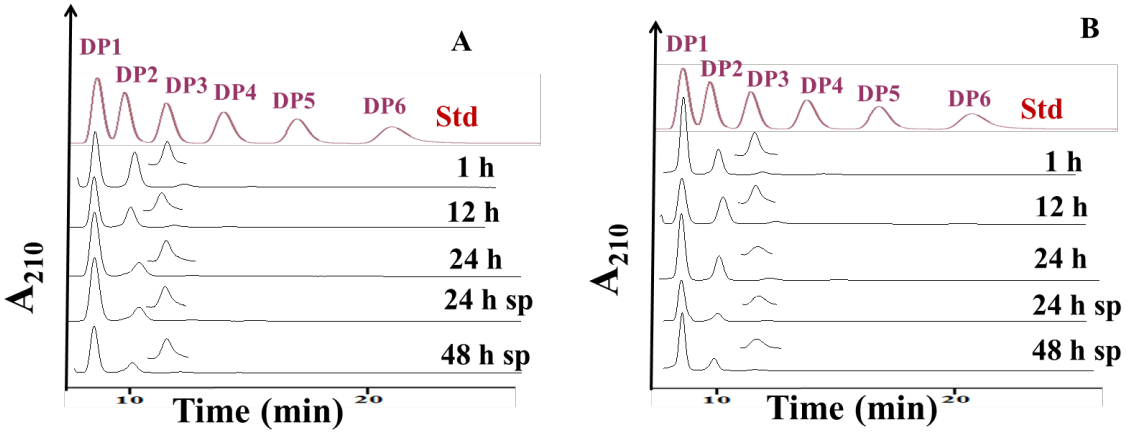

Fig. 5.4: Hydrolysis of 11KU2 by *Cs*ChiG: The *Cs*ChiG (5 µM) was incubated with 5 mg/mL of the substrate in the reaction mixture for different time periods at 50 °C. The reaction products were analyzed by binary gradient HPLC. The topmost profile shows a standard mixture of COS ranging from DP1 to DP6. The remaining profiles are the reactions at different incubation times. (A) Wet substrate (B) Dry substrate.

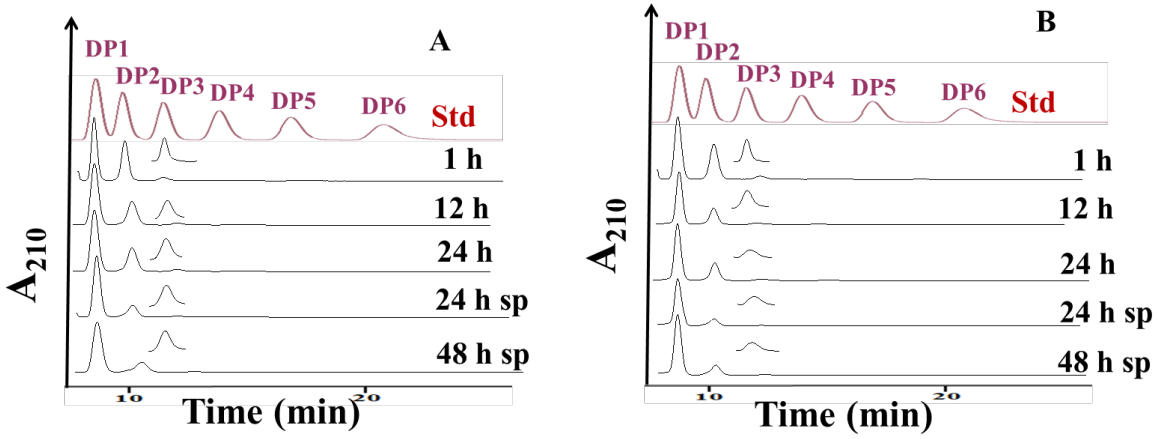

Fig. 5.5: Hydrolysis of 20K1 by *Cs*ChiG: The CsChiG (5 µM) was incubated with 5 mg/mL of the substrate in the reaction mixture for different time periods at 50 °C. The reaction products were analyzed by binary gradient HPLC. The topmost profile shows a standard mixture of COS ranging from DP1 to DP6. The remaining profiles are the reactions at different incubation times. (A) Wet substrate (B) Dry substrate.

Enzymatic hydrolysis of wet and dry pre-treated substrates of 11K1, 11KU1, 11K2, 11KU2, 20K1 and 20KU1 were shown in Fig. 5.1–5.6, respectively. Hydrolysis of all the treated chitin substrates generated DP1, DP2 and a small amount of DP3 as major products at 1 to 48 h sp. Sivaramakrishna et al. (2020a) also noticed similar products using this enzyme on incubation pre-treated 20K2 and 20KU2 substrates.

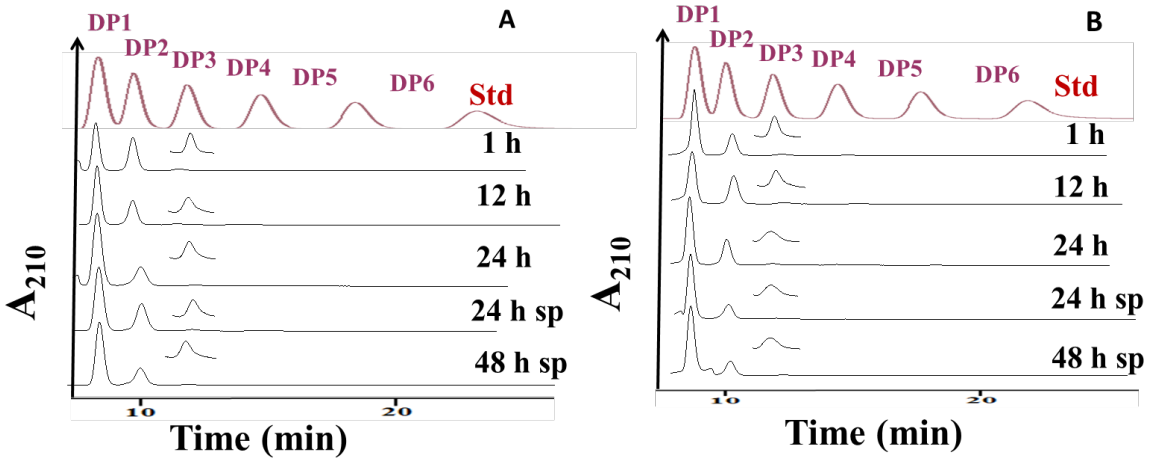

Fig. 5.6: Hydrolysis of 20KU1 by *Cs*ChiG: The *Cs*ChiG (5 µM) was incubated with 5 mg/mL of the substrate in the reaction mixture for different time periods at 50 °C. The reaction products were analyzed by binary gradient HPLC. The topmost profile shows a standard mixture of COS ranging from DP1 to DP6. The remaining profiles are the reactions at different incubation times. (A) Wet substrate (B) Dry substrate.

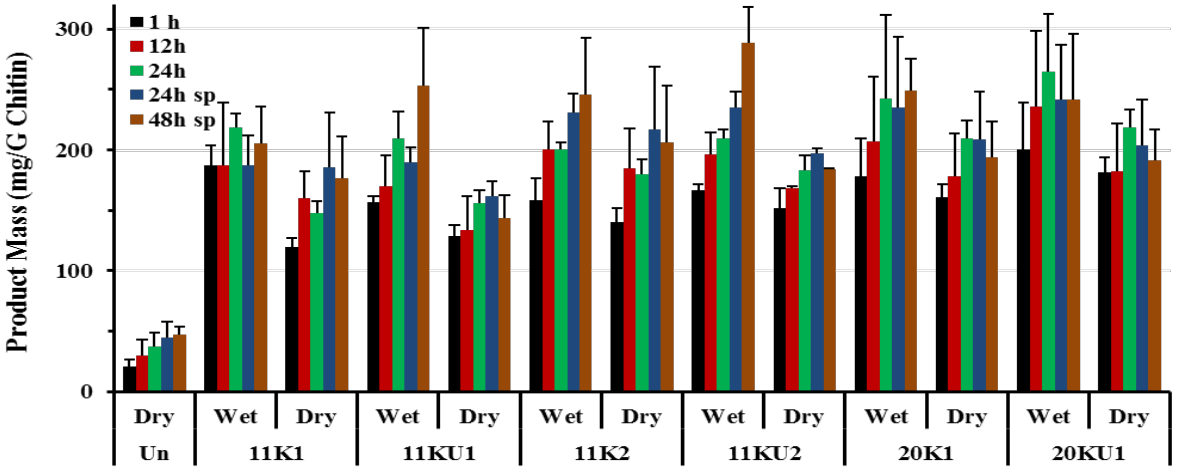

Fig. 5.7: Time-dependent hydrolysis of untreated (Un), 11K1, 11KU1, 11K2, 11KU2, 20K1, and 20KU1 substrates by *Cs*ChiG: Reaction: 5 mg/mL substrate and 5 µM enzyme in 50 mM sodium citrate buffer pH 4.0 at 50°C. The values are based on mean ± SD (of two identical experiments). Mass of product (mg/g chitin) for 1 h, 12h, 24 h, 24h sp, and 48h sp for wet and dry substrates. Sp: Additional 5 µM enzyme was added to the reaction mixture at 12 h.

Table 5.1. Mass values (individual and total) of DP1-DP3 products obtained by *Cs*ChiG on wet substrates.

Time/ Sample	Product	Wet					
		11K1	**11KU1**	**11K2**	**11KU2**	**20K1**	**20KU1**
1h	**DP1**	110.4(19.9)	90.4(5.8)	84.1(16.5)	91.2(7.1)	99.9(26.5)	116.0(37.7)
	DP2	70.3(2.5)	61.1(0.4)	67.5(3.3)	65.3(11.9)	70.5(6.5)	75.4 (2.3)
	DP3	6.6(0.9)	5.0(0.4)	6.8(1.4)	10.0 (0.8)	7.5(1.0)	9.0 (1.6)
	Total	**187.4(16.5)**	**156.5(5.0)**	**158.5(18.4)**	**166.5(5.6)**	**178.0(31.9)**	**200.4(38.4)**
12h	**DP1**	118.6(37.9)	109.1(19.9)	132.6(21.6)	129.4(13.3)	142.5(46.6)	164.7(58.8)
	DP2	63.6(11.6)	56.6(5.4)	61.0(5.2)	59.2(6.4)	57.5(7.2)	62.7(7.1)
	DP3	5.2 (2.2)	4.3(0.6)	7.3(2.0)	7.9(2.0)	7.4(1.8)	8.6(2.1)
	Total	**187.4(51.7)**	**170.0(25.8)**	**200.9(22.8)**	**196.4(18.3)**	**207.4(53.2)**	**236.0(62.8)**
24h	**DP1**	145.3(9.1)	139.3(16.0)	131.5(18.2)	135.4(5.0)	169.6(61.6)	187.3(52.5)
	DP2	67.6(2.4)	64.8(6.9)	61.8(10.2)	64.6(9.4)	65.3(8.0)	68.3(3.4)
	DP3	5.7(0.0)	5.1(0.6)	7.4(2.3)	9.3(2.9)	8.0(1.2)	9.5(1.3)
	Total	**218.6(11.6)**	**209.3(22.4)**	**200.7(5.7)**	**209.4(7.4)**	**242.9(68.5)**	**265.1(47.8)**
24h sp	**DP1**	138.2(20.6)	145.0(10.6)	177.5(7.0)	179.2(3.2)	187.1(45.9)	190.6(31.9)
	DP2	45.3(3.6)	41.9(0.8)	48.2(6.4)	49.5(8.4)	43.6(10.6)	45.9(11.1)
	DP3	3.5(1.0)	3.0(0.7)	5.5(2.2)	6.3(2.1)	4.5(1.8)	5.4(2.5)
	Total	**187.0(25.1)**	**189.9(12.2)**	**231.2(15.5)**	**235.0(13.7)**	**235.2(58.3)**	**241.8(45.5)**
48h sp	**DP1**	154.6(22.6)	191.1(59.1)	185.3(44.3)	217.6(42.5)	195.2(18.1)	187.7(35.8)
	DP2	47.0(7.2)	58.9(8.7)	54.9(20.7)	63.5(7.5)	49.5(7.7)	48.5(15.6)
	DP3	4.1(0.2)	3.6(0.2)	5.5(2.2)	7.5(0.2)	4.5(0.2)	5.8(3.1)
	Total	**205.8(30.0)**	**253.6(67.5)**	**245.8(67.1)**	**288.6(49.8)**	**249.3(26.0)**	**242.0(54.5)**

(Mass values (individual and total) of DP1-DP3 products obtained at 1 h, 12 h, 24 h, 24 h sp and 48 h sp. Sp: Additional 5 µM enzyme was added to the reaction mixture at 12 h. Hydrolysis of different chitin substrates by *Cs*ChiG. The *Cs*ChiG (5 µM) was incubated with 5 mg/mL of the substrate in the reaction mixture at 50 °C. The reaction products were analyzed by binary gradient HPLC. The eluted COS was monitored at 210 nm. Calibration curves were constructed separately for each COS in the mixture. The data points generated a linear curve for each COS with r^2 values of 0.997 to 1.0. Obtained milli Molar values were converted to mg/g using the following equation. [(Individual COS concentration*molecular weight)/1000]*200. Standard deviation is given in parenthesis.

5.2.2 Product mass of total COS (DP1- DP3)

The total product mass (DP1-DP3) of all the wet and dry pre-treated substrates was higher when compared to untreated substrate (Fig 5.7). The wet substrates produced greater mass when compared to the dry substrates. The untreated substrate has shown a gradual increase in COS production from 1 to 12 h and 24 h, and adding an extra enzyme also produced more products at 24 h sp and 48 h sp time points. The total products obtained by the 11K1 wet substrate have not changed from 1 to 12 h; however, they increased from 12 to 24 h. Adding an extra enzyme has not increased the products for 24 h sp and 48 h sp time points. Whereas, in the case of 11K1 dry substrate, the total product mass increased from 1 to 12 h, and upon adding an extra enzyme from

the 24 to 24 h sp and 48 h sp. The total products from the 11KU1 wet substrate gradually increased from 1 to 48 h sp. Whereas, in the case of the 11KU1 dry substrate, the total product mass increased from 1 to 24 h sp and then slight decreases in 48 h sp. Overall, the decline in conversion is very high than the 11KU1 wet substrate. The total products obtained by 11K2 wet substrate gradually increased from 1 to 48 h sp. Similarly, in the case of 11K2 dry substrate, a gradual increase is noticed from 1 to 12 h, and 24 to 24 h sp, suggesting the enzyme addition is beneficiary. The 11KU2 wet and dry substrates have shown a similar pattern exhibited by the 11K2 wet and dry substrate in total product generation. In the case of 20K1 wet and dry substrates, product mass increased gradually from 1 to 12 h and 24 h; however, adding an extra enzyme has not shown any increase in the product mass at 24 h sp and 48 h sp. 20KU1 wet and dry substrates exhibited a similar pattern in total product generation. From the above, it is evident that either the increase or decrease of product mass depends on the substrate state (dry/wet), the KOH concentration (11% or 20%) used for the pre-treatment and the presence of urea (4 M) in the KOH.

Table 5.2. Mass values (individual and total) of DP1-DP3 products obtained by *Cs*ChiG on dry substrates.

Time/ Sample	Product	Dry					
		11K1	**11KU1**	**11K2**	**11KU2**	**20K1**	**20KU1**
1h	**DP1**	53.7(3.6)	61.7(4.2)	67.1(5.7)	73.1(7.3)	79.2(5.3)	93.2(6.3)
	DP2	60.8(4.1)	62.4(4.2)	66.9(7.1)	71.1(11.4)	73.0(4.9)	78.7(5.3)
	DP3	4.9(0.3)	4.9(0.3)	6.5(1.2)	7.3(1.6)	9.0(0.6)	10.0(0.7)
	Total	**119.4(8.0)**	**129.0(8.7)**	**140.5(11.6)**	**151.5(17.1)**	**161.2(10.9)**	**181.9(12.2)**
12h	**DP1**	88.3(6.7)	80.9(15.2)	107.1(19.2)	103.9(3.0)	108.8(20.1)	113.7(25.7)
	DP2	69.7(18.4)	49.2(12.0)	68.6(10.9)	56.6(4.9)	61.3(14.0)	61.2(11.9)
	DP3	3.6(1.4)	3.5(1.3)	9.2(3.0)	7.6(0.2)	7.8(2.0)	7.5(1.9)
	Total	**159.8(22.6)**	**133.6(28.5)**	**184.9(33.1)**	**168.2(2.1)**	**178.0(36.1)**	**182.4(39.5)**
24h	**DP1**	85.6(5.8)	93.2(6.3)	104.0(7.0)	110.4(7.4)	127.9(8.6)	137.3(9.2)
	DP2	58.2(3.9)	58.7(4.0)	68.6(4.6)	65.6(7.4)	73.8(5.0)	72.7(4.9)
	DP3	4.0(0.3)	4.0(0.3)	7.3(0.5)	7.5(0.5)	8.3(0.6)	9.0(0.6)
	Total	**147.8(10.0)**	**155.9(10.5)**	**179.8(12.1)**	**183.5**(12.4)	**209.9(14.1)**	**219.0(14.7)**
24h sp	**DP1**	127.3(31.6)	116.3(2.8)	157.0(42.5)	144.5(6.2)	155.1(26.6)	151.3(25.9)
	DP2	53.1(10.8)	42.6(8.6)	52.4(5.1)	46.9(3.7)	49.0(11.4)	47.2(11.0)
	DP3	5.1(2.9)	2.7(1.0)	7.5(4.0)	6.0(1.7)	5.0(1.3)	5.1(1.0)
	Total	**185.5(45.3)**	**161.5(12.4)**	**216.9(51.6)**	**197.4(4.2)**	**209.2(39.3)**	**203.7(37.9)**
48h sp	**DP1**	121.1(24.5)	105.9(3.8)	147.8(37.4)	134.8(3.6)	143.0(19.6)	141.4(17.3)
	DP2	51.2(7.1)	35.6(14.4)	51.6(5.3)	44.1(5.7)	46.8(9.4)	45.6(7.7)
	DP3	4.4(2.8)	2.0(0.6)	7.1(4.3)	5.3(1.1)	4.3(0.4)	4.6(0.8)
	Total	**176.7(34.3)**	**143.5(18.8)**	**206.5(47.0)**	**184.2(1.1)**	**194.1(29.4)**	**191.5(25.8)**

(Mass values (individual and total) of DP1-DP3 products obtained at 1 h, 12 h, 24 h, 24 h sp, and 48 h sp. Sp: Additional 5 µM enzyme was added to the reaction mixture at 12 h. Hydrolysis of different chitin substrates by *Cs*ChiG. The *Cs*ChiG (5 µM) was incubated with 5 mg/mL of the substrate in the reaction mixture at 50 °C. The reaction products were analyzed by binary gradient HPLC. The eluted COS was monitored at 210 nm. Calibration curves were constructed separately for each COS in the mixture. The data points generated a linear curve for each COS with r^2 values of 0.997 to 1.0. Obtained milli Molar values were converted to mg/g using the following equation. [(Individual COS concentration*molecular weight)/1000]*200. Standard deviation is given in parenthesis.

5.2.3 Product mass of N-acetylglucosamine (DP1)

The formation of DP1 products was observed to be higher in all pre-treated substrates compared to the untreated substrate (Fig. 5.8). Specifically, for 11K1 wet substrates, the DP1 product mass showed a gradual increase from 1 to 24 h, later slightly decreased at 24 h sp, and then an increase again at 48 h sp. On the other hand, in the case of the 11K1 dry substrate, the DP1 product mass gradually increased from 1 to 24 h sp and a slight decrease at 48 h sp. Similarly, DP1 products obtained from 11KU1 wet substrate continuously increased from 1 to 48 h sp. However, for the 11KU1 dry substrate, the DP1 product mass gradually increased from 1 to 24 h sp, slightly decreasing at 48 h sp. For the 11K2 wet substrate, the DP1 product mass gradually increased from 1 to 48 h sp. In the case of the DP1 product, mass from 11K2 dry substrate gradually increased from 1 to 12 h, later a slight decrease at 24 h, an increase at 24 h sp, and a slight decline again at 48 h sp. As for the 11KU2 wet substrate, the DP1 product mass gradually increased from 1 to 48 h sp. In the case of the 11KU2 dry substrate, the DP1 product mass gradually increased from 1 to 24 h sp and then slightly decreased at 48 h sp. For the 20K1 wet substrate, the DP1 product mass gradually increased from 1 to 48 h sp. Similarly, in the case of 20K1 dry substrate, the DP1 product mass gradually increased from 1 to 24 h sp and then slightly decreased at 48 h sp. Finally, DP1 products obtained from 20KU1 wet substrate demonstrated a continuous increase from 1 to 48 h sp. In contrast, the DP1 product mass from 20KU1 dry substrate gradually increased from 1 to 24 h sp, slightly decreasing at 48 h sp.

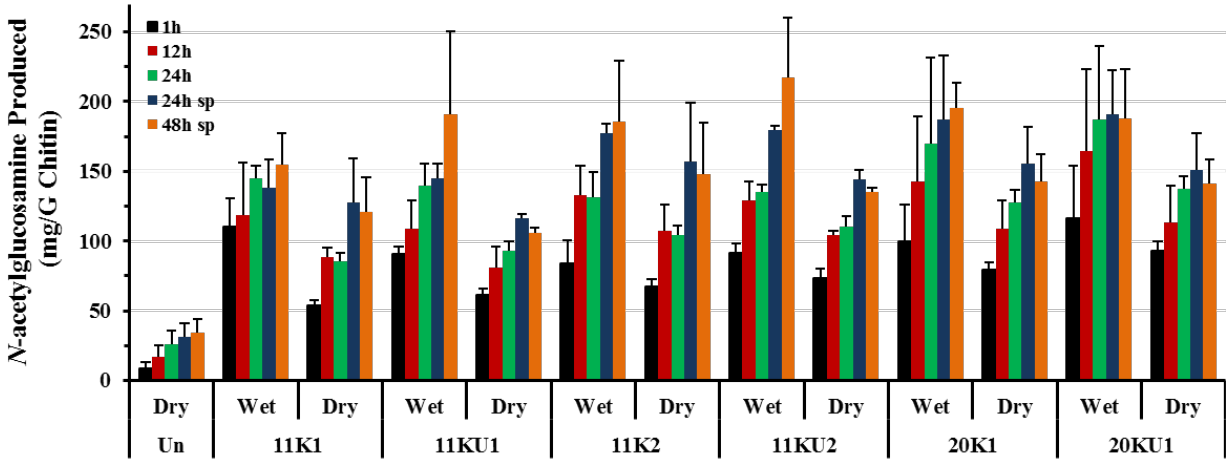

Fig. 5.8: Time-dependent *N*-acetyl glucosamine (DP1) generation by *Cs*ChiG: DP1 generated by *Cs*ChiG on dry and wet substrates of untreated, CC, 11K1,11KU1,11K2,11KU2, 20K1 and 20KU1 at 1 h, 12 h, 24 h, 24 h sp and 48h sp. Reaction: 5 mg/mL substrate and 5 µM enzyme.

5.2.4 Product mass of N, N'-diacetylchitobiose (DP2)

The production of DP2 products using different substrates (11K1, 11KU1, 11K2, 11KU2, 20K1, and 20K2) showed varying trends depending on whether wet or dry substrates are employed, as shown in (Fig 5.9). When utilizing the 11K1 wet substrate, there is a gradual decrease in DP2 product mass from 1 h to 12 h, and followed by an increase at 24 to 48 h sp. Addition of an extra enzyme had no impact on DP2 formation. Similarly, with the 11K1 dry substrate, the DP2 product mass slightly increased from 1 to 12 h but gradually decreased at 24 to 48 h sp. For the 11KU1 wet substrate, fluctuations were observed in DP2 product mass. It decreased from 1 to 12 h, increased at 24 h, then decreased again at 24 h sp, and finally increased at 48 h sp. Similarly, with the 11KU1 dry substrate, the DP2 mass decreased from 1 to 12 h, increased at 24 h, and decreased at 24 h sp and 48 h sp. The 11K2 wet substrate showed stable DP2 product mass at 1 to 24 h, but it decreased at 24 h sp and increased at 48 h sp. On the other hand, the11K2 dry substrate showed a gradual increase in DP2 product mass from 1 to 12 h, followed by stability until 24 h, then decreased at 24 h sp and 48 h sp. Using the 11KU2 wet substrate, the production of DP2 product decreased from 1 to 12 h and then increased at 24 to 48 h sp. However, with the 11KU2 dry substrate, the DP2 mass decreased at 1 to 12 h, increased at 24 h, and decreased at 24 h sp and 48 h sp. For the 20K1 wet substrate, the production of DP2 product gradually decreased at 1 to 12 h, then increased at 24 h, decreased again at 24 h sp, and slightly increased at 48 h sp. Similarly, with the 20K1 dry substrate, DP2 mass decreased at 1 h, increased at 12 h and 24 h, and gradually decreased at 24 h sp and 48 h sp. Regarding the 20KU1 wet substrate, the production of DP2 product gradually decreased at 1 to 12 h, then increased at 24 h, and then decreased at 24 h sp, with a slight increase at 48 h sp. Overall, the trends in DP2 product mass appear to be influenced by the type of substrate (wet or dry) and the specific substrate used.

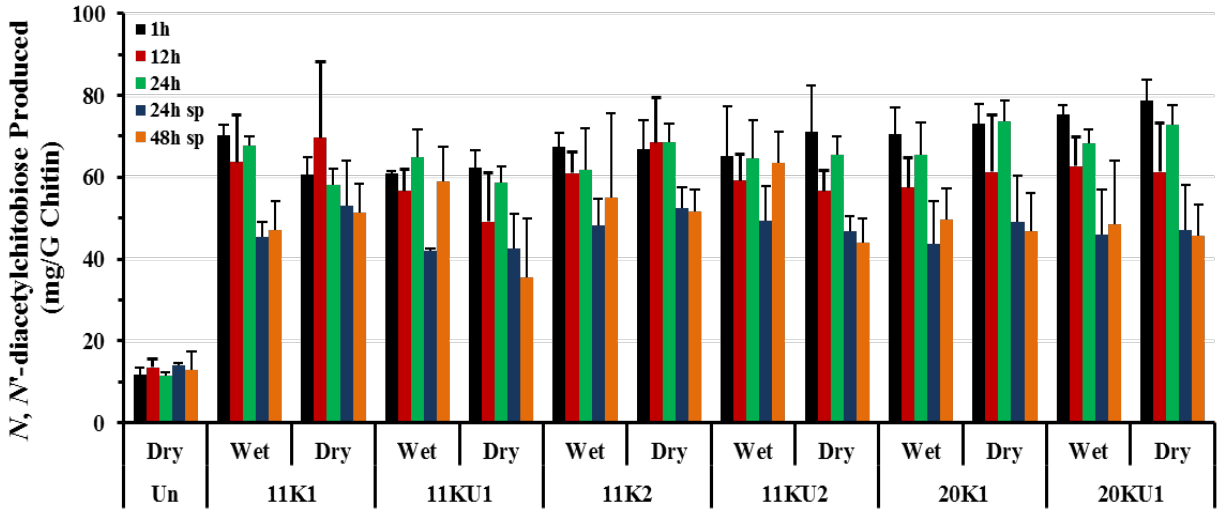

Fig. 5.9: Time-dependent *N*, *N*'-diacetylchitobiose (DP2) generation by *Cs*ChiG: DP2 generated by *Cs*ChiG on dry and wet substrates of untreated, CC, 11K1,11KU1,11K2, 11KU2, 20K1 and 20KU1 at 1 h, 12 h, 24 h, 24 h sp and 48 h sp. Reaction: 5 mg/mL substrate and 5 µM enzyme.

5.2.5 Product mass of N, N', N''-triacetylchitotriose (DP3)

All DP3 products were derived exclusively from pre-treated substrates and untreated substrate not given DP3 upon hydrolysis with *Cs*ChiG (Fig 5.10). When the 11K1 wet substrate was utilized, the mass of DP3 gradually decreased over time, showing a decline at 1 h and 12 h but a slight increase at 24 h compared to 12 h. However, there was a subsequent decrease at 24 to 48 h sp. In contrast, when the 11K1 dry substrate was used, the mass of DP3 remained relatively stable, with minor fluctuations observed from 1 to 48 h sp. The mass of DP3 decreased at 24 h sp and 48 h sp when using the 11KU1 wet substrate, compared to the time points from 1 to 24 h. Conversely, using the 11KU1 dry substrate resulted in a gradual decrease in the mass of DP3 over time, decreasing at 1 to 48 h sp. For the 11K2 substrate, DP3 products obtained from wet substrates displayed the same level, slightly increasing from 1 to 24 h, later a decrease at 24 h sp and 48 h sp. On the other hand, when the 11K2 dry substrate was used, the product mass of DP3 slightly increased from 1 to 12 h, later a decrease at 24 to 48 h sp. Using the 11KU2 wet substrate resulted in a decreased mass of DP3 products from 1 to 48 h sp. a subsequent decrease at 24 h sp and 48 h sp. When the 20K1 wet substrate was used, the DP3 product mass remained stable at 1 to 24 h but decreased at 24 h sp and 48 h sp. Likewise, employing the 20K1 dry substrate resulted in a reduction in DP3 mass from 1 to 48 h sp. Using the 20K2 wet substrate, the DP3 product mass remained stable from 1 to 24 h, with a subsequent decrease at 24 h sp. Similarly, when the 20K2 dry substrate was utilized, the product mass of DP3 remained constant from 1 to 24 h, later decreased at 24 h sp and 48 h sp.

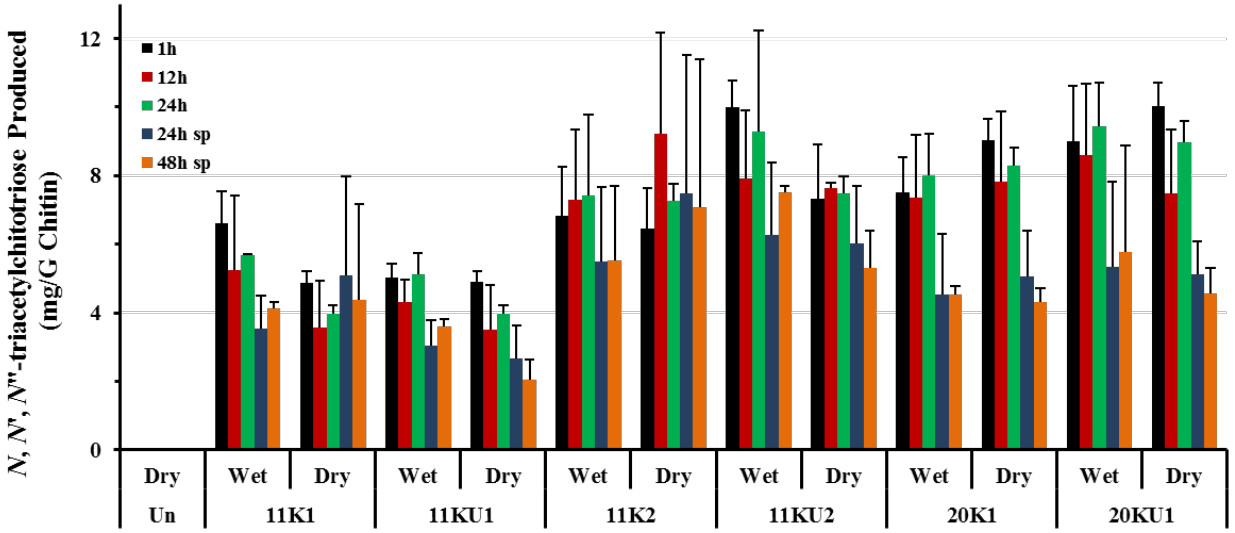

Fig. 5.10: Time-dependent *N*, *N*', *N*''-triacetylchitotriose (DP3) by *Cs*ChiG: DP3 generation by *Cs*ChiG on dry and wet substrates of untreated, 11K1, 11KU1, 11K2, 11KU2, 20K1 and 20KU1 at 1 h, 12 h, 24h, 24h sp and 48h sp. Reaction: 5 mg/mL substrate and 5 µM enzyme.

5.2.6 Percentage conversion

The overall conversion percentage of chitin to COS products from untreated and six pretreated substrates is given in Table 5.3. The results indicate that the percentage of products formed through hydrolysis follows the DP1 > DP2 > DP3 order. For the untreated substrate, the percentage of DP1 produced is 2.0%, 3.0%, 3.8%, 4.5%, and 4.8% at 1 h to 48 h sp, respectively. Similarly, for the 11K1 wet substrate, the COS percentage is 18.7%, 18.7%, 21.9%, 18.7%, and 20.6%, and for the 11K1 dry substrate, it is 11.9%, 16.0%, 14.8%, 18.5%, and 17.7% at the same time points in the same order. The COS percentage of the 11KU1 wet substrate was 15.6%, 17.0%, 20.9%, 19.0%, and 25.4% for the mentioned time points, and for the 11KU1 dry substrate, it is 12.9%, 13.4%, 15.6%, 16.2%, and 14.3% at the corresponding time points. COS percentage for 11K2 wet substrates was 15.8%, 20.1%, 20.1%, 23.1%, and 24.6%, and for 11K2 dry substrates, is 14.0%, 18.5%, 18.0%, 21.7%, and 20.7% at the same time points. The COS percentage for 11KU2 wet substrates was 16.6%, 19.6%, 20.9%, 23.5%, and 28.9%, and for 11KU2 dry substrate, was 15.1, 16.8, 18.3, 19.7 and 18.4 at the same time points. While for the 20K1 wet substrate, it was 17.8%, 20.7%, 24.3%, 23.5%, and 24.9%, and for 20K1 dry substrate was 16.1%, 17.8%, 21.0%, 20.9% and 19.4% at 1 h to 48 h sp, respectively. Notably, a high percentage of COS was produced through the hydrolysis of the 20K1 wet substrate. The COS percentage for 20KU1 wet substrates was 18.1%, 18.6%, 20.5%, 21.7%, and 23.6%, and 20KU1 dry substrates was 15.3%, 15.4%, 17.9%, 19.9%, and 18.3%.

Pre-treatment has increased the percentage of COS production and upon further addition of extra enzyme resulted in an increased amount of DP1 that inturn increased with extended incubation

time. These findings indicate that the chitotriosidase activity depended on the crystalline nature and type of substrate. The percentage of COS produced remained the same at 12 h and 24 h but increased upon adding additional enzymes to the reaction mixture. These findings suggest that addition of extra enzymes and extended incubation period promoted COS formation in pre-treated substrates.

In biocatalytic reactions, the crystalline nature of biopolymers plays a crucial role on the distribution and yield of products. Previous studies on chitinases from *S. griseus, S. albolongus,* and *P. pasadenensis* have shown that they produced low amount of DP1 compared to pre-treated substrates with low crystallinity (Husson et al. 2017; Berton et al. 2018; Li et al. 2019; Xu et al. 2019).

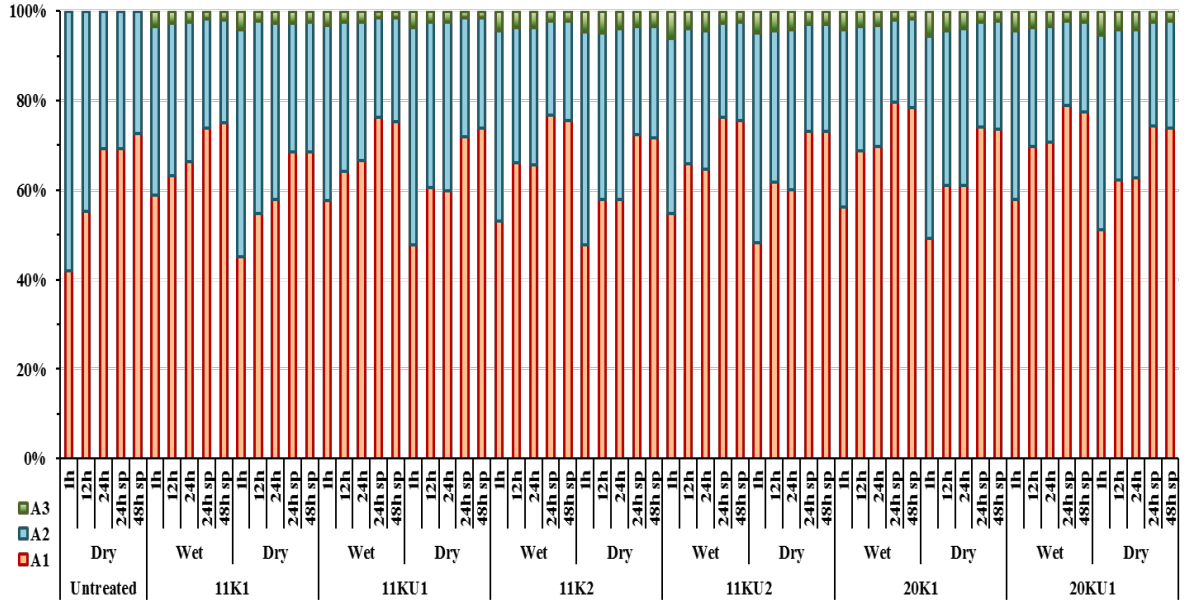

Fig. 5.11: Time-dependent percentage of COS: COS (DP1, DP2 and DP3) produced in the reaction mixtures of untreated (Un) and treated 11K1, 11KU1, 11K2, 11KU2, 20K1 and 20KU1 chitin substrates by *Cs*ChiG for wet and dry substrates.

Table 5.3. Percentage conversion of wet and dry chitin substrates into chitooligosaccharides by *Cs*ChiG

Substrate	1 h		12 h		24 h		24 h sp		48 h sp	
	Wet	Dry	Wet	Dry	Wet	Dry	Wet	Dry	Wet	Dry
Untreated	2.0 (0.6)		3.0 (1.3)		3.8 (1.1)		4.5 (1.3)		4.8 (0.6)	
11K1	18.7 (1.7)	11.9 (0.8)	18.7 (5.2)	16.0 (2.3)	21.9 (1.2)	14.8 (1.0)	18.7 (2.5)	18.5 (4.5)	20.6 (3.0)	17.7 (3.4)
11KU1	15.6 (0.5)	12.9 (0.9)	17.0 (2.6)	13.4 (2.8)	20.9 (2.2)	15.6 (1.0)	19.0 (1.2)	16.2 (1.2)	25.4 (6.8)	14.3 (1.9)
11K2	15.8 (1.8)	14.0 (1.2)	20.1 (2.3)	18.5 (3.3)	20.1 (0.6)	18.0 (1.2)	23.1 (1.6)	21.7 (5.2)	24.6 (6.7)	20.7 (4.7)
11KU2	16.6 (0.6)	15.1 (1.7)	19.6 (1.8)	16.8 (0.2)	20.9 (0.7)	18.3 (1.2)	23.5 (1.4)	19.7 (0.4)	28.9 (5.0)	18.4 (0.1)
20K1	17.8 (3.2)	16.1 (1.1)	20.7 (5.3)	17.8 (3.6)	24.3 (6.8)	21.0 (1.4)	23.5 (5.8)	20.9 (3.9)	24.9 (2.6)	19.4 (2.9)
20KU1	18.1 (1.3.)	15.3 (1.4)	18.6 (4.1)	15.4 (2.3)	20.5 (5.4)	17.9 (0.2)	21.7 (2.3)	19.9 (2.1)	23.6 (1.5)	18.3 (3.1)

24 h sp indicates the result obtained at 24 h in which 5 µM additional enzyme *Cs*ChiG was added to the reaction mixture at 12 h and incubated up to a total of 24 h, which means an extra 12 h after adding additional enzyme), and 48 h sp indicates the result obtained at the prolonged incubation of 24 h sp (it means extra 36 h after adding additional enzyme).

5.2.7 Fold-increase of products

Considering the yield of COS produced by *Cs*ChiG using the untreated substrate as a reference, we calculated the relative fold increase for six pre-treated substrates. The results are shown in Table 5.4 and Figure 5.12. 11K1 wet substrate yielded 9.7, 7.4, 6.1, 4.2, and 4.3-fold higher amounts of COS, while the 11K1 dry substrate produced 6.1, 6.0, 4.1, 4.4, and 3.8-fold higher amounts at 1 h to 48 h sp time points, respectively. Similarly, the 11KU1 wet substrate resulted in 8.0, 6.6, 5.7, 4.3, and 5.5-fold amounts of COS, while the 11KU1 dry substrate produced 6.6, 4.7, 4.4, 3.7, and 3.0-fold amounts at similar time points. For the 11K2 wet substrates, hydrolysis produced 7.9, 7.8, 5.5, 5.3, and 5.1-fold higher amounts of COS, and 11K2 dry substrate produced 7.2, 7.0, 5.0, 5.2, and 4.4-fold amounts at the mentioned time points. Similarly, the 11KU2 wet substrate resulted in 8.5, 7.6, 5.8, 5.4, and 6.2-fold amounts of COS, while the 11KU2 dry substrate yielded 7.8, 6.1, 5.1, 4.6, and 3.9-fold amounts at different time points. For the 20K1 wet substrates, hydrolysis produced 8.8, 8.1, 6.5, 5.2, and 5.3-fold higher amounts of COS, and 8.3, 6.2, 5.9, 4.7, and 4.1-fold amounts at the mentioned time points. 20KU1 wet substrate resulted in 9.9, 9.0, 7.1, 5.4, and 5.1-fold amounts of COS, while the 20KU1 dry substrate yielded 9.4, 6.3, 6.1, 4.6, and 4.0-fold amounts at different time points.

Overall results indicate that the highest fold increase of 9.9 was recorded for the pre-treated 20KU1 wet substrate, while the lowest fold increase of 3.7 in the 11KU1 dry substrate. The production of COS by chitin-degrading enzymes depends on various factors, including the mode of action (endochitinase or exochitinases), processive and non-processive nature, and the form of chitin (α or β), as well as the treated or untreated substrate. *S. griseus*, *P. psadensis* and *S. albolongus* chitinases increases the COS production by 1.5 to 2.0 and 7.4-fold with ionic liquid pre-treatment compared to untreated chitin (Husson et al. 2017; Berton et al. 2018; Li et al. 2019; Xu et al. 2019). Our study focused on using a less harsh and less expensive KOH and KOH-urea combination, compared to other harsh treatments, like HCl and expensive ionic liquids.

Table 5.4. Relative fold increase of chitooligosaccharides on wet and dry chitin substrates.

Substrate	1 h		12h		24h		24h sp		48h sp	
	Wet	Dry	Wet	Dry	Wet	Dry	Wet	Dry	Wet	Dry
Untreated	1		1		1		1		1	
11K1	9.7 (3.6)	6.1 (2.2)	7.4 (1.7)	6.0 (3.3)	6.1 (2.1)	4.1 (1.5)	4.2 (0.7)	4.4 (2.3)	4.3 (0.1)	3.8 (1.2)
11KU1	8.0 (2.6)	6.6 (2.3)	6.6 (2.3)	4.7 (1.0)	5.7 (1.1)	4.4 (1.5)	4.3 (1.0)	3.7 (0.8)	5.5 (2.1)	3.0 (0.1)
11K2	7.9 (1.4)	7.2 (2.7)	7.8 (3.5)	7.0 (4.1)	5.5 (1.5)	5.0 (1.8)	5.3 (1.2)	5.2 (2.6)	5.1 (0.7)	4.4 (1.6)
11KU2	8.5 (2.7)	7.8 (3.1)	7.6 (3.8)	6.1 (2.5)	5.8 (1.9)	5.1 (1.8)	5.4 (1.3)	4.6 (1.4)	6.2 (1.9)	3.9 (0.5)
20K1	8.8 (1.0)	8.3 (2.9)	8.1 (2.0)	6.2 (1.4)	6.5 (0.1)	5.9 (2.1)	5.2 (0.2)	4.7 (0.5)	5.3 (0.1)	4.1 (0.1)
20KU1	9.9 (1.0)	9.4 (3.3)	9.0 (1.6)	6.3 (1.4)	7.1 (0.8)	6.1 (2.2)	5.4 (0.6)	4.6 (0.5)	5.1 (0.5)	4.0 (0.1)

Note: Considering the total amount of products generated from the untreated substrate as one unit, the relative increase in the total amount of products generated from treated substrates was calculated using Table 5.3 and 5.4 values. Values in the parenthesis indicate standard deviation.

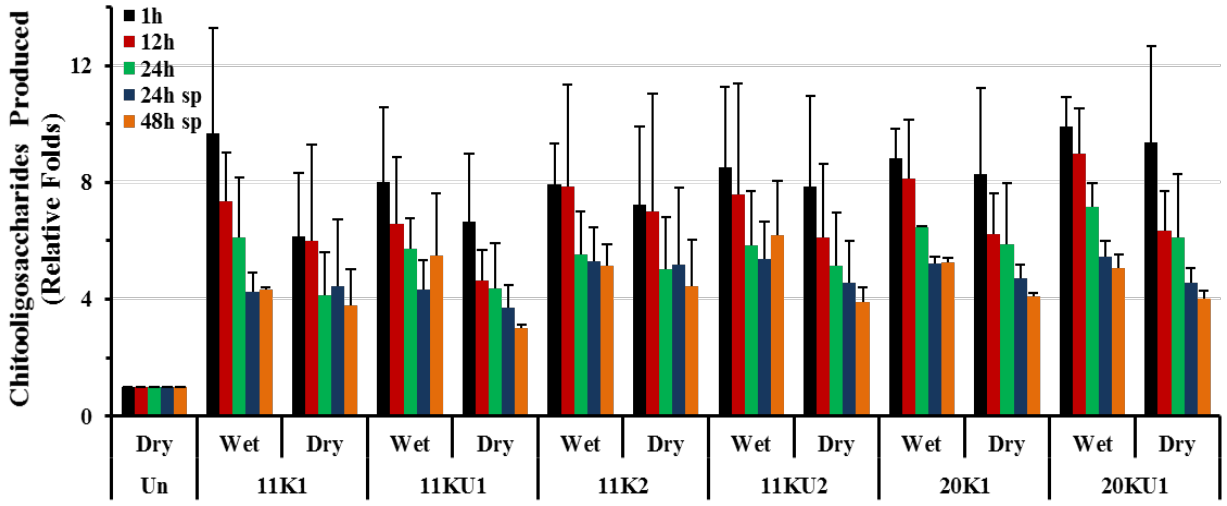

Fig. 5.12: **Time-dependent relative fold increase of COS yields:** Increase in the COS yields in the reaction mixture of untreated, 11K1, 11KU1, 11K2, 11KU2, 20K1 and 20KU1 chitin substrates by *Cs*ChiG Reaction; 5mg/ml substrate and 5 µM enzyme in 50 mM sodium citrate buffer, pH 4.0 at 50°C. The values are based on mean ± SD (of two identical experiments). Mass of product (mg/g chitin) for 1 h, 12h, 24 h, 24h sp and 48h sp for both wet and dry substrates.

Chapter VI

Testing the plant strengthening activity of the COS on tomato

6.1. Introduction

Synthetic chemicals are not only threat to environment but also equally harmful to human health. The chemical fertilizers, when used, tend to change the soil environment and microbiome that in course of ages has negative effect on plants growing in the soils (Katiyar et al. 2014). Chitin and its derivatives have many potential applications in the field of medicine, biotechnology and agriculture because of their bioactivity, biocompatibity and biodegradability. The main application of chitin derivatives in agriculture is their usefulness as an alternative to chemical fertilizers and pesticides for better plant health. Earlier studies indicate foliar application of COS has showed plant growth promotion and also elicited plant defence response (Fajardo et al. 1998; Yue et al. 2001; Khan et al. 2003; Ali et al. 2012; Dzung et al. 2017; Ramakrishna et al. 2021).

Tomato is an important vegetable crop not only because it has several important nutrients but it gives fruits in short time. With increasing pest incidence and due to environmental changes the yields of tomato have decreased. Therefore, to protect the crop and to increase the yield, farmers depend on chemical fertilizers and pesticides. COS could be the solution for having organic way of cultivation (Thapa et al. 2011). COS are harmless to humans, crops or other animals because of their biodegradable, nontoxic and biocompatibility nature. Chitosan application helped to overcome drought stress and also increased water use in coffee and pepper (Bittelli et al. 2001; Dzung et al. 2011). In the present study, COS produced *via* microbe-assisted SSF was used to enhance growth and defence response in tomato.The methodology followed for plant growth conditions, COS treatment, relative water content measurement, chlorophyll estimation, determination of PSII and PSI photochemical efficiencies, RNA isolation, cDNA synthesis and quantitative real-time PCR (qRT-PCR), estimation of amino acid content and FE-SEM of chitin and tomato leaves are described in the materials and methods (Sections 2.16, 2.17, 2.18, 2.19, 2.20, 2.21, 2.9).

6.2. Results and Discussion

6.2.1. Plant height in COS treated plants

The growth parameters like shoot height and length of the plants treated with water and COS (produced by ANU and CAI) were measured after 10 days of foliar spray. Tomato plants were 0.5-fold taller in ANU-COS treated and 0.25-fold taller in CAI-COS treated when compared to

control.

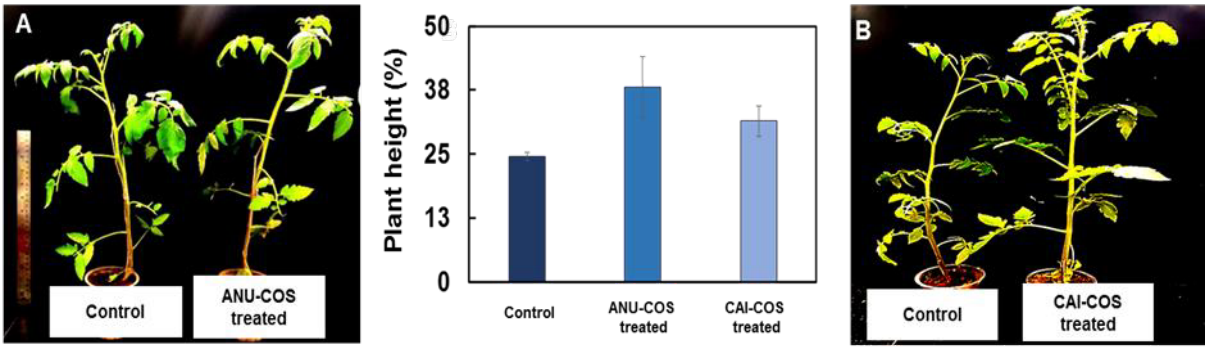

Fig. 6.1: Plant height in control and COS treated plants: Shoot height of tomato plants was recorded 10 days after foliar spray centimetres. Data represent the mean of the three independent experiments. Error bars indicate standard deviation (n=3). The data was subjected to one-way ANOVA followed by Dennett's multiple comparisons test. *$P \leq 0.05$, ** $P \leq 0.01$.

Plant height is a key aspect of plant development, which influence the ability of the plant to compete for light, access nutrients, and reproduction. Plant height slightly increased in COS pre-treated plants compared to control. ANU-COS treated plants were 0.5 fold taller and CAI-COS treated plants were 0.25 fold taller than control plants. Similar results were obtained in COS treated chilli plants (Dzung et al. 2017), and in bean plant (Sheikha and Al-Malki, 2011). Growth promotion of COS is by induction of tryptophan dependent pathway of auxin biosynthesis (Lopez et al. 2017). It is also suggested that COS may trigger a signal for the biosynthesis of hormones involved in plant growth promotion and development (Uthairatanakij et al. 2007).

6.2.2 Leaf relative water content in control and COS treated plants

The physiological water content of the plant is estimated through relative water content (RWC). It is calculated as the ratio of the water content of a sample (measured as a change between the fresh weight and the dry weight of the sample) to its maximum water-holding capacity (measured as the change between the turgid weight and the dry weight of the sample). RWC is used in to quantify the effects of different treatments or environmental conditions on plant water status, and can also be used in crop production to monitor water stress and optimize irrigation management. RWC remained same in COS treated and untreated plants. In comparison the relative water content remained constant in COS treated and untreated plants.

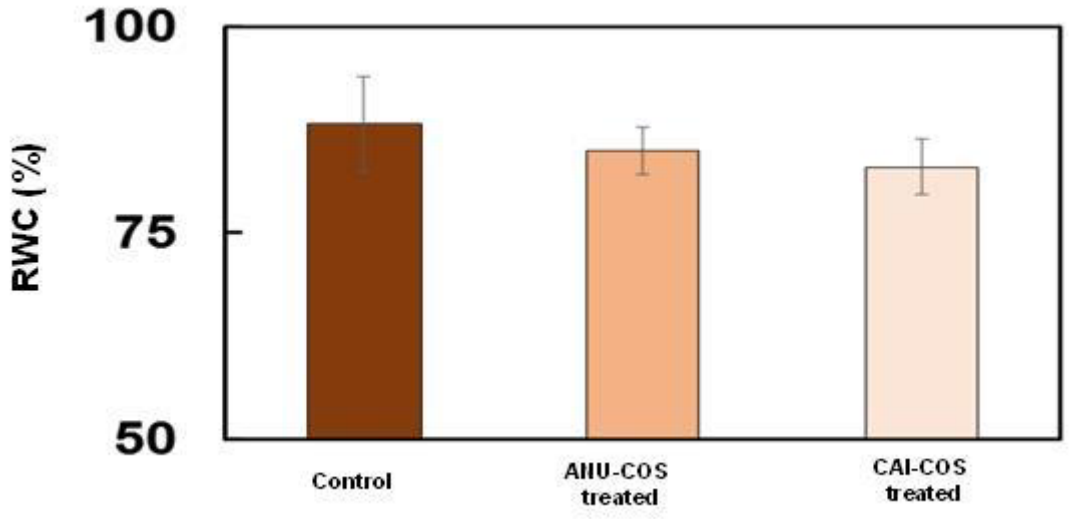

Fig. 6.2: Leaf Relative water content in control and COS treated plants: Relative water content of control and treated plants with COS. Values represent mean ± SD of three separate experiments, each in triplicate.

RWC remained same in control and COS treated plants. Similarly, COS has reverted the RWC values of drought stress plants on foliar application (Bittelli et al. 2001). Upon COS treatment RWC was reverted in stress exposed garlic (Abdelaal et al. 2021) and summer savory plants (Alizadeh et al. 2022).

6.2.3 Chlorophyll content

The chlorophyll content (chlorophyll a, chlorophyll b and total chlorophyll) of ANU-COS and CAI-COS treated plants along with control is shown in the Fig 6.3. Chlorophyll a content of ANU-COS treated showed no changes in the chlorophyll composition when compared with control plants. While chlorophyll content in CAI-COS treated was lower when compared to control plants. Similarly, chlorophyll b and total chlorophyll content in ANU-COS treatment was higher and in CAI-COS treatment was lower when compared to control plants. However, the chlorophyll a/b ratio in case of CAI-COS treated was 2-fold higher than in control plants. Whereas, in ANU-COS treated plants there was no change in chlorophyll a/b content. The relative chlorophyll contents (Chl a, Chl b, total Chl and Chl a/b) of control and ANU-34 were measured. A significant change in chlorophyll content was not observed in ANU-34 treated and untreated plants. While there is a reasonable enhancement of chlorophyll content seen in CAI treated plant (chl a/b).

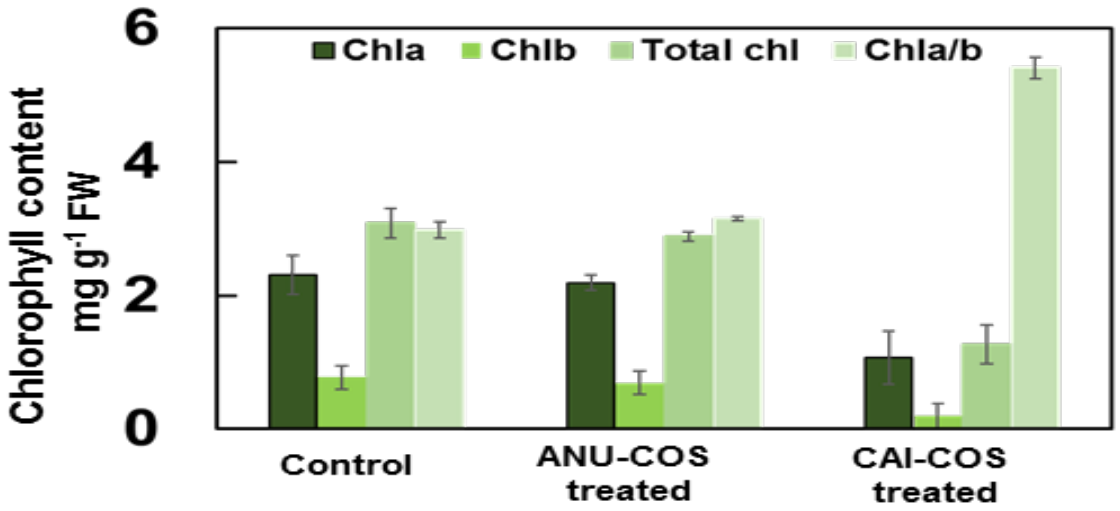

Fig. 6.3: Measurement of chlorophyll-chla, chlb, total chl and chl a/b content in control and COS treated plants: Relative chlorophyll content in tomato leaves from control plants and COS treated plants with. Values represent mean ± SD of three separate experiments, each in triplicate.

Chlorophyll content refers to the amount of chlorophyll present in a given plant tissue or sample. Spectrophotometry or fluorimeter can be used to measure chlorophyll content. These methods involve extracting the pigments from the sample and measuring their absorbance or fluorescence properties to estimate their concentration. A higher chlorophyll a/b ratio typically indicates that an organism is adapted to low light conditions, while a lower ratio indicates adaptation to high light conditions. For instance, shade-tolerant plants often have a higher chlorophyll a/b ratio than sun-loving plants. Chlorophyll content was almost same in case of control and ANU-COS treated plants but less in CAI-COS treated plants but chlorophyll a/b ratio seems to be very high when compared to ANU-COS treated and control plants.

Chlorophyll measurement was used in defining the photosynthesis rate in plants (Dalio et al. 2011). Application of COS has increased the photosynthetic pigments in many plants (El-Tantawy, 2009). Foliar application COS has increased the chlorophyll content in chilli, bean, and mungbean (Sheikha and Al-Malki, 2011; Mondal et al. 2013; Dzung et al. 2017). Similarly, foliar spray of COS of DP (8-16) improved chlorophyll content in coffee and peanut (Dzung et al. 2011). In edible rapeseed, foliar spray of 80% DD increased the chlorophyll content (Zong, et al. 2017). In wheat, COS decreased the effect of drought and increased the chlorophyll content (Zeng & Luo, 2012; Zou et al. 2017). Yue et al. (2001) reported that COS has a positive effect as foliar application on maize seedlings. Overall, COS has increased the chlorophyll content there by increased the photosynthetic rate.

6.2.4 Photosystem-II parameters

Dual PAM was used to measure the photosynthesis parameters of COS treated and control plants. The PAM fluorescence parameters studied were Y (II) - Efficient quantum yield, ETRII Electron transport rate, Y(NO) -Non-regulated energy dissipation, Y(NPQ)- Non-regulated non-photochemical quenching, qP-Coefficient of photochemical quenching, qN-Coefficient of non-photochemical quenching and qL -Redox state of PSII. The control and COS-treated *Lycopersicon esculentum* plants were illuminated with a constant PAR intensity and difference in light change were observed in COS treated and control plants.

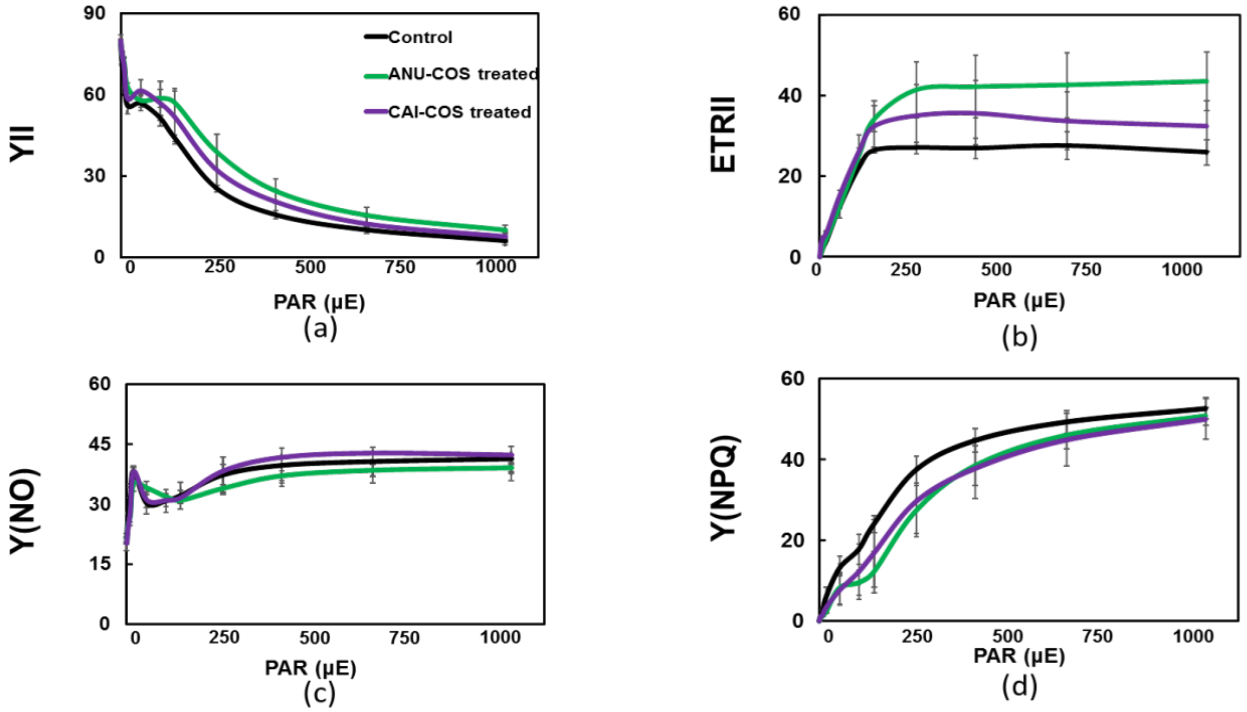

Fig. 6.4: Induction curves of control and COS treated tomato plants: Tomato plant illuminated with a constant PAR intensity and difference in light change of: (a) Y(II)- Efficient quantum yield of PSII; (b) ETRII- Electron transport rate of PSII; (c) Y(NO)- the yield of non-regulated energy dissipation of PSII; (d) Y(NPQ) - the yield of regulated energy dissipation of PSII.

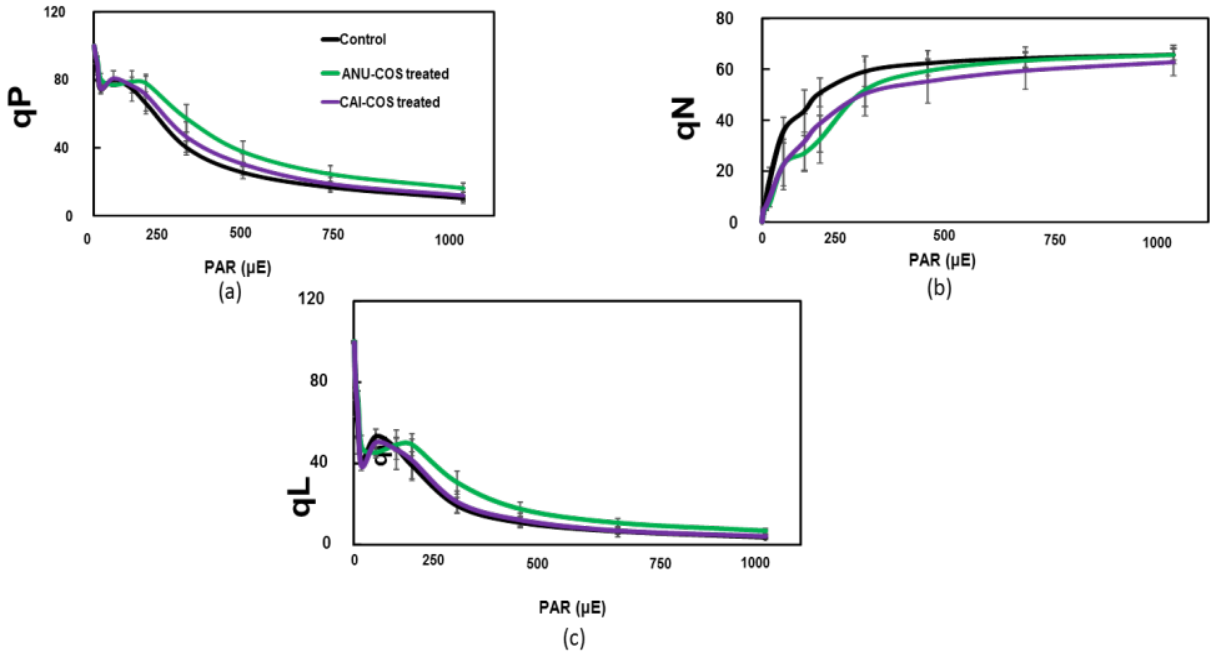

Fig. 6.5: Induction curves of control and COS treated tomato plants: Tomato plant illuminated with a constant PAR intensity and difference in light change of: (a) qP-coefficient of photochemical chemical quenching; (b) qN-coefficient of non-photochemical quenching of excess excitation energy; (c) qL-redox state of PSII.

Chlorophyll florescence in photosynthesis is an ideal parameter for measurement. Maximum PSII efficiency (Fv/Fm) is an indicator for effectiveness of PSII and is related to photosynthesis in leaf. Efficiency of photosynthesis of a leaf is related to Fv/Fm. COS treated plants had higher Fv/Fm when compared to control plants. However, the Fv/Fm values decreased under stress condition and revived back to normal in seedling of wheat treated with COS (Zou et al. 2015). An increase in Fv/Fm values were also observed in lemongrass and mentha after COS treatment (Jaleel et al. 2017). Similarly, 50 mg L^{-1} COS improved the PSII photochemical efficiency in cucumber (Xue et al. 2004) and in edible rape seed (Zong et al. 2017).

YII indicates the efficient quantum yield *i.e.,* the actual amount of light energy absorbed by PSII. ANU and CAI COS treated plants have higher values when compared to control plants similar result were also seen in wheat plant. A higher quantum yield was reported in COS treated wheat plants (Zou et al. 2015). Conversion of light energy to chemical energy by absorbed pigments is called photochemical quenching (qP) (Ritchie, 2006). In ANU-COS and COS treated plants qP values of where higher than control. The high qP value indicates the effective use of light (Olvera-González et al. 2013). Coefficient of photo chemical quenching qP at normal condition should be higher and COS treated plants where having even higher values. Similar reports where observed upon chitosan treatment in cucumber plants (Xue et al. 2004) and in wheat seedlings

(Zou et al. 2015). Excess light energy absorbed by the pigments is dissipated as heat and it is known as non-photochemical quenching (NPQ). At normal conditions the NPQ values should be lower and COS treated plants have even lower values compared to control plants. Similar results were observed in wheat (Zou et al. 2015). A decrease in NPQ was reported in edible rape seed upon COS treatment with increase in the chlorophyll parameters (Fv/Fm, qP, ϕPSII and ETR) and high rate of ATP and NADPH generation (Zong et al. 2017).

6.2.5 Defence response in plants

Relative mRNA expression levels of defence responsive genes in both COS-treated and control plants were analysed. The defence responsive genes like phenylalanine ammonia-lyase gene (PAL), catalase, peroxidase, polyphenol oxidase (PPO), lineolate lipoxygenase (LOX), cinamoyl-CoA reductase (CCR), chitinase, WRKY and MYB transcription factors were identified. Peroxidase, catalase, PPO are involved in maintain redox status of the cell. LOX is involved in the biosynthesis of jasmonic acid (JA); CCR is an enzyme involved in the biosynthesis of lignin; PAL plays a key role in the production of various secondary metabolites such as flavonoids and lignin (Dixon et al. 2002) CCR is an enzyme involved in the biosynthesis of lignin, a complex organic polymer found in the cell walls of many plants. WRKY and MYB are transcription factors involved in regulation of defence response genes (Jiang et al. 2013).

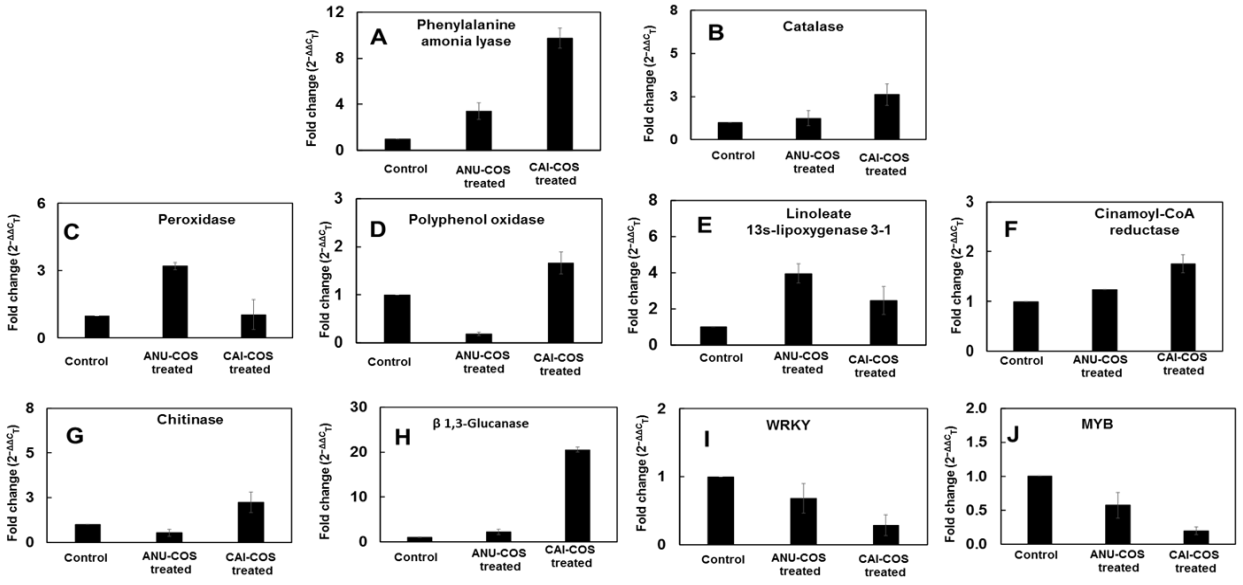

Fig. 6.6: Relative mRNA expression levels of genes in response to COS treatment: Relative mRNA expression levels of defence responsive genes in both COS treated and control plants. The defence responsive genes like PAL, catalase, peroxidase, PPO, LOX, CCR, Chitinase, WRKY and MYB transcription factors were identified. The significant results observed in the plant defence response genes were expressed in COS-treated plants.

PAL is the first gene to be activated upon infection by pathogens (Nichoson and Hammerschmidt, 1992). In CAI-COS treated plants, PAL gene expressed 8-fold higher and 4-fold in ANU treated plants compared to control plants. Similar results were reported in COS treated soya bean plants and the expression level of PAL depends upon chain length (Khan et al. 2003). COS treatment also elevated the PAL level that leads to increase in phenolic compounds in plants like sunflower (Cho et al. 2008), papaya (Ali et al. 2012) and tomato (Badawy and Rabea, 2009)

Catalase gene expression of control and ANU-COS treated were almost same but CAI-COS treated showed 2-fold upregulation in expression. POD gene expression in control and CAI-COS treated plants remained same whereas, in ANU-COS treatment POD upregulated by 2-fold. Upon treatment of low molecular weight COS, POD upregulated on resistant and susceptible varieties of wheat seedlings (Burkhanova et al. 2007). Similarly, PAL and POD were enhanced by COS treatment in the cell suspension culture of citrus, wheat and soybean (Vander et al. 1998; Jung et al. 2008). PPO gene expression in CAI-COS treatment was double compared to control plants and was down regulated in ANU-COS treated plants. LOX gene expression was higher in CAI-COS treated and higher in ANU-COS treatment. CCR gene expression during both ANU-COS showed half fold increase and CAI-COS treatment showed 1-fold increase compared to control plants. Chitinase gene expression of ANU-COS treated was down regulated but 2-fold increase in CAI-COS treatment compared to control plants. Similar results were observed when *Arabidopsis* seedling were treated with microbe digested shrimp shell extract (Ilangumaran et al. 2017). There was no significant change in β-1,3 glucanase expression during ANU-COS treatment whereas, a 20-fold upregulation occurred in CAI-COS treated plants compared to control plants. COS treatment has induced antifungal enzymes (chitinase, β-1,3-glucanase, and chitosanases) in rapeseed, and tomato (El Ghaouth et al. 1992; Vander Peter et al.1998; Benhamou et al. 2001). Elevation of β, 1-3 glucanase and chitinases in orange plants after COS treatment after infection with *P. digitatum* was reported (Fajardo et al. 1998). In raspberries and strawberries also increase of chitinases and β-1,3-glucanase were reported (Zhang and Quantick, 1998). WRKY and MYB transcription genes were downregulated in COS treatment compared to control plants. A low expression of WRKY upon COS treatment in rice seedling was reported (Ramakrishna et al. 2021).

6.2.6 COS-induced decrease in transpiration rate by stomatal closure

The transpiration rate of COS treated and control plants were examined by the stomatal aperture by using FE-SEM. Figure 6.7 shows the stomata of control and treated plants after COS

treatment. The zoomed image shows the stomatal aperture of control plant, where it was open normally but in case of COS-treated plants the stomatal aperture is closed.

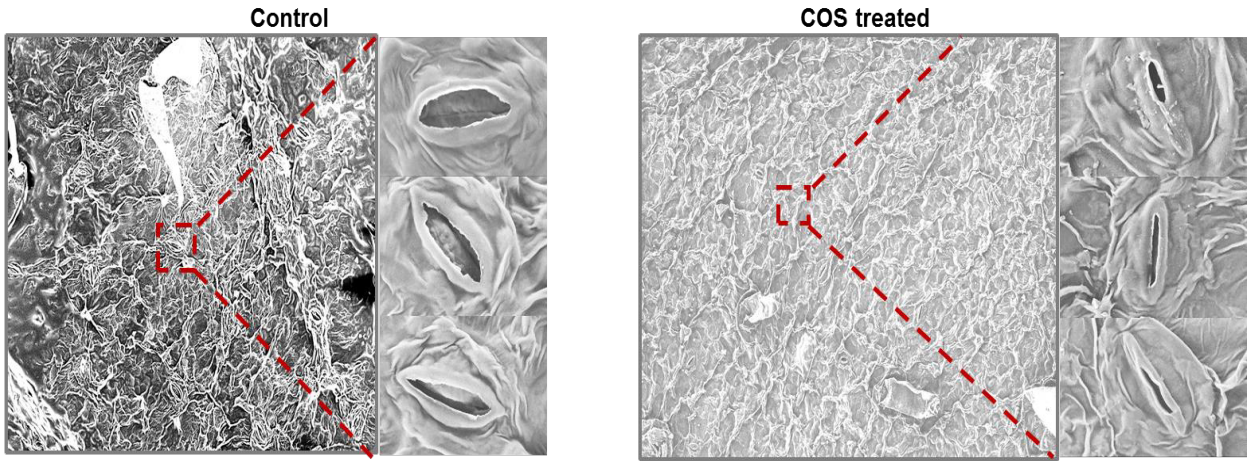

Fig. 6.7: FE-SEM images of stomatal opening of tomato leaves in control and partially opened in COS treated plants

COS treatment has led to stomatal closure there by reducing the rate of transpiration. Closure of stomata depends on the levels of abscisic acid (ABA), which in turn can lead to a reduction in transpiration rate. Chitosan can increase the concentration of ABA in the treated leaves when applied as a foliar spray. When plants experience water stress, ABA levels increase, leading to stomatal closure and a reduction in transpiration rate. By increasing the concentration of ABA in the treated leaves, chitosan can mimic the effects of water stress and reduce transpiration rate. The closure of stomata depends upon the increase in the levels of ABA in stomata (Iriti et al. 2009). It is not clearly known how the stomata closure occurs upon COS treatment. ABA signally pathway might be the reason based on phenotypic and transcriptomic analysis on COS treatment (Hidangmayum et al. 2019). The important role of stomatal closure is associated with ABA signaling pathway (Kuyyogsuy et al. 2018). Previous reports suggest that closure of stomata and transpiration reduction were initiated by ABA (Leung and Giraudat, 1998). COS-treated bell pepper showed 26–43% lower water intake due to stomatal closure compared to control plants (Bittelli et al. 2001). This implies that the anti-transpiration activity by COS treatment was mediated by closing of stomata. Similar observations were also reported in bean and barley (Khokon et al. 2010; Koers et al. 2011).

6.2.7 Amino acids analysis in tomato leaves after COS treatment

Tomato leaves after COS treatment were analysed for amino acids composition. The effect of COS on the composition of amino acids is represented in the form of heat map (Fig 6.8). In ANU-COS-treated plants histidine, glycine, valine, isoleucine and proline remained same but aspartate, glutamate, seine and alanine the fold values decrease compared to control plants. In CAI-COS-treated plants, when compared to control plants glycine and proline were increased whereas, glutamate, serine, histidine and alanine were decreased.

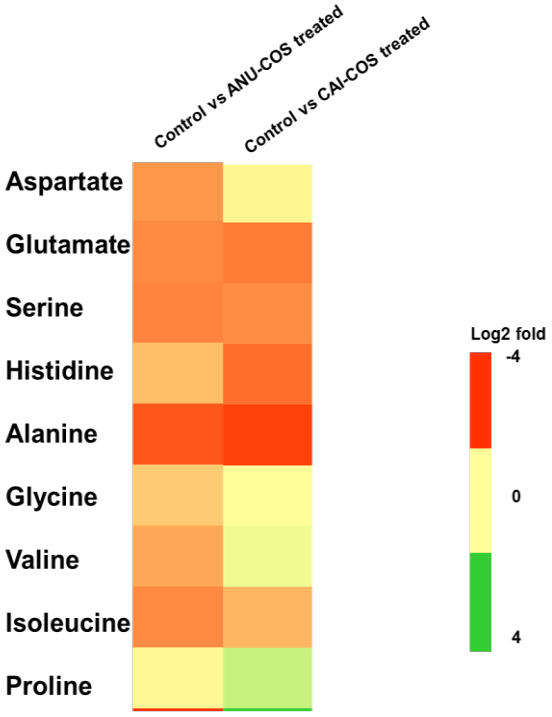

Fig. 6.8: Status of relative fold changes of amino acids content in COS treated and control plants: Fold changes of different amino acids in control and ANU-COS-treated and CAI-COS-treated plants.

The obtained data shows elevation of proline in the COS-treated plants. Proline is known to be act as a osmoprotectant (Gorham et al. 1987; Abdelaal et al. 2019) and protect many biological molecules under stress condition. Similar upregulation of proline occurred in plants treated with chitosan in combination with *B. thuringenisis* under saline conditions (Egamberdieva et al. 2017).

Chapter VII

Summary and conclusions

7. Summary

Chitin the second most abundant polymer on earth, after cellulose. COS are highly useful molecules obtained from chitin, that are known to have a wide range of applications in different fields including medicine and agriculture. Therefore, there is a great interest in exploring the opportunities to produce COS for different applications. One of the applications of COS in agriculture is crop protection *via* enhancement of plant growth and boost the immunity, thus strengthen the plant. Different physio-chemical methods are available for production of COS but they are either expensive or generate enormous waste which can cause environmental pollution. The need for an ecofriendly method for production of COS has, therefore, gained importance. Chitinases are the enzymes that act on chitin and can produce COS. Only chitinases with TG activity can synthesize highly valuable COS of higher chain length. Some chitinases can also act on nonspecific substrate like chitosan and produce COS. However, the main hurdle in COS production is the crystalline nature of chitin which makes it a major problem to be acted up on by the enzymes.

In search of a process that is ecofriendly and cost effective, without compromising on the quality and quantity of COS, we have designed an SSF process for the production of COS in a very economical process without harming the nature. We tried to increase the TG activity of enzymes by using different solvents and salts. We also used nonspecific substrate acting chitinases that could act on a polymer which is a hybrid of chitin and chitosan that produce products of different pattern of acetylation. We also tried to decrease the crystalline nature and increase the solubility of polymer making feasible for the chitinases to act on and produce COS. Further, the obtained COS were tested for the plant strengthening activity on tomato plants.

7.1 SSF for the production of COS

In the present study, combined use of lower amounts of fungal chitin as inducer, along with chitin flakes, was studied for the COS production. The biomass mixed with chitin flakes was seeded with *S. proteamaculans* to produce chitinases by SSF. The major end products on α-chitin were DP2 and DP3. While, DP1 and DP4 were the major end products on α-chitin with 1% fungal chitin inducer. Initial time points yielded only DP1, and the major product at 21h was DP4. Finally, DP1-DP4 were the major products on α-chitin (alone and in combination with fungal chitin). COS longer than hexamer (DP6) were also observed as low peak areas.

Similarly, chitin flakes were inoculated with *Streptomyces* in SSF. DP1, DP3, DP4 and DP5 were the major end products produced by *Streptomyces* ANU-34 strain. DP4 and DP5 were the major end products produced by *Streptomyces* CAI-21 strain. Further optimization of conditions like

inducer concentration, moisture concentration, salt and sugar concentration necessary for the higher yields of COS were also studied through SSF. COS of DP2-DP4 were detected in ANU-34 incubated samples at inducer concentration of 25-50%. DP1, DP3, and DP4 were detected in CAI-21 incubated samples at inducer concentration of 40-50%. Increase in salt concentration had no impact on COS production by both ANU-34 and CAI-21 strains. DP2 and DP4 were detected in ANU-34 incubated samples. DP1, DP3 and DP4 were detected in CAI-21 incubated salt treated samples. Increase in moisture content from 40-50% had positive impact on COS production by both ANU-34 and CAI-21 strains. DP2 and DP4 were detected in ANU-34 incubated samples. DP1, DP3 and DP4 were detected in CAI-21 incubated for different moisture content samples. Increase in sugar concentration had no impact on COS production by both ANU-34 and CAI-21 strains. DP4 and DP5 were detected in ANU-34 incubated samples. DP1 and DP3 were detected in CAI-21 incubated samples with different sugar concentration. Further optimization and extended incubation of SSF followed by HPLC and MALDI-TOF-MS analysis may enable us to generate and detect long chain COS.

7.2 Use of ionic liquid, osmolytes and humectants to improve transglycosylation

Enzymes are generally substrate specific and some enzymes can act on similar substrates and hydrolyze the substrates to form products. *Pe*chi3 being a chitinase enzyme also acted non-specifically on chitosan substrate of different degree of acetylation to yield COS products that are >DP8, which are the useful molecules that can elicit defence response in plants. Enzymes with TG activity can be used to produce COS of higher chain length. Several attempts were made to increase the TG activity and decrease the hydrolysis activity of hyper TG mutant of *Sp*ChiD by using different types of solvents like ionic liquids, osmolytes and humectants were used at different concentration. Effect of ionic liquid on the TG activity of *Sp*ChiD-Y28A was assessed at 10% and 15% v/v concentrations. TG activity of *Sp*ChiD-Y28A decreased with increase in hydrolytic activity. With the use of 15% v/v, both hydrolytic and TG activities decreased drastically. Over all, the ionic liquids had similar effect on TG of *Sp*ChiD-Y28A possibly due to the similar type of cationic (imidazolium) components. The reduction of TG in presence of these ionic liquids, although hydrolysis was retained at lower %, is not clearly understood. The reduction in hydrolysis at 15% (v/v) of ionic liquids may be due to the destabilizing effect of both imidazolium cations and anions on *Sp*ChiD-Y28A.there was no improvement in TG with tested osmolytes (proline, and *myo*-inositol) and humectants (glycerol). Myo inositol had neither increased nor decreased both TG and hydrolytic activity. However, proline and glycerol decreased the TG activity but inversely increased the hydrolytic activity. Therefore, proline and glycerol can be used for other enzymes to increase their hydrolytic activity.

7.3 Improvement of COS production by KOH and KOH-urea pre-treatment method

The major hurdle in the production of COS is the crystalline nature of chitin. Crystallinity of chitin can be reduced by KOH and KOH-urea treatment, followed by freeze thaw method that helps in loosening the chitin fibers and increase the solubility of chitin. Wet and dry forms of substrates were selected to study the difference in the crystalline nature of chitin in presence and absence of moisture. Pre-treated wet and dry substrate was acted upon by *Cs*chiG enzyme to obtain products. Time course hydrolysis of both wet and dry of 6 different substrates produced mainly DP1and DP2 as major product minor amount of DP3 was also produced in all the 6 pre-treated substrates. Pre-treatment has increased COS production in comparison to untreated substrates. Wet form of substrate has produced more amount of COS when compared to dry form of substrate in all the 6 pre-treated substrates. DP1 yielded in high amount in treated substrates when compared to untreated substrates. The yield of DP1 was higher in special treated substrates (24hsp-48hsp) when compare to normal substrates. Pre-treatment has also enhanced the DP2 products and higher amount were seen in the initial time points of 1h-24h when compared to (24hsp and 48hsp) time points. DP3 products were not observed in untreated substrates only found in α-chitin pre-treated substrates. DP3 yield was more in 11K2, 11U2, 20K1, 20KU1 substrates when to compared with 11K1 and 11KU1 pre-treated substrates. DP3 yield was higher in the initial time point of 1h-24h incubated samples when compared with sp samples (24hsp and 48h sp). Percentage of COS produced in 6 different substrates in both wet and dry substrates at different time points is of the order DP1>DP2>DP3. Fold increase in COS production was approximately 5 times in pretreated substrates when compared to untreated substrates and fold increase is seen in higher amount in initial time points of incubation. Pre-treatment of insoluble α-chitin with KOH and KOH-urea has decreased the incubation time required for COS production by *Cs*ChiG. The current investigation confirms that substrates pre-treated with KOH or KOH-urea have greater conversion potential for chitinase.

7.4 Plant strengthening activity of COS pre-treatment on tomato plants

COS obtained by different methods can be used to elicit plant defence response and plant growth promotion. In our study COS obtained by SSF was used in plant strengthening activity. Foliar application of COS on five- to six-leaf stage tomato leaves has showed increased plant growth. The treated plants were checked for plant height, relative water content and chlorophyll contents in COS treated plants with control plants. Plant PS-II photosynthesis parameters, defence response gene expression, transpiration rate and amino acids content in COS treated plants were analysed.

Plant height was more in ANU-COS-treated and CAI-COS-treated compared to control. ANU-COS treated plants were 1-fold higher when compared to CAI-COS treated plants. Relative water content remained same in control and COS-treated plants. A remarkable change was not observed in chlorophyll content in control and ANU-COS treated plants but was less in CAI-COS treated plants. However, chlorophyll a/b ratio seems to be very high in CAI-COS treated plants when compared to ANU-COS treated and control plants. Chlorophyll a/b represents the photosynthetic efficiency of the plants that means these plants can effectively perform photosynthesis in low light conditions. Photosynthetic PS-II parameters of Y(II)-efficient quantum yield values for both ANU-COS & CAI-COS treated are elevated as compared to control treated plants that represent higher quantum yield in treated plants related to control plants. The ETRII-electron transport rate values for both ANU-COS & CAI-COS treated were also higher when compared to control plants. The YNO-non regulated heat dissipation values for both ANU-COS & CAI-COS treated were constant for control values. The YNPQ-regulated heat dissipation values of ANU-COS treated and CAI-COS treated are very less related to control plant indicates plants are photo protective after COS treatment. The qP-photochemical quenching values of ANU-COS treated and CAI-COS treated were higher in comparison to control. The qN-non-photochemical quenching values of ANU-COS treated and CAI-COS treated were lesser when compared to control. The qL-redox state represents the balancing of oxidation and reduction state of PS-II values of ANU-COS treated were slightly higher when compared to CAI-COS and control plants. Stomatal behavior of COS-treated plants and control plants were considerable closed in. Plant defence responsive genes were highly expressed in COS-treated plants. In addition, stress responsive amino acids like proline got elevated in COS-treated plants.

Conclusions

- ✓ DP1, DP4 were the major end products on α-chitin alone
- ✓ DP2 and DP3 were produced in combination with fungal chitin when grown with *S. proteamaculans*
- ✓ ANU-34 produced DP2, DP3 and DP4 and CAI-21 produced DP1, DP3 and DP4 as major end products on α-chitin
- ✓ *Pe*Chi3 has produced different chitosan oligomers with different patterns of acetylation that can bind to immune receptors
- ✓ TG activity of *Sp*ChiD-Y28A decreased with increase in hydrolytic activity on ionic liquid treatments
- ✓ *Myo*-inositol had no effect on TG activity of *Sp*ChiD-Y28A while proline and glycerol decreased the TG but increased the hydrolytic activity

- ✓ Percentage of COS produced in six different substrates in both wet and dry substrates at different time points is of the order DP1>DP2>DP3 by pre-treatment method
- ✓ The time required to generated COS from insoluble α-chitin on pre-treated substrates decreased several folds compared to the untreated substrate
- ✓ KOH or KOH-urea treatment has better conversion capability on crystalline chitin
- ✓ Relative water content remained same but stomata were closed in COS treated plants
- ✓ Plant height, chlorophyll content, plant defence response genes and stress response amino acids like proline were elevated in COS-treated tomato

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