Chitin hydrolysis with chitinolytic enzymes for the production of chitooligomers with antimicrobial properties

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BY

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Declaration

This research has not been previously accepted for any degree and is not being currently considered for any other degree at any other university.

I declare that this MSc thesis contains my own work except where specifically acknowledged.

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Abstract

There are many diseases and illnesses in the world that require new drug treatments and chitin has been shown to produce chitooligomeric derivatives which exhibit promising antimicrobial and immune-enhancing properties. However, the rate-limiting step is associated with the high recalcitrance of chitinous substrates, and low hydrolytic activities of chitinolytic enzymes, resulting in low product release. To improve and create a more sustainable and economical process, enhancing chitin hydrolysis through various treatment procedures is essential for obtaining high enzyme hydrolysis rates, resulting in a higher yield of chitooligomers (CHOS).

In literature, pre-treatment of insoluble biomass is generally associated with an increase in accessibility of the carbohydrate to hydrolytic enzymes, thus generating more products. The first part of this study investigated the effect of alkali- (NaOH) and acid pre-treatments (HCl and phosphoric acid) on chitin biomass, and chemical and morphological modifications were assessed by the employment of scanning electron microscopy (SEM), Fourier Transform Infrared Spectroscopy (FTIR), Energy-Dispersive X-ray spectrometery (EDX) and x-ray diffraction (XRD). Data obtained confirmed that pre-treated substrates were more chemically and morphologically modified. These results confirmed the fact that pre-treatment of chitin disrupts the structure of the biomass, rendering the polymer more accessible for enzymatic hydrolysis.

The commercial chitinases from *Bacillus cereus* and *Streptomyces griseus* (CHB and CHS) are costly. Bio-prospecting for other chitin-degrading enzymes from alternate sources such as *Oidiodendron maius,* or the recombinant expression of CHOS, was a more economically feasible avenue. The *chit1* gene from *Thermomyces lanuginosus,* expressed in *Pichia pastoris,* produced a large range CHOS with a degree of polymerisation (DP) ranging from 1 to above 6. TLC analysis showed that *O. maius* exhibited chitin-degrading properties by producing CHOS with a DP length of 1 to 3. These two sources were therefore successful in producing chitin-degrading enzymes.

The physico-chemical properties of commercial (CHB and CHS) and expressed (Chit1) chitinolytic enzymes were investigated, to determine under which biochemical conditions and on which type of biomass they can function on optimally, for the production of value-added

products such as CHOS. Substrate affinity assays were conducted on the un-treated and pre-treated biomass. TLC revealed that chitosan hydrolysis by the commercial chitinases produced the largest range of CHOS with a DP length ranging from 1 to 6. A range of temperatures (35-90^{o}C) were investigated and CHB, CHS and Chit1 displayed optimum activities at 50, 40 and 45 ^{o}C, respectively. Thermostability studies that were conducted at 37 and 50^{o}C revealed that CHB and CHS were most stable at 37^{o}C. Chit1 showed great thermostablity at both temperatures, rendering this enzyme suitable for industrial processes at high temperatures. pH optima studies demonstrated that the pH optima for CHB, CHS and Chit1 was at a pH of 5.0, with specific activities of 33.459, 46.2 and 5.776 µmol/h/mg, respectively. The chain cleaving patterns of the commercial enzymes were determined and exo-chitinase activity was exhibited, due to the production of CHOS that were predominantly of a DP length of 2.

Enzyme binary synergy studies were conducted with commercial chitinases (CHB and CHS) on colloidal chitin. Studies illustrated that the simultaneous combination of CHB 75%: CHS 25% produced the highest specific activity (3.526 µmol/h/mg), with no synergy. TLC analysis of this enzyme combination over time revealed that predominantly chitobiose was produced. This suggested that the substrate crystallinity and morphology played an important role in the way the enzymes cleaved the carbohydrate.

Since CHOS have shown great promise for their antimicrobial properties, the CHOS generated from the chitinous substrates were tested for antimicrobial properties on *Bacillus subtilis*, *Escherichia coli*, *Klebsiella* and *Staphlococcus aureus*. This study revealed that certain CHOS produced have inhibitory effects on certain bacteria and could potentially be used in the pharamceutical or medical industries.

In conclusion, this study revealed that chitinases can be produced and found in alternate sources and be used for the hydrolysis of chitinous biomass in a more sustainabe and economically viable manner. The chitinases investigated (CHB, CHS and Chit1) exhibited different cleaving patterns of the chitinous substrates due to the chemical and morphological properties of the biomass. CHOS produced from chitinous biomass exhibited some inhibitory effects on bacterial growth and show potential for use in the medical industry.

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List of abbreviations

A.U	Arbitrary units
°C	Degree(s) Celsius
CBD	Chitin-binding domain
CBM	Chitin-binding module
CC	Colloidal chitin
CHB	Chitinase from *Bacillus subtilis*
chit1	Chitinase1 gene from *Thermomyces lanuginosus*
Chit1	Chitinase enzyme expressed in *Pichia pastoris*
CHO	Chitooligomer
CHS	Chitinase from *Streptomyces griseus*
CHTSN	Commercial chitosan from shrimp shell powder
CI	Crystallinity Index
DA	Degree of acetylation
DNS	Dinitrosalicylic
DP	Degree of polymerisation
DS	Degrees of synergy
EC	Enzyme Commission number
EDX	Energy-Dispersive X-ray spectrometery
F_A	Fraction of acetylation
FTIR	Fourier Transform Infrared Spectroscopy
g	Gram
g	Gravity
GH	Glycoside hydrolase
h	Hour
HPLC	High-performance liquid chromatography
kDa	Kilo Daltons
L	Litre
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass
MS	Spectrometry
mg	Milligram
min	Minute
mL	Millilitre

mM	Millimolar
MMN	Modified Melin Nokrans
MW	Molecular weight
nm	Nanometer
NMR	Nuclear Magnetic Resonance
P_A	Pattern of acetylation
PA	Phosphoric acide treated chitin A, from shrimp shell powder
PAS	Phosphoric acide treated chitin B, from shrimp shell powder
rpm	Revolutions per minute
SD	Standard deviation
SEM	Scanning electron microscopy
SHRMP	Chitin from shrimp shell powder
TLC	Thin layer chromatography
w/v	Weight per volume
XRD	X-ray diffraction
YPD	Yeast potato dextrose
YPG	Yeast-extract peptone glycerol
µg	Microgram
µL	Microlitre
µM	Micromolar
µmol	Micromole

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List of research outputs

a. National conference proceedings

25th South African Society for Biochemistry and Molecular Biology (SASBMB) Congress. 40th Anniversary 2016. East London ICC, East London, 10-14th July 2016. Oree G., Plaatjie S, Dames J.F. and Pletschke B.I. Investigation of the anti-microbial and physico-chemical properties of enzymes isolated from *Leohumicola minima and Cryptosporiopsis erica*. (Oral Presentation).

b. Anticipated publications

Oree, G., Mafa, M. S., Malgas, S. and Pletschke, B. I. (2018). The morphological and chemical characterisation of pre-treated and un-treated chitin containing substrates. Article in preparation for submission.

Oree, G., Mafa, M. S. and Pletschke, B. I. (2018). The production, activity and anti-microbial properties of chitooligosaccharides produced from *Pichia pastoris* expressed Chit1. Article in preparation for submission.

Chapter 1: General Introduction and Literature Review

1.1. Introduction

Chitin is the second most abundant biopolymer after cellulose and acts as a structural component in fungal cell walls, algae, nematodes and arthropods (Hamid *et al*., 2013). This demonstrates that this polymer is widely distributed in nature, with an estimate of chitin comprising 20 to 58% of dry weight waste produced from shell fish and thus poses a problem for the environment (Ramli *et al*., 2011). Shellfish waste comprises of valuable components such as protein and chitin which are employed in many biotechnological applications and are used in various industries ranging from pharmaceutical, agricultural, chemical to food industries.

Chitin is an insoluble polysaccharide and chemical modifications are required for the production of more soluble derivatives such as deacetylation into chitosan (Muzzarelli, 1977 and Roberts, 1992). Chitin and chitosan derivatives, such as chitooligomers (CHOS) have gained the attention of many researchers and have become a sensation due to their increased solubility and applicability in the medical industry due to their ability to exhibit various bio-activity properties, which include the treatment of a range ailments and their antimicrobial, immune-enhancing and anti-tumour properties (Chung *et al*., 2004; Je *et al*., 2004; Quan *et al*., 2009 Remunan-Lopez *et al*.,1998; Tsai *et al*., 2002; Xia *et* al., 2011and Zhang *et al*., 2010).

Enzyme technology is an environmentally-friendly discipline that is commonly employed in industries and is used for the effective degradation of biomass (Dahiya *et al*., 2006). However, enzymes are costly and hydrolysis of crystalline substrates such as chitin requires high enzyme loadings which results in the process not being economically viable. The recalcitrance of this biomass therefore necessitates pre-treatment prior to enzymatic digestion. Biomass pre-treatment often results in the increased exposure of the biomass surface for effective enzyme-binding, subsequently increasing hydrolysis (Pletschke *et al*., 2016). Reduction in the costs for the production of CHO value-added products can be achieved via the reduction in enzyme loading by synergistic hydrolysis (Van Dyk and Pletschke, 2012; Klein-Marcuschamer *et al*., 2012). This can be accomplished by using a combination of enzymes in an optimal ratio to improve the yield of hydrolysis products (Van Dyk and Pletschke, 2012; Klein-Marcuschamer *et al*., 2012).

1.1.1. Chitinous biomass

According to Ramesh and Tharanathan (2003), natural polymers such as chitin have many benefits such as their availability from marine waste, biocompatibility and biodegradability. This ensures conservation of the marine environment, with marine waste being efficiently hydrolysed via chemical or enzymatic avenues for the production of useful chitin derivatives such as chitosan or CHOS, which are used in many biotechnological applications (Ramesh and Tharanathan, 2003). The bio-activity of chitin and its derivatives is dependent on properties such as: the degree of polymerization (DP), degree of acetylation (DA), pattern of acetylation (P_A) and molecular weight (MW) (Madhuprakash *et al.*, 2015). These characteristics influence pH-dependent solubility and inter-chain interactions between the non-polar acetyl groups and hydrogen bonds within the biomass (Zhang *et al.*, 2010).

1.1.1.1. Chitin

Chitin can be described as a crystalline, non-elastic, tough and insoluble β-(1,4)-linked *N*-acetylglucosamine (GlcNAc) polymer, which is the second most abundant biopolymer after cellulose (see Fig. 1.1 for the structure of chitin) (Kumar, 2000). This polymer can also be described as a cellulose with the substitution of the hydroxyl at C-2 of a glucose residue with an acetyl amine group, which attributes to the matrix strength of the polymer, due to increased hydrogen bonding (see Fig. 1.1) (Kumar, 2000). According to Madhavan (1992), this hydrophobic polymer is insoluble in most organic solvents with some of the following exceptions: hexafluoroisopropanol, hexafluoroacetone and chloroalcohols in combination with acids.

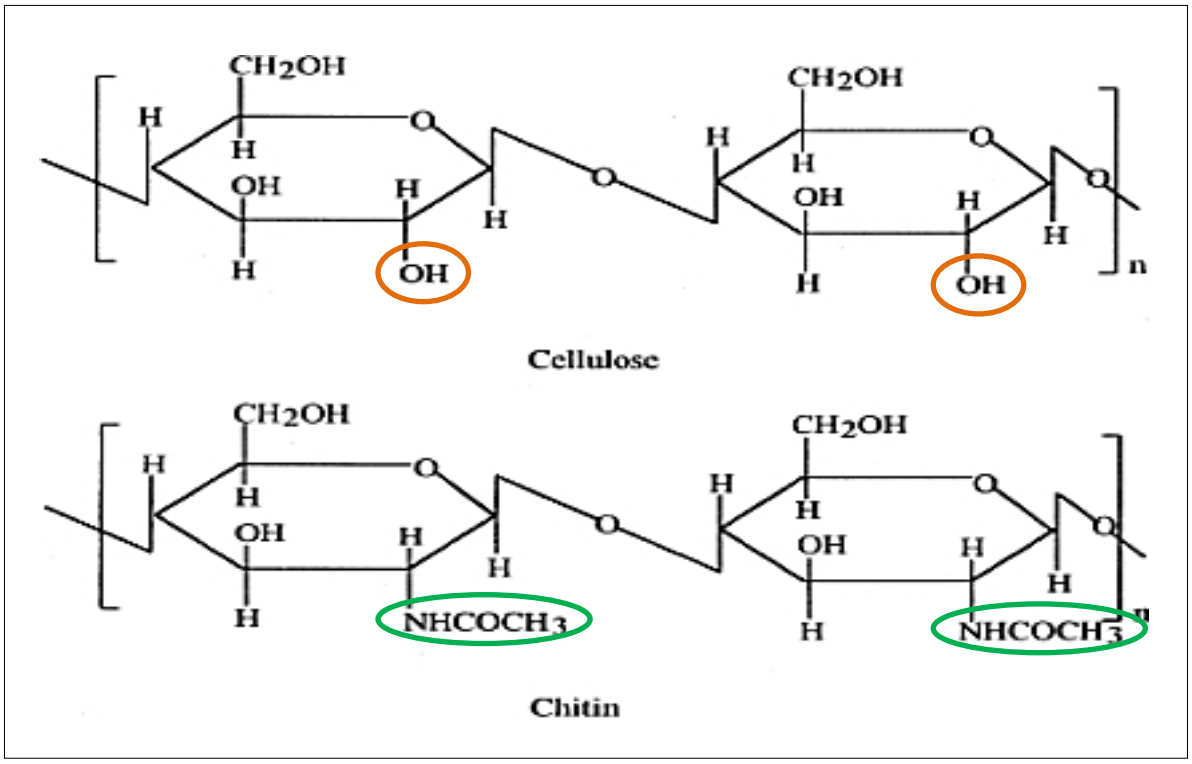

Figure 1.1. An illustrative structure of cellulose and chitin (adapted from Kumar, 2000)

X-ray crystallography reveals that chitin exists in three different polymorphic forms which differ in the orientation of peptide chains: α-chitin, β-chitin and γ-chitin (Tharanathan, 2003). The β-polymorph is predominantly found in mollusks and comprises of antiparallel chains and weak intermolecular interactions rendering it unstable, with a higher solubility and swelling capacity than α-chitin (Kim *et al*., 1996 and Kurita *et al*., 1993). On the other hand, α-chitin is prevalent in fungi, insects and crustaceans. This polymorph is insoluble, crystalline, and stable due to the parallel chains and strong hydrogen bonding (Focher *et* al., 1992; Kameda *et* al., 2005; Rathore and Gupta, 2015 and Zhang *et al*., 2014). In contrast, γ-chitin consists of a mixture of parallel and antiparallel chains and is commonly found in cocoons (Merzendorfer and Zimoch, 2003).

1.1.1.2. Colloidal chitin

The morphological and chemical nature of biomass is important as these features have a direct effect on the enzymatic hydrolysis of the biomass. This makes biomass pre-treatment essential, for surface exposure subsequently increases enzyme binding, which results in an increase of hydrolysis and a reduction in the enzyme loading quantity required (Pletschke *et al*., 2016). Since chitin is insoluble and inert when suspended in water and some acids, a chemically modified form of chitin is used as it has a higher solubility and is easily

manipulative, known as colloidal chitin (Murthy and Bleakley, 2012). A more efficient protocol of producing colloidal chitin, using crab shell flakes, has been developed by combining protocols by Wen and colleagues (2002) and Kuzu *et al*. (2012), which requires less cost, material and effort (Murthy and Bleakley, 2012). There have been reports of chitinases being produced at higher yields in the presence of 1 - 1.5 % (w/v) colloidal chitin, in comparison to other carbon sources (Nampoothiri *et al.*, 2004; Sharaf, 2005).

1.1.1.3. Chitosan

Chitosan is an *N*-deacetylated derivative of chitin (Fig. 1.2), that is produced by alkali or enzymes such as chitin deacetylase from glycoside hydrolase (GH) family 4 (Caufrier *et al*., 2003 and Muzzarelli, 1997). This is a heteropolymer that consists of randomly distributed β-linked D-glucosamine and N-acetyl-D-glucosamine residues (Fig. 1.2) (Lodhi *et al*., 2014).

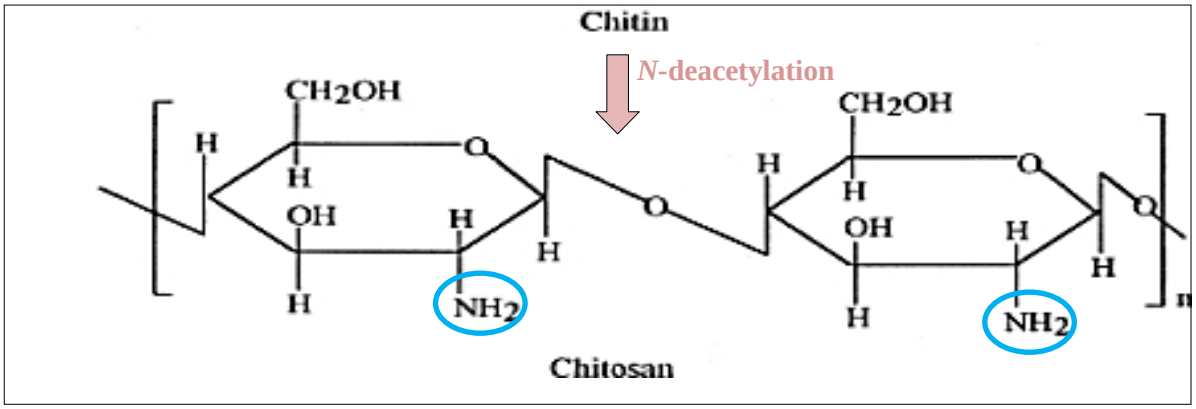

Figure 1.2. An illustrative structure of chitosan (adapted from Kumar, 2000)

According to Kim and Rajapakse (2005), alkali treatment of chitin for the generation of chitosan subsequently increases the degree of deacetylation (DD), shifting the charge distribution. The degree of acetylation is a factor that easily distinguishes chitosan from chitin, with chitosans exhibiting a DA below 50% after chitin deactylation with alkali treatment (Al Sagheer *et al*., 2009 and Chatelet *et al*., 2001). Chitosan is thus a more soluble derivative and exhibits polycationic behaviour, biocompatibility, bio-degradability and is more readily used in various applications for its bio-activities. (Kumar, 2000 and Muzzarelli, 1997). In addition, chitosan solubility is dependent on the free amino and N-acetyl groups which become protonated in acidic conditions, resulting in an increase in solubility (Madihally and Matthew, 1999). Glycol chitin is another derivative generated by the acetylation of glycol chitosan (Trudel and Asselin, 1989).

1.1.1.4. Biomass pretreatment

Chitin and chitosan are crystalline substrates that have a limited number of solvents in which they can be dissolved. Their extensive hydrogen bonding influences the typical characteristic of degradation before reaching a melting point (Kumar, 2000). Although some solvent systems assist in the use of these substrates, there is no comparative data available on the different solvents (Kumar, 2000). There are only a few standardised pre-treatment methods available for chitin biomass, which necessitates the use of different pre-treatment methods to disrupt chitin crystallinity to increase the porosity, to effectively allow enzymes more accessibility to the biomass (Karp *et al*., 2013; Van Dyk and Pletschke, 2012).

1.1.2. Enzymatic hydrolysis of chitinous biomass

According to Rathore and Gupta (2015), the use of enzymatic degradation of chitinous waste is a key aspect in reducing environmental impacts, due to enzymes being environmentally friendly, produced by a range of organisms and having the capacity to exhibit hydrolytic activities against a wide range of substrates (Patil *et al*., 2000). A number of different enzymes exhibit chitin degrading properties such as cellulases, papains and chitinolytic enzymes, that are preferably obtained from cheap fungal or plant sources, in a crude form (Sashiwa *et al*., 2003; Terbojevich *et al*., 1996 and Xie *et al*., 2010).

1.1.2.1. Glycoside hydrolases

Chitin is a complex, tough and insoluble substrate and requires an array of enzymes to degrade it into monomeric or oligomeric sugar residues (Kumar, 2000; Sashiwa *et al*., 2003; Terbojevich *et al*., 1996 and Xie *et al*., 2010). Chitin-degrading enzymes comprise a collection of glycoside hydrolases and are produced by fungi, bacteria, insects, vertebrates and some plants (Rathore and Gupta, 2015). These carbohydrate degrading enzymes are classified into Glycosyl Hydrolase (GH) families and clans based on amino acid sequence, structural and mechanistic similarities (Aam *et al*., 2010). Glycoside hydrolases are classified through a Carbohydrate-Active database (CAZy) that is continuously updated (http://www.cazy.org).

1.1.2.2. Classification of chitinases

The complete hydrolysis of chitinous biomass occurs by the concerted action of enzymes, collectively known as the chitinolytic system (see Fig. 1.3 for enzymes required for chitin degradation). This is described as both synergistic and consecutive (Deshpande, 1986; Shaik

and Deshpande, 1993). Chitinases belong to GH families 18, 19 and 20 according to the CAZy database (Dahiya *et al*., 2006). These enzymes are categorised into two groups: endo-chitinases (EC 3.2.1.14), which randomly cleave the internal glycosidic bonds of substrates in a non-processive manner, for the production of lower molecular mass multimers of glucosamine residues (Rathore and Gupta, 2015; www.cazy.com). Exochitinases, the second group of chitinases are sub-catergorised into two groups. The first group are chitobiosidases (EC 3.2.1.30, now included in EC 3.2.1.52), which cleave in a processive manner from the non-reducing ends of the polymers to produce diacetylchitobioses and the second sub-group, β-(1,4) N-acetyl glucosaminidases (EC 3.2.1.52), responsible for cleaving products generated by endochitinases and chitobiosidases into monomers (Rathore and Gupta, 2015; Sahai and Manocha 1993).

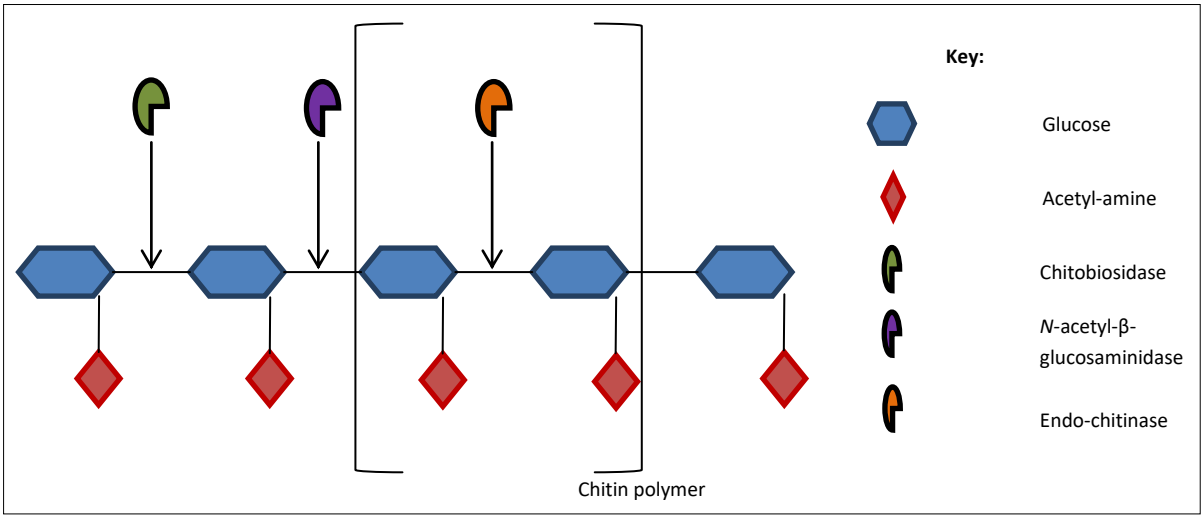

Figure 1.3. Schematic illustration of the enzyme sites of the enzymes required to completely degrade chitin

1.1.2.3. Enzyme families

As already mentioned, based on amino acid sequences, chitinases have been classified into GH families 18, 19 and 20 (Henrissat and Bairoch, 1993). Family 18 comprises of chitinases from bacteria, fungi, viruses, animals and some plants, whereas family 19 contains chitinases from *Streptomyces* (Hart *et al*., 1995 and Patil *et al*., 2000). Family 20 includes chitinases from some fungi and *N*-acetylglucosaminidases from bacteria and humans (Patil *et al*., 2000). According to literature, the different amino acid sequences and 3-dimensional structures of family 18 and 19 chitinases infer that they originate from different ancestors (Rathore and Gupta, 2015). Family 18 contain a $(\beta/\alpha)_8$ TIM-barrel catalytic domain, whereas

family 19 is comprised of a high α-helical content (Van Aalten *et al*., 2000; Hahn *et al*., 2000 and Hart *et al*., 1995).

1.1.2.4. Catalytic mechanisms of chitinases from GH families 18 and 19

Important biochemical differences exist between GH families 18 and 19 chitinases. Family 18 chitinases catalyse the hydrolysis between GlcNAc-GlcNAc and GlcNAc-GlcN residues (Mitsutomi *et* al., 1996). Family 18 employs the double displacement catalytic mechanism with retention of an anomeric configuration, illustrated in Figure 1.4 (Armand *et al*., 1994). This mechanism involves the protonation of the β -(1,4) glycosidic oxygen which leads to the formation of an oxazoline intermediate (Armand *et al*., 1994; Brameld and Goddard, 1998a). The nucleophilic attack involves water attacking the anomeric centre of the intermediate, thus yielding the product and releasing the enzyme to its original state (Armand *et al*., 1994; Brameld and Goddard, 1998a).

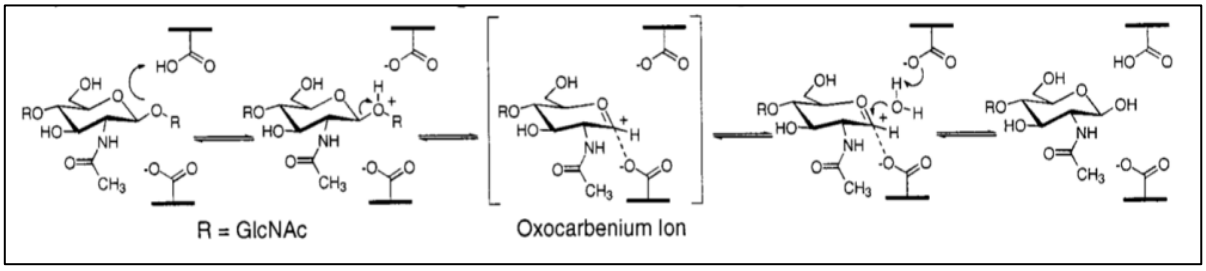

Figure 1.4. Double displacement catalytic mechanism by chitinase from GH family 18 (obtained from Brameld and Goddard, 1998a).

According to Ohno *et* al. (1996), family 19 is responsible for the hydrolysis of GlcNAc-GlcNAc and GlcN-GlcNAc linkages. The single displacement mechanism was proposed for family 19 chitinases, involving two acid residues in the active site, with the inversion of the anomeric configuration (Fig. 1.5) (Brameld and Goodard,1998b and Hart *et al*., 1995). This involved the nucleophilic attack by a molecule of water.

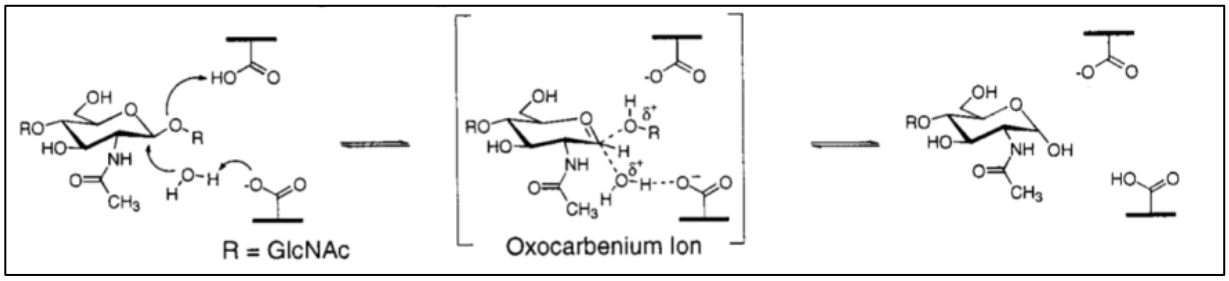

Figure 1.5. Single displacement catalytic mechanism by chitinase from GH family 19 (obtained from Brameld and Goddard, 1998b).

1.1.2.5. Molecular structure of chitinases

According to literature, many bacterial chitinases produced belong to GH family 19. However, exceptions such as the production of chitinase families 18 and 19 from *Streptomyces sp.* do exist (Dahiya *et* al., 2006). The well-investigated chitinolytic bacterium *Serratia marcescens* hosts four types of chitinases (ChiA, ChiB, ChiC and ChiD) which belong to family 18 and are comprised of a (β/α) 8 TIM-barrel catalytic domain that has a deep substrate binding cleft above the barrel (Aalten *et al*., 2000). Family 18 chitinases are described as multi-modular and the presence of chitin-binding modules (CBMs) is distinguished by the presence of interactions between tryphtopan residues and the substrate, which is essential for an increase in substrate binding affininty and hydrolysis (Chern and Chao, 2005 and Vaaje-Koltstad *et al.*, 2013). Chitin binding domains (CBDs) function to assist in the correct orientation between the substrate of interest and the catalytic domains, thus resulting in increased affinity with a subsequent increase in hydrolysis of crystalline chitinous biomass (Yan and Fong, 2015). The most common domain that has been described is the fibronectin III domain (Fn III) (Yan and Fong, 2015). According to Uchiyam *et* al. (2001), this domain in *S. marcescens* comprises of aromatic residues which assists substrate-binding by facilitating the positioning of the catalytic and chitin binding domains, leading to effective chitin hydrolysis.

1.1.2.6. Application of chitinases

A review conducted by Rathore and Gupta (2015) revealed that chitinases are employed in a large number of biotechnological applications in various industries and, as a result, have gained much more attention from researchers in the field. According to Sakhai *et al.* (1998), chitinases can potentially be employed for the reduction of chitinous waste from a range of organisms, including marine organisms on coastlines, this resulting in a decrease in water pollution. The hydrolysis of chitinous waste can be used as a nutritional source for the production of single cell proteins or in biofertlizers (Revah-Moiseev and Carroad, 1981; Sakhai *et al*., 1998).

Chitin is present in the gut linings and exoskeletons of many insects– as a result, chitinolytic enzymes have displayed anti-fungal and anti-pathogen activities and have shown promise as effective bio-control agents or bio-pesticides for the control of a range of fungi and insects (Ahmadi *et al*., 2008; Melchers and Stuiver, 2000 and Sakuda, 1996). Chitinases can potentially be used in the medical industry to treat an array of conditions, from being used as

an anti-fungal treatment and the employment of chitin hydrolysis products (CHOS) in the pharmaceutical industry for the treatment of various human ailments from anti-tumour activity to wound healing properties (Oranusi and Trinci, 1985).

1.1.3. Enzyme synergy

There are many challenges associated with the economical use of chitinases, including and not limited, to their low hydrolytic activities on chitinous waste to produce useful products and the high costs of these enzymes. Therefore, the focus area of many studies is to improve chitinase yield or catalytic activity (Rathore and Gupta, 2015). This is achieved by investigating biochemical and physico-chemical characteristics, which influence activity. This consequently provides a platform to understanding other conditions such as enzyme synergy. This is supported by a review conducted by Pletschke *et al*. (2016), which elaborates on how enzyme loading can be reduced by combining enzymes in an optimal ratio to improve the yield of hydrolysis products, consequently reducing biomass processing to value added product costs.

Since many organisms produce a variety of chitinases, only the synergism between their multi-chitinolytic systems have been investigated. The synergism between chitin-degrading enzymes from different sources has not been studied extensively and could be more sustainable and economical. Van Dyk and Pletschke (2012) describe simultaneous synergy as the effective hydrolysis of biomass using a combination of enzymes. Sequential synergy on the other hand, requires the addition of one enzyme for substrate hydrolysis, followed by enzyme heat inactivation and then the addition of a second enzyme, whilst successive synergy does not entail heat inactivation of the first enzyme after the addition of the second enzyme. Beukes and Pletschke (2011) indicated that synergistic associations between enzymes are present only when the degree of synergy is greater than 1.0. Any degree of synergy equal or lower than 1.0 implies no synergy or anti-synergy between enzymes as a result of substrate competition.

1.1.4. Production of CHOS with enzymes

Chitooligomers are derivatives of chitinous biomass and are generated via acid hydrolysis or by enzymatic degradation (Aam *et al.*, 2010). According to Dahiya *et al.* (2006), CHOS, glucosamines and GlcNAc have immense potential in the pharmaceutical industry and have gained the attention of many researchers due to their increased solubility and preference for environments with neutral pH values in comparison to chitin and other chitin-derivatives. The

production of CHOS with specific chain lengths requires various combinations of chitinolytic enzymes. This can be demonstrated by the addition of high quantities of endo-chitinases in combination with low concentrations of exo-chitinases and *N*-acetylglucosaminidase for the production of CHOS (Aloise *et al*., 1996). In contrast, the production of GlcNAc requires high concentrations of exo-chitinases and *N*-acetylglucosaminidase (Aloise *et al*., 1996). Studies conducted by Usui and colleagues (1990) demonstrated that transglycosylation can be utilised for the production of the desired CHOS by employing endo-chitinases and *N*-acetylglucosaminidases. Their study illustrated tetra-saccharides in combination with enzymes predominantly yielded bioses and hexoses (Usai *et al*., 1990).

1.1.4.1. Medical uses of CHOS

Most reports in literature have focused on the biological activity of CHOS, which are extensively used in many medical or pharmaceutical applications, for the treatment of illnesses and for drug delivery purposes.

Anti-tumour studies which have been conducted indicate that CHOS are responsible for the activation of intestinal immune functions and are useful in the treatment of tumours (Qin *et al.*, 2002; Pangestuti *et al*., 2011). These studies suggest that the cationic characteristics or molecular weights of CHOS played a vital role in anti-tumour activity (Qin *et al.*, 2002; Pangestuti *et al*., 2010). CHOS are also responsible for improving the functions of inflammatory cells and repairing cells, which result in the acceleration of the wound healing process (Maeda and Kimura, 2004).

It has been shown that the inhibition of acidic mammalian chitinases (AMCase) with a chitin inhibitor such as allosamidin leads to a decrease in inflammation. Therefore, partially deacetylated CHOS which act as inhibitors, can potentially be used to treat inflammation during asthma (Letzel *et al*., 2000; Zhu *et al*., 2004). Similarly, chitinase inhibitors used for the inhibition of chitinases found in *Plasmodium* result in a decrease in infection (Letzel *et al.*, 2000).

CHOS can be used for the prevention of adhesion of bacterial cells to lectins that facilitate binding to host cells (Rhoades *et al*., 2006). Studies indicate that CHOS also increase the differentiation of mesenchymal cells to osteoblasts in order to form bone tissue, by increasing the calcium concentration in bones (Ratanavaraporn *et al*., 2009). It was also demonstrated that CHOS reduce the loss of calcium and increase calcium retention, which reduces osteoporosis in mice and facilitates the strengthening of bone (Xia *et al*., 2011). Other

biological activities of CHOS are involved in lowering blood pressure, cholesterol and controlling arthritis (Lodhi *et al*., 2014).

1.1.4.2. Purification of CHOS

Chitooligomers generated enzymatically, or via chemical hydrolysis, normally constitute a mixture of CHOS that differ in degree of polymerization (DP), degree of acetylation (DA), pattern of acetylation (P_A), molecular weight (MW) and charge distribution (Madhuprakash, *et al.*, 2015 and Muzzarelli, 1997). Therefore, purification and characterisation of CHOS is required. Many methods have been proposed for CHOS purification such as gel filtration, ultrafiltration, ion exchange and metal exchange chromatography (Haebel *et al.*, 2007; Le Dévédec *et al.*, 2008 and Lopatin *et al.*, 2009). Techniques such as Nuclear Magnetic Resonance (NMR), High Performance Liquid Chromatography (HPLC) and Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) can be employed for the quantification and characterisation of the generated CHOS upon chitin degradation (Haebel *et al.*, 2007; Malviya *et al*., 2010 and Sorbotten *et al*., 2005).

1.1.5. Problem Statement

Chitin is present as a structural component in fungal cell walls and is associated with the exoskeletons of insects, nematodes and arthropods (Hamid *et al*., 2013). This polymer comprises 20 to 58% of dry weight waste produced from shell fish (Wang and Chang, 1997). A reduction in chitinous waste can be achieved by the hydrolysis of this biomass by the employment of eco-friendly methods, such as enzymatic hydrolysis for the production of value added products. A large demand exists for new treatment methods and antibiotics for the management of various ailments in the health sector (Aam *et al*., 2010). Many studies that have been conducted indicate that chitin-derivatives such as chitooligomers (CHOS) have the potential to treat different ailments, due to their promising antimicrobial, immune-enhancing and anti-tumour activities. CHOS show potential in the treatment of various ailments such as asthma, tumour growth, brittle bones, diabetes and many more. However, CHOS have mostly been produced using chitosanase enzymes and chitosan substrates, which is not economically viable, due to high cost of these compounds. It is therefore feasible to investigate chitinolytic hydrolysis of pre-treated chitinous biomass for the generation of CHOS, in a cost-effective manner and to investigate the bio-activities exhibited by the generated CHOS.

1.1.6. Hypothesis

Chitinolytic enzymes (CHS, CHB and Chit1) effectively and synergistically hydrolyse pre-treated chitinous biomass into chitooligomeric products that exhibit bio-activities.

1.1.7. Aims and Objectives

- To pre-treat chitinous biomass to improve enzymatic hydrolysis and product yields;
- To morphologically and chemically characterise un-treated and pre-treated chitin biomass using SEM, FTIR, EDS, XRD and activity assays;
- To conduct synergy studies using commercial chitinases (CHB and CHS), for optimal degradation of colloidal chitin
- To bio-prospect for chitin degrading enzymes that can be easily expressed in high yields for the production of CHOS;
- To determine the physico-chemical properties of chitinases for optimal hydrolysis activity;
- To produce a range of CHOS with at least a DP of 1-6 and detect this CHOS with TLC;
- To assess some anti-microbial properties of CHOS.

1.1.8. Overview of thesis

The characterisation of un-treated and pre-treated chitinous biomass was successful and is described in Chapter 2. In Chapter 3, Chit1 from *Thermomyces lanuginosus* expressed in *Pichia pastoris* was investigated for optimal hydrolysis of the chitinous biomass, and enzyme bio-prospecting for chitin-degrading enzymes from the ericoid mycorrhiza *Oidiodendron mauis* was explored. Chapter 4 focused on the chitinolytic enzymes used in this study and these were characterised in terms of their physico-chemical properties and chain-cleaving patterns of chitin. Chapter 5 of this thesis dealt with assessing the effectiveness of the synergistic combination of commercial chitinases on colloidal chitin hydrolysis. In Chapter 6, the antimicrobial activity of chitooligomeric products generated during chitinolytic degradation of biomass was investigated. Finally, Chapter 7 of the thesis provides a general discussion, and highlights the conclusions and future perspectives regarding the study undertaken.

Chapter 2: Characterization on un-treated and pre-treated chitin containing substrates

2.1. Introduction

Chitin is present as a structural component in fungal cell walls and is also associated with the exoskeletons of insects, nematodes and arthropods (Hamid *et al*., 2013). This polymer comprises 20 to 58% of dry weight waste produced from shell fish (Ramli *et al*., 2011). Chitin is a complex substrate, which is identified as a crystalline, linear polymer of β-(1,4)-linked N-acetylglucosamine residues, and is found in three polymorphic forms: α-chitin, β-chitin and γ-chitin (Lodhi *et al.*, 2014). The type of bonding that is present in each of these substrates is an important factor, as the bond type often contributes to hydrolysis and solubility of the substrate. According to Rathore and Gupta (2015), α-chitin has strong hydrogen bonds and is considered to be insoluble in comparison to β-chitin, which is less stable due to the presence of weaker intermolecular forces.

It has been found that the hydrolysis of chitin biomass produces products that are relevant to the medical, agricultural and food industries (Lodhi *et al*., 2014). Therefore, the degradation of chitin biomass is crucial for the production of value added products such as oligomers. However, chitin substrates are insoluble and recalcitrant for enzymatic degradation. This then necessitates the use of various pre-treatment methods, to produce a soluble, inexpensive substrate, which is be accessible and easily hydrolysed by chitinolytic enzymes. Therefore, a number of pre-treatment methods have been developed to make chitin more soluble.

It has been reported that the presence of a high content of hydrogen bonds has a direct influence on the permeability and the swelling of chitinous substrates in water (Synowiecki and Al-Khateeb, 2003). Alpha chitin has a high number of hydrogen bonds in its solid state and has been reported to be completely insoluble, therefore, highly concentrated solutions such as HCl, H_3PO_4, H_2SO_4, formic acid, di- or trichloroacetic acid have been used to dissolve the substrate (Roberts, 1997). These chemical modifications produce water soluble chitin via the disruption of inter- and intra- hydrogen bonds, without the cleavage of glycosidic linkages (Synowiecki and Al-Khateeb, 2003). The degree of N-acetylation (ratio of 2-acetamido-2-deoxy-D-glucopyranose to 2-amino-2-deoxy-D-glucopyranose units) is another factor that has an effect of the solubility and the physico-chemical properties of chitin derivatives (Lodhi *et al.*, 2014).

Chitin and chitosan are crystalline substrates that dissolve in a limited number of solvents. Their extensive hydrogen bonding influences the typical characteristic of degradation before reaching a melting point (Kumar, 2000). Although some solvent systems assist in the use of these substrates, there is no comparative data available on the different solvents (Kumar, 2000). A knowledge of pre- and un-treated chitin containing biomasses is essential in understanding how the structures and properties of biomasses impact the activity of enzymes. This necessitates the use of different pre-treatment methods to disrupt chitin crystallinity to increase the porosity, to effectively allow enzymes more accessibility to the biomass (Karp *et al*., 2013; Van Dyk and Pletschke, 2012).

2.2. Aims and Objectives

2.2.1. Aim

To develop a pre-treatment method which will effectively produce a modified chitinous substrate that is highly soluble for efficient hydrolysis with chitinolytic enzymes.

2.2.2. Objectives

- To conduct different pre-treatments on commercial chitin from shrimp powder;
- To morphologically characterise the pre-treated substrates using scanning electron microscopy (SEM);
- To chemically characterise components of pre-treated substrates using Fourier Transform Infrared Spectroscopy (FTIR), Energy-Dispersive X-ray spectrometery (EDX) and x-ray diffraction (XRD);

2.3. Methods and Materials

2.3.1. Pre-treatment of substrates

2.3.1.1. Colloidal chitin

Protocols from Wen and colleagues (2002) with modifications from Kuzu *et al.* (2012) were used for generating colloidal chitin. Commercial chitin from shrimp shells (5 g), was added to 60% HCl (v/v). This mixture was further stirred at 4^{o}C overnight. A volume of 2 L of 95% ethanol (v/v) was added to the mixture and was stirred overnight at room temperature (25^{o}C).

The precipitate was obtained by centrifugation at 6000 *g*, for 25 minutes at 4^{o}C. The precipitate was washed with distilled water, followed by washing with a potassium phosphate buffer (pH 7), until the filtrate from the colloidal chitin reached a neutral pH. The colloidal chitin was then dried overnight at 100^{o}C and was ground into a fine powder, and then stored at room temperature.

2.3.1.2. Phosphoric acid treated chitin A and B

A deacetylated phosphoric acid treated biomass (phosphoric acid treated chitin A) was produced using the protocol of Rojas Avelizapa *et al.* (1999), with some modifications. A mass of 10 g of commercial chitin from shrimp shells was mixed with 100 mL phosphoric acid (145.35 g) and was stored at 4^{o}C for 24 hours. To partially solubilise chitin (phosphoric acid treated chitin B) a protocol of Zhang *et al*. (2006) was employed, whereby 5 g of commercial chitin from shrimp shells was made moist with distilled water. Then 150 mL of concentrated phosphoric acid ≥85% and 50 ml of acetonitrile were used to dissolve the moist chitin at 4^{o}C for 24 hours. The precipitate was washed with distilled water, The pH was adjusted to neutral pH with sodium hydroxide (5 M) and was followed by the final washing step. The substrate was air dried, ground into a fine powder and stored at room temperature.

2.3.1.3. Sodium hydroxide

Sodium hydroxide pre-treatment involved the addition of 5 g of commercial chitin from shrimp shells powder to 150 mL of 40% NaOH and was stirred for 10 minutes. The mixture was incubated at 150^{o}C, for 2 hours and with intermitted stirring every 20 minutes. The substrate was washed with distilled water until the pH of the filtrate was in the neutral range. The substrate was air dried overnight and was then ground and stored at room temperature.

2.3.2. Morphological characterisation and elemental composition

All pre-treated chitin samples (section 2.3.1), commercial chitin from shrimp shells powder and commercial chitosan from shrimp shells powder, were each fixed onto metal stubs. Samples were coated with a thin layer of gold under vacuum and were then observed using SEM (Scanning electron microscope, Vega© Tescan). The SEM coupled to an EDX (energy-dispersive X-ray spectrometer) system was used to determine elements present in the un- and pre-treated chitin samples (in triplicate). Substrates were fixed onto metal stubs, with copper as a standard. Spectrograms produced were analysed and documented using EDX Oxford INCA version 4.02 software.

2.3.3. Fourier transform infrared spectroscopy (FTIR)

The mid-infrared absorption frequencies (4000 to 650 cm^{-1}) of all pre-treated samples (section 2.3.1) and untreated samples (commercial chitin from shrimp shells and chitosan from shrimp shells) were obtained using a Spectrum 100 Perkin Elmer FTIR spectrometer (Perkin Elmer, USA), at room temperature. FTIR spectra runs were each conducted in triplicate, with four scans for each replicate. Spectrum One Software was used to conduct baseline and ATR corrections for penetration depth and frequency vibrations. Degree of acetylation as described by Miya *et al*. (1980) was calculated according to the following equation with the corresponding baselines of the amide I band, for each spectra.

$$\mathbf{DA\ (\%) = (A_{1655} / A_{3450}) \times 115}$$

Whereby: *DA- Degree of acetylation and

*A- Transmittance (%)

2.3.4. X-ray diffraction

Pre-treated (section 2.3.1) and un-treated samples of chitin and chitosan from shrimp shells, were analysed by XRD. The samples were all scanned using a Bruker D8 Discover diffractometer coupled to a sealed tube Cu Kα source (0.15405 nm), equipped with a proportional counter. Scans were run from $2\theta = 0^{o}$ to 50^{o}. Crystallinity index (CI) was calculated with the Scherrer equation (Struszczyk, 1987):

$$\mathbf{CI\ (\%) = [(I_{110} - I_{am}) / I_{110}] \times 100}$$

Whereby: *I_{110} (arbitrary units) is the crystalline diffraction at $2\theta = 19°$ and

*I_{am} (arbitrary units) is the amorphous diffraction at $2\theta = 12.6°$

2.4. Results

2.4.1. SEM of pre- and untreated chitin substrates

The pre- and untreated chitin substrates were coated with a gold layer on metal stubs and were each analysed under various magnifications during SEM (Fig 2.1).

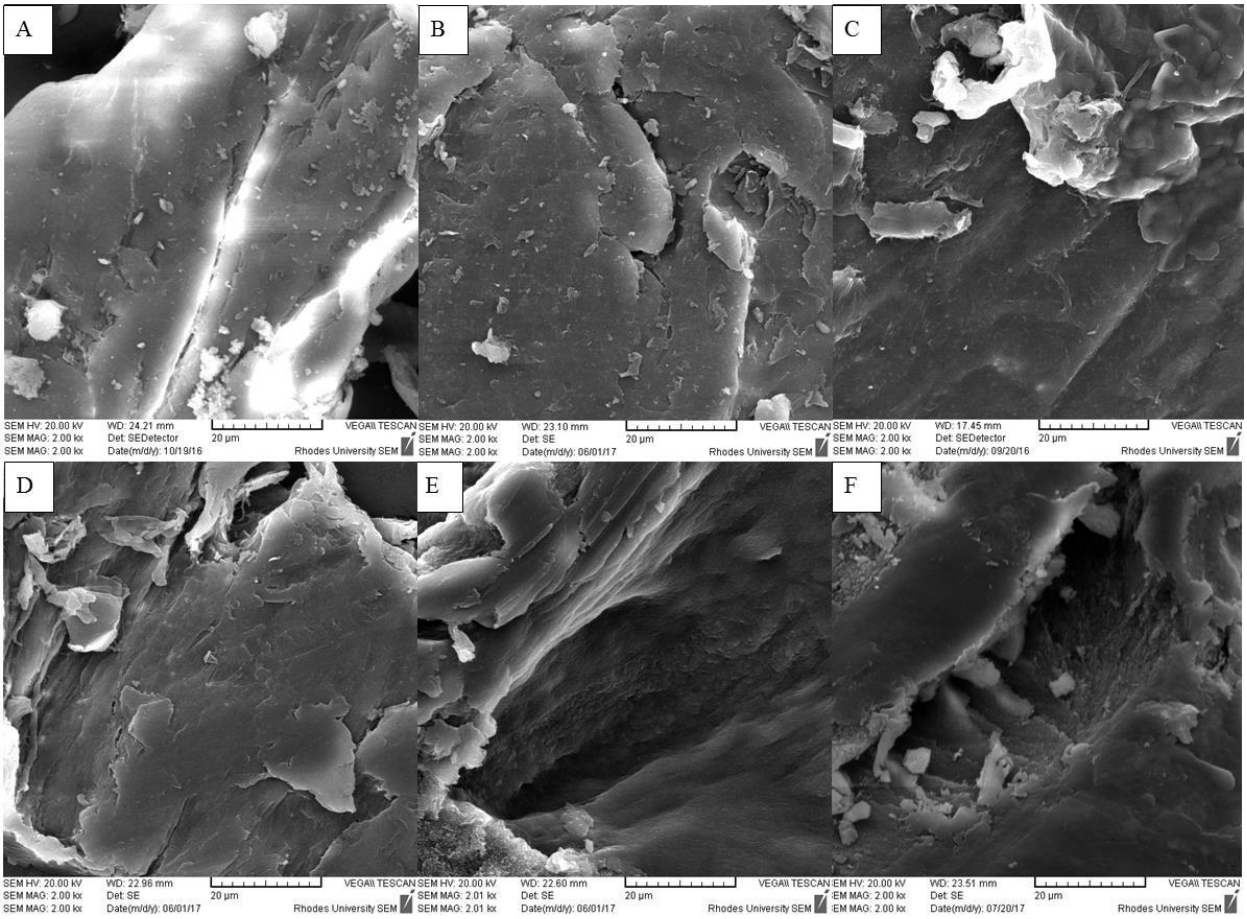

Figure 2.1. SEM analysis of un- and pre-treated chitin substrates at 20 µm at a magnification of 2 kx. (A) chitin from shrimp shells; (B) chitosan from shrimp shells; (C) HCl treated chitin from shrimp shells; (D) NaOH treated chitin from shrimp shells; (E) phosphoric acid treated chitin A chitin from shrimp shells and (F) phosphoric acid treated chitin B from shrimp shells.

Based on the SEM images shown in Fig 2.1, it was observed that the commercial chitin from shrimp shell powder (A) had an encapsulated, enclosed structure similar to commercial chitosan (B). NaOH treated chitin (D) showed that the structure of the top layer of the substrate started to unravel, exposing a rough texture; in contrast the HCl treated substrate (C) unravelled the substrate and exposed a smooth surface. Phosphoric acid treated chitin A (E) showed that the structure of the substrate was completely disrupted and exposed a smooth surface with grooves and some exposed fibrils, similar to (F) which was smoother. In summary, a noticeable change in morphological structure of the pre-treated biomass was observed, due to partial hydrolysis of the substrate.

2.4.2. Elemental analysis of un- and pre-treated chitin substrates

An energy-dispersive X-ray spectrometer system was used to determine chemical changes in the chitin by focusing on the presence of carbon, nitrogen and oxygen elements in the un- and pre-treated chitin substrates (Table 1). The rationale to focus on these elements was based on the fact that these elements constitute the major part of the biomass. Hydrogen was not quantified because it could not be detected with the equipment used in this study.

Table 1: Elemental analysis of nitrogen, carbon and oxygen content of pre- an un-treated chitin substrates

Substrate*	**Element (%)**		
	C	N	O
Shrimp chitin	53,060 ± 5,751	8,863 ± 1,248	38,077 ± 6,935
Chitosan	57,903 ± 2,039	13,14 ± 1,824	28,62 ± 1,539
Colloidal chitin (CC)	41,367 ± 8,013	6,76 ± 5,874	31,733 ± 5,152
NaOH chitin	62,223 ± 1,382	11,087 ± 0,988	26,69 ± 1,027
PA chitin	66,7 ± 3,943	12,123 ± 1,928	20,863 ± 3,571
PAS chitin	64,703 ± 1,531	9,337 ± 3,166	24 ± 3,335

Values are represented as mean values ± SD (n=3).

**Shrimp chitin- chitin from shrimp shells; Chitosan chitin- chitosan from shrimp shells; Colloidal chitin- HCl pre-treated chitin from shrimp shells; NaOH chitin- NaOH pre-treated chitin from shrimp shells; PA chitin- phosphoric acid treated chitin A from shrimp shells; PAS chitin- phosphoric acid treated chitin B from shrimp shells.*

Table 1 illustrates that the NaOH, PA and PAS pre-treated substrates have a higher carbon content, compared to colloidal chitin and un-treated chitin from shrimp shells and chitosan. However, the nitrogen content between un- and pre-treated substrates displayed similar values, with colloidal chitin having the least % N (6,76 ± 5,874). The oxygen content for NaOH, PA and PAS pre-treated substrates, were observed to be lower than that of the un-treated substrates and colloidal chitin.

2.4.3. FTIR of un- and pre-treated chitin substrates

Figure 2.2 displays the FTIR analysis of pre- and un-treated chitin containing substrates. This technique was used to determine chemical changes in chitin by focusing on the functional groups that are represented by bands.

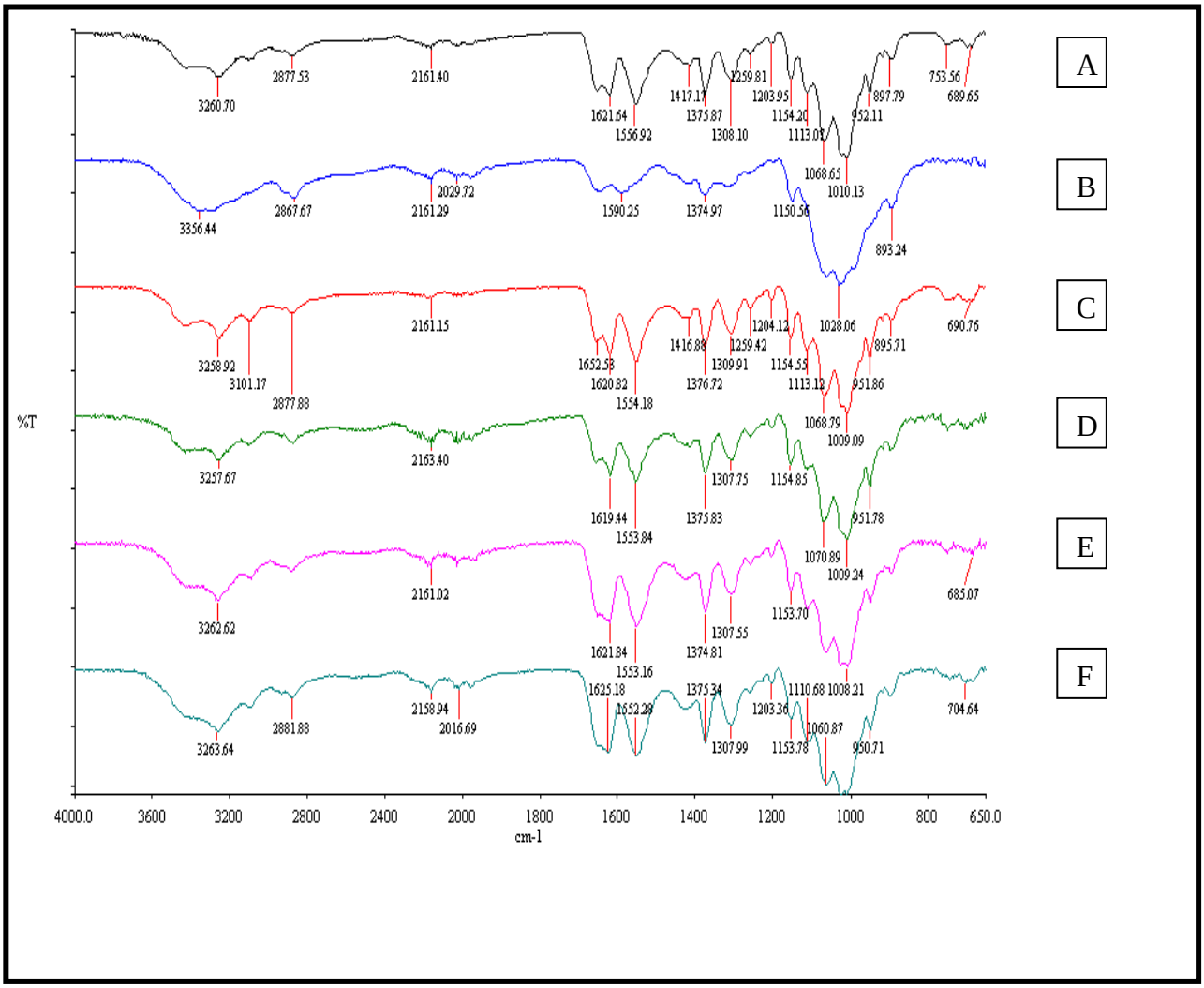

Figure 2.2. FTIR analysis of pre- and un-treated chitin substrate. (A) chitin from shrimp shells; (B) chitosan from shrimp shells; (C) HCl pre-treated chitin from shrimp shells; (D) NaOH pre-treated chitin from shrimp shells; (E) phosphoric acid A treated chitin from shrimp shells; (F) phosphoric acid B treated chitin from shrimp shells.

The above figure illustrates that commercial chitin (A) and commercial chitosan (B) from shrimp shells are slightly different in structure, with chitin (A) having numerous functional groups that are retained in comparison to chitosan (B). Pre-treated substrates C to D were observed to have similar peaks to (A). However, with each type of pre-treatment (C - D), there were slight shifts in the peaks, indicating structural modification of the substrate. The phosphoric acid treated biomasses (E and F) showed slightly modified peaks that were similar to (A), with fewer retained peaks in comparison to C - D.

2.4.4. Degree of acetylation of pre- and un-treated chitin substrates

The degree of acetylation (DA) was calculated for the various substrates, through the use of FTIR spectra absorbance values of specific bands that reflect chitin characteristics. The DA of the substrates were calculated through the use of the following equations:

Table 2: Degree of acetylation of pre- and un-treated chitin substrates

Substrate *	Degree of acetylation (%)
Shrimp chitin	69.60
Chitosan chitin	41.75
Colloidal chitin	28.75
NaOH chitin	61.42
PA chitin	38.46
PAS chitin	21.09

**Shrimp chitin- chitin from shrimp shells; Chitosan chitin- chitosan from shrimp shells; Colloidal chitin (CC)- HCl pre-treated chitin from shrimp shells; NaOH chitin- NaOH pre-treated chitin from shrimp shells; PA chitin- phosphoric acid treated chitin A from shrimp shells; PAS chitin- phosphoric acid treated chitin B from shrimp shells; DA- degree of acetylation.*

It was observed from Table 2, that the acid pre-treated substrates (CC, PA and PAS) exhibited the lowest degrees of acetylation (28.75, 38.46 and 21.09% respectively), in comparison to the NaOH and un-treated substrates. This demonstrated that the acid pre-treatments modified the biomass to a greater degree, increasing solubility and porosity.

2.4.5. X-ray diffraction of un- and pre-treated substrates

X-ray diffraction was conducted on the various substrates to determine the CI (%) and the shift in peaks of the substrates with the respective pre-treatment methods (Fig. 2.3). This method was important in determining the effect of substrate crystallinity on enzyme activity later in the study.

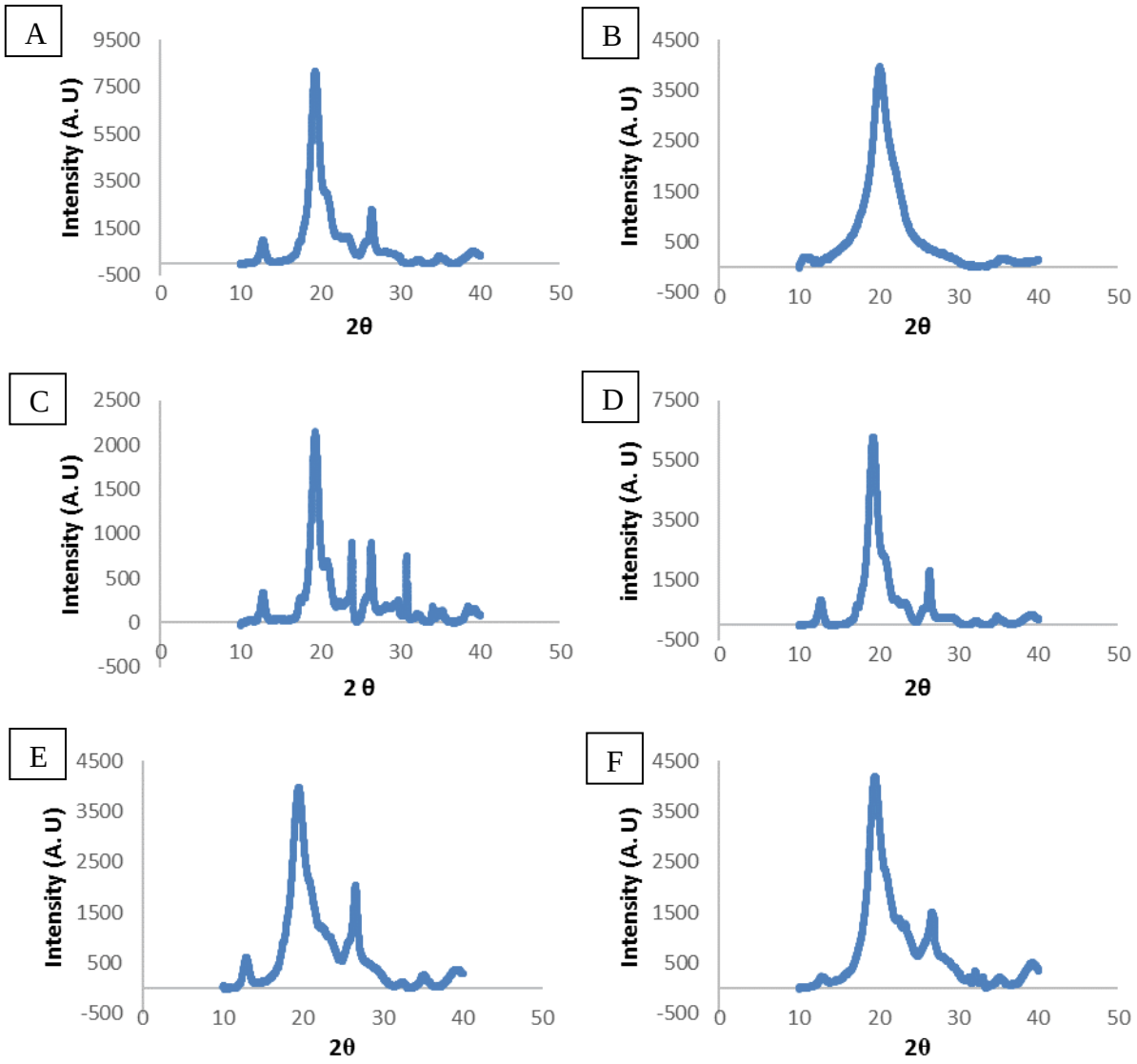

Figure 2.3. X-ray diffraction analysis of pre- and un-treated chitin substrate. (A) chitin from shrimp shells; (B) chitosan from shrimp shells; (C) HCl pre-treated chitin from shrimp shells; (D) NaOH pre-treated chitin from shrimp shells; (E) phosphoric acid A pre-treated chitin from shrimp shells; (F) phosphoric acid B pre-treated chitin from shrimp shells.

Figure 2.3 shows that A and D had the highest crystallinity peaks (8144 and 6275 A.U) and displayed crystallinity indexes of 97.94 and 98.09%, respectively. However, crystallinity peaks for B, E and F were similar with intensities of 3966, 3965 and 4201 A.U, respectively. The calculated CI for B, E and F were 95.53, 97.20 and 98.43%, respectively. The crystallinity peak of C was shown to be the lowest (2087 A.U) with a CI of 98.43%.

2.5. Discussion

2.5.1. Scanning electron microscopy and elemental composition of un- and pre-treated biomass

SEM analysis exhibited a noticeable change in the morphological structure of the pre-treatments, with the surface of the biomass becoming more exposed or unravelled and porous in comparison to the encapsulated un-treated biomasses (Fig. 2.1). The commercial chitin/chitosan from shrimp shells (A and B) displayed a similar encapsulated structure, with slight disruption of the chitosan structure. The commercial chitosan displayed a slightly exposed surface due to 70-75% deacetylation, resulting from the industrial processing of chitin from shrimp to produce chitosan; which involves washing with strong organic solvents, followed by treatment with sodium hydroxide (Sigma Aldrich, CAS: 9012-76-4).

Similarly, NaOH pre-treatment of chitin from shrimp shells is known to produce chitosan (C), therefore showing a larger amount of the structure unravelled and exposed with a rough surface of crumbling layers of flakes, which corresponds with the description of chitosan morphology that was produced by Yen and colleagues (2009). This can also be explained through the de-proteinisation of chitin via alkali treatment, being less harsh than the demineralisation of chitin by using acids (Roberts, 1992). Therefore, the acid pre-treatments of the chitin biomass (E - F) resulted in the complete disruption of the surface and unravelled the structure, producing a smooth surface (Zhang *et al*., 2006). Whereas alkali pre-treated biomass (D) resulted in the partial disruption of the surface structure. The phosphoric acid pre-treatments (E and F) produced a larger surface area on the biomass, with a smooth architecture, containing grooves and pores, with some chitin microfibrils being exposed (Zhang *et al*., 2006). It is thus evident that both the phosphoric acid pre-treatments were successful in the disruption and partial hydrolysis of the biomass, which should result in less recalcitrance and thus improve enzyme activity by increasing the accessibility of the biomass (Zhang *et al*., 2006; Van Dyk and Pletschke, 2012).

EDX results (Table 1) shows that all pre- and untreated substrates contained the greatest quantity of carbon, followed by oxygen and lastly, nitrogen. The CC pre-treated biomass contained the lowest carbon. In contrast, NaOH and both phosphoric acid pre-treated biomasses contained the higher quantities of carbon. However, the nitrogen content of NaOH and PA pre-treated substrates, were similar to chitosan. The CC and PAS pre-treatments had

nitrogen contents similar to chitin from shrimp. The pre-treated NaOH, PA and PAS biomass samples all displayed a similar oxygen contents to chitosan, whereas CC displayed the highest oxygen content of all pre-treated biomasses. The commercial chitosan (that is deacetylated) served as the positive control. Therefore, the observed similarities of the pre-treated biomasses and chitosan, suggests that the deacetylation of chitin does not necessarily imply the removal of amide functional groups. This was shown by the increase of carbon and nitrogen, with a decrease in oxygen in pre-treated substrates in comparison to un-treated chitin from shrimp.

Generally, all pre-treated biomasses contained higher quantities of carbon and nitrogen, and lower quantities of oxygen, than un-treated chitin from shrimp shells. It is thus evident that there was biomass modification, as a result of the different pre-treatments. This phenomenon has also been demonstrated on chitin from crab, by Yen *et al.* (2009), which explained that this was as a result of an increase in purity and N-deacetylation of the substrates. Due to EDX being unable to detect hydrogen, other available techniques such as FTIR were used to validate the chemical changes in the biomass and to further analyse the extent of modification of the biomasses, with respect to the degree of acetylation.

2.5.2. FTIR and determination of DA of un- and pre-treated biomasses

Chemical modifications of chitin or chitosan containing substrates may improve their solubility and this is fundamental in their applicability in industry (Sajomsang, *et al*., 2008; Silva *et al*., 2003 and Zhao *et al*., 2009). Studies indicate that α-chitin is represented by the presence of four characteristic amide bands around the following values: amide I at 1660/1627, amide II at 1558, amide III at 1312 and amide V at 698 cm^{-1} (Cardenas *et al*., 2004 and Paulino *et al*., 2006). All pre- and un-treated biomass samples (Fig. 2.2) showed the amide I band at around 1660 and 1627 cm^{-1} and the presence of the CH deformation of the β-glycosidic bonds band around 895 cm^{-1}. The peaks at approximately 3650 cm^{-1} and 1650 cm^{-1} of all biomasses imply that there was a presence of free hydroxyl groups or fewer interactions of hydrogen (Duarte *et al*., 2002). These bands are structural characteristic markers of chitin, which suggests that these biomasses are all α-chitins, with intra- and inter-sheet hydrogen bonding.

Degree of acetylation (DA) is an important factor to consider when working with chitin containing substrates, as the DA has a direct effect on the solubility and reactivity of the

substrates (Duarte *et al*., 2002). According to literature, approximately three infrared spectroscopy (IR) absorbance band ratios have been proposed for the determination of DA: A_{1655}/A_{3450}, A_{1550}/A_{2878} and A_{1655}/A_{2867} (Domard and Rinaudo, 1983; Domszy and Roberts, 1985; Miya *et al*., 1980; Moore and Roberts, 1980; Roberts, 1997). However, each of these ratios have their own limitations with regards to over- or under-estimating the DA, leading to inaccuracies (Baxter *et al*., 1992). The DA was calculated from each FTIR spectrum by employing the A_{1655}/A_{3450} ratio. The only inaccuracy of this particular band ratio is that the incorrect baseline was previously used for amide I, creating large discrepancies in DA%, in the presence of low levels of N-acetylation (Baxter *et al*., 1992). Therefore, to eliminate these inaccuracies, this band ratio was used in combination with the baseline method used by Miya *et al.* (1980) for the amide I band, which enables the detection of a wider range of values and for the N-acetylation to be determined accurately.

The results obtained in Table 2 illustrate that the un-treated chitin from shrimp shells had the highest DA (69.6%), followed by the NaOH pre-treatment (61.42%). This was expected as the shrimp sample had undergone no pre-treatment and the NaOH pre-treatment only resulted in de-proteinisation of the un-treated biomass (Roberts, 1992). The commercial un-treated chitosan was purchased from Sigma Aldrich and was obtained from the N-deacetylation of chitin from shrimp shells, using NaOH and organic solvents (Sigma Aldrich, CAS: 9012-76-4). This biomass had a DA of 41%, corresponding with reports stating that a substrate is considered chitosan only when the DA is below 50% (Chatelet *et al*., 2001). Lastly, the biomass that was pre-treated with organic solvents such as HCl, $\geq 85\%$ or $< 85\%$ phosphoric acid showed the lowest DAs which were 28.75, 38.46 and 21.09%, respectively. This confirmed that the organic solvents effectively demineralised and deacetylated the biomass samples, consequently increasing their solubility.

According to Focher and colleagues (1990), the appearance of the weak amide I band, as well as the amide stretch at 3265 and 3100 cm^{-1}, signifies deactylation. This was indicated in Fig. 2.2, where chitosan displayed this phenomenon (in contrast to the un-treated chitin from shrimp shells). The chitosan amide II band shifts up from 1558 to approximately 1590 cm^{-1}, with the appearance of a new band around 3356 cm^{-1} suggesting the occurrence of NH_2 groups (Darmon and Rudall, 1950; Focher *et* al., 1990). The decrease in acetylation implies that strong intermolecular forces between hydrogen bonds have been disrupted, directly affecting how polymers interact and results in the increase of solubility of the biomasses. The

FTIR spectra and calculated DAs therefore confirmed that the biomasses that were treated with strong acids displayed the highest degrees of acetylation, which suggests that the biomass structure had been modified to a greater extent, corresponding with the SEM data. The degrees of crystallinity were then investigated to analyse the morphology of the biomasses further, by determining whether pre- and un-treated biomasses were crystalline or amorphous.

2.5.3. X-ray crystallography of pre- and untreated biomasses

The XRD profiles (Fig. 2.3) exhibited intense peaks and no visible scattering, suggesting that the chitin source is a crystalline polymorph, such as α-chitin from shrimp (Al-Sagheer *et al*., 2009). The "crystallinity index", described as the percentage of the crystalline region in a particular biomass, was obtained by the employment of the Scherrer equation (section 2.3.4). The calculated CI was the highest after phosphoric acid pre-treatment B (98.43%), followed by the NaOH pre-treatment, un-treated chitin from shrimp, phosphoric acid pre-treatment A, chitosan and HCl pre-treated chitin, respectively. This showed that the HCl pre-treated biomass was more amorphous (CI: 85.72%) than all the other biomass samples. However, in contrast to the calculated CIs, the individual crystallinity peaks for each biomass at $2\theta = 19°$, suggest that the un-treated chitin from shrimp and the NaOH pre-treated chitin were the highest and the acid pre-treatments, together with chitosan had lower crystallinity peaks. This demonstrates that the acid pre-treatments disrupted the structure of the biomass, resulting in a decrease in crystallinity (Kumar, 2000). This phenomenon is in agreement with the DAs exhibited by these pre-treated biomass samples. This indicates that the acid pre-treatments disrupt the strong hydrogen bonds that exist in the α-chitin biomass, rendering the biomass less stable (Synowiecki and Al-Khateeb, 2003).

2.5.4. Conclusion

The characterisation of pre- and un-treated chitin biomasses was conducted in order to investigate a cheaper and more efficient way of producing a soluble chitin substrate that could potentially be used in industry. Therefore, the structural characteristics of various pre- and un-treated chitin biomass samples were investigated, to develop an understanding of the effects of the treatments on the chemical and physical properties of the samples. The data obtained from SEM, FTIR, DA and XRD analyses of the biomasses showed that acid pre-treatment disrupted the biomass the most, resulting in a more unstable α-chitin polymorph. This allowed the surface of the biomass to be exposed due to the disruption of

crystallinity and an increase in porosity, potentially resulting in greater accessibility for enzyme binding. Therefore, the significance of this study is self-evident in the biotechnological industry, where the chemical and physical properties of the biomass samples are required in order for chitin degrading enzymes to effectively hydrolyse these substrates into specific value added products, which have a direct effect on biological activity. This therefore necessitates the search for new, more readily available and cheaper sources of chitin degrading enzymes for the production of valuable products that can be utilised in industry.

Chapter 3: Prospecting for chitin degrading enzymes

3.1. Introduction

Chitin is one of the most abundant biopolymers found in nature, with its occurrence being reported in fungal cell walls, algae, insect exoskeletons and crustaceans. The hydrolysis of chitin produces chitooligosaccharides (CHOS) which are used in a many different applications; ranging from food, pharmaceutical and agriculture, to waste water management (Lodhi *et al*., 2014). However, chitinases or chitosanase enzymes which are commercially available are costly and as a result CHOS cannot be produced on an industrial scale. This therefore necessitates bio-prospecting for chitinolytic and other chitin degrading enzymes from alternate sources, which have the ability to efficiently hydrolyse chitin into value added products (Rathore and Gupta, 2015).

Ericoid mycorrrhizal (ERM) fungi are known to be associated with ericaceous plant roots (Bizabani *et al*., 2016). This facilitates the growth and survival of the plant by providing the necessary organic and inorganic nutrients, and water, in exchange for energy rich compounds such as carbohydrates (Finlay, 2008; Janerette, 1991). This nutrient transfer between the plant and the mycorrhiza occurs through the action of extracellular enzymes that are secreted by the fungus, such as in the case of *Oidiodendron maius* (CafRu082b) (Bizabani *et al*., 2016; Cairney and Meharg, 2003). Fungal chitinases are abundant in the cell wall and play an important role in nutrition, autolytic, parasitic morphogenesis and fungal development (Bhattacharya *et al*., 2007). Chitinases and other chitin degrading enzymes are responsible for the generation of carbon and nitrogen in ecosystems and are utilised in pathogen control (Hamid *et al*., 2013). This supported the rationale to investigate whether *Oidiodendron maius* could produce chitin degrading enzymes extra-cellularly.

Advances in the cloning, expression and isolation of proteins is extremely beneficial to research and industrial applications, where large quantities of proteins are required. A review by Ahmed *et al.* (2014) found that protein expression systems such as the methylotrophic yeast, *Pichia pastoris* (*P. pastoris*), exhibit a high growth rate and have the ability to produce high yields of certain proteins intra- and extra-cellularly, which is regulated by the *AOX1* promoter. Therefore, a cheaper avenue to explore was to clone the chitinase genes (*chit1*, *chit2*, *chit3* and *chit4*) from the recently sequenced *Thermomyces lanuginosus* SSBP genome and heterologously express these enzymes in *P. pastoris* (Singh *et* al., 2000 and Zhang *et al*.,

2015). These enzymes were successfully cloned, expressed and characterised by Zhang and colleagues (2015) and displayed the ability to hydrolyse colloidal chitin into chitooligosaccharide product(s). This showed that these enzymes have the ability to degrade chitinous waste and were therefore tested on different un- and pre-treated chitinous substrates. *Pichia pastoris* with the constitutive *chit1* gene that encodes a 44.1 kDa chitinase was of special interest (Zhang *et al*., 2015). This *P. pastoris* was cultivated in media containing the different chitin substrates to determine whether this recombinant strain could release/excrete the chitinase enzymes, which could effectively hydrolyse the chitinous substrates into products. This phenomenon is known as simultaneous saccharification (Bratheur, 2017).

The work in this chapter investigated the ability of *P. pastoris* expressed chit1 enzymes to degrade chitin biomass samples into CHOS and explored *O. maius* as a source of chitin degrading enzymes. The degradation of chitin on a large scale requires large quantities of enzyme – this therefore justifies the search for new sources and avenues to produce these enzymes which can degrade this polymer into value added products.

3.2. Aims and Objectives

3.2.1 Aim

To bio-prospect for enzymes that exhibit chitin-degrading properties, with the ability to produce chito-oligosaccharides.

3.2.2 Objectives

- To culture and express the *chit1* gene/s in *P. pastoris*;
- To perform simultaneous saccharification using *P. pastoris* with the *chit1* gene/s on different substrates;
- To determine if CHOS are produced by Chit1 enzymes using thin layer chromatography (TLC);
- To determine the chit1 enzyme activity of the intra- and extracellular factions of Chit1;
- To perform induction studies with the Chit1 enzyme from *P. pastoris* grown using different concentrations of various substrates;

- To culture *O. maius* in growth media containing chitin based substrates;
- To conduct activity assays with the intra- and extracellular fractions of *O. maius* on various chitinous substrates;
- To determine if CHOS are produced by the chitin degrading enzyme(s) from *O. maius* using TLC.

3.3. Methods and Materials

3.3.1. Culturing and expression of the *chit1* gene in *P. pastoris*

chit1 transformed *Pichia pastoris* transformants for chitinase (Chit1) production were provided by Zhang and colleagues (2015) (Professor K. Permaul from Durban University of Technology). Fresh colonies of transformed *P. pastoris* were regrown on YPD plates according to Zhang et al. (2015) with slight modifications. The 2% (w/v) peptone, 1% (w/v) yeast extract, 2% (w/v) glucose, 2% (w/v) bacteriological agar, 0.2% (w/v) colloidal chitin and 100 μg/mL zeocin (Thermo Fisher Scientific) were added to Milli-Q H_2O and the mixture was autoclaved. Transformants were grown in YPG media to produce seed cultures (10 g/L yeast extract, 20 g/L peptone, 10 g/L glycerol) with 100 μg/mL zeocin, at 28^{o}C and 200 rpm until an OD_{600} of 1.5 was reached. A negative control was included with the absence of transformants being inoculated into the media.

3.3.2. Simultaneous saccharification studies with Chit1enzymes from *P. pastoris*

Experimental controls were produced, each composed of YPG media (as prepared in section 3.3.1.). Control 1 contained reactions in the absence of yeast extract and glycerol in YPG media, with duplicate reactions containing 0.5% (w/v) individual substrates: commercial chitin from shrimp shell powder, commercial chitosan from shrimp shells, colloidal chitin, NaOH treated chitin and phosphoric acid treated chitin A prepared in chapter 2, section 2.3.1. Control 2 comprised of YPG media without glycerol and 0.5% of each of the individual substrates and *P. pastoris* in each flask. Control 3 comprised of YPG media with *P. pastoris* and with no substrate added. Control 4 comprised of YPG media with no *P. pastoris inoculum* and no substrate, which served as a blank for the dinitrosalicylic acid (DNS assay). Control 5 comprised of YPG media with colloidal chitin without *P. pastoris* inoculum. Test samples contained YPG media, 0.5% of each of the individual substrates and inocula. All reactions were conducted in duplicate for a period of 4, 7 and 10 days at 28^{o}C with shaking at

200 rpm. Samples were centrifuged at 13000 rpm for 5 minutes and the quantity of reducing sugar released was then measured using the DNS method (Miller, 1959), whereby 300 µL of DNS was added to 100 µL of the supernatants. Hydrolysis was terminated by boiling at 100ºC (Labnet AccuBlock digital dry bath) for 5 minutes, followed by cooling on ice for 5 minutes (Miller, 1959). Reactions were monitored using a 96-well plate at 540 nm with a Power $Wave_x$ Spectrophotometer and analysed on Kc Junior software, with N-acetylglucosamine (0.1 to 1.2 mg/mL) used as a suitable sugar standard (Appendix 2B).

3.3.3 Protein determination

The Bradford method was used to determine the protein concentration of fractions from section 3.3.2 (Bradford, 1976). A standard curve was constructed with protein concentrations from 0.1 to 0.8 mg/mL of protein using bovine serum albumin (BSA) as standard (Appendix 2A). For protein sample analysis, 25 µL of sample was added to 230 µL of Bradfords reagent. The blank consisted of citrate-phosphate buffer (pH 5, 0.2 M dibasic sodium phosphate- 0.1 M citric acid monohydrate) and Bradford reagent. After 15 minutes at room temperature, the absorbance values of the samples were measured at 595 nm with a Power Wavex Spectrophotometer using KC Junior software.

3.3.4. Determination of CHOS produced using thin layer chromatography

Protocols were modified to detect chitooligomeric sugars with TLC (Baron and Economidis, 1963; Smith, 1960). Each sample (section 3.3.2) and a mixture containing 10 mg/ml of standards ranging from N-acetylglucosamine to chitohexaose, was spotted on a silica plate. The mobile phase consisted of n-butanol: methanol: 32% ammonia water: dH_2O (5:4:2:1). The TLC was run for 2 hours, followed by drying for 10 minutes and a re-run in the mobile phase. The staining solution comprised of a mixture of solution 1 and 2. Solution 1 comprised of 20 mL of 5% (w/v) alanine in acetone, added to 20 mL of 5% (w/v) diphenylamine in acetone; solution 2 comprised of: 85% phosphoric acid. Solution 1 and 2 was mixed at a 5:1 ratio to produce the staining solution. The plate was dried for 10 minutes, stained, dried for 10 minutes and baked at 120^{o}C for 10 minutes.

3.3.5. Determination of intra- and extracellular activity of Chit1 from *P. pastoris*

Pichia pastoris inocula were each added into YPG media and YPG media with phosphoric acid treated chitin (Chapter 2, section 2.3.1.2.), and were grown for 8 days at 28^{o}C with

shaking at 200 rpm. Samples were centrifuged at 10000 x *g* for 1 hour. The supernatant was stored as the extracellular fraction. The pellets were re-suspended in 10 mL lysis buffer (50 mM sodium dihydrogen phosphate, 300 mM sodium chloride and 10 mM imidazole, pH 7), followed by a 5 minute rest period on ice. Samples were then sonicated for 10 seconds with a 10 seconds rest period on ice for 8 repeats. A volume of 5 mL lysis buffer was added to the sample, followed by centrifugation at 10000 *g* for 1 hour. The supernatant was considered to be the intracellular fraction. The intra- and extracellular fractions obtained from the two types of growth media were used to conduct activity assays on various substrates, using 300 µL reactions that comprised of 200 µL of the fractions and 100 µL of 1.33% stock of substrates (commercial chitin from shrimp shell powder, commercial chitosan from shrimp shells, colloidal chitin, NaOH treated chitin and phosphoric acid treated chitin A) dissolved in citrate-phosphate buffer (pH 5, 0.2 M dibasic sodium phosphate- 0.1 M citric acid monohydrate). The samples were incubated at 37^{o}C, at 25 rpm, for 48 hours, followed by centrifugation at 13000 rpm for 5 minutes at the end of the reaction. The activities of the intra- and extracellular fractions of *P. pastoris*, grown in the presence of the various chitinous substrates, were determined by the DNS method (section 3.3.2.). TLC (section 3.3.4.) was performed with the hydrolysates from the activity assays to determine the type of CHOS which were produced during hydrolysis.

3.3.6. Induction study of extracellular Chit1

The induction study used 50 mL of YPG media, each with various concentrations [0.1, 0.4, 0.6, 0.8 and 1% (w/v)] of colloidal chitin or phosphoric acid treated chitin A (section 2.3.1.). *Pichia* pastoris inocula were each added into the media and were grown for 7 days at 28^{o}C with shaking at 200 rpm. YPG media without any added substrate and with only *P. pastoris* served as a positive control. Samples were filtered with a Buchner Flask and funnel under vacuum, and the flow-through was filtered using 10 kDa Amicon cut-off filters, which were centrifuged at 4000 *g* for 45 minutes. Activity assays reactions were prepared as mentioned in section 3.3.5, using 400 µL reactions comprised of 100 µL of enzyme samples added to 300 µL of substrate, the total volume was 400 µL. Enzyme controls for the activity assay were comprised of YPG media with colloidal chitin or phosphoric acid, and phosphate-citrate buffer (pH 5.0, 0.2 M dibasic sodium phosphate- 0.1 M citric acid monohydrate). The DNS method was employed to detect the amount of reducing sugars (section 3.3.2.).

3.3.7. Culturing of *Oidiodendron maius* (CafRu082b)

Pure cultures of *O. maius* were provided by Professor Dames in the Department of Biochemistry and Microbiology, Rhodes University. The culture was maintained on Potato Dextrose Agar at 28^{o}C. The fungal isolate was sub-cultured in Modified Melin Norkrans (MMN) medium containing the following (L): 10 g peptone, 3 g yeast extract, 1 g malt extract, 1 g glucose, 0.5 g KH_2PO_4, 0.25 g $(NH_4)_2HPO_4$, 0.15 g $MgSO_4.7H_2O$, 50 mg $CaCl_2$, 25 mg NaCl, 3 mg $ZnSO_4$, 1.2 mL of iron chloride solution and 100 ug thiamine hydrochloride, pH adjusted to pH 5.0 (using 1 M HCl) prior to autoclaving (Personal Communication: Professor Dames, Dr Bizabani and Colleen Manyumwa). One disc of inoculum was inoculated per 50 mL MMN broth. In order to stimulate chitin degrading enzyme activity, 3 g chitin from shrimp shell powder was added to the MMN. Three conditions were tested, *O. maius* grown in: 1) MMN; 2) MMN without glucose and 3) MMN without peptone. Samples in Schott bottles were covered with foil and were incubated at room temperature for 2 and 3 weeks on a rotary shaker at 150 rpm.

3.3.8. Quantification of reducing sugars in intra- and extracellular fractions of *O. maius* growth media

After 2 and 3 weeks, all MMN media were centrifuged at 5000 *g* for 30 minutes. The supernatant was considered to be the extracellular fraction. Intracellular fractions were produced by employing the lysis method (section 3.3.5.). Fractions were concentrated using 10 kDa filters, at 4000 *g*, for 45 minutes and activity assays (section 3.3.6.) were conducted, where each concentrated fraction was tested on 1.33% phosphoric acid treated chitin A, followed by the DNS protocol (section 3.3.2.). Determination of the CHOS produced during activity assays was performed using TLC (section 3.3.4).

3.4. Results

3.4.1. Culturing of *P. pastoris*

A seed culture of *P. pastoris* was produced in YPG media until an OD of 1.5 was reached when reading at 600 nm. The seed culture was then discontinuously streaked onto YPD plates to produce an inoculum (Fig. 3.1).

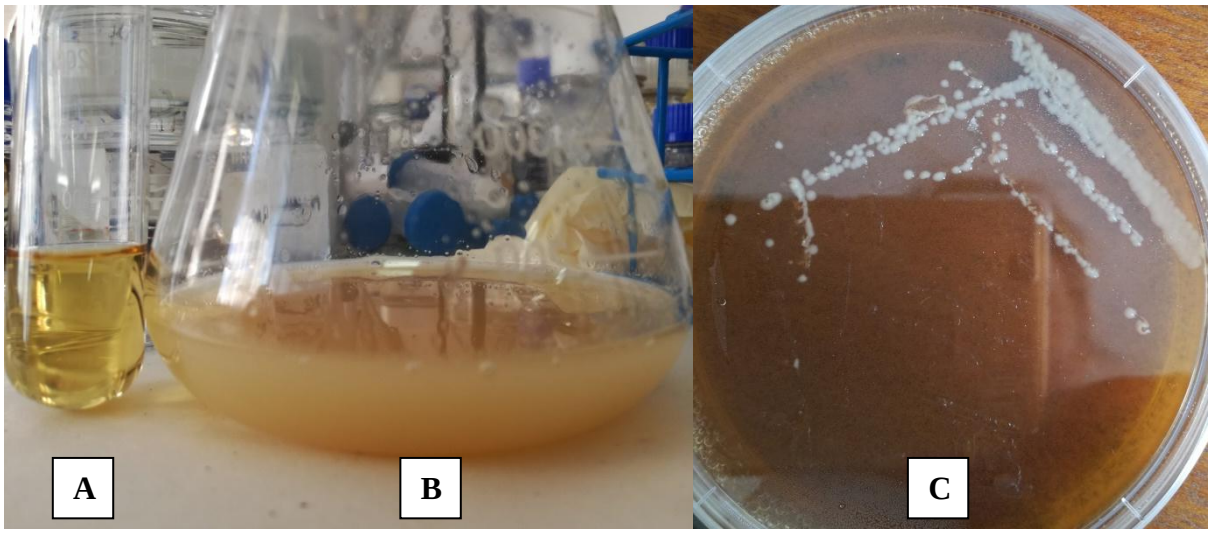

Figure 3.1. Seed culture of *P. pastoris* in YPG media, sub-cultured onto YPD plates. (A) YPG media negative control; (B) YPG media with *P. pastoris* at OD_{600} and (C) YPD plate with *P. pastoris* transformants.

Fig. 3.1 illustrates that the negative control (A) is a clear golden colour - in contrast to the media with the *P. pastoris* culture (B) which has a cloudy/opaque appearance characteristic of microbial growth. The streaked transformants were observed as small, circular colonies on YPD plates (C).

3.4.2. Simultaneous saccharification studies of Chit1 from *P. pastoris*

Simultaneous sacchrification studies were performed to determine whether Chit1 enzymes secreted from *P. pastoris* can immediately hydrolyse substrates present in the growth media more efficiently. The results are depicted in Figure 3.2.

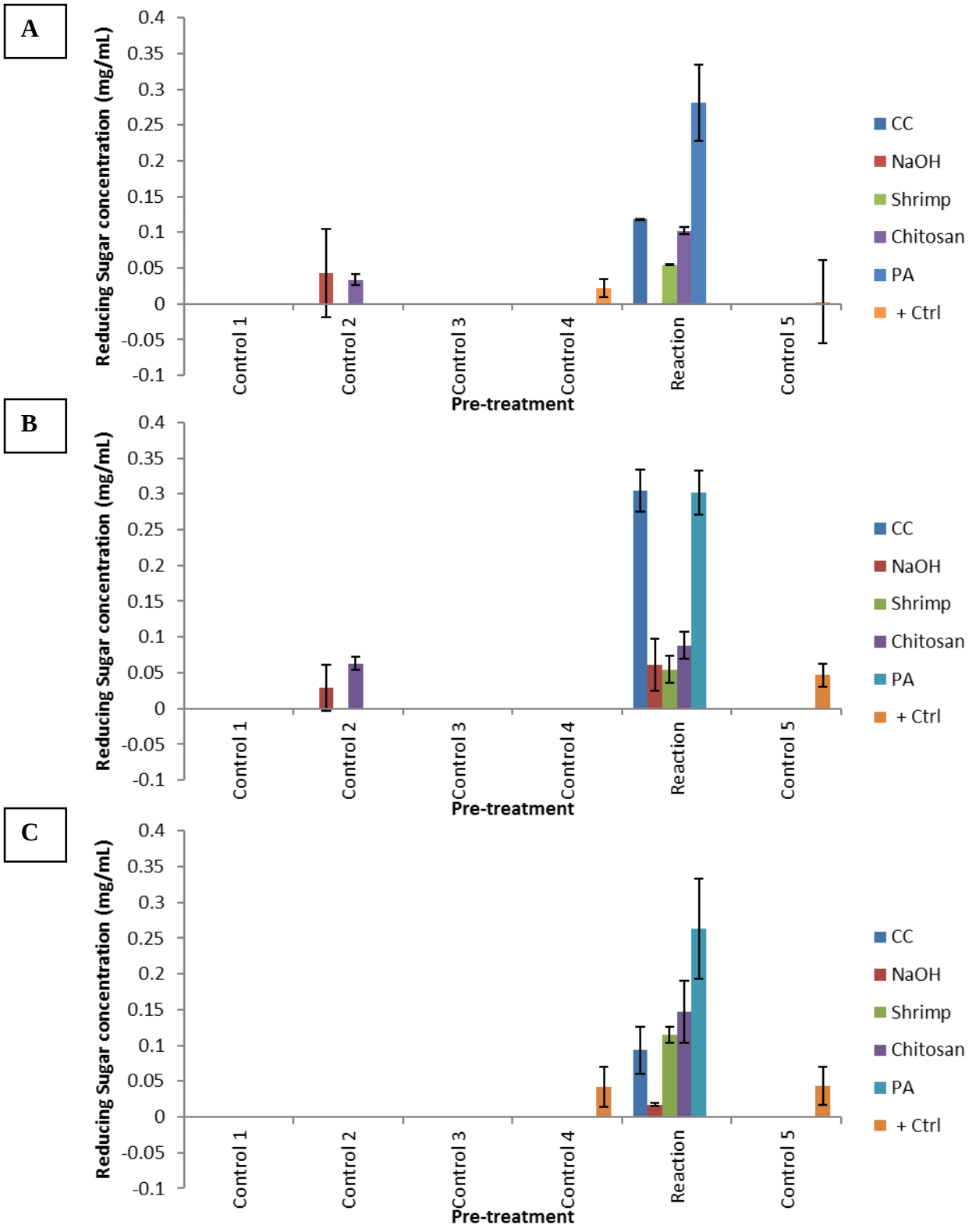

Figure 3.2. Simultaneous saccharification studies of *P. pastoris* on various substrates. Values are represented as means ± SD, n=2. (A) Simultaneous saccharifiaction study for 4 days; (B) simultaneous saccharification study for 7 days and (C) simultaneous saccharification study for 10 days. CC: colloidal chitin; NaOH: NaOH treated chitin; Shrimp: commercial chitin from shrimp shell powder; Chitosan, commercial chitosan from shrimp shell powder; PA: phosphoric acid trated chitin A and + Ctrl: positive control.

All of the above figures illustrate that there was no product formation in the absence of glycerol and yeast from the media, for control 1. When only glycerol was omitted from the media (control 2), it was observed that assays conducted with NaOH treated chitin or chitosan produced reducing sugars. In contrast, Fig 3.2C showed the no reducing sugars produced from the hydrolysis of chitosan or NaOH substrates, which suggests *P. pastoris* starts to consume the reducing sugars as a source of nutrients only after 7 days. Control 3 for Figures 3.2A, 3.2B and 3.2C demonstrated that, in the absence of substrate, Chit1 secreted from *P. pastoris* produced no reducing sugars. Some auto-hydrolysis (control 4) of the media was observed in Figures 3.2A and 3.2C. Samples after 4 days, showed the highest quantity of reducing sugars (0.281 mg/mL) produced with phosphoric acid treated chitin A, and NaOH treated chitin produced no product. Colloidal chitin and phosphoric acid treated chitin A, both produced high quantities of reducing sugars after simultaneous saccharification for seven days with 0.305 and 0.302 mg/mL of reducing sugars, respectively. The NaOH treated substrate started to release reducing sugars (0.061 mg/mL) after 7 days as shown in Fig 3.2B. In contrast, Fig 3.2C demonstrates a rapid decrease in reducing sugars produced for colloidal chitin and NaOH treated chitin 0.093 and 0.017 mg/mL, respectively, after seven days of simultaneous saccharification. Control 5 showed little activity, which suggests some auto-hydrolysis of colloidal chitin in Figs 3.2A, B and C (0.003, 0.047 and 0.043 mg/mL, respectively).

3.4.3. Identification of CHOS produced

To detect the presence of the different chitoligomers that may have been produced by Chit1 with different sources of substrate, TLC was used as a qualitative technique (see Figure 3.3 below).

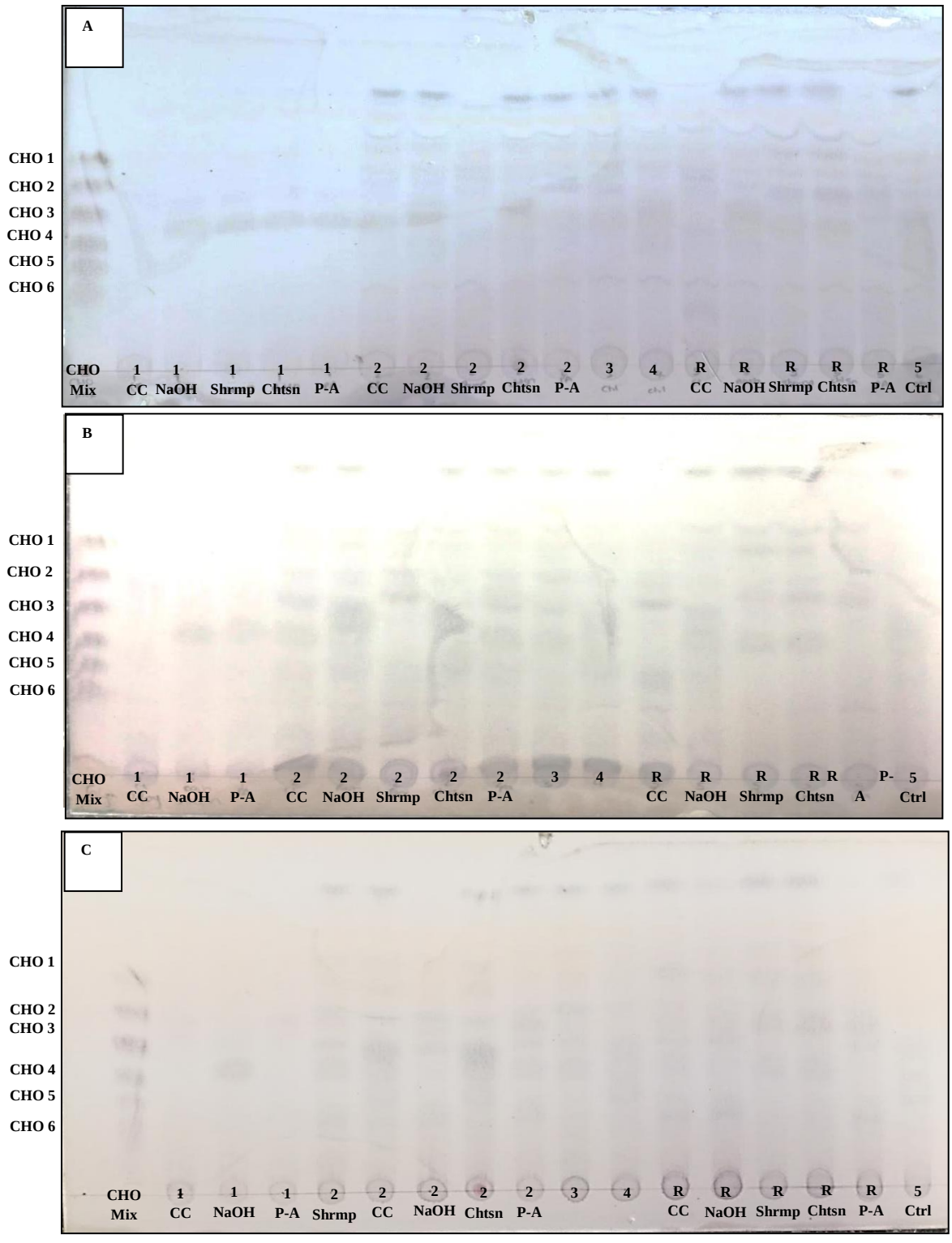

Figure 3.3. Thin layer chromatography of simultaneous saccharification reactions to detect chitooligomers after (A) 4 days; (B) after 7 days and (C) 10 days. CHO Mix: chitooligomer mix; CHO1-CHO6: N-acetylglucosamine- hexaacetyl-chitohexose; 1: control 1; 2: control 2; 3: control 3; 4: control 4; R: reactions; 6: control 5; CC: colloidal chitin; NaOH: NaOH treated chitin from shrimp shells; shrmp: chitin from shrimp shells; chtsn: commercial chitosan from shrimp shell powder and P-A: phosphoric a0cid treated chitin from shrimp shells A.

Figure 3.3 shows that control 1 in Fig. 3.3A, B and C only have one prominent band with a degree of polymerisation (DP) of 4, in the absence of yeast and glycerol from the media. In contrast, control 2 produced an array of bands (A, B and C) in the absence of only glucose, which suggested that yeast may have sugars present that are used as an energy source (Li *et* al., 2011; Matsumura *et al*., 1999; Yamatsu *et al*., 2006). Control 3, as expected, did not show any prominent bands due to no substrate being available for hydrolysis. Similarly, control 4, composed of YPG media only, showed no prominent bands. Reactions samples of A, B and C all showed a range of chitooligomers that were produced, illustrating *P. pastoris'* ability to hydrolyse various chitin substrates. Control 5 showed no prominent bands, due to the absence of *P. pastoris*, and thus confirming that no Chit1 enzymes were produced and no biomass was hydrolysed. Bands produced in all lanes (except for control 1 samples) that were lower than a DP of 1, were sugars that may have been derived from the yeast extract.

3.4.4. Determination of intra- and extracellular activity of Chit1 from *P. pastoris*

To investigate whether Chit1 was secreted in higher concentrations intra- or extra-cellularly from *P. pastoris*, activity assays with both these fractions were conducted on different substrates to determine the location of Chit1.

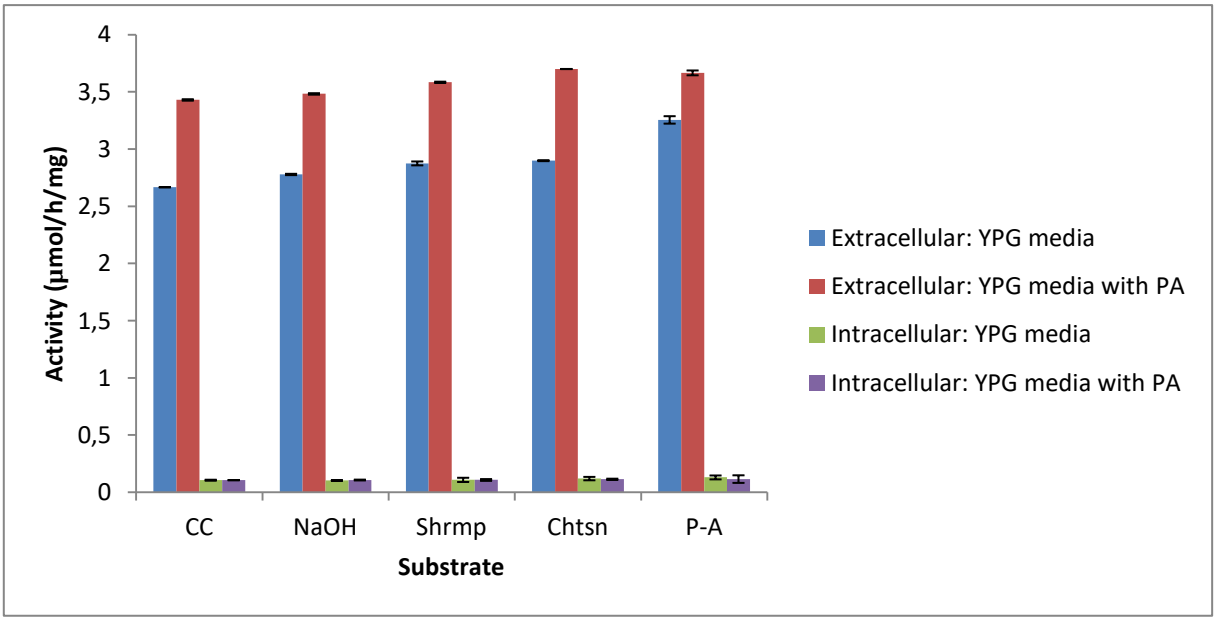

Figure 3.4. Activity of intra- and extracellular Chit1 from *Pichia pastoris* grown in YPG media and YPG media with phosphoric acid treated chitin A from shrimp shells, on different substrates in assays. Values are represented as means ± SD, n=3. CC: colloidal chitin; NaOH: NaOH treated chitin from shrimp shells; shrmp: chitin from shrimp shells; chtsn: commercial chitosan from shrimp shell powder and P-A: phosphoric acid treated chitin from shrimp shells A.

Based on Fig 3.4, it was observed that Chit1 displayed the highest activity in extracellular fractions in comparison to intracellular fractions. A higher activity was observed when *P. pastoris* was cultured in YPG media with phosphoric acid treated chitin A, as an inducer for Chit1. This illustrates that extracellularly produced Chit1 from both extracellular cultures exhibit the highest specific activity on phosphoric acid treated chitin A as a substrate.

To determine the hydrolysis products formed by the different fractions of *P. pastoris*, TLC performed with the hydrolysates from the activity assay (see Fig. 3.5). Activity assays using the extracellular fractions of *P. pastoris* grown in YPG media or YPG media with phosphoric acid treated chitin A as an inducer, exhibited the highest chitinase activities and were run on TLC.

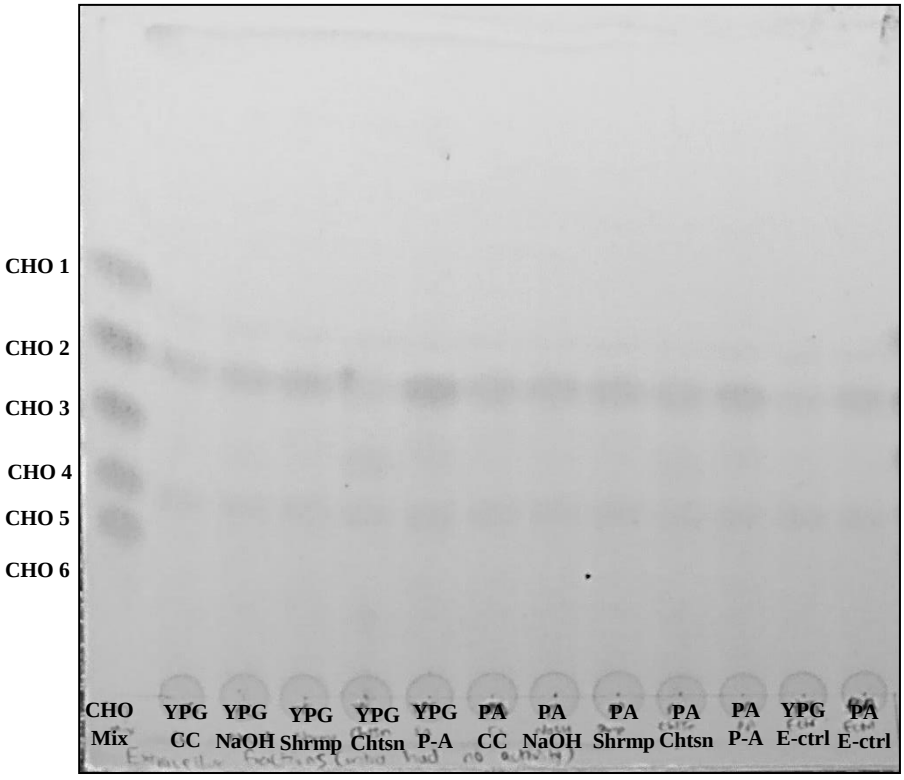

Figure 3.5. Thin layer chromatography on extracellular activity of Chit1 from *Pichia pastoris* grown in normal YPG media and YPG media with phosphoric acid treated chitin A from shrimp shells on different substrates. CHO Mix; chitooligomer mix; CHO1-CHO6: N-acetylglucosamine- hexaacetyl-chitohexose; YPG: Chit1 from cultured in YPG media; PA: Chit1 cultured in YPG media with phosphoric treated chitin A as a inducer; CC: colloidal chitin; NaOH: NaOH pre-treated chitin from shrimp; Shrmp: chitin from shrimp shells; Chtsn: commercial chitosan; P-A: phosphoric acid treated chitin A from shrimp shells and E-ctrl: enzyme control.

TLC revealed that both the enzyme controls displayed a few light bands, which showed the presence of a few CHOS. This finding correlates with Fig. 3.4, where enzyme controls were taken into consideration before determining specific activities. Chit1 enzymes produced in

media with phosphoric acid treated chitin A as an inducer showed the darkest bands when activity assays were run with phosphoric acid treated chitin A or chitosan as substrates. Similarly, Chit1 enzymes cultured in YPG media, show dark bands when these two substrates were also used in activity assays. The intensity of these bands suggested high activity and corresponds to activities illustrated in Fig. 3.3.

3.4.5. Induction study of extracellular Chit1 10 kDa fractions

Induction studies of Chit1 from *P. pastoris* were performed at different concentrations of the two acid pre-treated chitin substrates: colloidal and phosphoric acid treated chitin A (Fig. 3.6). *Pichia pastoris* was grown in YPG media to produce Chit1 as a positive control for comparison. Chit1 was then concentrated from the different media using 10 kDa Amicon cut-off filters and activity assays were conducted on 1% (w/v) colloidal chitin or phosphoric acid treated chitin A.

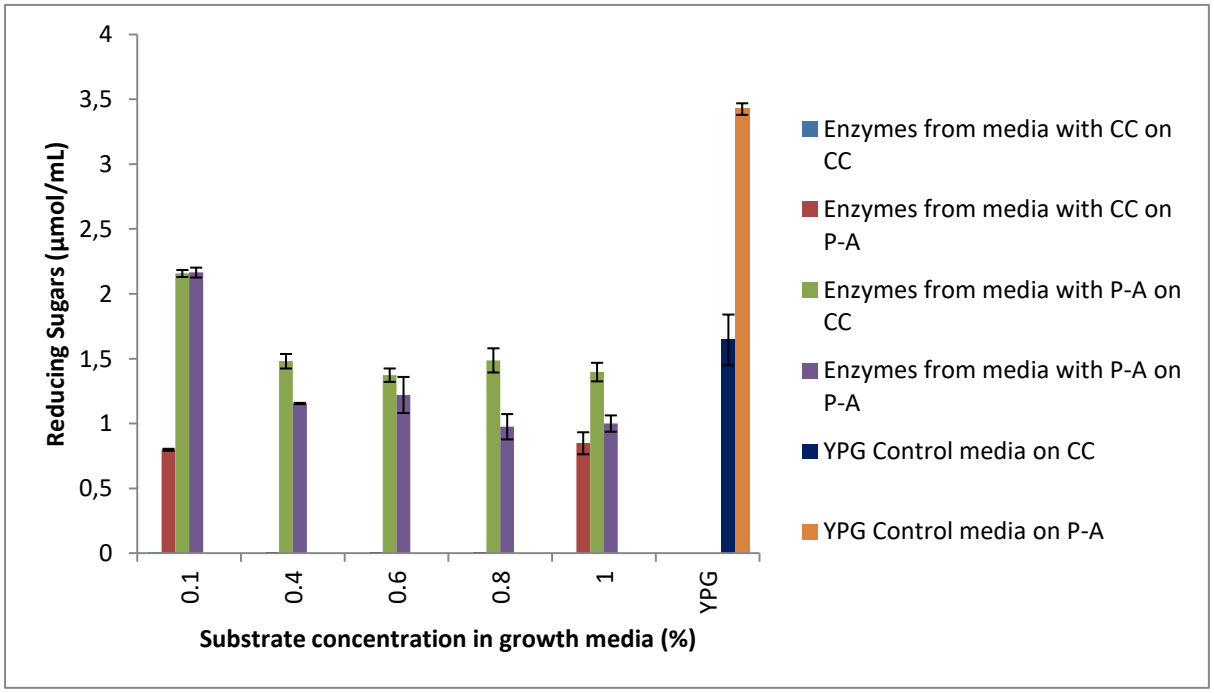

Figure 3.6. Induction study of Chit1 10 kDa fractions from *Pichia pastoris* grown in media with different concentrations of colloidal chitin and phosphoric acid pre-treated chitin from shrimp shells. With activity assay conducted on 1% colloidal chitin or phosphoric acid treated chitin A from shrimp shells. Values are represented as means ± SD, n=3. CC- colloidal chitin; P-A: phosphoric acid treated chitin A from shrimp shells.

It was evident from Fig. 3.6 that as the induction concentration of phosphoric acid treated chitin A (P-A) decreased, the quantity of Chit1 enzymes increased, thus producing a higher activity in comparison to colloidal chitin (CC), that showed no activity. The Chit1 enzymes induced by P-A showed activity in assays that used P-A or CC as the substrate. However, for

the positive control, whereby YPG media was used with no (substrate) inducer, the highest quantity of Chit1 was produced. YPG produced Chit1 exhibited the highest reducing sugar concentrations in the activity assays, when P-A was used as the substrate. This indicated that Chit1 does not require induction and can be cultured in YPG media, Chit1 activity assays could be performed on P-A and CC substrates.

Hydrolysates from activity assays performed using Chit1 fractions from the induction study were then analysed for CHOS production using TLC (Fig. 3.7). This was used to indicate what products were formed and to validate the results obtained in Fig. 3.6.

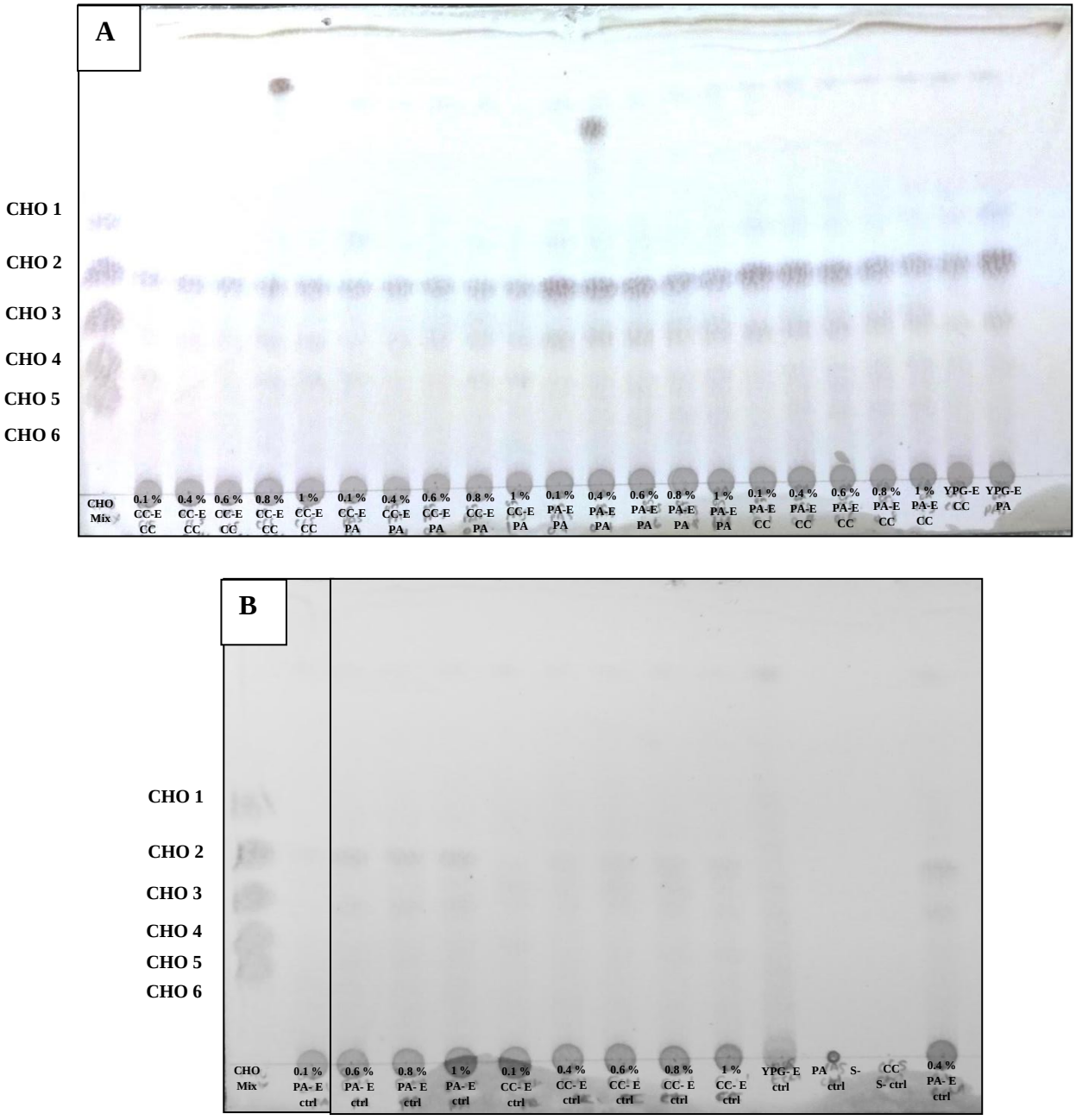

Figure 3.7 Thin layer chromatography of an induction study of Chit1 10 kDa fractions from *Pichia pastoris* grown in media with different concentrations (0.1- 1%; w/v) of colloidal chitin and phosphoric acid treated chitin A from shrimp shells. (A)Activity assays conducted on 1% colloidal chitin or phosphoric acid pre-treated chitin from shrimp shells and (B) enzyme controls for the activity assays. CHO Mix; chitooligomer mix; CHO1-CHO6: N-acetylglucosamine- hexaacetyl-chitohexose; CC- colloidal chitin; P-A: phosphoric acid pre-treated chitin from shrimp shells; CC-E: enzyme produced in media containing colloidal chitin; PA-E: enzyme produced in media containing phosphoric acid treated chitin A from shrimp shells; YPG-E: enzyme produced in YPG media with no substrate added; E-ctrl- enzyme control and S-ctrl: substrate control.

Figure 3.7 demonstrates that Chit1, produced in YPG media or in YPG media with the chitin substrates as inducers, has the ability to hydrolyse chitin into various sizes of chitooligomers.

Figure 3.7A shows that the bands observed in the last two lanes, whereby Chit1 from YPG media on colloidal chitin and phosphoric acid treated chitin produce the darkest bands, which validated the data shown in Fig. 3.5, wherein the highest activity was observed. Dark, prominent bands were also observed in lanes where 0.1% (w/v) phosphoric acid treated chitin was used as an inducer for Chit1 production, with the Chit1 enzymes obtained showing high activity on colloidal chitin and phosphoric acid treated chitin, correlating with Fig. 3.5. The controls (Fig. 3.7B) also showed lighter bands than the reaction bands, as expected.

3.4.6. Culturing of *Oidiodendron maius* (CafRu082b)

Another source of chitin degrading enzymes was investigated -the ericoid mycorrhizal fungus *Oidiodendron maius*. Modified Melin Norkrans media (MMN) (Fig. 3.8) was used to culture this mycorrhizal fungus, with chitin from shrimp shells used as a carbon source to induce the secretion of chitin degrading enzymes.

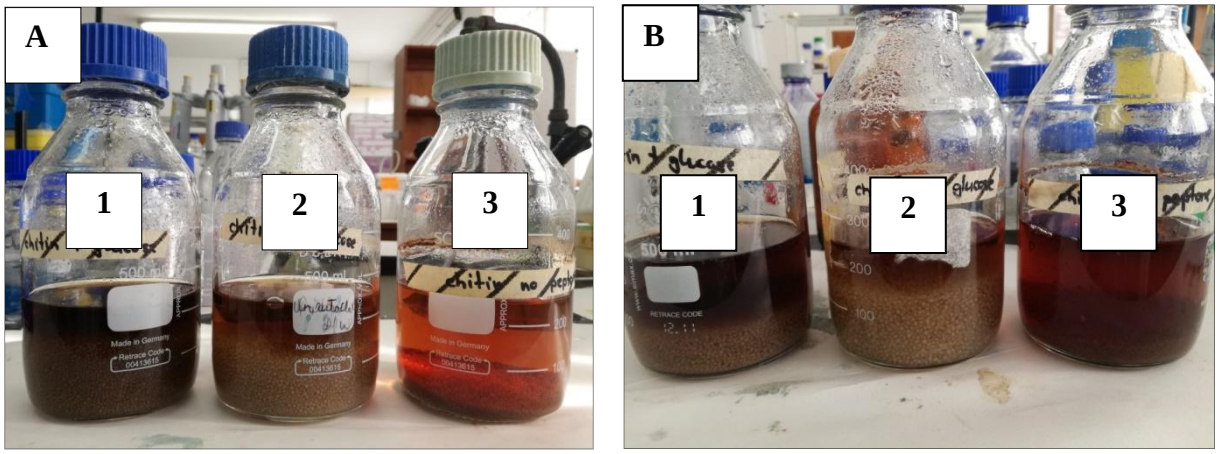

Figure 3.8. *Oidiodendron maius* cultured in Modified Melin Norkrans media with chitin from shrimp shells for a period of: (A) 2 weeks and (B) 3 weeks. 1: MMN with chitin; 2: MMN with no glucose and 3: MMN with no peptone.

Figure 3.8 showed that all isolates in (A) and (B) were circular and of a hazel wood colour. However, more growth was observed after 3 weeks, with larger isolates (B). It was seen that after 2 weeks (A) there was a change in the colour of the media with the different conditions, the media was: (1) dark brown/black, (2) light brown and (3) orange. Similar observations were made of the media in (B). However, bottle (3) showed a change in colour and became dark brown, after 3 weeks.

3.4.7. Quantification of reducing sugars released by intra- and extracellular fractions of *O. maius* on phosphoric acid treated chitin A

The intra- and extracellular enzyme fractions of *O. maius* grown under different conditions were tested on phosphoric acid treated chitin A for chitin degrading activity (Figure 3.9 below). Phosphoric acid treated chitin A was used as the main substrate in the activity assays as this substrate was seen to produce the highest activity in comparison to the other substrates that were previously tested using the *P. pastoris* Chit1 enzyme.

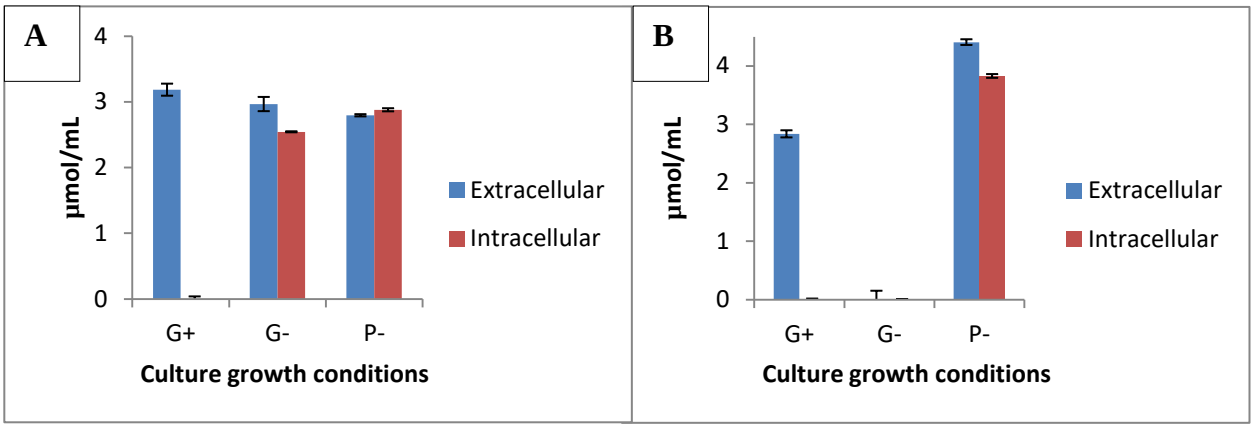

Figure 3.9. Activity assays of the 10 kDa intra- and extracellular fractions from the different ericoid mycorrhizal growth media on phosphoric acid pre-treated chitin from shrimp shells, after a growth period of (A) 2 weeks and (B) 3 weeks. Values are represented as means ± SD, n=2. G+: glucose present in media; G-: glucose absent in media; P-: peptone absent in media

It was observed, that after two weeks (Fig. 3.9A), both the intra- and extracellular fractions without glucose, showed reducing sugars with no significant difference between these fractions in terms of enzyme production. In contrast, the absence of glucose in intra- and extracellular fractions in Fig, 3.9B, showed no activity after 3 weeks. Interestingly, the intra- and extracellular fractions with the absence of peptone, showed a high reducing sugar content (Figs. 3.9A and B), illustrating high enzyme production. No activity was elucidated in the intracellular glucose fraction after 2 and 3 weeks. However, the glucose extracellular fractions after 2 and 3 weeks demonstrated the presence of enzyme activity.

To confirm that *O. maius* had the ability to produce chitin degrading enzymes that hydrolyse chitin containing substrates into useful chitooligomers, TLC of activity assay fractions was conducted (see Fig. 3.10).

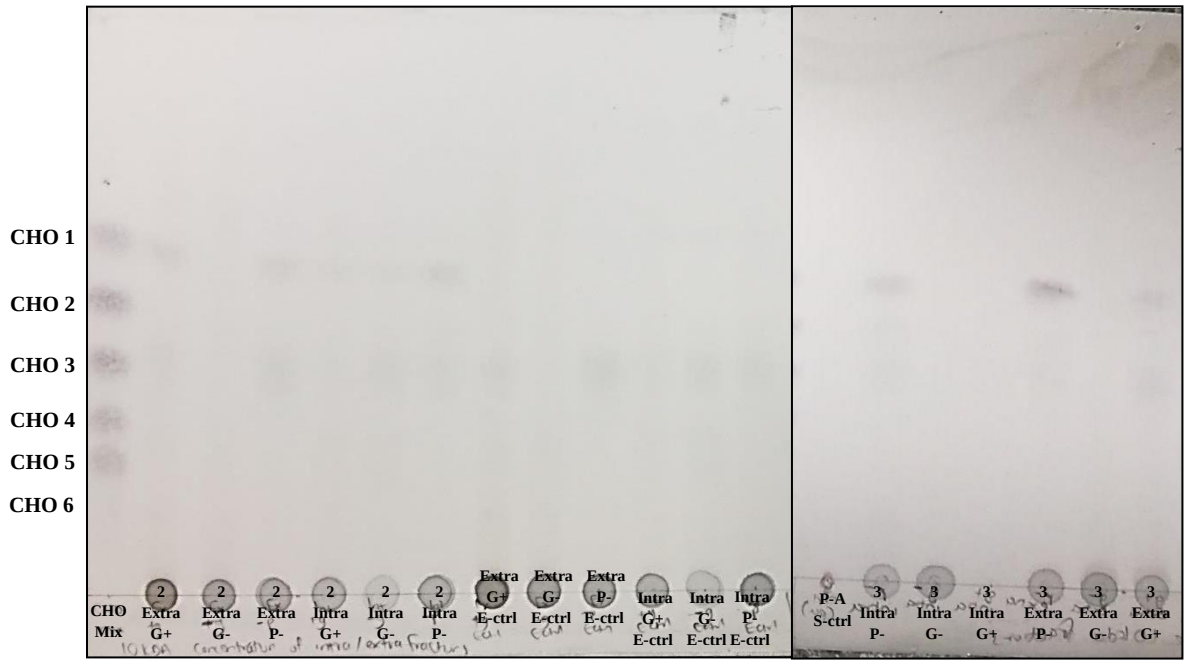

Figure 3.10. Thin layer chromatography of 10 kDa intra- and extracellular fractions, from the different ericoid mycorrhizal growth media on phosphoric acid treated chitin A (after a growth period of 2 and 3 weeks). CHO Mix; chitooligomer mix; CHO1-CHO6: N-acetylglucosamine- hexaacetyl-chitohexose; G+: glucose present in media; G-: glucose absent in media; P-: peptone absent in media; Intra: intracellular fraction; Extra: extracellular fraction; S-ctrl: substrate control; P-A: phosphoric acid treated chitin A, 2: 2 weeks and 3: 3 weeks.

Figure 3.10, illustrated that it was evident that *O. maius* had the ability to produce chitin degrading enzymes that exhibited chitin degrading properties. All the intra- and extracellular activity assay fractions from 2 and 3 weeks both illustrated that the most prominent hydrolysis products had a DP length of 1 to 3. All enzyme controls showed a single light band at a DP length of 3.

3.5. Discussion

3.5.1. Culturing of *Pichia pastoris*

Pichia pastoris was used as a protein production host for recombinant *chit1* (Zhang *et al*., 2015). This expression host is preferred due to its high growth density and tight regulation by promoters, which renders this system successful for the expression of various proteins on an industrial scale (Ahmad *et al*., 2014). This expression system can produce recombinant proteins and can also act as a host for membrane bound proteins, peptides or bioactive compounds (Hirz *et al*., 2013 and Zhang *et al*., 2015). However, a review by Ahmad and colleagues (2014) suggested that, depending on the protein of interest, there is scope to

optimise expression and secretion of the desired protein. *P. pastoris* transformants were cultured in YPG media (Fig 3.1B), with conditions favourable for high Chit1 secretion being previously optimised by Zhang and colleagues (2015). The opaque colour of the media in Fig 3.1B showed that there was growth of the culture and therefore it could be assumed that this growth was accompanied by the secretion of Chit1.

3.5.2. Simultaneous saccharification of chitin substrates by Chit1 and identification of produced CHOS

According to Vyas and Deshpande (1989), chitinases can be produced constitutively; however, the addition of chitinous substrates to the culture media has the ability to enhance the production of these enzymes. Therefore, for the optimal production of Chit1 from *P. pastoris* and optimum hydrolysis of chitin substrates, simultaneous saccharification was investigated. According to studies in literature, this method is current and has been used for the degradation of biomass in the presence of the fungus with a preferred substrate, resulting in the simultaneous secretion of enzymes and effective saccharification of the substrate by the extracellularly secreted enzymes (Shirkavand *et al*., 2016; Sindhu *et al*., 2016). Zhang and colleagues (2015) suggested that Chit1 was secreted extracellularly from *P. pastoris*, this method was therefore employed in our study. Results from Fig. 3.2 (A, B and C) indicated that glycerol and yeast (controls 1 and 2) are important for promoting fungal growth, which directly affects the secretion of enzyme due to the yeast containing some nutrients that act as a source of carbon. This observation is supported by studies conducted on polyvinyl alcohol degrading enzymes (PVAases) that described using a yeast extract as an energy source, which stimulated cell growth, resulting in the increased production of PVAase enzymes (Li *et* al., 2011; Matsumura *et al*., 1999; Yamatsu *et al*., 2006).

This study (Fig. 3.2) demonstrated that Chit1 enzymes are secreted into media containing the substrate of interest, which can be hydrolysed more effectively. The addition of substrates to the culture media does not necessarily mean that the enzyme secretion will be higher than in media with no additional substrate. This study indicated that the enzyme's ability to hydrolyse substrate immediately after being secreted from an organism is effective and thus deemed simultaneous biomass hydrolysis a success.

For the identification of the sugars produced in all of the simultaneous saccharification studies, TLC was conducted (Fig. 3.3). The different bands produced (with a range of DP

lengths), confirmed that Chit1 is a true endo-chitinase which has the ability to produce a range of CHOS (Zhang *et al*., 2015).

3.5.3. Determination of intra- and extracellular activity of Chit1 from *P. pastoris*

According to Ahmad *et* al. (2014), *P. pastoris* has the ability to produce recombinant proteins intra- and extracellularly, with some proteins being hetero-oligomers or membrane attached. Figure 3.4 confirmed and validated findings by Zhang *et al.* (2015) which stated that *P. pastoris* secreted Chit1 extracellularly. However, constitutively produced Chit1 and Chit1 production (in the presence of phosphoric acid treated chitin A) displayed the highest activities, when phosphoric acid treated chitin A was used as the substrate in activity assays. This illustrated that for the generation of higher sugar concentrations, phosphoric acid treated chitin A should be used in future.

TLC (Fig. 3.5) validated the data shown in Fig. 3.4, and showed that extracellularly produced Chit1 has the ability to hydrolyse chitin substrates into useful chitooligomers with a DP length of 1-6, which was also found by Zhang *et al*. (2015).

3.5.4. Induction study of Chit1 and identification of CHOS

According to Vyas and Deshpande (1989), for the optimal production of chitinase enzymes in the fungus *Myrothecium verrucaria*, chitin degrading activity was the highest when acid swollen chitin substrates were hydrolysed by extracellular secreted chitinases. Therefore, once it was established that Chit1 was extracellularly secreted by *P. pastoris*, an induction study was performed using different concentrations of the two acid treated chitin substrates (colloidal chitin and phosphoric acid treated chitin A) in YPG media to culture *P. pastoris.* The positive control (YPG media) produced the highest Chit1 activity on P-A treated chitin in activity assays (3.425 umol/mL) (Fig. 3.6). This illustrated that Chit1 does not require induction and can be cultured in YPG media. Chit1 activity assays could be performed on P-A and CC substrates for the production of CHOS. This also demonstrated the constitutive expression of high concentrations of Chit1 using YPG media. This therefore eliminates the use of substrates as inducers or other hazardous inducers such a methanol, which is undesirable on an industrial scale (Ahmad *et al*., 2014).

TLC data obtained (Fig. 3.7) showed that a range of CHOS were produced with a DP length of 1 to 6, along with other products below a DP of 1 (A). This indicated that Chit1 has the

ability to produce a range of CHOS from the two chitin substrates, with activity being the highest when grown in YPG media, which is economical on an industrial scale.

3.5.5. Culturing of *O. maius* (CafRu082b)

Endophytic fungi are often found in extreme conditions and are relatively unexplored as sources for obtaining novel enzymes. The fungus of interest was *Oidiodendron maius*, an ericoid mycorrhizal fungus that shares a symbiotic relationship with the roots of ericaceous plants (Rice and Currah, 2006). This fungus plays an important role in the protection of plants against high concentrations of metals and assists with the supply of nutrients to plants, suggesting that this type of fungus can be harvested for a range of enzymes (Daghino *et al*, 2016). Differences in the colour of the growth media (Fig. 3.8A and B) were observed under all the different conditions (1-3). This indicated that the growth period and the different media conditions had an effect on the growth of *O. maius*, which may have affected the production of chitin degrading enzymes. This could explain the colour change in the media, if the chitin degrading enzymes were secreted extracellularly.

3.5.6. Quantification of reducing sugars in the intra- and extracellular fractions of *O. maius* and identification of CHOS produced

Samples without peptone (3.9A and B) showed the highest activities in contrast to the media without glucose, showing no activity (Fig. 3.9B). This data suggests that glucose is an important carbon source in fungi and is thus crucial for the production of chitin degrading enzymes and that peptone as a nitrogen source is not important for enzyme production. According to studies in literature, the production of chitinase is influenced by the components of the growth medium, such as the carbon and nitrogen sources (Bhushan, 1998 and Dahiya *et* al, 2005). Bhushan (1998) established that the addition of glucose to media enhances the production of chitinases, which agrees with the data obtained in our study. TLC analysis (Fig. 3.10) confirmed that the enzymes exhibited chitin-degrading properties and were successful in producing CHOS with a DP of 1 and 3.

3.5.7. Conclusion

Commercial chitinolytic enzymes are expensive, hence there is a need to search for new sources of chitin-degrading enzymes. Heterologous expression of the recombinant protein in appropriate host systems can help reduce the cost of the chitin-degrading enzyme. In this chapter, the *chit1* gene was expressed in the *P. pastoris* system. *Pichia pastoris* can produce

high quantities of Chit1 that can be used on industrial scale to degrade chitinous waste and produce a range of CHOS from various chitin containing substrates. Another avenue to investigate are ericoid mycorrhizas, these are fungi that can produce an array of enzymes intra- and extracellularly. *O. maius* was investigated and it was found that mycorrhiza have the ability to produce chitinases or chitin degrading enzymes that hydrolyse chitin and produce CHOS. However, the chitin degrading enzymes produced by this organism requires further investigation (such as purification, zymograms and SDS-PAGE) to identify the enzyme(s) of interest. In conclusion, the two sources of chitin degrading enzymes that were investigated proved to be successful for supplying copious amounts of chitinolytic activity. Conditions for optimal chitinolytic activity for the production of value added products is an important factor to consider. Therefore, the characterisation of these enzymes via assay optimisation is an essential component and is discussed in the next chapter, Chapter 4.

Chapter 4: Characterisation of chitinolytic enzymes

4.1. Introduction

Chitin is a polysaccharide that is found abundantly in nature; existing in a range of eukaryotic and some prokaryotic organisms (Patil *et al*., 2000). To decrease the accumulation of chitinous waste in the environment, chitinolytic enzymes produced by various organisms are required for the hydrolysis of the β-(1,4)-linked *N*-acetylglucosamine (GlcNAc) residue composed polymer (Aly *et al.* 1997). The hydrolysis of the chitinous waste is generally conducted by a concerted action of enzymes working simultaneously or in a consecutive manner to produce useful chitooligomers (CHOS) (Deshpande, 1986; Shaik and Deshpande, 1993). CHOS are used in a number of applications; ranging from the health and food sector, to the bio-refinery industry (Patil *et al*., 2000). A review conducted by Rathore and Gupta (2015) revealed that there are challenges associated with the economical use of chitinases due to their low hydrolytic activities on chitnious waste and the high costs of these enzymes. Therefore, the focus area of many studies is to improve the yield of chitinase production or chitinase catalytic activity (Rathore and Gupta, 2015). This is directed by investigating biochemical and physico-chemical characteristics which influence activity. This consequently provides a platform to understanding the conditions that are required for optimal chitinolytic activity for the high production of CHOS from chitinous biomass.

Chitinases are glycosyl hydrolases belonging to glycosyl hydrolase families 18, 19 and 20 (Hamid *et al*., 2013). Two categories of chitinases exist; the endo-chitinases (EC 3.2.1.14) which initiate hydrolysis by binding to the substrate and then randomly cleaving the internal glycosidic bonds of substrates in a non-processive manner to produce lower molecular mass oligomers of glucosamine residues (Rathore and Gupta, 2015; www.cazy.com). The second category known as exochitinases are sub-catergorised into two groups. The first group consists of chitobiosidases (EC 3.2.1.29), which cleave in a processive manner from the non-reducing end of the polymer to produce diacetylchitobiose, and the second group of exochitinases are β-(1,4) N-acetyl glucosaminidases (EC 3.2.1.30), which cleave the products of endochitinases and chitobiosidases into monomeric products (GlcNAc) (Rathore and Gupta, 2015; Sahai and Manocha 1993).

In this study, chitinases from the following sources: *Streptomyces griseus*, *Thermomyces lanuginosus* (*Pichia pastoris* expressed) and *Bacillus cereus* (E.C. 3.2.1.14) were

investigated. Enzymes in general are predisposed to various factors, such as temperature and pH (Turon *et al.*, 2008; El-Hefnawy *et al.*, 2014). The majority of enzymes exhibit optimal hydrolytic activities at a specific temperature or pH (Jahangeer *et al.*, 2005). It is therefore crucial to determine these two conditions for the enzyme to function optimally (Jahangeer *et al.*, 2005). The thermal stability is an important factor to consider, especially for enzyme applications in industry, as the optimum temperature of the enzyme might not necessarily be the best for the sytem in which the enzyme is employed (Daniel *et al.*, 2008; Thomas and Scopes, 1998). These factors are important when designing experiments and for the application of enzymes in industry. Hence, these chitinases from different sources were characterised to determine which biochemical conditions and type of biomass they can function optimally on, in order to produce useful value-added products such as CHOS.

4.2. Aims and objectives

4.2.1 Aim

To conduct comparative physico-chemical characterisation of chitinases from different sources and identification of their hydrolysis products.

4.2.2 Objectives

- To determine substrate specificities of the chitinolytic enzymes;
- To determine temperature optima of the chitinolytic enzymes;
- To determine temperature stability of the chitinolytic enzymes;
- To determine pH optima of the chitinolytic enzymes;
- To identify the CHOS produced by the chitinolytic enzymes;
- To determine the chain specificity for enzyme cleaving by the chitinolytic enzymes.

4.3. Methods and Materials

4.3.1. Materials

Chitinase from *Streptomyces griseus* (chitodextrinase/poly(1,4-β-2-acetamido-2-deoxy-D-glucoside) glycanohydrolase, GH19) , chitin from shrimp shell powder and chitosan from

shrimp shell powder were purchased from Sigma Aldrich. Chitinase from *Bacillus cereus* (1,4-β-chitinase, GH18) was purchased from Nzytech. *Thermomyces lanuginosus* endochitinase transformants expressed in *Pichia pastoris* were supplied by Professor K. Permaul at the Durban University of Technology (Department of Biotechnology and Food Technology).

4.3.2. Substrate specificity study

Substrate specificity assays were conducted using 1.33% (w/v) un-treated and pre-treated chitin substrates (chitin from shrimp shell powder, commercial chitosan from shrimp shell powder, colloidal chitin, NaOH treated chitin and phosphoric acid treated chitin A) (Chapter 2, section 2.3.1). Reactions using 0.1 mg/mL chitinases from *S. griseus* (CHS) and *B. subtilis* (CHB) were prepared using the same parameters described in Chapter 3, section 3.3.6. Chitinase from *P. pastoris* (Chit1) was produced (Chapter 3, section 3.3.1) and the 10 kDa concentrated crude fraction (0.3 mg/mL) was used (Chapter 3, section 3.3.5 and 3.3.6), whereby the total reaction volumes of 90 µL were comprised of 60 µL enzyme (0.3mg/mL) and 30 µL of the various un- and pre-treated substrates. The DNS assay was employed to determine reducing sugars (Chapter 3, section 3.3.2) and N-acetylgucosamine was used as a suitable sugar standard (Appendix 2B).

4.3.3. Activity of chitinolytic enzymes on phosphoric acid treated chitin B (PAS)

Activity assays using CHB and CHS (0.1 mg/mL) were conducted as described in section 4.3.2 and 10 kDa fractions of Chit1 (0.3 mg/mL) (Chapter 3, section 3.3.6) were conducted on partially solubilised phosphoric acid treated chitin B (PAS) (Chapter 2, section 2.3.1.2). The reducing sugar content was determined by the DNS assay (Chapter 3, section 3.3.2).

4.3.4. Temperature optima determination

All chitinolytic enzyme assays were conducted on 1.33% (w/v) PAS, with reactions prepared as described in section 4.3.2, with the exception of the Chit110 kDa filtered crude being 0.54 mg/mL. A range of temperatures (30-90^{o}C) were used to determine the temperature optima of the chitinolytic enzymes (Labnet AccuBlock digital dry bath) at pH 5.0 using a citrate-phosphate buffer (0.2 M dibasic sodium phosphate and 0.1 M citric acid monohydrate). The activities of Chit1, CHB, and CHS were measured with the DNS protocol described in Chapter 3, section 3.3.2, with chit1 being added in a 2:1 ratio to the substrate.

4.3.5. Temperature stability determination

The thermal stability profiles of the chitiinolytic enzymes were determined by incubating the enzymes at 37 and 50^{o}C over time (conducted in triplicate). At designated time intervals, enzyme samples were drawn and used to conduct activity assays as described in section 4.3.3. The subsequent release of reducing sugars was measured using the DNS method as described in Chapter 3, section 3.3.2.

4.3.6. pH optima determination

To determine the pH optima of the chitinolytic enzymes, a range of pH values (3.5-8) were investigated. The chitinolytic enzymes were each incubated with PAS at the respective pH values, in a citrate-phosphate buffer (0.2 M dibasic sodium phosphate- 0.1 M citric acid monohydrate). The assays were performed in triplicate (section 4.3.3) and the reducing sugar content was measured by the DNS method using the protocol described in Chapter 3, section 3.3.2.

4.3.7. Identification of CHOS produced by the hydrolysis of substrates by chitinolytic enzymes

Hydrolysates from activity assays which were conducted in section 4.3.2 were used to perform TLC (as described in Chapter 3, section 3.3.4), to identify the CHOS produced by the chitinolytic enzymes on the various chitinous substrates.

4.3.8 Chain specificity study with chitinolytic enzymes

To determine whether the enzymes were processive or non-processive, chain cleaving pattern studies were conducted with the chitinolytic enzymes. Activity assays were conducted using 0.1% (w/v) N-acetylglucosamine to hexaacetyl-chitohexose oligosaccharides dissolved in citrate-phosphate buffer (pH 5, 0.2 M dibasic sodium phosphate- 0.1 M citric acid monohydrate) at 37^{o}C for 1 hour. The total reaction volume was 100 µL, comprised of 25 µL enzyme and 75 µL acetyl-chitooligosaccharide. Samples were centrifuged at 13000 rpm for 5 minutes and hydrolysates were used to conduct TLC as described in Chapter 3, section 3.3.4.

4.4. Results

4.4.1. Substrate specificity study of chitinolytic enzymes

The chitinolytic enzymes were subjected to various un- and pre-treated chitin substrates to determine which substrate would produce the highest quantity of reducing sugars for the

different enzymes and which substrate would be the most appropriate to use to physico-chemically characterise the chitinolytic enzymes . Figure 4.1 below demonstrates the specific activities of the various chitinolytic enzymes on various chitin substrates.

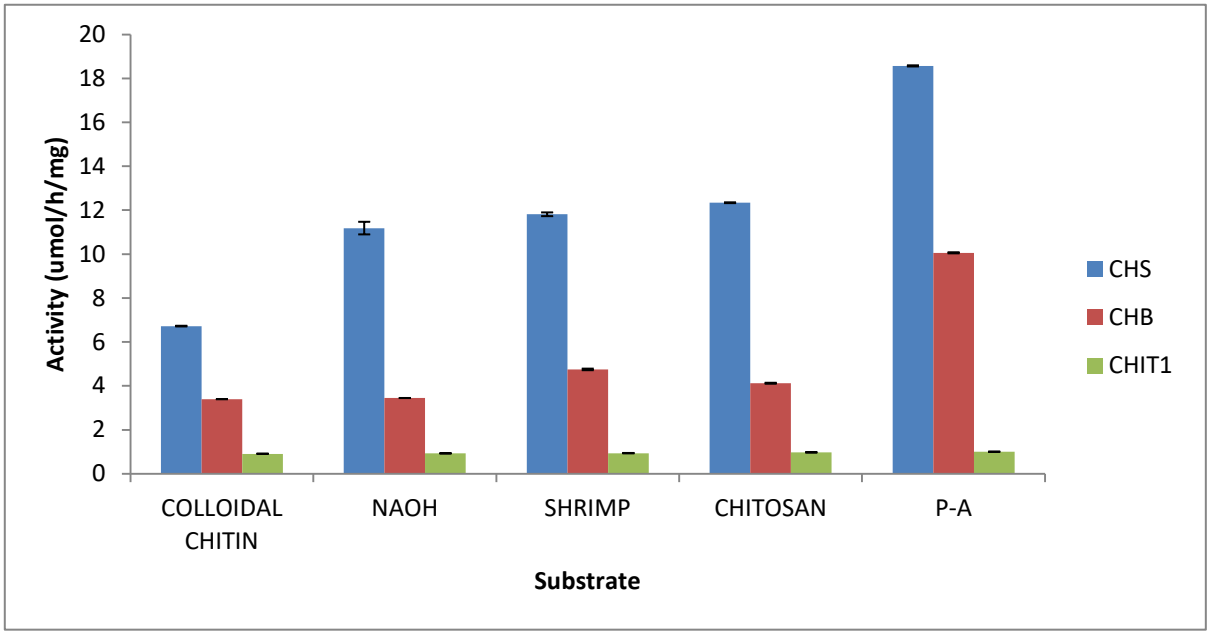

Figure 4.1. Activity assays of chitinolytic enzymes on un- and pre-treated chitin containing substrates. Values are represented as means ± SD, n=3. CHS: chitinase from *S. griseus*; CHB: chitinase from *B. cereus*; CHIT1: chitinase from *P. pastoris*, colloidal chitin: colloidal chitin; NAOH: NaOH treated chitin from shrimp; SHRIMP: chitin from shrimp shells; chitosan: commercial chitosan from shrimp shells and P-A: phosphoric acid treated chitin A from shrimp shells.

Figure 4.1 illustrates that CHS and CHB exhibited the highest activities on phosphoric acid treated chitin A, 18.56 and 10.06 µmol/h/mg, respectively. The un-treated commercial chitin from shrimp and chitosan both produced a higher quantity of reducing sugars (than colloidal chitin and NaOH treated chitin) upon hydrolysis by both commercial enzymes. It was shown that the Chit1crude fraction produced very low amounts of reducing sugars throughout.

Since the chitinolytic enzymes displayed the greatest activity on the de-acetylated phosphoric acid treated biomass (phosphoric acid treated chitin A), the enzymes were examined on partially solubilised chitin (phosphoric acid treated chitin B). This was performed to determine whether this substrate will be more appropriate for producing high quantities of reducing sugars in order to allow these enzymes to be easily characterised (see Fig. 4.2 below).

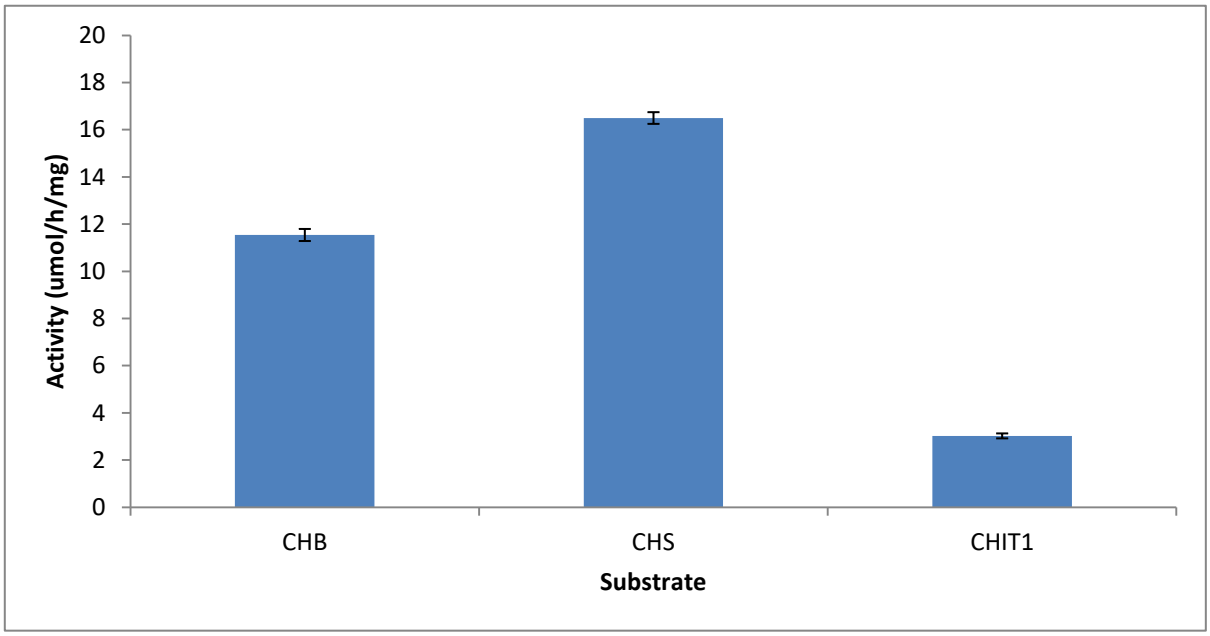

Figure 4.2. Activity assays of chitinolytic enzymes on phosphoric acid treated chitin B from shrimp shells. Values are represented as means ± SD, n=3. CHS: chitinase from *S. griseus*; CHB: chitinase from *B. cereus*; CHIT1: chitinase from *P. pastoris* and P-A: phosphoric acid treated chitin B from shrimp shells.

Figure 4.2 illustrated that the phosphoric acid treated chitin B substrate resulted in an increase of activity for Chit1 which was 3.025 µmol/h/mg. The activity for CHB and CHS was 11.54 and 16.498 µmol/h/mg, respectively - which was similar to the activity on phosphoric acid treated chitin A.

4.4.2. Temperature optima and stability determination

The temperature optimum and stability for each of the chitinolytic enzymes was determined on 1% (w/v) phosphoric acid treated chitin B. Figure 4.3 below illustrates the thermal-properties of CHB.

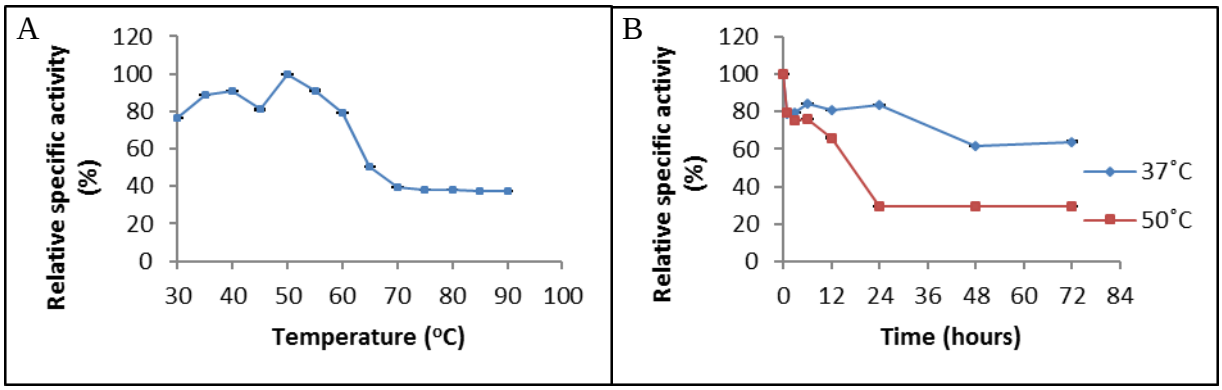

Figure 4.3. Temperature profile of CHB on ≥ 85% phosphoric acid treated chitin B from shrimp shells. (A) temperature optimum and (B) temperature stability at 37 (▲) and 50°C (●) over a 72 hour period. Values are represented as means ± SD, n=3.

Based on Fig. 4.3A, the temperature optimum for CHB was 50°C with a relative specific activity of 52.858 µmol/h/mg. The residual activity was ≥ 70% between 30 to 60°C. CHB (see Fig. 4.3B) was the most stable at 37°C, with a residual activity ≥ 60% after 72 hours, in contrast to the rapid decline in residual activity at 50°C after 12 hours.

Figure 4.4 below illustrates the thermal-properties of CHS, which was determined on 1 % (w/v) phosphoric acid treated chitin B.

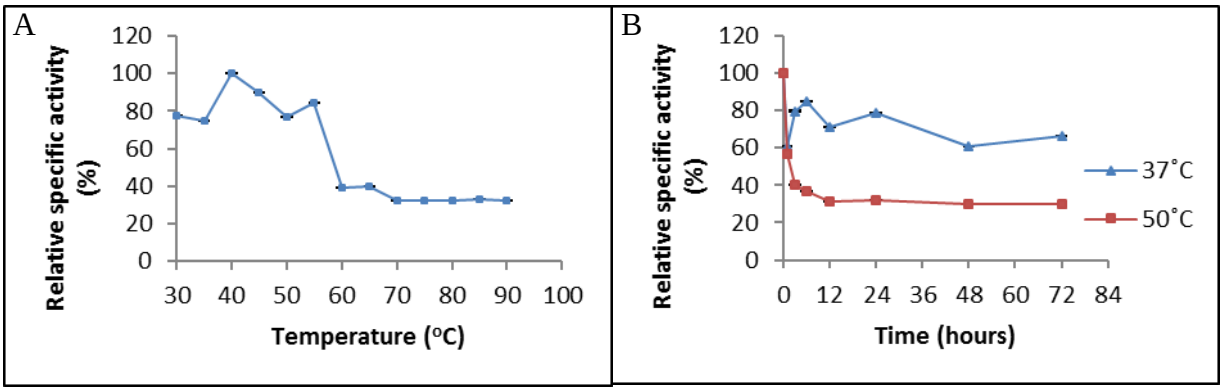

Figure 4.4. Temperature profile of CHS on ≥ 85% phosphoric acid treated chitin B from shrimp shells of; (A) temperature optimum and (B) temperature stability at 37 (▲) and 50°C (●) over a 72 hour period. Values are represented as means ± SD, n=3.

Figure 4.4A illustrates that CHS displayed optimum activity at 40°C (60.366 µmol/h/mg) and a residual activity of ≥ 70% between 30 to 60°C, with activity declining at higher temperatures. Temperature stability studies (Fig. 4.4B) revealed that the enzyme was most stable at 37°C and maintained a residual activity above 60% up to 72 hours, whereas at 50°C, residual activity rapidly declined after 1 hour.

Figure 4.5 below illustrates the thermal-properties of Chit1, which was determined on 1 % (w/v) phosphoric acid treated chitin B.

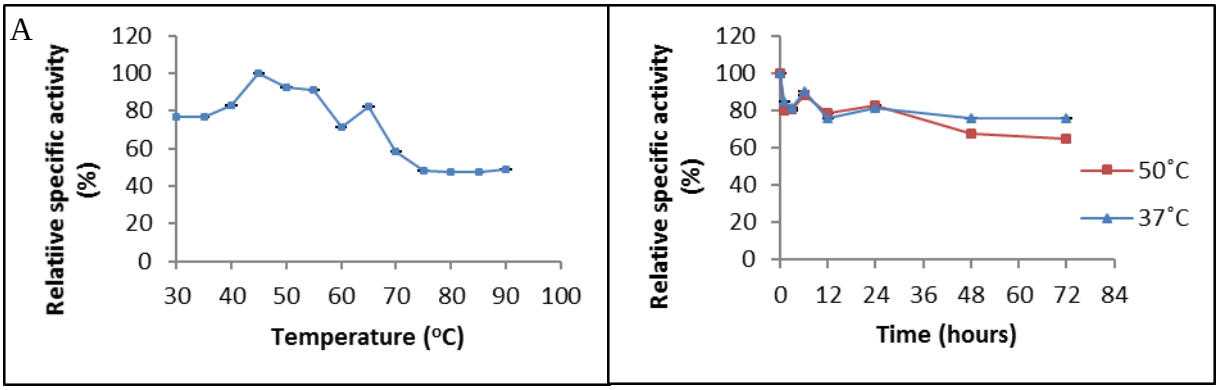

Figure 4.5. Temperature profile of Chit1 on ≥ 85% phosphoric acid treated chitin B from shrimp shells of; (A) temperature optimum and (B) temperature stability at 37 (▲) and 50°C (●) over a 72 hour period. Values are represented as means ± SD, n=3.

The Chit1 enzyme concentrated fraction exhibited an optimum activity of 3.992 µmol/h/mg at 45°C and demonstrated a residual activity of ≥ 47% from 30 to 90°C (Fig. 4.5A). Figure 4.5B indicates that Chit1 was stable at 37 and 50°C and maintained a residual activity above 64% throughout the 72 hour period (at both temperatures).

4.4.3. Determination of pH optima

The pH optima of the chitinolytic enzymes were determined by using a citrate-phosphate (0.2 M dibasic sodium phosphate- 0.1 M citric acid monohydrate) buffers in a pH range of 3.5 to 8 shown in Fig. 4.6. The enzyme reactions were conducted on 1% (w/v) phosphoric acid treated chitin B, at 37°C and the reducing sugar content was measured with the standard DNS protocol (Chapter 3, section 3.3.2).

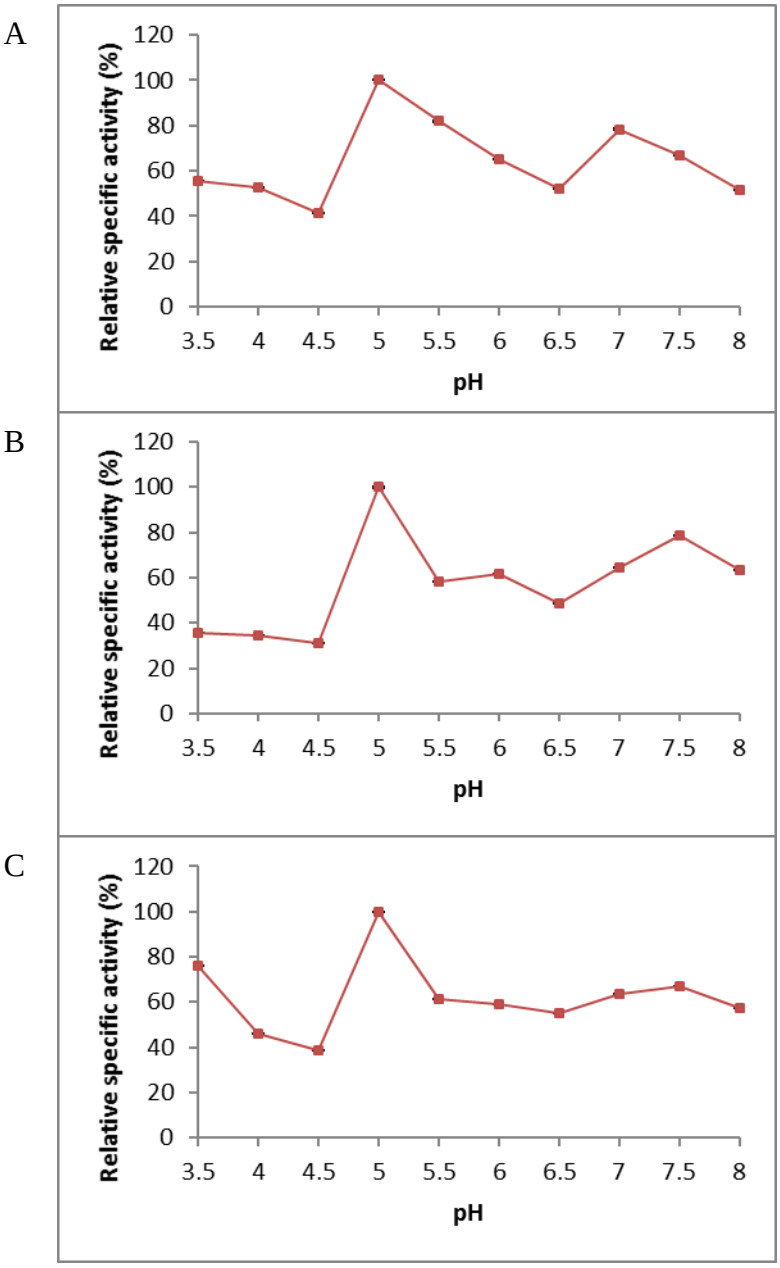

Figure 4.6. The pH optima profiles of the chitinolytic enzymes; (A) CHB, (B) CHS and (C) Chit1 on ≥ 85% phosphoric acid treated chitin B from shrimp shells, from pH 3.5 to 8.0 using a citrate-phosphate buffer (phosphate (0.2 M dibasic sodium phosphate- 0.1 citric acid monohydrate). Values are represented as means ± SD, n=3.

Figure 4.6. demonstrates that the pH optima for CHB, CHS and Chit1 was at pH 5.0 with specific activities of 33.459, 46.2 and 5.776 µmol/h/mg, respectively. All chitinolytic enzymes maintained a residual activity above 45% from pH 5 to 8.

4.4.4. Identification of CHOS produced from the hydrolysis of various chitinous substrates by chitinolytic enzymes

The chitooligomeric products produced from the hydrolysis of the chitinous substrates in section 4.1, were identified by TLC - see Figure 4.7.

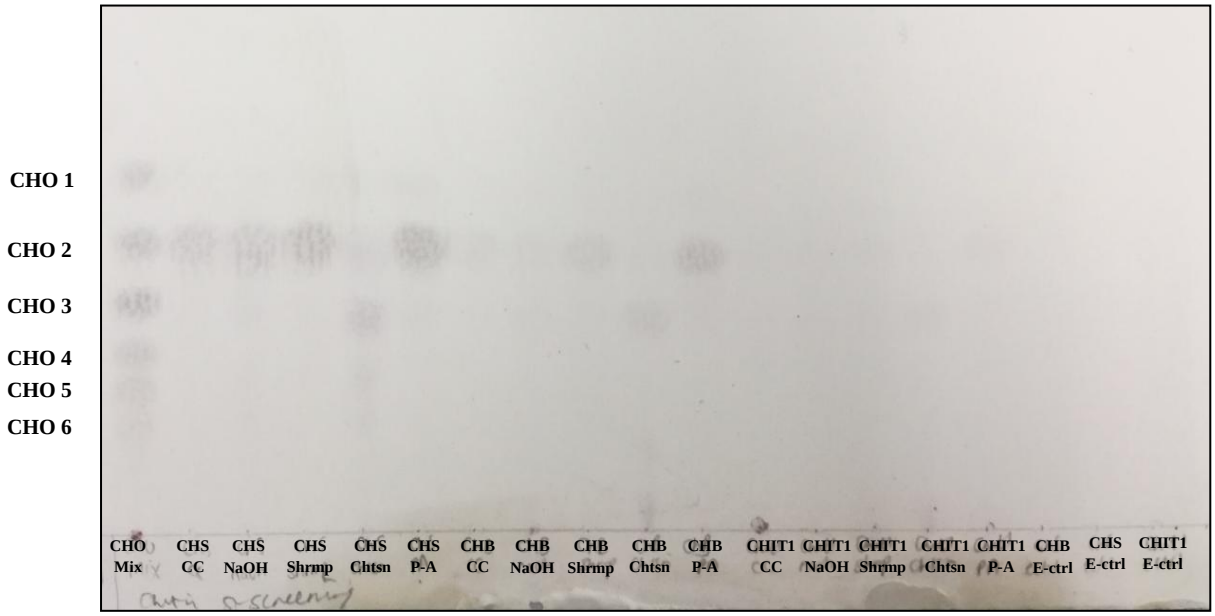

Figure 4.7. Thin layer chromatography of activity assays of chitinases on un- and pre-treated chitin containing substrates. CHO Mix: chitooligomer mix; CHO1-CHO6: N-acetylglucosamine- hexaacetyl-chitohexose; CC: colloidal chitin; NaOH: NaOH pre-treated chitin from shrimp shells; shrmp: chitin from shrimp shells; chtsn: commercial chitosan; P-A: phosphoric acid treated chitin A from shrimp shells; E-ctrl: enzyme control; CHS: chitinase from *S. griseus*; CHB: chitinase from *B. cereus*; CHIT1: chitinase from *P. pastoris.*

The above figure (Fig. 4.7) illustrates that CHB and CHS have the ability to hydrolyse the un- and pre-treated substrates into CHOS, with the prominence of CHOS with a DP length of 1 and 2. However, the hydrolysis of the commercial chitosan by CHB and CHS consequently produced a range of CHOS (DP1-6). Chit1 produced low quantities of CHOS with a DP range of 2 to 4.

4.4.5. Determination of chain cleaving pattern by commercial chitinolytic enzymes

TLC was conducted with the pure commercial chitinolytic enzymes on 10 mg/ml of each of the chitooligomeric standards (N-acetylglucosamine to hexaacetyl-chitohexose) to confirm whether CHS and CHB are endo-cleaving or exo-cleaving enzymes (refer to Fig. 4.8).

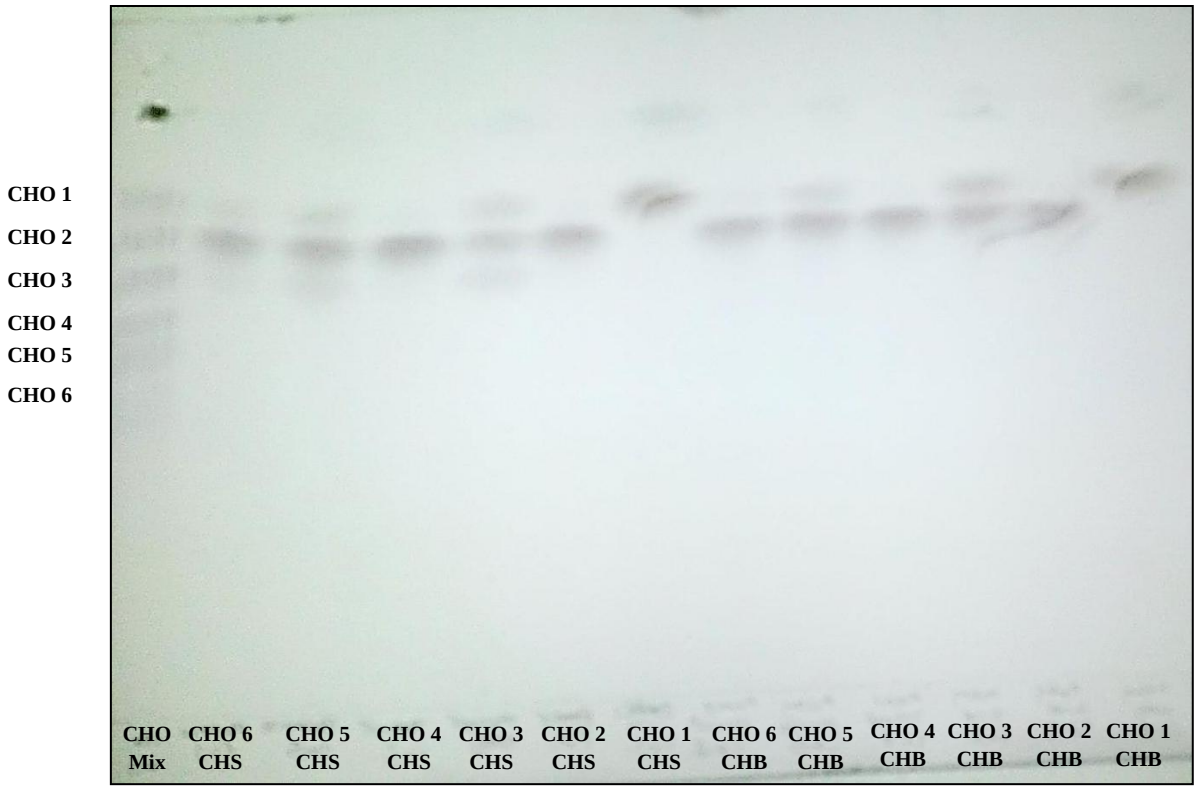

Figure 4.8. Thin layer chromatography of CHB and CHS action on chitooligomeric standards, with a degree of polymerisation of 1 to 6. CHO Mix: chitooligomer mix; CHO1-CHO6: N-acetylglucosamine- hexaacetyl-chitohexose; CHS: chitinase from *S. griseus* and CHB: chitinase from *B. cereus*.

Based on Fig. 4.8, it is evident that CHB and CHS predominantly produce CHOS with a DP of 2, with smaller quantities of CHOS with a DP of 1 and 3 and a product smaller than CHO 1.

4.5. Discussion

4.5.1. Hydrolysis of chitinous biomass with chitinolytic enzymes

The chitinolytic enzymes that were utilised in this study were from glycosyl hydrolase families 18 and 19, comprised of chitinolytic enzymes from viruses, bacteria, fungi, animals and some plants (Gooday, 1999). Family 18 chitinases contain a $(\beta/\alpha)_8$ TIM-barrel catalytic domain (Van Aalten *et al*., 2000). Chitinases from GH 18 generally hydrolyse glycosidic bonds through a retention mechanism, whereby a nucleophilic attack occurs at the anomeric carbon (via acid catalysis) (Koshland and Stein, 1954). This mechanism involves one carboxylic acid which acts as a proton donor (in close proximity of the glycosidic oxygen)

and the other carboxylic acid acting as a nucleophile (Koshland and Stein, 1954 and Sinnot, 1990). In contrast, GH 19 chitinases employ the inversion mechanism which involves the inversion of the anomeric configuration from a β- to an α-anomer (Kezuka *et* al., 2006). Chitinase from families 18 and 19 also possess multi-modular systems which have been reported to assist in the correct positioning of the catalytic domain, resulting in an increase in the de-crystallisation of substrates (Yan and Fong, 2015). This consequently increases the enzyme's substrate affinity for effective chitinolytic hydrolysis of crystalline chitin (Yan and Fong, 2015).

This phenomenon described by Yan and Fong (2015) above, contradicts what was observed in our study, based on the results demonstrated in Fig. 4.1, where the highest activity for CHS and CHB (18.565 and 10.056 μmol/h/mg, respectively) was observed on amorphous substrates such as phosphoric acid treated chitin A. Low activities of Chit1 were observed on all the substrates tested. Phosphoric acid treated chitin B (using a higher percentage of phosphoric acid to treat the biomass) was used to determine whether CHB, CHS and Chit1 would produce higher activities when the substrate was in a more amorphous state (Figure 4.2). This observation can be explained by the absence of any carbohydrate-binding molecules (CBM) in CHB and Chit1, therefore decreasing the ability of these enzymes to bind to crystalline substrates and increasing their affinity towards amorphous substrates (Hsieh *et* al., 2010 and Zhang *et al*., 2015). In comparison, CHS displayed the highest activity on all substrates. According to Kezuka *et al*. (2006), CHS contains a chitin-binding domain (CBM 5), whereby the Trp59 and Trp60 residues facilitate binding of the exposed binding-site on the substrates surface, with the Trp60 side-chain rotating the C2 angle to align the enzyme with the binding-site of crystalline chitinous substrates. This possibly explains why CHS has the highest activities on amorphous substrates, but also has the ability to efficiently hydrolyse the crystalline substrates (Fig. 4.1),

In Figure 4.1, it is illustrated that commercial enzymes (CHB and CHS) produced activities that were similar on phosphoric acid treated chitin A; however, Chit1 activity increased to 3.025 μmol/hr/mg on phosphoric acid treated chitin B (Fig. 4.2). All the chitinolytic enzymes exhibited optimum hydrolysis on phosphoric acid treated chitin B, followed by phosphoric acid treated chitin A and commercial chitosan. These results correlate with the crystallinity peaks observed in Chapter 2, Figure 2.3, where the crystallinity peaks for phosphoric acid treated chitin B and A, and chitosan were 4201, 3965 and 3966 A.U, respectively. This

suggests that these enzymes have a preference for substrates with a certain proportion of amorphous and crystalline regions.

4.5.2. Temperature profiles of chitinolytic enzymes

According to Pletschke *et al*. (2016), physico-chemical characterisation of enzymes is crucial in order to establish optimal conditions for the hydrolysis of biomass into value-added products at an industrial scale. In general, model substrates used for the characterisation of chitinolytic enzymes are costly for relatively small quantities and require long waiting periods from companies from which they are purchased. Since all chitinolytic enzymes hydrolysed the phosphoric acid treated chitin more efficiently compared to the other chitinous substrates (Fig. 4.2), this substrate was used as a more suitable substrate with which to characterise these enzymes.

Figure 4.3A illustrates that CHB displays optimum activity at 50^{o}C (52.858 μmol/h/mg), with a residual activity of ≥ 70% between 30 to 60^{o}C. The temperature optima for CHB is in agreement with many other reports on chitinolytic enzymes from various *Bacillus* strains that exhibited a temperature optima of 50^{o}C. Similarly, numerous studies have reported that chitinases from a *Bacillus* strain had an optimum temperature of between 45 and 55^{o}C (Bhushan and Hoondal, 1999; Senol *et al*., 2014 and Yan *et al*., 2011). The thermostability of CHB was investigated to determine whether CHB was stable across a range of temperatures which are applicable to industrial applications. Thermostability was investigated at 37 and 50^{o}C, as standard assays were performed at 37^{o}C and industrial applications are often conducted at 50^{o}C. Thermostability studies (Fig. 4.3B) revealed that CHB was most stable at 37^{o}C and produced optimal activity after 24 hours (56.3 μmol/h/mg).

A study conducted by Xiayun and colleagues (2012) on a chitinase from *Streptomyces roseolus* strain (GH 18) showed that this enzyme had a temperature optimum of 60^{o}C and a thermostability that retained ~90% of relative activity between 30 and 60^{o}C on colloidal chitin. In contrast, a chitinase that was isolated by Kim *et al.* (2003) from *Streptomyces sp.* M-20 demonstrated a low temperature optimum of 30^{o}C. A temperature optima of 40^{o}C (60.366 μmol/h/mg) was exhibited by CHS in this study, with a similar residual activity of ≥ 70% between 30 to 60^{o}C (Fig. 4.4A). Gomes *et* al. (2001) showed that the endochitinase from *Streptomyces* RC1071 exhibited a similar temperature profile, with an optimum of 40^{o}C and thermostability studies illustrating that the enzyme retained ~ 80% of relative activity between 30 to 70^{o}C. The chitinase from *Streptomyces sp.* M-20 was only thermostable up to

40^{o}C, whereas CHS was highly unstable at 50^{o}C and there was a rapid decrease in residual activity from time 0 to 12 hours (Fig. 4.4B) (Kim *et al*., 2003). In contrast, the enzyme showed good thermostability at 37^{o}C, with a relative activity that was maintained above 56% after 72 hours.

An investigation of the activity of *P. pastoris* expressed Chit1 on colloidal chitin by Zhang and colleagues (2015) showed that this enzyme displayed an optimum temperature of 50^{o}C and this enzyme was described as a more thermostable enzyme compared to other chitinase enzymes that were investigated (Zhang et al., 2015). This enzyme was used in our study, expressed in *P. pastoris* grown on 1% (w/v) phosphoric acid treated chitin B from shrimp powder as a carbon source. However, in our current study, Figure 4.5A illustrates that the enzyme exhibited a temperature optima of 45^{o}C (3.992 μmol/h/mg), with a residual activity that was above 47% from 30 to 90^{o}C. This illustrates that Chit1 crude enzyme is active over a range of temperatures. This illustrates that the Chit1 crude enzyme is active over a range of temperatures and that Chit1 is thermostable at 37 and 50^{o}C, as Chit1 maintained a residual activity above 64% at both temperatures (Fig 4.5B). Therefore, the data obtained for the temperature profile of Chit1 correlates with data obtained by Zhang *et al*. (2015) and suggests that this thermostable enzyme can be produced in high yields on an industrial scale and can potentially be used for the production of value-added products.

4.5.3. Determination of pH optima

Determining the pH optima for enzymes is crucial as the pH affects the catalytic activity and stability of enzymes, consequently limiting their use in industry (Suginta *et* al., 2004; Vaidya *et al*., 2003; Zhang *et al.*, 2000). The pH optima was determined for the different chitinolytic enzymes (Figs. 4.6A, B and C). The pH optima for CHB, CHS and Chit1 was pH 5.0, with specific activities of 33.459, 46.2 and 5.776 μmol/h/mg, respectively. It was observed that all chitinolytic enzymes maintained a residual activity above 45% from pH 5.0 to 8.0. Another bacterial strain known as *Bacillus subtilis* TV-125A produced a chitinase that exhibited an optimum pH of 4.0 and retained 71% of activity at a pH of 10.0, which is comparable to the pH optima of 5.0 which was obtained for CHB from *B. cereus* (Senol *et al*., 2014). On the other hand, studies conducted on chitinases from *S. griseus*, *S. reseolus* and *S. venezuele* displayed a slightly elevated pH optimum of 6.0 and were relatively stable at alkaline pH values. These findings were comparable to the pH optima and relative activities at alkaline pH reported for CHS (Mukherjee and Sen, 2006; Tanabe *et al*., 2000; Xiayun *et* al., 2012).

The recombinantly expressed Chit1 by Zhang and colleagues (2015) exhibited activity over a broad pH range (3-11). However, optimal activity was detected at pH 5.0, which was confirmed in our study.

4.5.4. Identification of CHOS produced from the hydrolysis of various chitinous substrates

The hydrolysis products produced from the degradation of chitinous substrates with chitinolytic enzymes was analysed by TLC. This study was conducted to determine which substrates the enzymes performed optimally on and whether a high activity implies the production of a greater range of CHOS. Figure 4.7 illustrates that all chitinolytic enzymes have the ability to hydrolyse the different biomasses. The highest activity of the chitinolytic enzymes was observed on phosphoric acid treated chitin A, followed by commercial chitosan from shrimp shells and chitin from shrimp shells powder (Fig. 4.1). However, Fig. 4.7 shows that high enzyme activity does not necessarily imply that a wider range of CHOS are produced. It was observed (Fig 4.7) that CHB, CHS and Chit1 facilitated the release of CHOS with a DP of 1 to 3, with chitobiose being predominantly produced from biomass hydrolysis. Hydrolysis of commercial chitosan with commercial chitinolytic enzymes generated CHOS with a DP of 2-6. This suggests that the polymeric chitosan substrate is the most suitable substrate for the production of a range of CHOS as value-added products (to potentially treat diseases and illnesses in the health sector). Phosphoric acid treated chitin B was hydrolysed the most effectively. This pre-treatment of chitinous biomass, with the concerted degrading action of chitinolytic enzymes, could potentially be used for the hydrolysis of chitinous biomass, resulting in a decrease in environmental pollution.

4.5.5. Chain cleaving patterns of commercial chitinolytic enzymes

For the production of CHOS, it is essential to analyse the cleaving patterns of the chitinolytic enzymes and to understand their mechanism of action. Only the cleaving patterns of commercial enzymes on CHO standards were analysed, as these enzymes were pure in contrast to the Chit1 crude fraction. A study conducted by Hsieh *et al*. (2010) on a *Bacillus cereus* NCTU2 chitinase (ChiNCTU2), revealed that this enzyme lacks any chitin binding or insertion domains and often functions as an exochitinase. Therefore, this enzyme contains two loops (Gly-67—Thr-69 and Ile-106–Val-112) which facilitates the interaction of the enzyme with NAG, consequently facilitating substrate binding and disruption for hydrolysis (Hsieh *et al*., 2010). This property permits the enzyme to hydrolyse other complex polymeric

substrates predominantly into bioses, which explains the other hydrolysis products produced by CHB in Fig. 4.7. ChiNCTU2 was described as a producer of predominantly chitobioses, which validates the results obtained in Fig. 4.8, illustrating that CHB cleaves CHOS with an even number of sugar residues into CHOS with a DP of 2, whereas CHOS with an odd number of residues were cleaved into CHOS with a DP of 1 and 2. This is indicative that CHB is an exo-cleaving enzyme, due to the processive chain cleaving pattern that was observed - which suggests cleavage from the non-reducing ends to predominantly produces bioses and monomers (Rathore and Gupta, 2015; Sahai and Manocha 1993).

According to Rathore and Gupta (2015), fungal chitinases belonging to group C are endo-acting enzymes as a result of their substrate binding site and generally comprise of a CBM5 on the N-terminal of the catalytic domain (Kezuka *et al*., 2006). The CBM5 domain contains important residues that are crucial for efficient binding to substrates (Kezuka*et al*., 2006). In contrast, it was shown that CHS cleaves some substrates in a processive manner (Fig. 4.8). CHB was observed to cleave CHOS containing an even number of sugar residues into CHOS with a DP of 2 and standards with an odd number of residues were cleaved into CHOS with a DP of 1 to 3, which is similar to CHB. However, due to the CBM5, CHS has the ability to hydrolyse crystalline substrates more efficiently, thereby producing a greater range of CHOS (1-6) from the hydrolysis of the chitosan biomass than CHB (Kezuka *et al*., 2006).

4.5.6. Conclusion

In this chapter, the substrate preference and physico-chemical characterisation of the chitiolytic enzymes for the production of a range of CHOS was determined. The temperature optima of the chitinolytic enzymes ranged from 40-50^{o}C, which correlated with data obtained in other studies. Thermal stability data indicated that these enzymes are stable at 37^{o}C, as opposed to 50^{o}C. Chit1 was observed to be stable at both temperatures and is a potentially viable option for industrial processes. The pH optima of the enzymes was at pH 5.0, with a good percentage of activity being retained under alkaline conditions. This consequently demonstrated that chitinolytic enzymes can be characterised using phosphoric acid treated chitin B as a cheaper and alternate substrate, and that the enzymes' biochemical properties correlated well with other studies using model substrates such as glycol-chitin. This alternative creates an avenue to reduce chitionous waste in the environment by using a recyclable method which involves treating the substrate with phosphoric acid, followed by degradation with enzymes into value-added products. This investigation illustrated that the

high affinity of an enzyme for a substrate and high enzyme activity on that substrate does not necessarily imply that a greater range of CHOS will be produced. It is evident from the data obtained that the commercial chitinolytic enzymes that were used for the hydrolysis of chitinous biomasses were both exo-chitinases and predominantly produced chitobiose. The ability of CHS to produce a larger range of CHOS than CHB can be explained by the presence of a CBM5 domain in the enzyme, which increases its binding and hydrolysis of crystalline substrates. Therefore, enzyme synergy studies between CHB and CHS on chitinous biomass were conducted to determine whether higher amounts of reducing sugars could be produced using an optimal enzyme ratio combination (Chapter 5).

Chapter 5: Synergy between commercial chitinolytic enzymes during the degradation of colloidal chitin

5.1. Introduction

Chitin is a complex substrate and requires a range of enzymes to hydrolyse it into monomeric or chitooligomeric residues. Chitin-degrading enzymes are responsible for the hydrolysis of glycosidic bonds in carbohydrates and comprise a wide selection of glycoside hydrolases (GHs). Glycoside hydrolases are classified through a Carbohydrate-Active Enzyme data base (CAZy) that is continuously updated (http://www.cazy.org). Enzymes are classified based on amino acid sequence, structural and mechanistic similarities (Aam *et al*., 2010). A more sustainable way of hydrolysing chitin is through the employment of chitin degrading enzymes, which are found in various organisms and include the following: cellulases, papains, lysozymes, proteases, chitinases, chitosanase, lipases and pectinases (Aam *et al*., 2010; Kumar *et al*., 2004; Lin *et* al., 2009 and Muzzarelli *et al*., 1995). However, chitin is a tough and insoluble substrate and enzyme hydrolysis of this substrate produces low yields of reducing sugars and requires high enzyme loadings, which is costly.

According to Pletschke *et al*. (2016), enzyme loading can be reduced by combining enzymes at an optimal ratio to improve the yield of hydrolysis products, making bioconversion more economical. Therefore, the substrate and enzyme loading during saccharification are important factors to consider (Van Dyk and Pletschke, 2012; Klein-Marcuschamer *et al*., 2012). Another important factor is the nature of the biomass which may contain morphological and chemical barriers, consequently reducing enzymatic hydrolysis. Which makes biomass pretreatment essential, for surface exposure subsequently increasing enzyme binding, resulting in an increase of hydrolysis and reducing the enzyme loading quantity required (Pletschke *et al*., 2016).

Enzyme synergy is a relatively efficient technique that is employed to reduce enzyme loading and costs. Enzyme synergy comprises of simultaneous, sequential and successive synergy. Van Dyk and Pletschke (2012) describe simultaneous synergy as the effective hydrolysis of biomass using a combination of enzymes at the same time, while sequential synergy requires the addition of one enzyme for substrate hydrolysis, followed by enzyme heat inactivation and then the addition of a second enzyme. Successive synergy, on the other hand, entails the degradation of biomass with one enzyme and then the addition of the second enzyme without

heat inactivation of the first deployed enzyme (Pletschke et al., 2016). However, enzyme synergy is complex and dependent on numerous factors such as the substrate composition, enzyme ratios, time of hydrolysis, enzyme loading and the enzyme specificities for interactions with a particular substrate (Pletschke *et al*., 2016).

Since many organisms host multi-chitinolytic systems for the effective degradation of chitin, it was of interest to determine whether the combination of the two commercial chitinolytic enzymes from *Bacillus cereus* and *Streptomyces griseus* would synergistically degrade colloidal chitin. Therefore, this study focused on investigating which type of enzyme synergism and which enzyme ratio would produce the highest reducing sugar release from chitinous biomass. We also wanted to determine whether time studies conducted with the best enzyme combination on colloidal chitin would produce a wider range of CHOS.

5.2. Aims and Objectives

5.2.1. Aim

To conduct synergy studies on colloidal chitin using chitinases from *Bacillus cereus* (CHB) and *Streptomyces griseus* (CHS) to establish an optimal enzyme combination (ratio) for the production of high amounts of reducing sugar with reduced enzyme loading.

5.2.2. Objectives

- To conduct binary simultaneous, successive and sequential synergy studies with the two commercial chitinolytic enzymes on colloidal chitin;
- To determine the effect of hydrolysis time (using simultaneous synergy) on the degree of polymerization (DP) of chitooligomers (CHOS) produced using the optimal enzyme combination.

5.3. Methods

5.3.1. Synergy studies

5.3.1.1. Simultaneous synergy

Binary simultaneous synergy studies between chitinases were conducted on colloidal chitin by varying their protein ratios (0-100%). Assays were made up to a final volume of 400 µL with a citrate-phosphate buffer (0.2 M dibasic sodium phosphate- 0.1 M citric acid monohydrate). The protein loading for each of the enzymes was 0.1 mg/mL which were

added in different ratios (0, 25, 50, 75 and 100 µL), with the substrate at 1% (w/v) concentration. Assays were performed in triplicate at 37^{o}C for 48 hours, at 25 rpm. After hydrolysis, samples were centrifuged at 13000 rpm for 5 minutes and the supernatants were assayed for the release of reducing sugars as described in Chapter 3, section 3.3.2. The degree of synergy (DS) was expressed by dividing the activities of the combined enzymes, by the theoretical sum of their individual activities.

5.3.1.2. Sequential synergy

Binary sequential synergism studies with varying protein ratios (identified in section 5.3.1.1) were conducted on colloidal chitin. Assays were made up to a final volume of 400 µL with citrate-phosphate buffer (0.2 M dibasic sodium phosphate- 0.1 M citric acid monohydrate). The total protein concentration of reactions was 0.1 mg/mL with 1% (w/v) substrate. A volume of 25 µL CHB or 75 µL CHS was added to the reaction and assays were performed in triplicate at 37^{o}C at 25 rpm and were heat-inactivated for 5 minutes at 100^{o}C (Labnet AccuBlock digital dry bath) after 24 hours. This was followed by the addition of 75 µL CHS or 25 µL CHB to the reaction samples, respectively, which were then incubated at 37^{o}C for 24 hours, at 25 rpm. After hydrolysis, samples were centrifuged at 13000 rpm for 5 minutes and the supernatants were then assayed for the release of reducing sugars as described in Chapter 3, section 3.3.2, and the DS was calculated.

5.3.1.3. Successive synergy

Binary successive synergism studies with varying protein ratios (identified in section 5.3.1.1) were conducted on colloidal chitin. Assays were made up to a final volume of 400 µL with a citrate-phosphate buffer (0.2 M dibasic sodium phosphate- 0.1 M citric acid monohydrate). The protein loading was 0.1 mg/mL with 1% (w/v) substrate. A volume of 25 µL CHB or 75 µL CHS enzyme was added to the reaction and assays were performed in triplicate at 37^{o}C for 24 hours at 25 rpm. This was followed by the addition of 75 µL CHS or 25 µL CHB to the reaction samples, respectively, followed by incubation at 37^{o}C for 24 hours at 25 rpm. After hydrolysis, samples were centrifuged at 13000 rpm for 5 minutes and the supernatants were assayed for the release of reducing sugars as described in Chapter 3, section 3.3.2, and the DS was calculated.

5.3.2. Time study with the best simultaneous synergistic combination of chitinases for the production of CHOS

The best simultaneous synergy combination of CHB and CHS on colloidal chitin (section 5.3.1.1) was investigated at different time intervals (3 to 72 hours). Thin layer chromatography (TLC) was performed (Chapter 3, section 3.3.4) to test the effect of hydrolysis time on the range of CHOS produced.

5.4. Results

5.4.1. Synergy studies

Figure 5.1 illustrates the different types of enzyme synergy assays that were performed using the 75% CHB: 25% CHS (protein ratio) combination on colloidal chitin. Colloidal chitin was used as the substrate of choice due to chitinase activity assays generally being conducted on glycol chitin or colloidal chitin. Colloidal chitin was the cheaper alternative. This study demonstrated which type of synergy was the most effective in producing the highest amount of reducing sugar by determining specific activities.

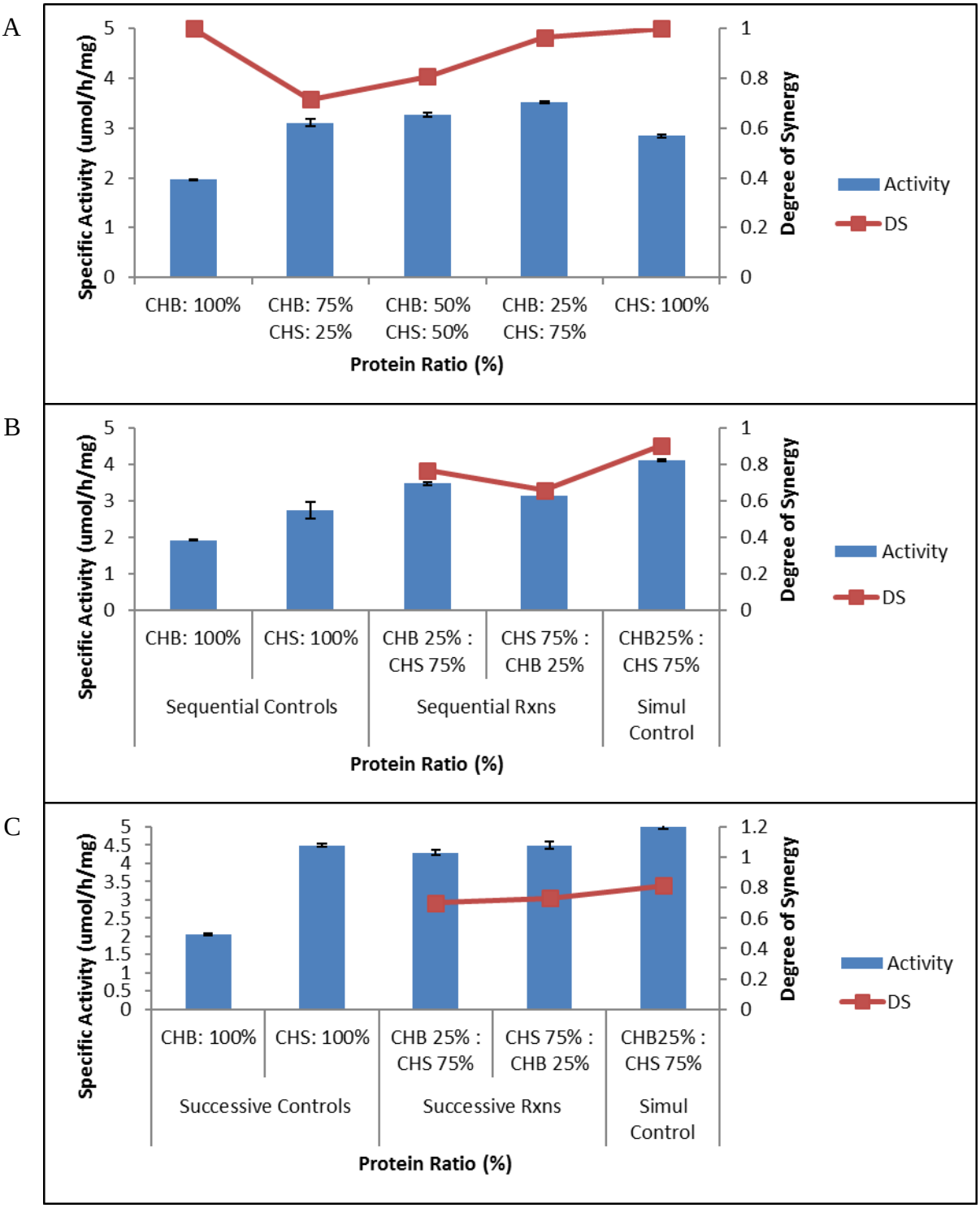

Figure 5.1. Synergy studies with commercial chitinolytic enzymes CHB and CHS (A) simultaneous synergy; (B) sequential synergy and (C) successive synergy. Values are represented as means ± SD, n=3. CHS: chitinase from *S. griseus*; CHB: chitinase from *B. cereus*; Sequential Rxns: sequential synergy reactions; Successive Rxns: successive synergy reactions; Simul Control: simultaneous synergy control and DS: degree of synergy.

Based on Fig. 5.1, the simultaneous (Fig 5.1A), sequential (Fig. 5.1B) and successive (Fig. 5.1C) synergies produced higher activities than the individual enzymes (each at 100%). Figure 5.1A demonstrated that the CHB 75%: CHS 25% combination produced the highest specific activity (3.526 umol/h/mg). It was observed that there was no synergy for all studies that were conducted (Fig. 5.1) and the simultaneous synergy controls in Fig. 5.1B and C, were seen to have a higher activities than the successive or sequential synergy reactions.

5.4.2. Time study of best synergistic combination of chitinases for the production of CHOS

The best simultaneous synergy combination (CHB 25%: CHS 75%) was tested over different periods of time to detect whether this enzyme combination would produce a broader range of CHOS over the course of hydrolysis, shown in Fig. 5.2.

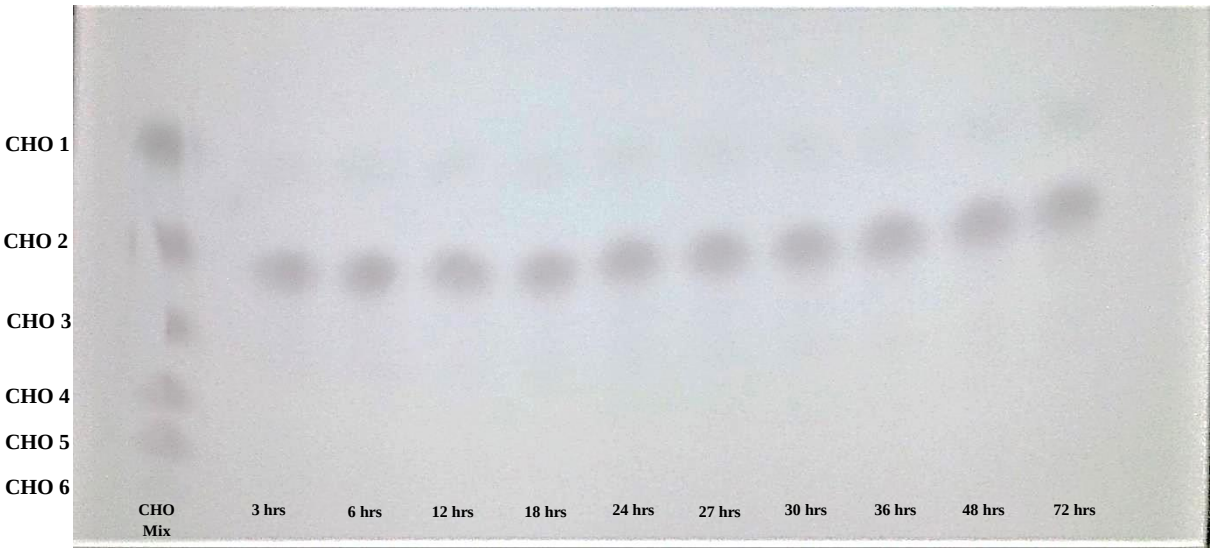

Figure 5.2. Thin layer chromatography of time study conducted with simultaneous synergy combination of CHB 25%:CHS 75%. CHO: CHO Mix: chitooligomer mix; CHO1-CHO6: N-acetylglucosamine- hexaacetyl-chitohexose and hrs: hours.

Figure 5.2 clearly demonstrated that the combination of CHB and CHS produced an identical range of CHOS (DP 1-3) throughout the study.

5.5. Discussion

5.5.1. Synergy studies

Studies conducted illustrated that the combination of CHB and CHS at an enzyme ratio of 25:75% protein loading, respectively, produced the highest specific activity in simultaneous synergy reactions in comparison to sequential and successive synergy (Figs. 5.1A, B and C).

These results demonstrated that the simultaneous combination of the two chitinolytic enzymes led to good cooperation with one another to produce a higher specific activity than when each enzyme was acting independently. This showed that the concerted action of the two enzymes produced a higher yield of CHOS as value added products. This study is in agreement with Van Dyk and Pletschke (2012), since the combination of CHB and CHS produced higher quantities of reducing sugars and was more economical as chitinases are generally expensive.

5.5.2. Time study for the production of CHOS, using simultaneous synergy between chitinolytic enzymes

Studies conducted by Vaaje-Kolstad *et al*. (2013) illustrated that the chitinolytic system of *Serratia marcescens* (comprising ChiA, ChiB and ChiC) hydrolysed insoluble biomass efficiently into CHOS. Their study indicated that the important difference between chitinolytic enzymes is their processivity and not whether they are exo- or endo-cleaving enzymes, due to non-processive enzymes occasionally having the ability to bind on biomass and initiating an endo-cleaving mode of when substrates are accessible (Vaaje-Kolstad *et al*., 2013). This is demonstrated by the combination of CHS and CHB, where the CHO products are predominantly chitobioses and N-acetylglucosamine (Fig. 5.2). These enzymes clearly demonstrated processive activity and this may be as a result of the substrate being extremely crystalline, resulting in colloidal chitin being highly inaccessible, thus the chitinases prefer to bind onto chain ends (Hult *et al*., 2005). Similarly, biomass components such as hemicellulose, act as barriers to cellulose fibrils which act as a limiting factor to accessibility for enzymatic hydrolysis (Banerjee *et al*., 2010; van Dyk and Pletschke, 2012; Varnai *et al*., 2011). Therefore, the depolymerisation of crystalline substrates (such as cellulose and chitin) is essential for reducing enzyme inhibition by the crystalline substrates, polymers or oligosaccharides that are released during hydrolysis. This creates efficient enzymatic hydrolysis and the generation of higher yields.

5.5.3. Conclusion

The synergy studies revealed that the simultaneous combination of CHB 25% : CHS 75% protein dosage produced the largest quantity of product in comparison to the other synergy studies. This demonstrated that the concerted hydrolysis of chitin by these enzymes may lead to a reduction in the quantities of enzymes required for the generation of value added products such as CHOS. Time studies revealed that the enzymes produced mainly

chitobioses. The production of mainly chitobiose may not directly infer that CHS is exo-acting; the substrate morphology of colloidal chitin may have resulted in the enzymes binding to the chain ends because the substrate was inaccessible due to its high crystallinity. Time therefore had no effect on the production of a wider range of CHOS. However, this study was important as it revealed that higher yields of CHOS with a DP of 1 to 3 can be produced with the combination of these enzymes using simultaneous binary-synergy.

Chapter 6: Antimicrobial properties of chitooligomers

6.1. Introduction

Chitosan and its acid or enzymatically derived chitooligomers (CHOS) have gained increasing attention over the years for a variety of biotechnological applications. The hydrolysis of chitin or chitosan leads to the generation of CHOS which are homo- or hetero-oligomers which are comprised of N-acetylglucosamine and D-glucosamine units (Aam *et* al., 2010; Kim and Rajapakse, 2005). Many studies have indicated that chitosan derived chitooligomers (CHOS) are extensively used in the pharmaceutical industry, as they have the potential to treat different ailments due to their ease in absorption through the intestine and their promising antimicrobial, drug delivery, immune-enhancing and anti-tumour activities (Chung *et al*., 2004; Je *et al*., 2004; Quan *et al*., 2009; Remunan-Lopez *et al*.¸1998; Tsai *et al*., 2002; Xia *et* al., 2011 and Zhang *et al*., 2010).

According to Jeon *et al*. (2000), chitosan derived CHOS are very attractive due to their neutral pH, greater solubility, free amino groups and shorter chain lengths. Many properties influence the bio-activities of CHOS, such as the degree of polymerization (DP), degree of acetylation (DA), pattern of acetylation (P_A), molecular weight (MW) and charge distribution (Madhuprakash, *et al.*, 2015 and Muzzarelli, 1996). These characteristics of CHOS have an effect on their pH-dependent solubility and their inter-chain interactions (Zhang *et al*., 2010).These characteristics are influenced by the interactions between the non-polar acetyl groups and hydrogen bonds, thus interfering with bio-activity (Zhang *et al*., 2010). In contrast to chitin and chitosan, CHOS have a greater solubility and lower viscosity at neutral pH, which has gained the attention of many researchers for a range of applications due to their non-toxic and progressive physiological characteristics (Lodhi, *et al*., 2014). Mourya and colleagues (2011) have stated that chitin or chitosan derivatives which have a degree of polymerization of <20 and a MW of 30 to 50 kDa are known as CHOS and generally possess biological activity (Shin *et al*., 2001).

A review conducted by Aam *et* al. (2010) indicated that many methods have been proposed and used for the production of CHOS, such as chemical synthesis of CHOS. However, this method is not used regularly, due to the high consumption and expense of organic solvents and the production of short chain CHOS. Chemical methods such as acid hydrolysis of chitosan for the production of CHOS are well known, where the increase of chitinous biomass degradation into CHOS is dependent on the initial fraction of *N*-acetyl residues

(Einbu *et al*., 2007). In contrast, a cheaper and more effective way of producing CHOS is to employ enzymes such as cellulases, papains and chitinolytic enzymes preferably from cheap fungal or plant sources in a crude form (Sashiwa *et al*., 2003; Terbojevich *et al*., 1996 and Xie *et al*., 2010). Therefore, enzyme technology is an alternate and efficient way to generate CHOS, considering important factors such as the: 1) crystallinity of the substrates, 2) the enzymes' affinity for the substrate, 3) the amount of time required for processivity of the substrate by the enzymes, and 4) the production of specific CHOS through the employment of a variety of enzymes (Sikorski *et al*., 2005).

According to Kim and Rajapakse (2005), waste materials such as crab and shrimp shells are often used as the major sources of biomass for the production of chitosan and chitin and eventually CHOS. The CHOS produced are utilised in a many applications, ranging from the food, pharmaceutical, agricultural to the environmental industry (Park and Kim, 2010). Studies that were previously conducted often used chitosan as biomass and chitosanases for the generation of CHOS, or derived the CHOS via acid hydrolysis (Xia *et al*., 2011). However, many problems exist during acid hydrolysis of chitinous biomass, such as environmental pollution, the degradation of sugar products, non-specific production and low yield of CHOS (Xia *et* al., 2011).

Therefore, the enzymatic production of CHOS has gained wide interest due to environmental safety and the production of specific CHOS in a controlled manner (Xia *et* al., 2011).The enzymatic hydrolysis of chitin containing biomasses, with chitinolytic enzymes, for the generation of CHOS with anti-microbial activities, is thus seen as a viable method which produces specific CHOS in a controlled environment. It is evident that there is a large knowledge gap in terms of the generation and bio-activities of chitin generated derivatives. Therefore, the prime focus of this study was to generate chitin and chitosan derived CHOS and to investigate their antimicrobial properties.

6.2. Aims and Objectives

6.2.1 Aim

To investigate the antimicrobial properties of CHOS generated from different chitinous biomass samples on *Bacillus subtilis* (*B. subtilis*), *Escherichia coli (E. coli*), *Klebsiella* and *Staphlococcus aureus* (*S. aureus*).

6.2.2. Objectives

- To conduct activity assays for the hydrolysis of various chitinous biomass samples with chitinolytic enzymes for the production of CHOS;
- To identify the chitooligomeric products produced by the chitinolytic enzymes;
- To determine the antibacterial activity of the CHOS.

6.3. Methods and Materials

6.3.1. Activity assay for the hydrolysis of chitinous biomass into CHOS

Activity assays were conducted using 1.33% (w/v) commercial chitosan, colloidal chitin, phosphoric acid treated chitin A and B (Chapter 2, section 2.3.1). Reactions using 0.1 mg/mL chitinases from *S. griseus* (CHS) and *B. subtilis* (CHB) were prepared using the same parameters as described in Chapter 3, section 3.3.6. Chitinase from *P. pastoris* (Chit1, 0.57 mg/mL) was used (Chapter 3, sections 3.3.5 and 3.3.6), where the total reaction volume of 90 µL was comprised of 60 µL enzyme (0.3mg/mL) and 30 µL of the various un- and pre-treated substrates. Enzyme controls were comprised of the same volumes of enzyme and were made up with citrate-phosphate buffer (pH 5.0) buffer to the final reaction volumes. The DNS protocol was employed to determine reducing sugars (Chapter 3, section 3.3.2) and N-acetylglucosamine was used as a suitable sugar standard.

6.3.2. Identification of CHOS produced by hydrolysis of chitinous substrates by chitinolytic enzymes

Hydrolysates from activity assays that were conducted in section 6.3.1 were used to perform TLC (as described in Chapter 3, section 3.3.4) to identify CHOS produced by the chitinolytic enzymes.

6.3.3. Determination of inhibition of bacterial growth by CHOS

A volume of 100 µL of bacterial glycerol stocks (*B. subtilis*, *E. coli*, *Klebsiella* and *S. aureus*) were each added to 5 mL of Luria broth (10 g/L tryptone, 10 g/L NaCl and 5 g/L yeast extract) and were grown overnight at 37^{o}C and 200 rpm. The OD600 for these samples were taken at 600 nm with the Power Wave$_x$ Spectrophotometer and analysed on Kc Junior software. The required absorbance was between 0.7 and 1.2 OD units. For bacterial growth inhibition studies, 250 µL of the hydrolysates (obtained from the activity assays in section 6.3.2) was added to 5 mL Luria broth inoculated with 50 µL of the bacterial cultures. Control 1 contained Luria broth only (negative control), control 2 contained Luria broth with bacteria

(positive control), control 3 comprised of Luria broth and citrate-phosphate buffer (pH 5.0) and control 4 comprised of Luria broth with a citrate-phosphate buffer (pH 5.0) that contained 0.03% (w/v) sodium azide . All samples and controls were incubated at 37^{o}C with shaking at 200 rpm. Absorbance of the samples was measured in triplicate at 600 nm after 3, 5 and 8 hours. Absorbance was expressed relative to control 3. Control 3 values were expressed as 100%. Percentages below and above 100% were considered as growth inhibition or activation, respectively. ANOVA analysis was applied to detect significant bacterial growth or inhibition.

6.4. Results

6.4.1. Identification of CHOS produced

Activity assays were conducted on acid treated substrates and commercial chitosan as these substrates were shown to produce a range of CHOS and high reducing sugar content in Chapter 4. Hydrolysates from activity assays were subjected to TLC for the identification of CHOS that were subsequently used in bacterial growth inhibition studies, see Fig 6.1 below.

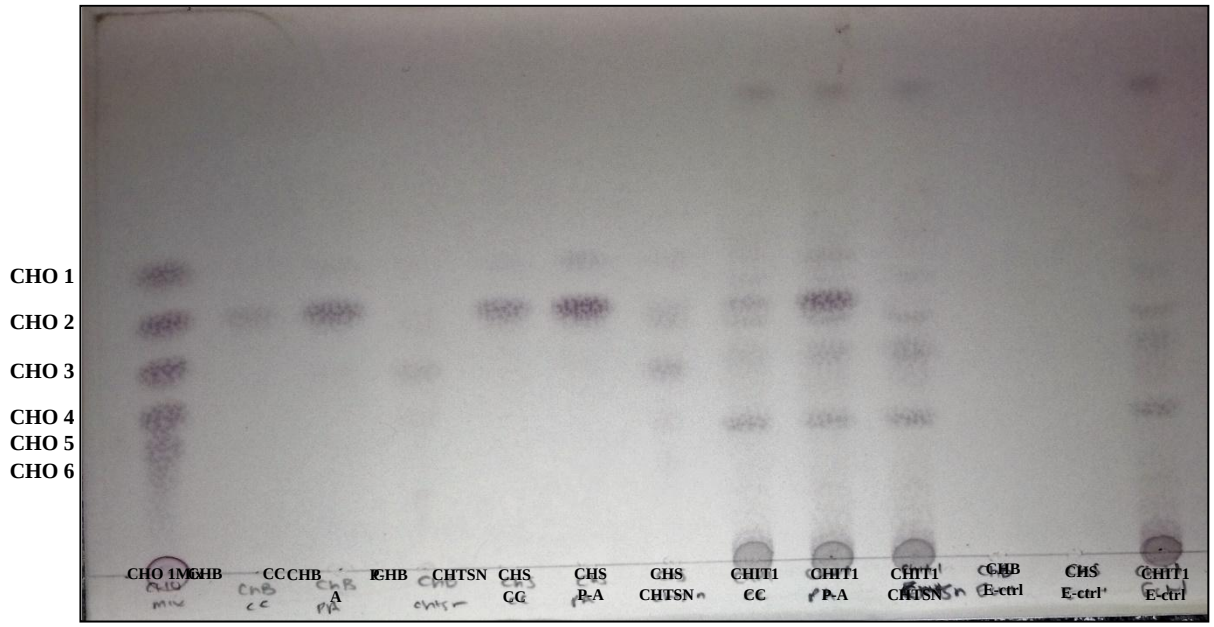

Figure 6.1. Thin layer chromatography of activity assays (of chitinases) on chitinous substrates. CHO Mix: chitooligomer mix; CHO1-CHO6: N-acetylglucosamine- hexaacetyl-chitohexose; CC: colloidal chitin; CHTSN: commercial chitosan; P-A: phosphoric acid treated chitin A from shrimp shells; E-ctrl: enzyme control; CHS: chitinase from *S. griseus*; CHB: chitinase from *B. cereus* and CHIT1: chitinase from *P. pastoris.*

Based on Fig. 6.1, CHB and CHS have the ability to hydrolyse the chitinous biomasses into CHOS, with the prominence of CHOS with a DP length of 1 and 2. However, the hydrolysis

of the commercial chitosan by CHB and CHS subsequently produced a range of CHOS (DP1-6). Chit1 produced CHOS with a DP of 1 to 4 on all biomasses, with some bands above DP 1. The enzyme control for Chit1 also exhibited bands on TLC, but the intensity of these bands were lighter than those observed for the reactions.

6.4.2. Inhibition studies conducted with CHOS on bacterial growth

The CHOS produced from the hydrolysis of chitinous biomasses with chitinolytic enzymes were identified by TLC (Fig. 6.1) (as described in section 6.4.1). These CHOS were then each screened for inhibition of bacterial growth against *B. subtilis*, *E. coli*, *Klebsiella* and *S. aureus* over time shown below in Figure 6.2.

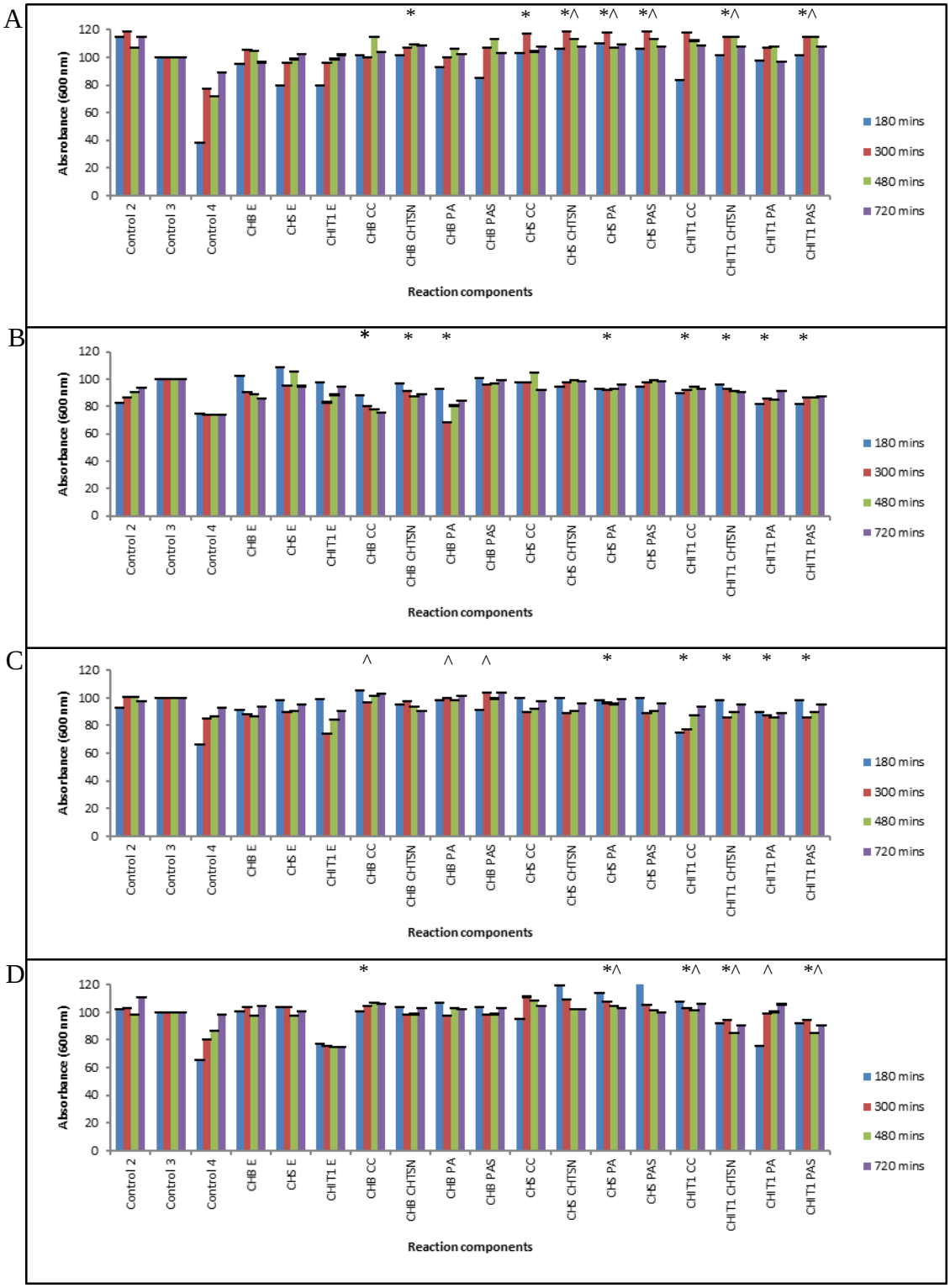

Figure 6.2. Antibacterial activity of CHOS produced from the hydrolysis of chitinous biomass, with chitinolytic enzymes against: (A) *B. subtilis*; (B) *E. coli*; (C) *Klebsiella* and (D) *S. aureus*. ANOVA analysis for the significance of inhibition or activation of bacterial growth, with respect to CHOS produced from various chitinous biomasses compared to control 3 and enzyme controls, keys: * (p value < 0.05 for activity of CHOS relative to control 3) and ^ (p value < 0.05 for activity of CHOS relative to their respective enzyme controls).Values are represented as means ± SD, n=3. Control 2: Luria broth with bacteria; Control 3: Luria broth and citrate-phosphate buffer (pH 5); Control 4: Luria broth with a citrate-

phosphate buffer (pH 5) and 0.03% (w/v) sodium azide; CHB E: CHB enzyme control; CHS E: CHS enzyme control; CHIT1 E: CHIT1 enzyme control; CC: colloidal chitin produced CHOS; CHTSN: commercial chitosan produced CHOS; PA: phosphoric acid treated chitin A produced CHOS and PAS: phosphoric acid treated chitin B produced CHOS.

It was evident from Fig. 6.2 that control 4, which was comprised of 0.03% (w/v) sodium azide (a well-known antimicrobial agent) in the buffer, inhibited all types of bacterial growth. It was observed that the CHOS produced had no inhibitory effect on the growth of *B. subtilis*, with the exceptions of some CHOS significantly activating growth in comparison to significantly lower growth observed in CHS and CHIT1 enzyme controls (Fig 6.2A). Figure 6.2B illustrates that all CHOS produced by substrate hydrolysis using CHB and CHIT1 exhibited inhibitory effects on the growth of *E.coli*. Based on Fig 6.2C, it was evident that CHIT1, CHB enzyme controls and CHOS produced by CHIT1 from the different chitinous biomass had a slight inhibitory effect on *Klebsiella* growth (as well as control 4). Lastly, Fig. 6.2D demonstrated that *S. aureus* was slightly inhibited by CHOS produced by CHIT1 and that the CHIT1 enzyme control significantly inhibited growth to a greater extent than CHOS.

6.5. Discussion

6.5.1. Identification of and analysis of CHOS produced from activity assays by TLC

The production of CHOS was only examined using acid treated chitin substrates and commercial chitosan, due to the high amounts of reducing sugars obtained, and in order to obtain a range of CHOS. Based on the TLC profile in Fig. 6.1, it is evident that the commercial enzymes CHB and CHS have the ability to hydrolyse the pretreated chitinous biomass into CHOS with a DP of 1-3, with a DP of 2 being the predominant CHO that was produced. Chitosan is a biopolymer produced by the deacetylation of chitin and comprises of three funtional groups that affect reactivity: amino groups, primary and secondary hydroxyl groups at C1, C2 and C6 positions (Lodhi *et al.*, 2014). This substrate is generally cationic due to positively charged amino groups and their distribution which subsequently affects the physico-chemical characteristics and reactivity of the substrate in terms of an increase in solubility in an acidic pH environment, chelation, bioactivities and flocculation (Lodhi *et al*., 2014 and Xia, 2003). Therefore, it was evident that the commercial chitinolytic enzymes had the ability to hydrolyse chitosan into a wider range of CHOS (DP of 1-6). This was expected, due to the properties of the pretreated chitinous biomass differing from the chitosan in terms of enzyme-biomass reactivity, where the amount of reducing sugars is not a reflection of the range of different products formed.

CHIT1 exhibited true endo-activity with its non-processive behaviour. The hydrolysis of all chitinous biomasses were tested and produced a range of CHOS with DP lengths from 1 to above 6 (Zhang *et al*., 2015). The enzyme control for CHIT1 exhibited the same bands as the reactions, however, the activity for the enzyme control was lower than that the reactions, which is validated by bands for CHIT1 enzyme control on TLC being of a lighter intensity than the reaction bands. The activity assay and TLC demonstrated that a range of CHOS was produced by the degradation of the chitinous biomasses with the chitinolytic enzymes. The antimicrobial properties of the produced CHOS could then be assessed against a range of bacteria.

6.5.2. Antimicrobial properties of CHOS

Since CHOS are known to exhibit a range of bio-activities, the CHOS, obtained from the hydrolysis of chitinous biomass with chitinolytic enzymes, were screened against various bacteria for any inhibitory effects by a series of turbidity tests of the culture media. Figure 6.2 illustrated that the CHOS exhibited both inhibitory and activatory effects on bacterial growth. Figure 6.2 illustrated that the CHOS produced from the different biomasses by the different chitinolytic enymes elicited different reponses in terms of their bio-activity. This can be explained by the varying DP lengths of CHOS that were produced in the activity assays, (which were used in the antimicrobial studies), consequently affecting the antimicrobial activities. However, according to Benhabiles *et al*. (2012), the molecular weight (MW) of CHOS and the degree of acetylation (DA) and other physico-chemical properties such as solubility and pattern of acetylation (PA) have been reported to have a major influence on the bio-acitivity of CHOS. Fernandez-Kim (2004) suggested that the molecular weight of chitin and chitosan are affected by the source in which they originate from and their preparation method, which can ultimately affect the MW of their produced derivatives (CHOS). Kumar *et al.* (2005) and Zheng and Zhu (2003) suggested that CHOS with a low DA and MW exhibit a greater degree of antimicrobial activity (Zheng and Zhu, 2003).

Benhabiles and colleagues (2012) suggested that chitin and chitosan substrates are bacterostatic in nature and are usually more active against Gram-negative bacteria. This phenomenon can be explained by the positively charged amine groups interacting with the negatively charged residues on the surface of the bacteria, such as lipids, proteins and carbohydrates, resulting in inactivation due to a change in membrane permeability (Tsai and Su, 1999 and Wu *et al*., 2006). Another possible explanation is that the low MW of the

substrates increases the flexibility of polymer chains between chitinous substrates and bacteria, consequently binding to more cells and inactivating them (Wu *et al*., 2006). In addition, a low DA of chitin and chitosan elicits antibacterial activity as a result of a decrease in the substrate's crystallinity, which allows greater flexibility of polmer chains. In contrast to the chitinous substrates, their derivatives (such as CHOS) exhibit bacteriocidal activity. Studies conducted by Eaton *et al*. (2008) demonstrated that CHOS have the capacity to compromise bacterial cell walls, resulting in some lesions, which consequently result in a reduction of stiffness and permeability, suggesting that low molecular weight CHOS have the ability to pass through the cell walls of certain bacteria.

CHOS were screened for growth inhibition of the Gram-positive bacterium, *B. subtilis* (Fig. 6.2A). It was observed that bacterial growth was not inhibited by the addition of CHOS produced by all chitinolytic enzymes from the hydrolysis chitinous substrates, with the exception of and CHS enzyme controls that showed greater inhibition of bacterial growth, in relation to CHOS produced from CHTSN and PAS hydrolysis by CHB and CHS. The inhibitory action of CHB and CHS enzyme controls showed ANOVA pvalues that were signifcant (p values < 0.05), in comparison to CHOS activity from PAS and CHTSN hydrolysis by CHB and CHS. CHIT1 is an endo-acting enzymeand cleaves randomly, which may result in its unique inhibitory effect.. According to Eaton *et al.* (2008), the peptidoglycan layer of Gram-positive bacteria are thicker than Gram-negative bacteria and provides strength and shape to the cell wall. This is demonstrated in Fig. 6.2A, where the addition of CHOS had no inhibitory effect and possibly used the escape of nutrients from CHOS through lesions in the cell wall for an increase in growth. Interestingly, other studies conducted by Benhabiles and colleagues (2012) revealed that *B. subtilis* growth was suppressed by the addition of a concentration of 0.01% (w/v) CHOS. In contrast, the pathogenic bacterium, *S. aureus*, is Gram-positive and round in shape, and was inhibited significantlyby CHOS produced from the hydrolysis of CC, CHTSN, PA and PAS by CHIT1 (p values < 0.02) in comparison to control 3 (Fig 6.2D). However ANOVA analysis revealed that the CHIT enzyme had a greater inhibitory effect (p values < 0.02), when compared to the inhibitory activities of the CHOS produced. Similar results were obtained in various studies that utilised low concentrationns of chitosan and chitin derived CHOS for the inhibition of *S. aureus* (Benhabiles *et* al., 2012 and Fernandes *et al*., 2008).

The well known Gram-negative, rod-shaped bacteria, *Klebsiella* was susceptible to some inhibition by the addition of CHOS produced from the hydrolysis of CHTSN by CHB and CC, CHTSN, PA and PAS by CHS in comparison to control 3 (with p values < 0.01) (Fig. 6.2C). All CHOS produced by hydrolysis using CHIT1, showed significant inhibory effects. It was also shown that all enzyme controls had superior inhibitory effects in comparison to the CHOS produced (p values < 0.05). Figure 6B illustrated similar activities and displayed inhibition of *E. coli* growth by CHOS produced fromsubstrate hydrolysis with CHIT1 (p values < 0.002) in comparison to control 3. The CHIT1 enzyme control inhibited bacterial growth similar to CHOS (p values > 0.05). This demonstrates that even though these Gram-negative bacteria possess an outer membrane, they have a thin peptidoglycan layer, which may have been the reason behind their susceptibility towards CHOS (Eaton *et al*., 2008). A similar study conducted on Gram-negative bacteria, including *E. coli*, demonstrated that CC and PA derived CHOS by CHB hydrolysis had a high inhibitory effect on the growth of *E. coli* (Benhabiles *et* al., 2012).

6.5.3. Conclusion

This chapter illustrated that the CHOS produced from the hydrolysis of chitinous biomass and chitosan with chitinolytic enzymes exhibited activatory or inhibitory effects on bacterial growth, and factors such as the type of bacteria, influenced the activity of the CHOS. It was also demonstrated that many other factors have to be considered. The characterisation of the CHOS produced, with respect to MW, DA and minimum inhibitory concentrations is essential and the generated CHOS should be confirmed and quantified via HPLC and/or NMR. To determine the effects of the CHOS produced, CHOS could be purified in future experiments to determine whether a specific DP length elicits activity against certain bacteria. The chitinolytic enzymes were observed to inhibit some bacterial growth to a greater extent than the CHOS produced and should therefore be investigated further for antimicrobial activities. However, this screening process was a preliminary study that indicated that these CHOS have antibacterial potential and warrant further investigation.

Chapter 7: General discussion, conclusions and future recommendations

7.1. General discussion and conclusions

Chitin is the second most abundant biopolymer and is often described as a tough, insoluble substrate that is found in many prokaryotic and eukaryotic organisms (Hamid *et al*., 2013). The majority of chitinous waste originates from shell fish which exists predominantly in the α-chitin polymorphic form (Lodhi *et al.*, 2014). This polymorph is reported to be the most insoluble form in comparison to β-chitin, due to the strong hydrogen bonds which attribute to the crystallinity of the biomass, consequently affecting solubility and hydrolysis (Lodhi *et al*., 2014; Rathore and Gupta, 2015).

The hydrolysis products of chitin are utilised in many biotechnological applications ranging from the medical, agricultural to food industry (Lodhi *et al*., 2014). The hydrolysis of chitinous biomass releases a range of chitooligomers (CHOS), which are of key interest to many researchers, due to chitin-derived CHOS eliciting a higher solubility and their easy absorption by the body. This has drawn attention towards CHOS and their applicability in terms of antimicrobial and immune-enhancing properties (Chung *et al*., 2004; Park *et al*., 2004; Quan *et al*., 2009; Remunan-Lopez *et al*.¸1998; Tsai *et al*., 2002; Xia *et* al., 2011 and Yang *et al*., 2010).

However, chitinolytic enzymes are expensive and the hydrolysis of crystalline substrates require high enzyme loadings, which are not economically feasible. Literature has revealed that enzyme loading can be reduced by combining enzymes in an optimal ratio to improve the yield of hydrolysis products, making bioconversion more economical (Van Dyk and Pletschke, 2012; Klein-Marcuschamer *et al*., 2012). This makes chitin biomass preteatment a necessity, for surface exposure subsequently increases enzyme binding, with a reduced enzyme loading required, resulting in an increase in hydrolysis and an increase in CHO value-added products (Pletschke *et al*., 2016).

Most of the studies performed on chitinous biomass has focused on the acid hydrolysis of chitin, using various solvents for the generation of CHOS or the use of high enzyme loadings for CHO generation (Xia *et al*., 2011). However, many problems exist, such as environmental pollution, the degradation of sugar products, non-specific production and low yields of CHOS (Xia *et* al., 2011). Therefore, this study investigated the following aspects:

1. Determining the morphological and chemical properties of all un- and pre-treated biomass, for efficient degradation by chitinolytic enzymes into value-added products.
2. Bio-prospecting for other chitin degrading enzymes from ericoid mycorrhiza and using recombinant expression, to produce high yields of chitin-degrading enzymes, as a more economical avenue for industrial application.
3. Investigating the physico-chemical properties of commercial (CHB and CHS) and crude enzymes (CHIIT1), to determine the optimal conditions for the generation of CHOS.
4. Studying the potential synergy between the two commercial chitinolytic enzymes for a reduction in enzyme loading and the generation of higher yields of hydrolysis products.
5. Investigating whether CHOS generated from the hydrolysis of chitinous biomass by chitinolytic enzymes elicit any antimicrobial properties against various bacteria.

Knowledge of pre- and un-treated chitin containing biomasses is essential for understanding how the structures and properties of biomasses impact on the activity of enzymes. This necessitated the use of different pre-treatment methods to disrupt chitin crystallinity to increase the porosity, to effectively allow enzymes more accessibility to the biomass for hydrolysis (Karp *et al*., 2013; Van Dyk and Pletschke, 2012). The data obtained from SEM, FTIR, DA and XRD demonstrated the disruption of crystallinity and increase in porosity, potentially resulting in greater accessibility for enzyme binding with subsequent biomass hydrolysis into value-added products.

Following biomass pre-treatments, new sources of chitin-degrading enzymes were explored, due to the high cost of commercial chitinases currently available. This study revealed that recombinantly expressed Chit1 from *P. pastoris* is produced in high yields and is feasible to use on an industrial scale (Zhang *et al*., 2015). Zhang and colleagues (2015) illustrated that Chit1 is an endo-cleaving enzyme that produces a range of CHOS that are applicable to various industries. Another economical avenue was to investigate the ericoid mycorrhiza as a potential source of chitin degrading enzymes, since mycorrhiza are found to produce an array of enzymes due to their saprotrophic habitat (Bizabani *et al*., 2016). *Oidiodendron maius* was screened for intra- and extracellular secretion of chitin-degrading enzymes and showed promise of producing chitin-degrading enzymes that hydrolysed chitinous biomass into CHOS with a DP of 1 to 3.

The biochemical characterisation of the chitinolytic enzymes was performed for the efficient production of CHOS. The temperature optima of the chitinolytic enzymes ranged from 40-50^{o}C, which correlated with data obtained in other studies (Bhushan and Hoondal, 1999; Gomes *et al.*, 2001; Senol *et al*., 2014; Yan *et al*., 2011 and Zhang *et al*., 2015). Thermal stability studies indicated that these enzymes are stable at 37^{o}C, as opposed to 50^{o}C. Chit1 was observed to be stable at both temperatures and is potentially a viable option for industrial processes. The pH optima of the enzymes was at a pH of 5.0, with a good percentage of activity being retained under alkaline conditions. This consequently demonstrated that chitinous substrates can be pre-treated using phosphoric acid treated chitin B, as a cheaper, alternate substrate correlated with the findings of other studies. This alternative creates an avenue to reduce chitinous waste in the environment, by using a recycling method, by treating the substrate with phosphoric acid, followed by degradation with enzymes into value-added products. TLC analysis illustrated that commercial chitinolytic enzymes exhibited processive activities and predominantly produced chitobiose. CHIT1 produced a range of CHOS from 1 to 10, and illustrated true endo-activity. The ability of CHS to produce a larger range of CHOS than CHB can be explained by the presence of a CBM 5 domain, that increases its binding and hydrolysis of crystalline substrates.

A review conducted by Pletschke *et al*. (2016) illustrated that enzyme loading can be reduced by combining enzymes in an optimal ratio to improve the yield of hydrolysis products, making bioconversion more economical. Synergy studies that were conducted revealed that a simultaneous synergistic combination of CHB 25% : CHS 75% produced the largest quantity of hydrolysis products. This demonstrated that the concerted hydrolysis of these enzymes increases yield and reduces the quantity of enzyme required, making the generation of value added products such as CHOS more economical. Even though the Nzytech and Megazyme information sheets suggest that CHB and CHS are endo-acting, time studies revealed that the enzymes produced mainly chitobioses which may not directly infer that CHS is exo-acting. The substrate morphology of colloidal chitin may have resulted in the enzymes binding to the chain ends, as a result of the substrate being inaccessible due to its high crystallinity.

CHOS has gained interest for the safe production of specific CHOS in a controlled manner (Xia *et* al., 2011). The enzymatic hydrolysis of chitin containing biomasses with chitinolytic enzymes, for the generation of CHOS with anti-microbial activities, is thus seen as a viable method which produces specific CHOS in a controlled environment. This study showed that

the CHOS, produced from the hydrolysis of acid treated chitinous and chitosan biomass with chitinolytic enzymes, exhibited activating or inhibitory effects on bacterial growth. Factors such as whether the bacteria are Gram-negative or Gram-positive influences the activity of CHOS. It was also demonstrated that many other factors have to be considered, with respect to MW and DA and minimum inhibitory concentrations of CHOS (Benhabiles *et al*., 2012).

In conclusion, this study demonstrated that simultaneous bi-synergy combnation of the commercial enzymes produced higher yields of reducing sugars and reduces enzyme loading, which is economically beneficial. It was also clearly illustrated that all chitinolytic enzymes have the capacity to hydrolyse un- and pre-treated chitinous biomasses to produce CHOS that exhibit antimicrobial properties. Other sources such as ericoid mycorhizza or recombinant expression elicited chitin-degrading activity and can be used to produce high yields of CHOS more economically.

7.2. Future recommendations

- The un- and pre-treated chitinous substrates could be analysed further by nuclear magnetic resonance (NMR) to develop an in depth understanding regarding the structure, dynamics, reaction state and their reactivity in various environments.
- The ericoid mycorrhiza *O. maius* should be explored further due to the secretion of chitinases or chitin degrading enzymes that hydrolyse chitin and produce CHOS. However, the chitin degrading enzymes produced by this organism requires further investigation and other techniques for confirmation, such as zymography, SDS-PAGE and enzyme purification to identify and characterise the enzyme(s) of interest.
- Since the crude enzyme CHIT1 produced a range of CHOS, enzyme crudes should rather be tested for chitinolytic activity for the production of CHOS. Some enzymes work more efficiently in a concerted manner than in their purified states.
- High performance liquid chromatography (HPLC) or matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) should be used in future for the quantification, separation and identification of biological CHO compounds.
- A wider range of bacteria with different properties should be tested against the mixture of CHOS produced. However, to determine the effects of CHOS with various DP lengths, CHOS must be purified in future studies to determine separate or combined inhibitory/activating effects of the different CHOS produced.

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Appendices

Appendix 1: List of reagents

Name of Reagent	Supplier (Catalogue number)
Acetic acid	Sigma Aldrich (Cat. No. 695092)
Acetone	Sigma Aldrich (Cat. No. 6050501)
Acetonitrile	Sigma Aldrich (Cat. No.271004)
Alanine	Sigma Aldrich (Cat. No. A7627)
Ammonia water (32%)	Sigma Aldrich (Cat. No. 338818)
Ammonium persulphate	Sigma Aldrich (Cat. No. A3678)
Bacteriological agar	Sigma Aldrich (Cat. No. A5306)
Bovine serum albumin (BSA)	Sigma (Cat. No. A7906)
Bradford reagent	Sigma (Cat. No. B6916)
Calcium chloride	Merck (Cat. No. 102378)
Chitin from shrimp	Sigma (Cat. No. C7170)
Chitinase from *Bacillus cereus*	Nzytech (Cat. No. CZ04382)
Chitinase from *Streptomyces griseus*	Sigma (Cat. No. C6137)
Chitosan	Sigma (Cat. No. 448869)
Citric acid	Merck (Cat. No. 1.00244)
D-Glucose	Saarchem (Cat. No. 2676020)
3,5-Dinitrosalicylic acid	Sigma (Cat. No. D0550)
Diphenylamine	Sigma (Cat. No. 242586)
Di-potassium hydrogen phoshate	Merck (1.05104.1000)
Di-sodium hydrogen orthophosphate	Saarchem (Cat. No. 5822860)
Ethanol	Merck (Cat. No. 8.18700)
Glycerol	Saarchem (Cat. No. 2676520)
Hydrochloric acid	Sigma (Cat. No. 320331)
Imidazole	Merck (Cat. No. 1.04716)
Iron chloride	Merck (Cat. No. 845124)
Luria Broth	Sigma Aldrich (Cat. No. L3522)
Magnesium sulphate	Merck (Cat. No. 106067)
Methanol	Merck (Cat. No. 8.22283)
1-Butanol	Sigma (Cat. No. 34867)
Orthophopshoric acid (≥ 85%)	Sigma (Cat. No. 438081)
Peptone	Merck (Cat. No. 102239)
Phenol	Sigma (Cat. No. P3653)
Sodium azide	Merck (Cat. No. 8.22335)
Sodium chloride	Saarchem (Cat. No. 5822320)
Sodium hydroxide	Saarchem (Cat. No. 5823200)
Sodium metabisulfite	Sigma-Aldrich (Cat. No.255556)
Sodium potassium tartrate	Merck (Cat. No. 1.08087)
Thiamine	Merck (Cat. No. 5871)
Yeast extract	Biolab (Cat. No. BX6)

Zeocin	Themo Fisher Scientific (Cat. No. R25001)
Zinc sulphate	Merck (Cat. No. 108883)

Appendix 2A: Protein standard curve

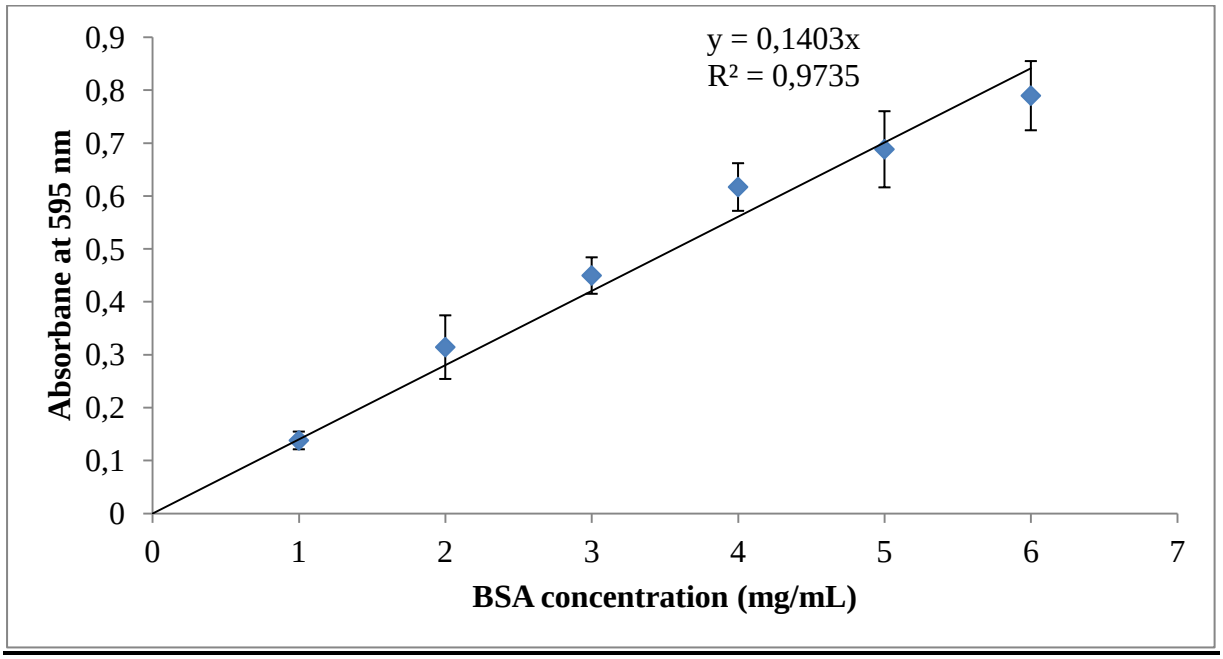

Figure 2A. Bradford standard curve to determine protein concentration. BSA was used as the protein standard. Values are represented as means ± SD, n=3.

Appendix 2B: *N*-acetylglucosamine standard curve

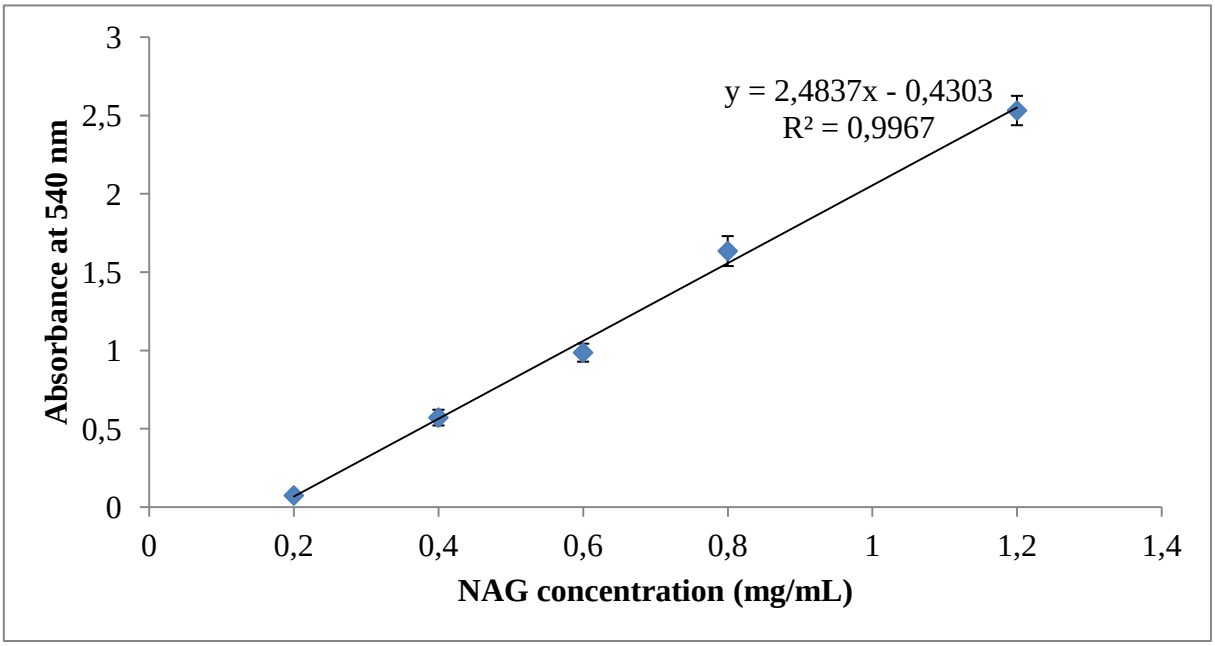

Figure 2B. *N*-acetylglucosamine standard curve using DNS assay. Values are represented as means ± SD, n=3.