Diogo Jorge Silva Ferreira

Candida albicans Cell Wall Surface Chitinase 3: Production, Purification, and Characterization

outubro de 2022

Universidade do Minho
Escola de Ciências

Diogo Jorge Silva Ferreira

Candida albicans **Cell Wall Surface**

Chitinase 3: Production, Purification, and

Characterization

Dissertação de Mestrado
Mestrado em Bioquímica Aplicada

Trabalho efetuado sob a orientação de
Doutor James Anthony Collins
e de
Professora Doutora Ana Paula Fernandes Monteiro
Sampaio Carvalho

outubro de 2022

Direitos de Autor e Condições de Utilização do Trabalho por Terceiros

Este é um trabalho académico que pode ser utilizado por terceiros desde que respeitadas as regras e boas práticas internacionalmente aceites, no que concerne aos direitos de autor e direitos conexos.

Assim, o presente trabalho pode ser utilizado nos termos previstos na licença abaixo indicada.

Caso o utilizador necessite de permissão para poder fazer um uso do trabalho em condições não previstas no licenciamento indicado, deverá contactar o autor, através do RepositóriUM da Universidade do Minho.

Licença concedida aos utilizadores deste trabalho

Acknowledgments

A minha dissertação foi uma jornada longa, e conheci muitas pessoas pelo caminho, e agarrei-me, entretanto, a outras já conhecidas, e queria agradecer a esse tal monte de pessoas aqui.

Queria agradecer ao professor Tony, por tudo o que me ensinou ao longo da minha dissertação, por ter-me ajudado a ser um melhor cientista, tanto a nível de dedicação como a nível de responsabilidade. Também lhe queria agradecer pela sua disponibilidade ao longo da minha jornada e por sempre me tentar motivar ao máximo para terminar este trabalho. Queria agradecer à professora Paula também, pelas palavras motivadores e preocupação que demonstrou sempre que pudemos falar.

Para o Augusto vai o meio maior dos obrigados, que foi o meu "mentor" de laboratório, por me ensinar a como trabalhar no laboratório e por estar sempre disponível a ajudar. Queria agradecer também o ambiente alegre que sempre trouxe a todos os laboratórios em que punha os pés, criava sempre um ambiente mais feliz e motivador em qualquer laboratório.

Também quero dar o meu obrigado a toda as pessoas que se cruzaram comigo pela Micro II, IB-S e LBM, e que sempre me fizeram sentir acolhido apesar de andar constantemente a saltar de um lado para o outro. Também gostava de agradecer a todo o departamento e técnicos pelo ambiente que criam.

Para os meus colegas de mestrado também deixo um obrigado, especialmente à Cristina e Pedro, que sempre me acompanharam durante ambos os anos de mestrado e foram sempre meus bons amigos e uma companhia interminável.

Queria agradecer ao Bano e a Machadinho, pela companhia e apoio durante este ano que vivemos juntos, ao Simão e ao Ricardo pelos intermináveis cafés e companhia, e a todos os outros amigos que estiveram presentes durante esta minha jornada.

Para a minha família, quero agradecer aos meus pais por me apoiarem incondicionalmente durante estes 2/3 anos e por terem sempre uma palavra carinhosa sempre que eu estava frustrado. Queria agradecer ao meu irmão também, Rafael, por toda a disponibilidade que sempre mostra quando eu preciso de algo, por todas as boleias, por toda a ajuda e apoio no geral, é clichê, mas é mesmo o melhor irmão que poderia pedir. Queria agradecer também à Andreia e à minha recém-nascida afilhada, Inês. E por final, um agradecimentozinho à minha gatinha Isabel, por todo o amor que transpira quer por ronronares quer por arranhadelas.

Statement of Integrity

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Quitinase 3 da Superfície da Parede Celular de *Candida albicans*: Produção, Purificação e Caracterização

Resumo

Candida albicans é uma das principais causas de infeções fúngicas a nível mundial e é a terceira causa mais comum de infeções nosocomiais da corrente sanguínea. É considerada um agente patogénico oportunista, que consegue causar infeções superficiais e crónicas, e em pacientes com imunidade comprometida pode levar a infeções graves com taxas de mortalidade elevadas. Num estudo prévio relativo a infeções por parte deste organismo, foi relatado que uma quitinase da superfície da parede celular não caracterizada, quitinase 3 (Cht3p), de *C. albicans*, demonstrou capacidade de induzir em ratos uma resposta imunológica contra estas infeções. O presente estudo visou investigar aprofundadamente esta quitinase, nomeadamente desenvolvendo protocolos de produção heteróloga desta proteína e purificação da mesma, assim como caracterizar esta enzima pura, focando na sua atividade, estabilidade e função.

Neste estudo, a levedura *Pichia pastoris* GS115 foi utilizada com sucesso para a expressão extracelular de Cht3p recombinante com um rendimento de ~100 mg/L de produção. Para a remoção de proteínas contaminantes vestigiais e pigmentos presentes, foi desenvolvido um protocolo de purificação contendo 4 etapas, incluindo um tratamento com carvão ativado, uma cromatografia de interações hidrofóbicas, uma precipitação com sulfato de amónio e uma cromatografia de filtração em gel, com um rendimento de ~50 %. A caracterização desta enzima demonstrou-a ativa e estável numa ampla gama de temperaturas e pHs, com ótimos a 40-50 ºC e pH 4.5, respetivamente. Esta enzima demonstrou ser uma verdadeira quitinase, atuando especificamente em polissacarídeos de quitina e de quitosana. Usando quitina como substrato, demonstrou-se que os parâmetros cinéticos de Cht3p (Km=37.5 $\pm$ 19 mg/mL, $kcat$ =7.5 s^{-1}) estão dentro da gama demonstrada por outras quitinases. Para além disso, foi demonstrado que esta tem uma preferência por quito-oligossacarídeos de cadeia longa, assim como uma ausência de atividade em dímeros e trímeros de quito-oligossacarídeos. Os principais produtos da hidrólise são monómeros, dímeros e trímeros de quito-oligossacarídeos, demonstrando assim que esta enzima é uma endoquitinase, clivando aleatoriamente ligações internas β-(1→4) da quitina e quito-oligossacarídeos. Estes resultados irão suplementar o uso desta enzima como um antígeno no futuro desenvolvimento de uma vacina contra infeções de *C. albicans*.

Palavras-chave: *Candida albicans*; Caracterização; Quitinase 3; Produção; Purificação.

Candida albicans Cell Wall Surface Chitinase 3: Production, Purification, and Characterization

Abstract

Candida albicans is one of the most common causes of fungal infections worldwide and is the third most common cause of nosocomial bloodstream infections. It is an opportunistic pathogen that can cause both superficial and chronic infections, and in patients with a compromised immunity can lead to life threatening systemic infections with high mortality rates, reaching 50% in many cases. In a previous study aiming to identify novel vaccine antigens against infection by this organism, an uncharacterized cell wall surface chitinase, chitinase 3 (Cht3p), from *C. albicans*, was reported to induce an immune response against infection in treated mice. The present project aimed to investigate this chitinase further, namely, to develop heterologous protein production and purification protocols, and to characterize the purified enzyme with a focus on determining its activity, stability, and function.

In this study, Pichia pastoris GS115 was successfully used as host for the extracellular expression of recombinant Cht3 with a production yield of ~100 mg/L of production culture. For removal of the trace protein contaminants and pigments present, a 4-step purification protocol with a yield of ~50 %, including activated carbon treatment, hydrophobic interactions chromatography, ammonium sulfate precipitation, and gel filtration chromatography, was developed. Characterization of the enzyme showed it to be active and stable over a relatively broad temperature and pH range, with respective optima at 40-50 ℃ and pH 4.5. The enzyme was shown to be a true chitinase, being specific for the polysaccharide chitin and the chitin derivative chitosan. With chitin as substrate, it displayed kinetic parameters (Km=37.5 ± 19 mg/mL, $kcat$ =7.5 s^{-1}) within the range of those reported for other characterized chitinases. Furthermore, it was shown to have a preference for longer chain chito-oligosaccharide substrates, with no activity on chito-oligosaccharide dimers and trimers. The principal end-products of hydrolysis are chito-oligosaccharide monomers, dimers, and trimers, thereby demonstrating this enzyme as being an endochitinase, randomly cleaving internal β-(1→4) linkages of chitin and chito-oligosaccharides. The results of this study will aid in the further application of this enzyme as a vaccine antigen against *C. albicans* infections.

Key words: *Candida albicans*; Characterization; Chitinase 3; Production; Purification.

Index

List of Abbreviations and Acronyms

AA – Auxiliary Activity

AC – Activated Carbon

APS – Ammonium Persulfate

BMGY – Buffered Glycerol Complex Medium

BMMY – Buffered Methanol Complex Medium

BSA – Bovine Serum Albumin

CAPS – N-cyclohexyl-3-aminopropanesulfonic Acid

CAZy – Carbohydrate-Active enZYmes

CBM – Chitin Binding Domain

CC – Colloidal Chitin

CE – Carbohydrate Esterase

CHES - N-cyclohexyl-2-aminoethanesulfonic Acid

CFP – Cellulose Filter Papers

Cht – Chitinase from *Candida albicans*

CMC – Carboxymethyl Cellulose

COS – Chito-Oligosaccharides

Cts – Chitinase from *Saccharomyces serevisiae*

Da – Dalton

DOBAB – Dioctadecyldimethylammonium Bromide

DNS – 3,4-Dinitrosalicylic Acid

FPLC – Fast Protein Liquid Chromatography

G418 – Geneticin

GFC – Gel Filtration Chromatography

GH – Glycoside Hydrolase

GlcNAc – N-Acetyl-Glucosaminide

GRAS – Generally Recognized as Safe

GT – Glycosyl Transferase

HEPES – 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic Acid

HIC – Hydrophobic Interactions Chromatography

HPLC – High-performance Liquid Chromatography

LPS – Lipopolysaccharides

MES – 2-(N-morpholino)ethanesulfonic Acid

MO – Monoolein

MOPS – 3-(N-morpholino)propanesulfonic Acid

MWM – Molecular Weight Marker

OD – Optical Density

PES – Polyethersulfone

p*Ka* – Logarithm of the acid dissociation constant ($-\log_{10}K$a)

PL – Polysaccharide Lyase

Sap – Secreted Aspartyl Proteinase

SDS-PAGE – Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

TAPS - N-[Tris(hydroxymethyl)methyl]-3-aminopropanesulfonic Acid

TCA – Trychloroacetic Acid

TEMED – Tetramethylethylenediamine

List of Figures

List of Tables

CHAPTER 1. INTRODUCTION

1.1. Endochitinases

Endochitinases (E.C 3.2.1.14) are glycoside hydrolyses that catalyze cleavage of the internal N-acetyl-β-D-glucosaminide (1→4)-β-linkages of the structural polysaccharide chitin and chito-oligosaccharides (Adrangi and Faramarzi, 2013; Hartl et al., 2012). Their accepted name is chitinase, but other alternative names have been used such as 1,4-beta-poly-N-acetylglucosaminidase, chitodextrinase, and poly-beta-glucosaminidase.

Chitinases are found in nature in a diverse range of organisms, such as fungi, bacteria, viruses, higher plants, and animals (Hamid et al., 2013), with bacteria and fungi being the main chitin decomposers. In their hosts, they have been shown to have a wide range of functions, including in nutrition, tissue degradation, developmental regulation, pathogenicity, and immune defense (Chen et al., 2020). While bacteria mostly utilize chitinases to obtain energy from chitin degradation, it has also been suggested that these bacterial chitinases, found in roots of cucumber and in tomato plants, might play a role in defense against phytopathogenic fungi in plants (Kavroulakis et al., 2010; Lu et al., 2018), and, more recently, have been linked to invasive listeriosis in humans by compromising their immune systems (Halbedel et al., 2019). Fungal chitinases have been shown to play crucial roles in parasitism, degradation, morphogenesis, cell division, autolysis, and nutrition (Langner and Göhre, 2016; Rathore and Gupta, 2015; Sharma et al., 2018; Shimono et al., 2002), while viral chitinases are important in weakening the host barrier structure and facilitating virus infection (Hawtin et al., 1997). Plant chitinases are present in stems, roots, latex, leaves, fruits, and seeds (Malik, 2019), being produced for physiological plant processes, including embryogenesis, and for self-defense in response to environmental stresses such as wounding or phytopathogen's attack (van Loon et al., 2006; Wu and Bradford, 2003). In insects, chitinases are involved indirectly in cuticle synthesis and play defensive roles against their parasites (Koga et al., 1997). Mammalians, such as humans, have also been reported to possess chitinases in their stomach (Paoletti et al., 2007), blood (Renkema et al., 1995), and in cartilage (Hakala et al., 1993), having anti-pathogenic functions through catalyzing the degradation of chitin upon inhalation or ingestion (Tang et al., 2015). Finally, recent studies have indicated chitinases and/or chitinase like proteins (lacking chitinase activity) as being important biomarkers for various diseases, such as inflammatory, autoimmune, and neurologic diseases, metabolic disorders, cancer, and asthma, etc. (Di Rosa and Malaguarnera, 2016; Mazur et al., 2021; Nagpure et al., 2014; Yeo et al., 2019).

1.2. The Substrate: Chitin

Chitin is the recognized substrate of chitinases, but in addition, they can also act on chitosan, a partially deacetylated chitin (Adrangi and Faramarzi, 2013; Hartl et al., 2012), while affinity towards peptidoglycans has also been reported for some chitinases (Bokma et al., 1997).

Chitin, initially discovered in 1811, is generally considered as the second most abundant natural polysaccharide after cellulose (Crini, 2019). It is an insoluble linear homopolymer composed of N-acetyl-glucosamine (GlcNAc) residues covalently linked by β-(1–4) glycosidic bonds (Figure 1A). Structurally, chitin can occur in three different allomorph forms: α-chitin (the most common), mainly present in fungi, crustaceans, and insects; β-chitin, present in tubes synthesized by the tube worms *vestimetiferan* and *pogonophoran*, squid pens, *Aphrodite chaetae*, and in monocrystalline spines excreted by the diatom *Thalassiosira fluviatilis*; and γ-chitin, found in *Loligo* squid pens and in several insect species (Akakuru et al., 2018; Jang et al., 2004; Kaya et al., 2017). These different forms present different orientations and packing of the chitin molecular chains, with α-chitin presenting antiparallel arrangements (Figure 1B), β-chitin presenting parallel arrangements (Figure 1C), and γ-chitin presenting an arrangement based on one antiparallel chain for every two parallel chains (Figure 1D) (Adrangi and Faramarzi, 2013; Jang et al., 2004; Seidl, 2008).
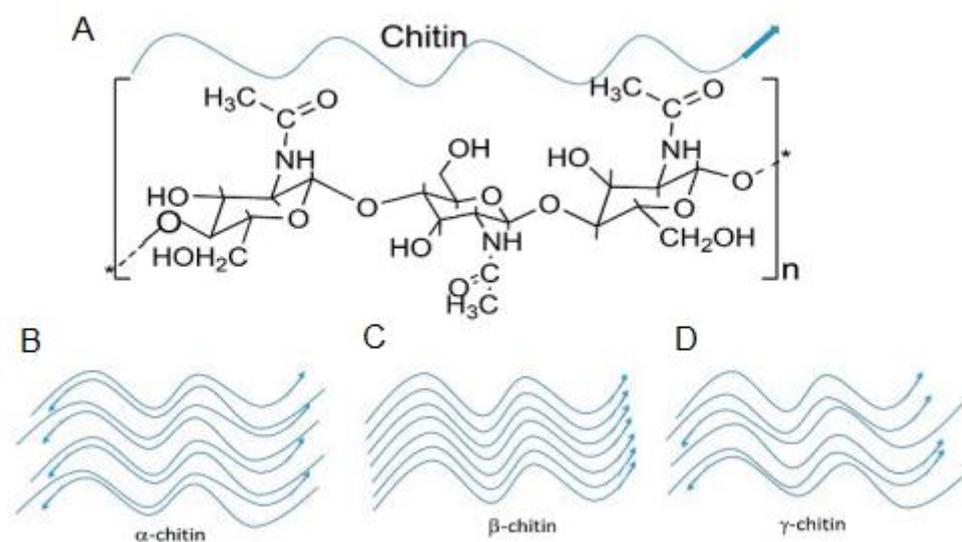

Figure 1. Chitin Structure. Schematic representations of chitin structure showing the β-(1–4) linked N-acetyl-glucosamine residues (A), the α-chitin domain conformation (B), the β-chitin domain conformation (C), and the γ-chitin domain conformation (D). Adapted from Roy et al., 2017.

In nature, natural chitin doesn't typically exist as free chitin fibers, but is commonly found covalently bound to proteins, glucans, and/or calcium carbonate. In arthropods in general, chitin is packed in fibers of 18 to 25 chains arranged in an antiparallel manner, and associated with proteins, forming a Bouligand-type structure (Minke and Blackwell, 1978) which plays a supportive and protective role. Fungal chitin content and localization can vary among fungi but is usually found in the internal layer of the cell wall, forming a scaffold with β-1,3-glucan, and with the external layer being formed by other polysaccharides and glycoproteins (Adams, 2004). Here, in fungi, the primary role of chitin is in maintaining structural integrity, with other roles being suggested such as epithelial adhesion, linkage between the cell wall and capsule, and antifungal resistance (Goldman and Vicencio, 2012).

Complete chitin degradation to monomers requires the concerted action of a variety of enzymes: endochitinases (EC 3.2.1.14), exochitinases (EC 3.2.1.200 and EC 3.2.1.201), N-acetyl-β-glucosaminidases (EC 3.2.1.52), and, to a lesser extent, endo-chitodextinases (EC 3.2.1.202), with lytic chitin monooxygenases sometimes also playing a role (EC 1.14.99.53). This latter group of oxido-reductases assists the action of the hydrolases via the oxidative breakdown of the crystalline structure of chitin to release chitin polymer chains with enhanced accessibility. The endo-acting endochitinases randomly cleave the internal β-(1→4) linkages of chitin and chito-oligosaccharides (COS) (Adrangi and Faramarzi, 2013; Hartl et al., 2012) to produce lower molecular mass COS, multimers of GlcNAc (Sahai and Manocha, 1993). On the other hand, the exochitinases systematically release chitobiose from either the reducing (EC 3.2.1.201) or the non-reducing (EC 3.2.1.200) ends of chitin and COS. N-acetyl-β-glucosaminidases are also exo-acting, but catalyze the release of terminal GlcNAc monomers from the non-reducing end of COS (Lorito et al., 1993; Sahai and Manocha, 1993). Finally, the endo-chitodextinase, which are rare enzymes currently only identified in members of the *Vibrionaceae*, act in a similar endo-manner to endochitinases but are only active on COS, with no activity on chitin being detected.
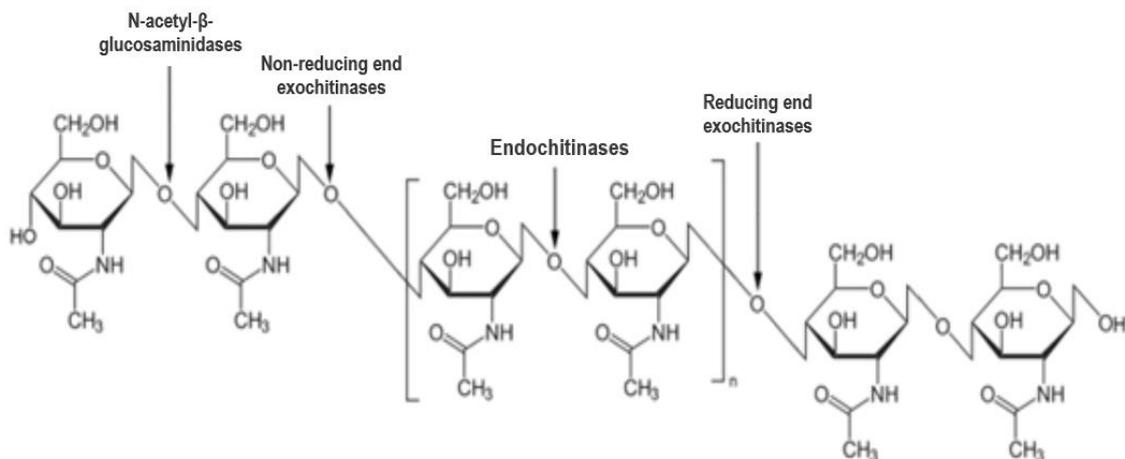
Figure 2. Chitinolytic enzymes. Mode of action of endochitinases (EC 3.2.1.14), non-reducing end exochitinases (EC 3.2.1.200), reducing end exochitinases (EC 3.2.1.201), and N-acetyl-β-glucosaminidases (EC 3.2.1.52). Adapted from Mathew et al., 2021.

1.2.1. Chitin: Market Value and Applications

Market predictions for the global value of chitin are 2,942 million US dollars by 2027, up from approximately 893 million US dollars in 2017, and the market for the chitin monomer glucosamine is expected to reach 1,840 million US dollars by 2027 (Vision et al ., 2017). The main commercial sources for chitin are shrimp and crab shells, while chitin and chitin derivates from a fungal source have also recently joined the market (Chibio Biotech, 2021; KitoZyme, 2021; Vegan, 2021).

Chitin and its derivates, especially chitosan, produced via the deacetylation of chitin by specific chitin deacetylases (EC.3.5.1.41) (Dutta et al ., 2003), have a wide range of potential applications in numerous areas. They are biodegradable and biocompatible and are available as a low-cost waste product of the fisheries industries. Indeed, approximately 6-8 million tons of shell waste are accumulated each year by the fisheries industries, of which chitin constitutes 20-58% of the overall dry weight (Chen et al ., 2016; Wang and Chang, 1997). Hence, its utilization and valorization would help solve current ecological, health, and economical problems associated with fisheries wastes and their disposal. Chitin and its derivatives can be used in carbon fixation due to their compostable character (Xie et al., 2006), and have the potential to be used in agriculture, as fertilizers and pesticides (Mukarram et al., 2021; Shamshina et al., 2019), they can also be used in the food industry, as food preservatives and quality enhancers, as in packaging (Lekjing, 2016; Malinowska-Pańczyk et al., 2009; Mat Amin and Kang, 2013). Furthermore, they've also been reported to have applications in cosmetics,

water purification, and health care (Barik et al., 2020; Morganti et al., 2013; Morin-Crini et al., 2019). Such applications are facilitated by the ease in which they can be processed into hydrogels, films, and microspheres (Freier et al., 2005; Frindy et al., 2017; Ladet et al., 2008). As such, they can be used in wound dressing, tissue engineering, bone regeneration, and in gene and drug delivery (Ali and Ahmed, 2018; Busilacchi et al., 2013; Muzzarelli et al., 2012; Sudheesh Kumar et al., 2012). Importantly also, they have been shown to display antimicrobial, antitumor and antioxidant activities (Elieh-Ali-Komi and Hamblin, 2016; Younes et al., 2014), thereby opening up high value markets for their utilization.

1.3. Classification of Chitinases

In addition to classification by their site of action into either endo- or exo-acting enzymes, two other classification systems have been commonly used for chitinases. The first is based on the primary sequence of the whole protein and takes into account domain architecture, sequence features, and sequence homology, namely the presence/absence of various domains, the number and positioning of these, and their degrees of identity. Indeed, chitinases are often multi-domain structures, containing in addition to the catalytic domain(s), carbohydrate/chitin binding domain(s) (CBM), lysin motif(s), FnIII domain(s), transmembrane domain(s), serine-threonine-rich domain(s), and signal peptides. With this classification system, different classifications are used for different source organisms. The system was initially developed for classification of plant chitinases (Fukamizo, 2000; Hamel et al., 1997), for which 7 classes (I to VII) are currently known. Four subgroups (A-D) are suggested for fungi, eleven for insects (I to h), and three for bacteria (A-C) (Chen et al., 2020).

Recently, a more simplified classification system has become widely adapted for chitinase classification. This system, known as the CAZy (Carbohydrate-Active enZYmes) classification system has been developed for the classification of enzymes that catalyze the breakdown, biosynthesis or modification of carbohydrates or glycoconjugates, i.e., glycoside hydrolases (GHs), glycosyl transferases (GTs), polysaccharide lyases (PLs), carbohydrate esterases (CEs), and auxiliary activities (AAs). Enzymes are grouped into families based on the primary sequence of the catalytic domain only, and families with related structural folds are further grouped into clans. As the primary sequence of enzymes determines their 3D-structure, function, specificity, and mechanism of action, this system groups proteins with similar properties into families. For GHs, currently 173 glycoside hydrolase families have been

6

described (http://www.cazy.org/Glycoside-Hydrolases.html), with chitinases being found in GH families 18, 19, 23, and 48 (Drula et al., 2022).

Chitinases from GH family 18 belong in clan GH-K and comprise fungal, mammalian, insect, bacterial, and viral chitinases, as well as both class III and V plant chitinases. Both non-processive endochitinases and processive exo-chitinases have been reported for this family. The catalytic domain of members of this family display a $(\beta/\alpha)_8$ barrel fold in which the substrate-binding clefts is lined with aromatic residues (Horn et al., 2006). Hydrolysis is carried out with retention of the anomeric configuration, using a double-displacement mechanism that is distinguished by the utilization of the *N*-acetamido carbonyl oxygen of the substrate as a nucleophile, instead of the most common enzyme-derived nucleophile, in what is called neighboring group participation (Terwisscha van Scheltinga et al., 1995). Furthermore, in members of this family, the proton donor has been shown to be a conserved glutamic acid residue.
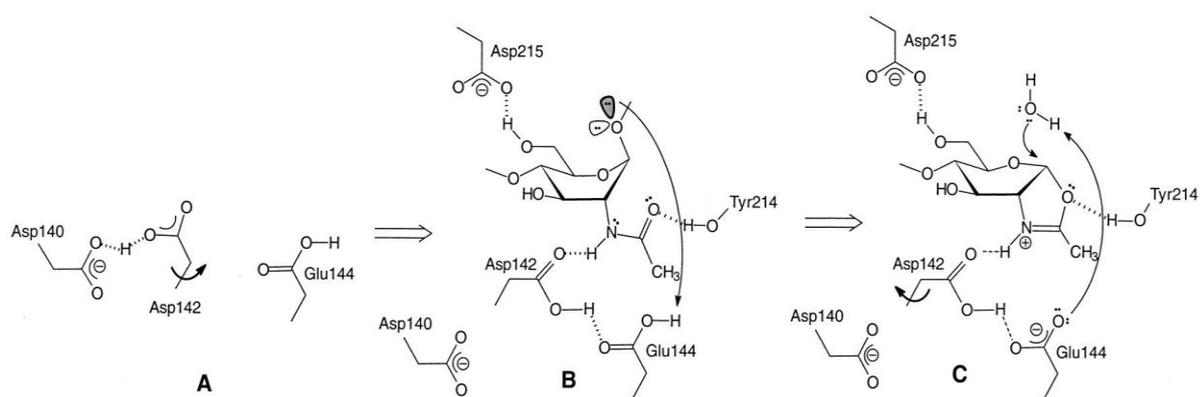
Figure 3. Catalytic retaining mechanism of GH-18 family enzymes proposed for ChiB from *Serratia marcescens*. A. The Asp142 and Glu144 are very distanced and therefore unable to interact. B. The binding of the sugar distorts the pyranose ring to a boat or skewed boat conformation, rotating Asp142 in the direction of Glu144 and allowing hydrogen bonds to occur. C. The oxazolinium ion intermediate is hydrolyzed, intermediating the protonation of Glu144 and taking Asp142 back to its origin position in A, sharing a proton with Asp140. Mechanism proposed by Tews et al., 1997 and by Brameld and Goddard, 1998, and optimized by van Aalten et al., 2001. Taken from van Aalten et al., 2001.

GH family 19 chitinases comprise bacterial chitinases, namely actinobacteria and purple bacteria (Udaya Prakash et al., 2010), as well as class I, II, and IV plant chitinases. Their catalytic domain displays a fold that is known as a lysozyme-like fold, they employ an inverting mechanism of action (Fukamizo et al., 1995; Iseli et al., 1996) in which two glutamic acids are believed to be the essential catalytic residues (Figure 4) (Andersen et al., 1997; Hart et al., 1993; Hoell et al., 2006; Huet et al., 2008) and are thought to be mainly non-processive endo-acting

enzymes (Heggset et al., 2009; Sasaki et al., 2006). Furthermore, GH family 19 chitinases, especially those lacking a CBM, are often observed to be less active on crystalline chitin than are GH family 18 chitinases.
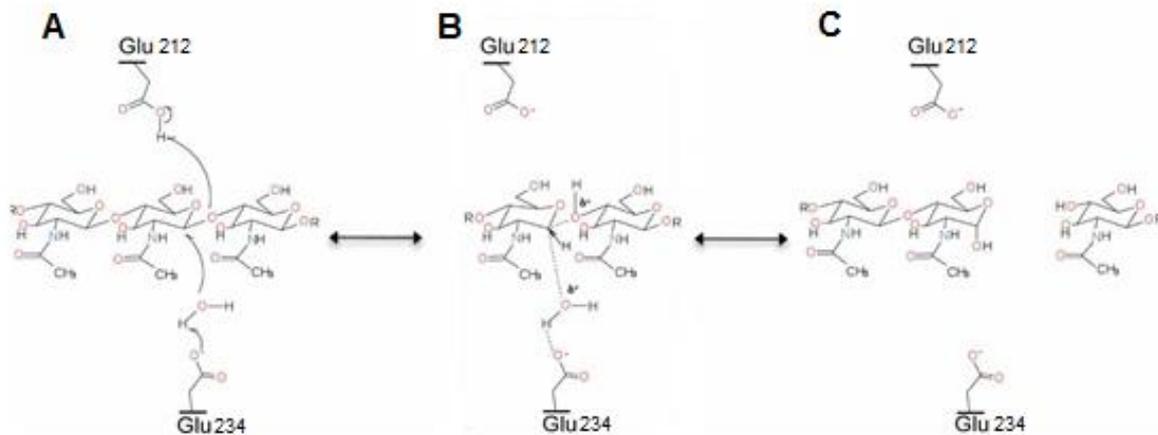
Figure 4. Catalytic inverting mechanism of GH-19 family enzymes proposed for the chitinase OsChia1c from rice, *Oryza sativa* L.. A. Glu212 activates the sugar by donating a proton to its O1, moving the loop and bringing Glu234 closer to the substrate enables the removal of the proton from the water molecule. B. The activated water's O attacks the sugar, inverting its configuration. C. Release of the hydrolyzed product. Adapted from Ubhayasekera, 2011.

To date, only one GH23 bacterial chitinase has been identified, that from *Ralstonia* sp. A-471, comprising a N-terminal chitin-binding-domain linked to a C-terminal catalytic domain homologous to the g-type lysozymes belonging to this family (Ueda et al., 2009). Nevertheless, no lysozyme activity, as determined by a lack of activity on the cell wall of *Micrococcus lysodeikticus*, has been detected, yet an endochitinase action was clearly shown. Like GH family 19 members, GH family 23 members also display an α+β lysozyme like fold and are thought to use an inverting mechanism (Helland et al., 2009; Hirakawa et al., 2008) of action, using a glutamic acid as its catalytic proton donor.

Only one insect chitinase, isolated from the leaf beetle *Gastrphysa atrocyanea*, has been described in GH family 48, whereas all other insect chitinases are restricted to GH family 18 (Fujita et al., 2006). The catalytic domain of GH family 48 enzymes displays an $(\alpha/\alpha)_6$ barrel fold and belongs to clan GH-M. These enzymes are described as employing an inverting mechanism, using one as yet unknown basic residue to extract the proton from the nucleophilic

water molecule, and an acidic proton donor, thought to be a glutamate residue, to protonate the glycosidic oxygen atom (Parsiegla et al., 2008).

1.3.1. Applications of Chitinases

Chitinases from several different sources have been reported to have huge commercial potential. They are used not only for their direct hydrolysis effect, but also for the products they produce. They have found application in agriculture, nutrition, medicine, industry and in the production of value-added products, namely COS and GlcNAc, which themselves have numerous potential uses.

Phytopathogenic bacteria and fungi are major causes of agriculture crops and vegetable losses every year, with fungi alone representing more than 70 % of crop diseases which include white rot, leaf mold, black mold, rust, wilt, basal stem rot, and southern leaf blight. Chitinases have been reported to possess strong antifungal activity and are already being used in crop protection against fungal damage (J. Liu et al., 2019). They have been shown to be active against *Fusarium roseum* in the treatment of dry rot in potato tubers (Sadfi et al., 2001), against *Fusarium oxysporum* in the treatment of vascular wilt in date palm (Djenane et al., 2017), and against *Sclerotinia sclerotiorum* and *Sclerotinia minor* in lettuce drop disease (Shrestha et al., 2015). This antifungal activity is thought to be due to the inhibition of mycelial growth, the suppression of spores germination, the reduction of germ tube elongation, and/or the lysis of hyphal tips (Öztopuz, et al., 2018), by affecting several parts of the complex fungal body and in particular the chitin in the cell wall. In a similar manner, this antifungal activity can also be of benefit in the food industry, wherein chitinases, or chitinase producing organisms, can be used in place of chemical preservatives in food preservation against fungal spoilage (Castillo et al., 2016; Sharma et al., 2009).

Insects pests are another major agriculture problem, and chitinases active against the chitin in the cuticle and peritrophic matrix of these (Berini et al., 2018), offer opportunities as low toxicity, biodegradable, eco-friendly, biopesticides (Bonanomi et al., 2018). Indeed, they have already been shown to be effective against tea mosquito bug (100 % mortality) (Suganthi et al., 2017) and *Helicoverpa armigera* larvae (J. Liu et al., 2019).

Regarding medical applications, chitinases have been shown to be effective against several fungal infections, especially when coupled with other treatments, such as the chitinase

coupled with the lactic acid bacterium *Lactobacillus casei*, enabling a much-enhanced antifungal effect on *Candida albicans* (Allonsius et al., 2019). Nematode eggs have been shown to be one of the most resistant biological structures, but have been found to be vulnerable to egg-parasitic fungi as a result of their high abundance of chitin (Halder et al., 2019)(Halder et al., 2019). As an example, Gan and collaborators (2007) demonstrated that the eggs from the nematode *Meloidogyne incognita* had their development significantly hindered by the action of a fungal chitinase and protease which were shown to interact and be more effective against egg infection when coupled. Furthermore, the biopesticide properties of chitinases can be beneficial in control of the spread of disease via an activity against insect vectors, such as a chitinase from *Myrothecium verrucaria* which was shown to be effective in killing of the mosquito *Aedes aegypti* larvae, responsible for dengue and yellow fever (Souza-Neto et al., 2003). Other important potential medical uses for chitinases include as anticancer agents, with chitinases from *Streptomyces griseus* and *Serratia marcescens* being reported to destroy MCF-7 cancer cells and B_{11}-2 xenograf tissue in mice (Pan et al., 2005), in mediating allergic inflammation (Goldman and Vicencio, 2012), and asthma (Shuhui et al., 2009).

The products of chitinase action, COS and GlcNAc, have been found to have numerous potentially high-value applications. Indeed, as already discussed above, the utilization of chitinous wastes, principally produced from aquatic organisms, or their conversion to high value products, is of importance in the aquatic solid waste accumulation problem (Khan et al., 2017), this waste being considered hazardous to both human health and the global climate. Use of enzymes for conversion of chitinous wastes to high value products is more favorable than chemical or physical conversion processes due to a higher specificity and control, the possibility for use of mild conditions, and a reduced environmental impact (Jung and Park, 2014). The principal products of endochitinase action, COS, have been found to have several potential applications: as prebiotics (Selenius et al., 2018), anticancer agents, antimicrobials, and anti-inflammatory compounds. They have been shown to enhance probiotic populations in the ileum and colon (Kong et al., 2014), and have already been used in the preparation of foods such as milk, yogurt, tofu, and some fruit juices (Patel and Goyal, 2011). Anti-obesity properties have also been assigned to COS, with experiments in rats recording an inhibition of the increase in body weight and of the serum levels of total cholesterol and triglycerides under a high-fat diet (Zhou et al., 2020). The antimicrobial activities of COS against food pathogens, such as *Escherichia coli*, *Salmonella typhimurium*, *Shigella dysenteriae*, and *Vibrio cholerae* (Benhabiles et al., 2012), has promoted their use in food preservation. Interestingly also, these

oligosaccharides are also reported to have potential for use in cancer treatment, by enhancing an innate immune response through activation of cytokine secretion and the proliferation of cytotoxic T lymphocytes, with suppression of tumor cells (Mattaveewong et al., 2016; Park et al., 2011). Multiple studies have shown COS to have anti-inflammatory and antioxidant properties, this being believed to be due to inhibition of TNF-α in LPS-stimulated inflammation, as well as by induction of an increase in intracellular glutathione levels, in addition to direct intracellular radical scavenging effects, inhibiting cellular oxidative stress and DNA and protein oxidation (Park and Kim, 2010). Finally, COS are believed to provoke immunostimulatory activities, thereby revealing their potential for use in vaccines (Zhang et al., 2014). The chitin-breakdown monomer-product, GlcNAc, and its deacetylated form, is also seen as a potential high value product, with potential for use as a platform intermediate in the production of bio-based organonitrogen compounds such as amino sugars, heterocyclic compound, and various acids and alcohols (Cao et al., 2022) Furthermore, its use in the production of single cell protein (Nasseri et al., 2011) has also been demonstrated, while it has also been reported to play a positive role in the treatment of osteoarthritis (Shiro et al., 1996), and also possesses antitumor activity on colon carcinoma cell lines (CT26) (Liang et al., 2007; Nagpure et al., 2014).

1.4. Chitinase 3 from *Candida albicans*

The present study is based on a chitinase, titled Chitinase 3 (Cht3p), from *C. albicans*, and this enzyme will be now presented. Chitinase 1 (Cht1p), chitinase 2 (Cht2p), and Cht3p, initially identified by McCreath and collaborators (1995), along with the later identified chitinase 4 (Cht4p) (Selvaggini et al., 2004), comprise the known chitinases encoded by *C. albicans*. The gene of Cht3p, *CHT3*, has an open reading frame of 1704 base pairs encoding a protein of 567 amino acids, with a predicted size of 60,092 Da and predicted isoelectric point of 4.65 (McCreath et al., 1995). Cht3p is a member of glycoside hydrolase family 18, having 1 potential N-glycosylation site (Asn-Xaa-Ser/Thr), and, like Cht2p, has a region near the C-terminus end in which >70 % of the amino acids are either serines or threonines. This domain was also observed for the *S. cerevisae* chitinase Cts1p and has been suggested to act as potential 0-mannosylation sites (McCreath et al., 1995). In further agreement with Cts1p, Dünkler and collaborators (2005) discovered a functional homology between Cts1p and Cht3p, concluding that they might have a similar role in aiding in cell division and in the separation of cells following division. Measurements of chitinase activity in yeast cells of *C. albicans* revealed Cht3p to display the highest activity of the four chitinases produced by this organism and to be

located in the extracellular medium, indicating secretion of this protein (Dünkler et al., 2005). Studies indicated that the *CHT3* gene is expressed periodically in *C. albicans*, with maximal expression in the M/G1 phase of the cell cycle along with the endo-β-1,3-glucanase gene *ENG1* and the β-1,3-glucan-modifying gene *SCW11* (Klis and Brul, 2015). These genes are regulated by the transcription factor Ace2, a regulator of cell separation that controls the expression of M/G1-specific genes (Mulhern et al., 2013; Kelly et al., 2004), further confirming Cht3p's role in cell division, degrading the chitin present in the primary septum between mother and daughter cells (Klis and Brul, 2015).

Carneiro and collaborators (Carneiro et al., 2015) have identified Cht3p as a potential antigen against *C. albicans* infections. Mice immunizations with liposomal formulations containing Cell Wall Surface Protein extracts of *C. albicans* led to the detection of IgG1 and IgG2 production and IFN-γ and IL-17A secretion in treated mice, suggesting induction of a TH1/th17-TYPE response. Western blot analysis of the antibodies produced indicated Cht3p as the principal antigen inducing the immune response. To investigate this further, utilization of Cht3p in a vaccine formulation with a delivery system and adjuvants to assure efficient antigen delivery and enhance the immune response was investigated. A variety of delivery systems are currently known but the principal systems used are based on liposomes, polymer-based micro- and nanoparticles, immune stimulation complexes (ISCOMS), and virosomes, etc. (García and De Sanctis, 2014; Gregory et al., 2013; Joshi et al., 2013; Mohammed et al., 2010). Of these, liposomes have the additional advantage of functioning as an adjuvant and are the main delivery system currently used. Liposomes are regarded as highly valuable carrier systems due to their versatility, effectively improving antigen stability and presentation to immunocompetent cells according to their specific characteristics such as composition, size, and surface properties. In addition, they provide a controlled and slow release of antigens and demonstrate the capacity to pass through several biological barriers, including mucosa and skin (Schwendener, 2014). Indeed, cationic liposomes, constituted by positively charged lipids, have already been widely used as vaccine and drug delivery systems (Davidsen et al., 2005; Ingvarsson et al., 2013). A good example of these lipids is the cationic surfactant dioctadecyldimethylammonium bromide (DODAB), a synthetic amphiphilic lipid, with a positively charged dimethylammonium group (head) attached to two hydrophobic 18-carbon alkyl chains (tail). However, cationic liposomes have been associated with *in vivo* cytotoxicity (Carmona-Ribeiro, 2003; Lincopan et al., 2009) and aggregation in salt buffers (Ogris et al., 1998), despite assembling into closed liposomal vesicular bilayers (Silva et al., 2008). To overcome this problem, 1-monooleoyl-rac-glycerol

(MO) has been used as a stabilizer to enhance fluidity via induction of lipid chain mobility (Silva et al., 2014), with successful results being observed for use as a DOBAB:MO mammalian cell transfection system and as an *in vivo* gene silencing system (Oliveira et al., 2014; Silva et al., 2011). This DOBAB:MO system has also been employed successfully with Cht3p (Carneiro et al., 2015), and its use with Cht3p associated with a well-studied antigen, Sap2p, has also been recently investigated (Barbosa et al., 2017). Ongoing work is currently focused on continuing the study to better understand and develop this system via optimization of the protein formulation and use in in vivo rat trials.

1.5. Heterologous Protein Production

Cht3p has been identified as a potential antigen of interest and thus further studies of this protein and its potential for use in vaccine formulations are required. For this, high levels of pure protein are required. Production from the wild type host, *C. albicans*, is limited by safety concerns associated with the pathogenic nature of this organisms, as well as low protein yields, and high levels of other contaminants. Heterologous protein expression, in which the gene encoding the protein of interest is introduced into another host species in such a way that the host expresses the protein of interest, offers advantages of enabling the use of non-toxic hosts, enabling potentially high protein productivity, and use of cells with high growth rates, a capacity to attain high cell densities with inexpensive media, and a propensity to scale up to industrial scale production processes (Yesilirmak and Sayers, 2009).

The host organism most used in heterologous protein production is the gram-negative bacterium *E. coli*. It is a very well-established host for this purpose, with the availability of multiple host and plasmid variants, and is commonly used in numerous large-scale, production systems for industrial and pharmaceutical proteins. It is fast growing, and relatively inexpensive media can be used, but it presents the disadvantage of an accumulation of endotoxins (LPS), which are pyrogenic in humans and, hence requires an additional purification step to completely remove these endotoxins (Petsch and Anspach, 2000). Previous studies by Barbosa and collaborators (2017) investigated the use of *E. coli* for heterologous expression of Cht3p but numerous attempts with the pET22b(+)-*E. coli* BL21(DE3) (Novagen) expression system proved unsuccessful. Constructs for both intracellular and extracellular production were prepared but Cht3 production was not detected, neither by activity analyses nor SDS-PAGE analyses. Various process parameters were investigated, including production medium, growth

temperature, inducer concentration, time of induction, induction period, and aeration rate, but Cht3p production was not detected. It is postulated that this may be due to the inability of *E. coli* to perform post-translational modifications (El-Battari et al., 2003; Rosano and Ceccarelli, 2014), such as glycosylation, or due to differences in codon usage preferences. Indeed, as indicated above, Cht3p has numerous potential N-glycosylation and 0-mannosylation sites, which may be necessary for proper protein folding. Furthermore, comparison of *E. coli* codon usage and analysis of the Cht3p sequence indicates a reduced Codon Adaptation Index (CAI: 0.65). This would indicate a reduced suitability of the Cht3p gene for expression in this host (Terpe, 2003), with potential for translational stalling, premature translation termination, translation frameshift, and/or amino acid misincorporation (Kurland and Gallant, 1996).

Due to the inability to produce Cht3p in *E. coli*, attention was turned to eukaryotic expression systems, namely yeasts, which may be better suited to expression of the eukaryote derived Cht3p. Yeasts, such as *Saccharomyces cerevisiae* and *Pichia pastoris*, present several advantages over bacterial expression platforms; these include the ability to carry out eukaryotic post-translational modifications such as glycosylation, the possibility for secretory expression, and an increased safety, these hosts being classified as GRAS hosts (Generally Recognized As Safe). While *S. cerevisiae* is a well-studied organism suited to heterologous protein expression, it often faces problems related with hyper-glycosylation of proteins, and a fermentative mode of respiration, translating in a low growth and, consequently, lower yields (Liu et al., 2012).

Pichia pastoris, also known as *Komagataella pastoris*, is a methylotrophic yeast with a similar codon usage to *C. albicans*. It has the capacity to attain high cell densities with minimal media (Veenhuis et al., 1983; Wegner, 1990), and to express high concentrations of recombinant protein, either intra- or extra-cellularly (Cereghino and Cregg, 2000), with little or no contaminant endogenous proteins being directed to the extracellular environment. It can perform higher eukaryotic protein modifications, including transglycosylation, disulphide bond formation, and proteolytic processing (Cregg et al., 2000), and is apt for high-frequency DNA transformation and cloning by functional complementation. A variety of variants are available, with some being metabolically engineered to present various types of post-translational modifications patterns, thereby making them suited to a wide range of target proteins (Laukens et al., 2015; Wildt and Gerngross, 2005).

Barbosa and collaborators (2017) used *P. pastoris* GS115 for the successful production of extracellular Cht3p in a glycosylated and active form. The Cht3p gene was cloned in the

expression plasmid pPIC9K and multiple plasmid-construct copies integrated into the *P. pastoris* GS115 genome. This expression system makes use of the strong inducible alcohol oxidase 1 (A0X1) promotor (P_{A0X1}), that can be induced by methanol to direct expression of the target protein to as much as 30% of the total protein content (Daly and Hearn, 2005). Furthermore, the system enables for the incorporation of an N-terminal α-mating factor secretion signal peptide for extracellular protein production. Finally, the system also facilitates multiple integration events, thereby enabling constructs with multiple numbers of gene copies and hence potentially higher production levels (Aw and Polizzi, 2013). *P. pastoris* is known to produce low levels of extracellular endogenous proteins and indeed SDS-PAGE analyses indicated extracellular Cht3p production in a relatively pure form (Barr et al., 1992; Furqan and Akhmaloka, 2019). Nevertheless, closer examination, with higher levels of protein product, indicated the presence of trace amounts of protein contaminants as well as solution pigmentation. The present study is focused on this problem, on developing and optimizing a Cht3p purification protocol while also determining the physicochemical and functional properties of this protein.

CHAPTER 2. OBJECTIVES

The *Candida albicans* cell wall surface chitinase, Cht3p, has been shown to have a high potential for application as a novel vaccine antigen (Barbosa et al., 2017; Carneiro et al., 2015). For further investigation and development of the potential of this enzyme in this application, it needs to be successfully produced, purified, and characterized. Indeed, little is known of the physicochemical and functional properties of this enzyme and the product of the current purification protocol contains trace amounts of protein and pigment contaminants (Barbosa et al., 2017). The current study aims to address these shortcomings by focusing on the following:

- Production of recombinant Cht3p with the previously prepared *Pichia pastoris* GS115-pPIC9K-Cht3 expression construct
- Development of a protein purification protocol enabling removal of protein and pigment contaminants.
- Physicochemical and functional characterization of Cht3p
    - o Determination of the pH and thermal activity of Cht3p
    - o Determination of the substrate specificity of Cht3p
    - o Identification of the hydrolysis pattern of Cht3p

CHAPTER 3. MATERIALS AND METHODS

3.1. Protein Production in *P. pastoris* GS115

The previously prepared recombinant expression construct *P. pastoris* GS115-pPIC9K-Cht3 (Barbosa et al., 2017), containing multiple copies of the pPIC9K-Cht3 vector construct integrated in the yeast host genome, was used for Cht3p production. A schematic representation of this construct and of the multi genome integration mechanism are given in Annexes 1 and 2, respectively.

P. pastoris GS115-pPIC9K-Cht3 growth and Cht3p expression were carried out by batch culture at 30 °C with a rotation speed of 200 rpm and with use of two different media: BMGY (Buffered Glycerol Complex Medium: 1% (w/v) yeast extract (Panreac), 2% (w/v) bacto tryptone (Difco), 1.34% (w/v) yeast nitrogen base w/o amino acids (Difco), 4×10^{-5} % (w/v) biotin, 1% (v/v) glycerol, 100 mM potassium phosphate buffer (KH_2PO_4/K_2HPO_4), pH 6.0), for repression of the A0X1 promotor and to enable biomass accumulation; and BMMY (Buffered Methanol Complex Medium: 1% yeast extract (Panreac), 2% bacto tryptone (Difco), 1.34% (w/v) yeast nitrogen base w/o amino acids (Difco), 4×10^{-5}% (w/v) biotin, 1% (v/v) methanol (Carlo Erba), 100 mM potassium phosphate buffer (KH_2PO_4 and K_2HPO_4), pH 6.0) for induction of protein expression.

The transformant growth and enzyme production protocol used was that recommended by the host/vector manufacturer (Invitrogen), as follows. Firstly, a pre-pre-culture was prepared by inoculation of 25 mL BMGY in a 250 mL Erlenmeyer flask with a single transformant colony and incubation overnight at 30 °C/200 rpm. Following measurement of cell density at 600 nm (OD_{600nm}), this pre-pre-culture was used to inoculate a 50 mL BMGY pre-culture in a 500 mL Erlenmeyer to an initial OD_{600nm} of 0.1. Following another overnight incubation, once the OD_{600nm} reached 2-6 (log-phase of growth), cells were harvested by centrifugation at 3,500 x *g* for 3 minutes. Thereafter, for induction of Cht3p expression, the harvested cells were resuspended at an initial OD_{600nm} of 1.0 in 100 mL BMMY in a 1000 mL Erlenmeyer and incubated for 96 h with addition of 100% methanol to a final concentration of 1% every 24 h. The supernatant was then recovered by centrifugation at 9,500 x *g* for 30 min at 4 °C, filtered through a 0.2 μm polyethersulfone (PES) membrane filter (Whatman) and stored at 4 °C until further use.

3.2. Protein Purification

For purification of the extracellular produced Cht3p, treatment with activated carbon (AC), hydrophobic interaction chromatography, and gel filtration chromatography were investigated and optimized.

3.2.1 Activated Carbon Treatment

Extracellular extracts were incubated with AC for removal of pigment contaminants and, following incubation, the AC was then removed by filtration through a 0.2 µm PES membrane filter (Whatman). For process optimization, the following process parameters were investigated: AC concentration (0.5, 1, 1.5, and 2%), AC manufacturer/product (Sigma-Aldrich granules 4-14 mesh, Merck powder, and Panreac powder), treatment durations (30, 60, and 90 min), incubation temperature (18, 30, and 37 °C), and sample treated (production culture before cells removal, and after cells removal). A one-factor-at-a-time (OFAT) approach was used wherein one process parameter was varied over the range indicated while all other parameters were maintained constant. Levels of pigments were monitored by absorbance measurements at 400 nm with a Shimadzu UV-1700 spectrophotometer. Cht3p concentrations were determined by activity measurements with chitin as substrate according to the modified Bernfeld reducing sugar assay (Bernfeld, 1955) as described in 3.3.1.

3.2.2. Hydrophobic Interaction Chromatography

A phenyl sepharose high performance hydrophobic interaction column (GE Healthcare, 12.5 X 2.2 cm) was employed for hydrophobic interaction chromatography with a Fast Protein Liquid Chromatography (FPLC) system (Pharmacia Biotech) made up of an LCC-501 Plus system controller, two P-500 pumps, a UV MII detector at 280 nm, and a Frac-100 fraction collector. Ammonium sulphate was added to filtered protein samples prior to loading to the HIC column, which itself was pre-equilibrated in the same concentration of ammonium sulphate in 25 mM HEPES buffer, pH 7.5. Samples were eluted by either batch or gradient elution with decreasing ammonium sulphate concentrations in 25 mM HEPES buffer, pH 7.5. For process optimization, various initial ammonium sulphate concentrations (1, 2, and 2.5 M) and flow rates (2 and 5 mL/min) were investigated. Collected fractions were analyzed for Cht3p

concentration by activity measurements with the modified Bernfeld reducing sugar assay (Bernfeld, 1955), and for purity by SDS-PAGE as described in 3.3.1 and 3.2.5, respectively.

3.2.3. Ammonium Sulfate Precipitation

80% ammonium sulfate (Panreac) was used for protein precipitation and concentration as defined previously by Barbosa and collaborators (2017). The salt was added slowly to the sample with constant mixing, and the mixture incubated for 1.5 to 2h at 4 °C with constant mixing before centrifugation at 18,000 rpm for 30 min at 4 °C. The supernatant was discarded, and the precipitate resuspended in 25 mM HEPES buffer, pH 7.5 to a final volume of 1-1.5 mL.

3.2.4. Gel Filtration Chromatography

Gel Filtration Chromatography (GFC) was carried out with a Sephacryl S-100 High Resolution column (GE Healthcare, 95 X 2.2 cm, 360 mL), using the same FPLC device mentioned in 3.2.2.. 25 mM HEPES buffer at pH 7.5 was used as the mobile phase at a flow rate of 1 mL/min.

3.2.5. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Protein purity was assessed by 10% Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) with either silver or copper staining. SDS-PAGE gels composed of a lower running gel and upper stacking gel were prepared as described in Table 1.

Table 1. 10% SDS-PAGE gel contents (1 gel).

Reagents	Running Gel	Stacking Gel
Acrylamide 40%	1.35 mL	0.216 mL
Bisacrylamide 2%	0.75 mL	0.117 mL
0.25 M Tris-HCl 0.2% SDS pH 6.8	2.8 mL	
0.75 M Tris-HCl 0.2% SDS pH 8.8		1.1 mL
dH$_2$O	0.65 mL	0.75 mL
APS 10%	32.5 μL	15 μL
TEMED	5 μL	4 μL

Samples were concentrated before SDS-PAGE gel loading by 10% trichloroacetic acid (TCA) treatment for ~30 minutes at room temperature with occasional vortexing, followed by centrifugation at maximum speed for 15 mins. The precipitated protein pellet was resuspended in 20 µL of 5x loading buffer (10% (w/v) SDS, 40% (v/v) glycerol, 0.2 M tris-HCl, 50 mM β-mercaptoethanol, and 0.5% bromophenol blue), heated for 5 mins at 98 °C, and centrifuged for 25 mins at maximum speed. 10 µL of the supernatant was loaded on the 10% SDS-PAGE gel which was run at 20 mA/gel (Amp constant).

For copper staining of SDS-PAGE gels, gels were incubated with gentle agitation in ~50 mL of 0.3 M $CuCl_2$ solution for 10 minutes, the $CuCl_2$ solution was then removed, and the gel washed with distilled water. The resulting negatively stained gels were viewed against a dark background.

For silver staining of SDS-PAGE gels, gels were initially incubated for 20 minutes in fixing solution (50% (v/v) methanol, 50% (v/v) acetic acid), then 20 minutes in 50% (v/v) methanol, before being washed with dH_2O for 10 minutes and treated for 1 min with 0.02% (w/v) sodium thiosulfate solution. Gels were then washed twice for 1 minute with dH_2O, and then incubated for 20 minutes with 0.1% (w/v) silver nitrate solution. Gels were washed with ~5 mL of 3% (w/v) sodium carbonate/0.1% (v/v) formaldehyde solution and following solution removal were incubated with 40-50mL of the same solution with gently agitation, until the desired band intensity was achieved (3-7 minutes approximately). The staining reaction was stopped by incubation with a 5% (v/v) acetic acid solution for 5 minutes and stained gels were preserved in distilled H_2O.

3.2.6. Protein Concentration

Protein samples were concentrated by ultrafiltration with a Pierce™ PES Protein Concentrator with a 3kDa molecular weight cut off (MWCO) as recommended by the manufacturer (Thermo Scientific). All centrifugations were carried out at 6000 x g and 4 °C.

3.2.7. Protein Quantification

For protein quantification the Pierce™ BCA Protein Assay Kit (Thermo Scientific) was used as recommended by the manufacturer with a Molecular Devices SpectraMax Plus

microplate reader being employed for absorbance readings at 562 nm. A standard curve of BSA concentrations from 25 to 2000 µg/mL was used for concentration determination (Annex 3).

3.3. Protein Characterization

3.3.1. Cht3p Activity Assay: Bernfeld Reducing Sugar Assay

Unless otherwise stated, all Cht3p activity assays were carried out in triplicate using a modified Bernfeld reducing sugar assay (Bernfeld, 1955), as described below:

50 µL of enzyme solution was added to 200 µL of 10 mg/mL Colloidal Chitin (CC) in 0.5 mM McIlvaine's buffer at pH 4.5, briefly vortexed, and then incubated for 5 minutes at 50 ℃. The reaction was stopped by addition of 500 µL of DNS solution (43.8 mM 3,5-Dinitrosalicylic acid, 0.4 M NaOH, 1.1 M potassium sodium tartare tetrahydrate) and briefly vortexing. Samples were then boiled for 10 minutes and finally cooled in ice for 10-15 minutes. Cooled samples were centrifuged for 5 minutes at maximum speed, removing any existing any insoluble fractions, and the absorption at A_{546nm} of the supernatant read with a Thermo Spectronic Genesys 20 visible spectrophotometer.

The colloidal chitin (CC) used in the activity assay was prepared from Chitin flakes (Tokyo Chemical Industry) using a protocol developed by Hsu and Lockwood in 1975 (Hsu and Lockwood, 1975). 50 g chitin from shrimp cells was dissolved in 150-300 mL of cold 12M HCl by stirring for 1 hour. The chitin solution was then slowly filtered through a strainer into 2 liters of cold dH_2O and left overnight at 4 ℃ for chitin precipitation. The suspension was then centrifuged at 11,000 rpm for 8 minutes at 4 ℃, and the pellet washed with water until the pH reached ~3.5 to remove residual HCl from the solution. Chitin was stored at 4 ℃ or lyophilized and stored at room temperature.

3.3.2. Chitinase Kinetics

The kinetic parameters of Cht3p were determined at 50 ℃, pH 4.5 by non-linear regression, using the Michaelis-Menten equation, of initial reaction rates at 5 min, as determined by the Bernfeld reducing assay with 2.5 to 40 mg/mL CC and a Cht3p concentration of 0.3 mg/mL**.**

3.3.3. Temperature Dependence of Activity

The temperature-activity profile of Cht3p was determined by activity measurements at temperatures between 10 and 70 ℃. The Bernfeld reducing sugar assay was used as described above (2.3.1) with a 5 min incubation with 10 mg/mL CC in 0.5 mM McIlvaine's Buffer at pH 4.

3.3.4. pH Dependence of Activity

The pH dependence of activity between pH 1.5 and 12.5 was determined using a buffer mix composed of 100 mM citrate, 20 mM MES, 20 mM TAPS, 20 mM CAPS, 20 mM CHES and 20 mM MOPS (Barroca et al., 2017), with pH adjustment being made with HCl or NaOH as required. Activity was measured with a 5 min Bernfeld reducing sugar assay at 50 ℃ with 10 mg/mL CC and the buffer mix.

3.3.5. Substrate Specificity

To examine the substrate specificity of purified Cht3p, activity on a variety of macrosubstrates was tested with the Bernfeld reducing sugar assay as described in 3.3.1 above. In all cases, 10 mg/mL substrate in 50 mM McIlvaine's buffer at 50 °C and pH 4.5 was used, with incubation for 30 min, and this being increased to 4 hours when an absence of activity was seen. The following substrates were examined: colloidal chitin (total and soluble fractions), chitin powder (obtained by homogenizing the chitin flakes using a Freezer/Mill® Cryogenic Grinder (SPEX® SamplePrep)), chitosan (from shrimp shells, 70% deacetylated), xylan (extracted in-house from *Palmaria palmata*), pectin (practical grade (~50 µm particle size) (Acros Organics)), low-viscosity carboxymethyl cellulose (CMC), avicel (Fluka), and cellulose filter papers (CFP) (Whatman No. 1).

3.3.6. Lysozyme Activity Assay

Lysozyme activity of purified Cht3p, and lysozyme as a positive control, were analyzed according to the "Enzymatic Assay of Lysozyme (EC 3.2.1.17)" by Sigma Aldrich with *Micrococcus lysodeikticus* cells (Sigma Aldrich) as substrate. Optimal conditions for chitinase activity (pH 4.5, 50 ℃, 2 mg/mL enzyme) and lysozyme activity (pH 6.2, 25 ℃, 200-400

units/mL) were employed. Following pre-equilibration of 2.5 mL *M. lysodeikticus* cell suspensions (0.015% [w/v]) at the reaction temperatures, 0.1 mL enzyme solutions were added, the solutions was mixed, and the A_{450nm} recorded over 5 minutes in a temperature-controlled spectrophotometer to determine the maximum linear rate ($\Delta A450/min$).

3.3.7. Activity on Chito-Oligosaccharides

Cht3p activity on the COS chitobiose, chitotriose, chitotetraose, chitopentaose, and chitohexaose (Megazyme) was investigated and the products profiles identified by HPLC. Hydrolysis reactions were carried out with 0.06 mg/mL purified Cht3p and 1 mg/mL COS in 0.5 mM McIlvaine's buffer at pH 4.5 and 50 °C. At 5 minutes, 1 hour and 2 hour, 100 µL samples were taken and the reaction stopped by addition of trichloroacetic acid to a final concentration of 10% and centrifugation for 15 minutes at 17,000 x g. Samples were then diluted tenfold in ultrapure water, filtered through a 0.2 µm PES membrane filter and analyzed on an RSO-Oligosaccharide Ag+ (4%) HPLC column (Phenomenex) at 80 °C with ultrapure water as the mobile phase. An Elite LaChrom (VWR Hitachi) HPLC system with an Elite LaChrom L-2490 RI detector (VWR Hitachi) at 40 °C and the EZChrom Elite 3.3.2 SP2 software was used for HPLC analysis, and data collection, treatment, and analysis. Standard curves with known concentrations (($GlcNAc)_{1-3}$ – 0.25-1 mg/mL; $(GlcNAc)_4$ – 0.5-8 mg/mL; $(GlcNAc)_5$ – 0.5-7 mg/mL; $(GlcNAc)_6$ – 0.5-5 mg/mL) of each of the COS studied were prepared for determination of retention time and concentration.

CHAPTER 4. RESULTS AND DISCUSSION

4.1. Protein Production

Cht3p was successfully produced in *P. pastoris* GS115 using the pre-prepared expression construct *P. pastoris* GS115-pPIC9K-Cht3 and a previously developed production protocol (Barbosa, 2017). Methanol induction of recombinant cell cultures resulted in Cht3p accumulation in the extracellular medium as determined by chitinase activity measurements and the appearance and accumulation of a protein band of ~150 kDa on SDS-PAGE. While the expected size of Cht3p is 60,092 Da, mobility on SDS-PAGE equivalent to ~150 kDa has already been observed for this protein (Barbosa et al., 2017; Carneiro et al., 2015) and is believed to be due to glycosylation effects. Following cell harvest, analysis of the supernatant indicated production of ~100 mg of Cht3p per liter of production culture, this productivity being similar to that reported for the batch production of other proteins in *P. pastoris* (Díaz-Jiménez et al., 2012; Duan et al., 2015; Mansur et al., 2005).

Analysis of SDS-PAGE gels of supernatant samples indicted highly pure (>90%) Cht3p with one intense protein band at ~150 kDa being visible, but with trace amounts of various protein contaminants becoming visible upon loading of the SDS-PAGE gels with large amounts of sample and use of the highly sensitive silver nitrate protein stain. Unexpectedly, a contaminant with cellulase activity was detected, this being in disagreement with the general understanding that *P. pastoris* GS115 does not produce extracellular cellulases in methanol inducing conditions and indeed this yeast has even been used as host for production of cellulases (de Amorim Araújo et al., 2015; Javanmard et al., 2021). Furthermore, in addition to these trace protein contaminants, supernatant samples were characterised by a yellow colouration, that turned into an intense brown-green color when concentrated, indicative of pigment contamination as has previously been reported for *P. pastoris* extracellular extracts (Fortis et al., 2006; Jozala et al., 2016).

4.2. Protein Purification

To purify the produced Cht3p, we investigated both chromatographic and non-chromatographic approaches, namely, activated carbon (AC) treatment and both hydrophobic interaction and gel filtration chromatography's.

In addition to leading to a decreased purity of protein product, pigments produced by *P. pastoris* have been reported to have a negative impact on downstream purification processes due to a protein and chromatographic matrix binding ability, thereby reducing the protein yield and the loading capacity and effective life span of chromatographic matrices (Hao et al., 2013;

Minyasab et al., 2010; Whittaker and Whittaker, 2002). Indeed, the pigments produced in the extracellular extract during Cht3p production were found to negatively impact subsequent chromatographic steps, namely by strongly binding to the hydrophobic interaction chromatography matrix and reducing its load capacity, with resultant losses of up to 40% Cht3p in the non-binding fraction. In addition, this strong binding of pigments led to intense column coloration and the necessity for additional and more frequent column matrix cleaning with aggressive agents such as ethanol and NaOH that reduce matrix lifetime.

To remove the pigments produced by *P. pastoris* we investigated the use of activated carbon, a non-specific adsorbent that is frequently for pigments removal from various samples (Demirbas, 2009). Both direct treatment of the whole cell culture and of the extracellular fraction obtained following centrifugation were investigated. In addition, to optimize the process, various treatment conditions were compared: activated carbon particle size, AC manufacturer, AC concentration, incubation temperature, and incubation time. As can be seen in Figure 5A, powdered AC is more effective in pigments removal, as measured by solution absorption at 400 nm, than is granular AC, with a slight difference in performance being also seen with different manufacturers. The higher effectiveness observed with the powdered AC is probably due to the increased surface area available for pigments binding. AC contact time with the sample, i.e., the incubation time, and incubation temperature, showed no effect under the conditions studied (Figure 5B). This being in disagreement with previous studies which identified an increased pigments adsorption at higher temperatures (Al-Degs et al., 2008), which was postulated as being resultant of an increased penetration of reactive dyes inside the AC micropores at higher temperatures. Nevertheless, it is also worth noting that increasing temperature could also have a hindering effect on the adsorption process by increasing interactions between solute and solvent more than between solute and adsorbent (Foo and Hameed, 2010). Increasing the AC concentration showed a positive effect on pigments removal (Figure 5C) but was also characterized by increasing losses of Cht3, due probably to non-specific binding or perhaps also Cht3p inactivation. Based on the observed results, the optimal AC concentration for treatment was set at 1% (w/v) AC, this permitting a high retention of Cht3p activity (~90%) and enabling an acceptable level of pigments removal (~60% reduction in A_{400nm}), while also reducing Cht3p losses in hydrophobic interaction chromatography from ~40 to ~25%, and leading to less visual column coloration and damage. Furthermore, similar results were observed when using the whole cell extract or the cell free extract for treatment, in disagreement with previous results which indicated an increased efficiency with whole cell extracts. Due to the increased simplicity and reduced number of steps, and hence also reduced

costs, for the direct AC treatment of the whole cell culture, this approach was adapted in our study.
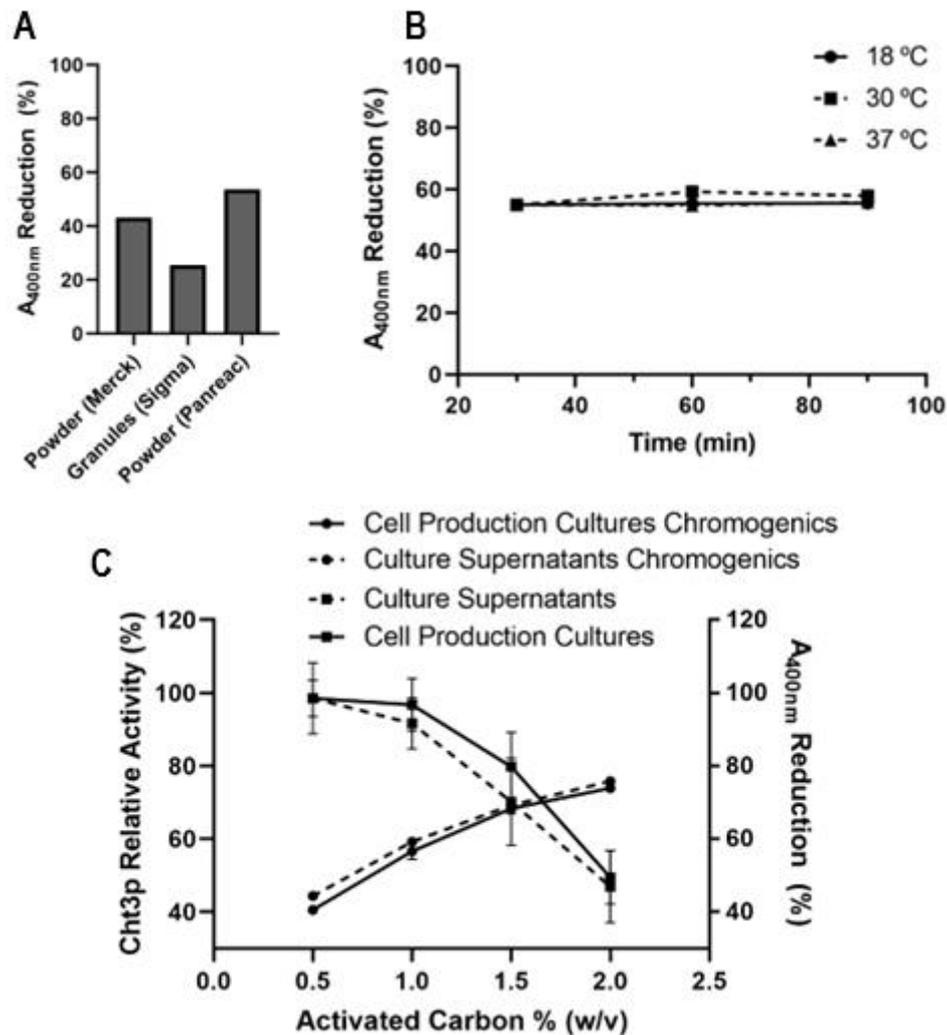

Figure 5. Treatment of Cht3p production cultures with activated carbon. A. Pigments removal as measured by a reduction in A_{400nm} of the cell supernatant treated with 1 % (w/v) activated carbon powder from Merck and Panreac and activated carbon granules 2-14 mesh from Sigma. B. Effect of incubation temperature and incubation time on pigments removal as measured by reduction in A400nm following treatment with 1% Panreac activated carbon. C. Effect of activated carbon concentration on Cht3p relative activity (left axis, squares) and A_{400nm} reduction (right axis, circles) for treatment of the whole cell production cultures (solid lines) and culture supernatants (dashed lines).

Hydrophobic interaction chromatography (HIC) was next investigated for further purification of AC treated samples and, in this study, various initial ammonium sulphate concentrations (1, 2 and 2.5 M) and flow rates (2 and 5 mL/min.) were examined. Interestingly, while an initial ammonium sulphate concentration of 1 M is commonly used in HIC, we observed losses of up to 25% Cht3p in the non-binding fraction. We thus examined higher

initial ammonium sulphate concentrations and found that both 2 and 2.5 M were effective in increasing Cht3p binding to ~90%. Hence, a concentration of 2M was used. Furthermore, our study of flow rates showed no differences between 2 and 5 mL/min. and hence the latter faster rate was employed. Following protein binding to HIC in the presence of 2M ammonium sulphate, various linear decreasing ammonium sulphate concentration gradients were examined for protein elution, with highest resolution being observed with a 2 to 0 M gradient in 350 mL. During elution, 5 mL fractions were collected for SDS-PAGE and chitinase activity analysis. The results of the SDS-PAGE analysis can be seen on Figure 6 A and B noting that, due to the low amounts of protein contaminants present, samples were ~80x –fold concentrated by TCA precipitation and stained with the highly sensitive stain silver nitrate so as to enable visualization of these contaminants but with this leading to intense saturation of the Cht3p protein band and reduced gel quality. Chitinase activity was detected in the eluting fractions from 61 to 86 (corresponding to the range of ammonium sulphate concentrations from 0.8 to 0.3M), in agreement with the observation of an intense protein band at ~150 kDa corresponding to Cht3p in these same sample fractions. Numerous contaminants were eluted outside this range of fractions, in particular a protein band at ~50kDa, but contaminants with molecular weights of ~25 kDa, 35 kDa, and to a lesser extent, 45 kDa, remained. Hence, gel filtration chromatography with a Sephacryl S-100 column with a fractionation range of 1 to 100 kDa was investigated.
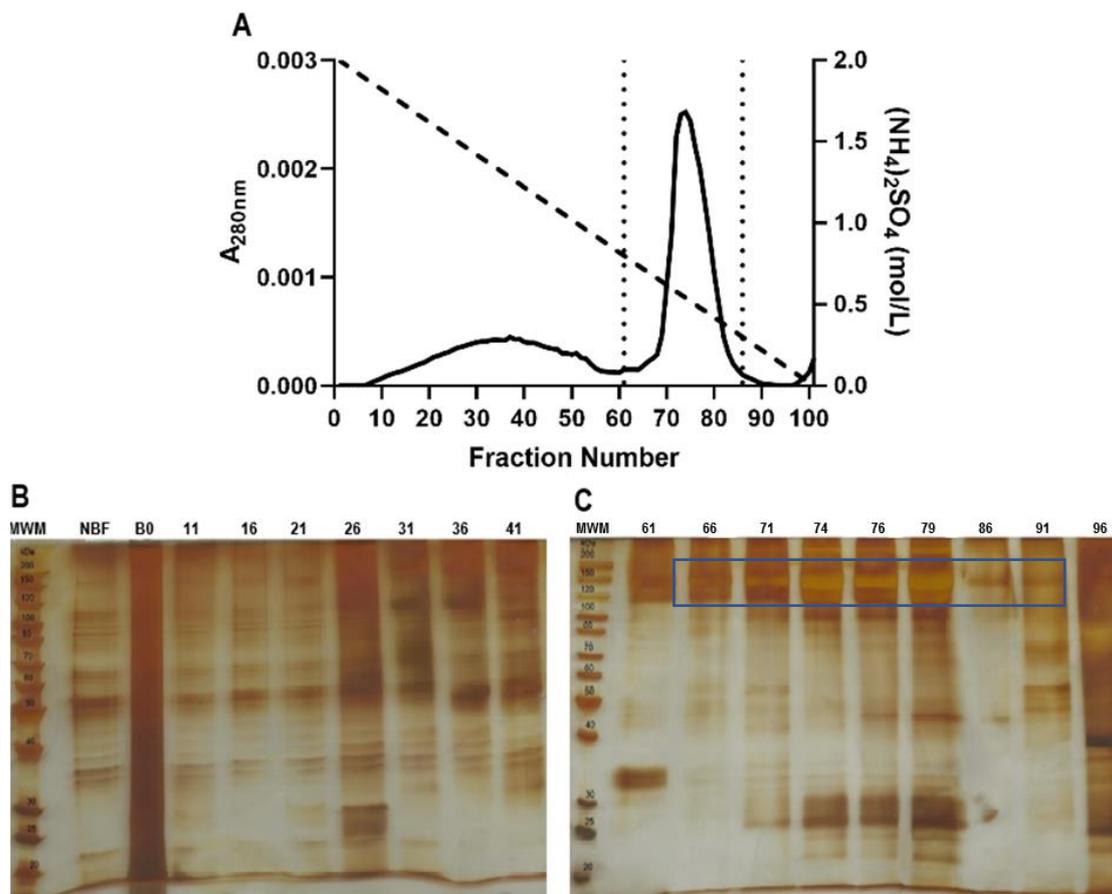
Figure 6. Hydrophobic Interaction Chromatography of Cht3p. A. Chromatogram of the purification showing the absorption at 280 nm (left axis, solid line) and ammonium sulphate gradient (right axis, dashed line) versus the collected numbered fractions. The dotted vertical lines indicate the fractions used for the HIC pool of samples (61-86). B and C. SDS-PAGE analysis of HIC fractions as identified by their fraction numbers, with silver staining. Blue square identifying Cht3p band at ~150 kDa size. MWM – Thermo Scientific PageRuler Unstained Protein Ladder. NBF – Non-binding fraction.

Prior to gel filtration chromatography, the HIC pooled sample was concentrated to ~1.5 mL by 80% ammonium sulphate precipitation and resuspension in 1.5 mL buffer. Indeed, this treatment not only enabled for sample concentration for loading to the gel filtration column but also reduced pigments levels. $A_{280 \, nm}$ measurements showed one broad protein peak eluting over many fractions (Figure 7A) and SDS-PAGE analysis (Figure 7B, C, D, and E) showed these to correspond to Cht3p but with several lower molecular weight contaminants at the peak tail. The elution of Cht3p over such a broad range of fractions indicates some type of interaction or retardation on the column matrix, due the protein itself or of the glycans of this glycosylated protein.
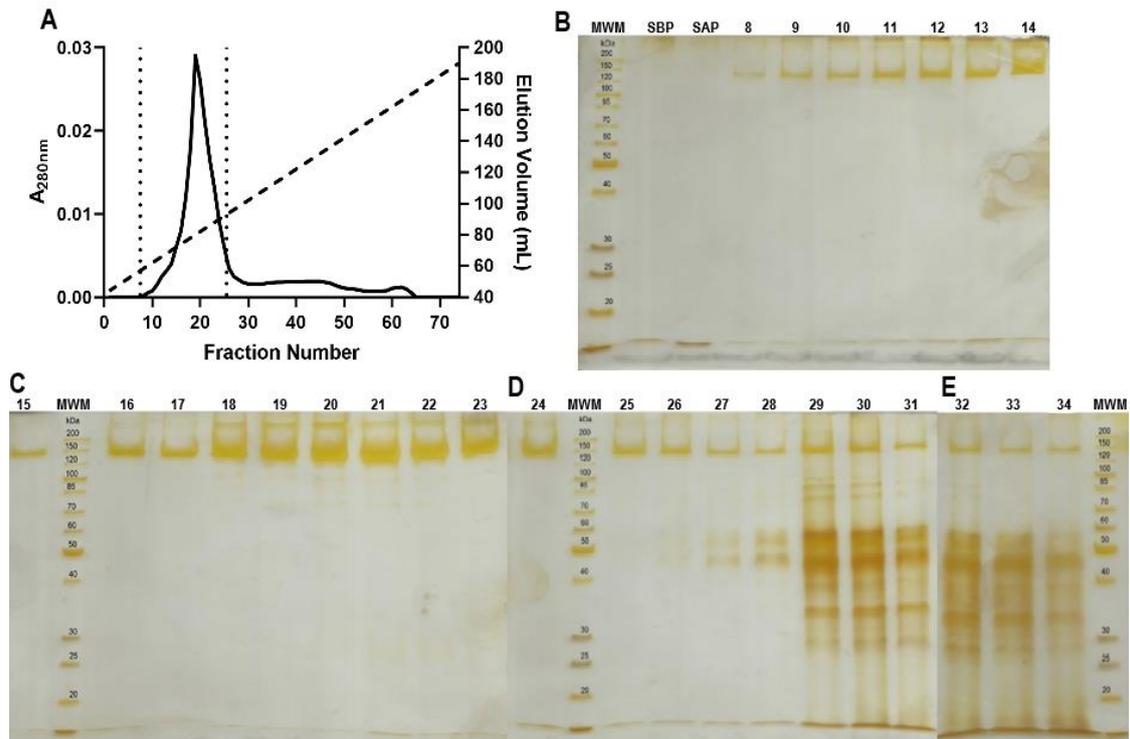
31

Figure 7. Gel Filtration Chromatography of Cht3p. A. Chromatogram of the purification showing the absorption at 280 nm versus (left axis, solid line) and the elution volume (right axis, dashed line) versus the collected numbered fractions. The dotted vertical lines indicate the fractions used for the GFC pool of samples (8-26) B, C, D, and E. SDS-PAGE analysis of GFC fractions as identified by their fraction number, with silver staining. MWM – Thermo Scientific PageRuler Unstained Protein Ladder. SBP – Pool of samples eluted before Cht3p peak. SAP – Pool of samples eluted after the Cht3p peak.

All fractions containing pure Cht3p (i.e., fractions 8 to 25 in Figure 7B, C, and D) were pooled and SDS-PAGE analysis showed a highly pure protein. Indeed, the developed 4-step purification protocol also enabled effective pigments removal as measured by A_{400nm} readings with a final Cht3p yield of ~50%.
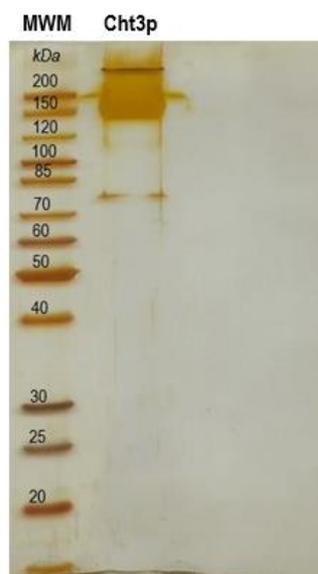
Figure 8. SDS-PAGE analysis of the GFC pooled samples, with silver staining. MWM – Thermo Scientific PageRuler Unstained Protein Ladder. Cht3p – GFC pooled samples.

4.3. Protein Characterization

Following purification, the activity of Cht3p as a function of pH and temperature and its functional properties were examined. The enzyme was found to be active over a broad range of pHs and temperatures (Figure 9). It is optimally active at pHs 3.5-5.5, but also shows a small activity shoulder at pHs 7.5-8.5 (Figure 9A). The wild-type host of this enzymes, *C. albicans*, has been reported to grow over a wide range of extracellular pHs, from pH 2 to 10, this being related to the wide range of pHs of the environments colonized by this yeast, e.g., the vaginal mucosa has an acidic pH of ~4.5, whereas the bloodstream has a pH of ~7.4 (Davis, 2003). Therefore, to enable its function in chitin degradation for cell separation in these different environments, the observed wide pH range of activity for Cht3p was to be expected (Klis and Brul, 2015). Furthermore, the bimodal distribution of pH-activity observed for this enzyme is not uncommon, having already been reported for GH-18 chitinases (Barboza-Corona et al., 2003; Suginta and Sritho, 2012; Wang et al., 2009; Zhang et al., 2002). The exact reason for this remains uncovered but it has been hypothesized that such enzymes might be secreted from cells with 2 different conformations with different optimum pHs (Suginta and Sritho, 2012), while also the existence of multiple catalytic residues with different $pKa′$s and different ionization pHs has also been suggested as playing a role (Barboza-Corona et al., 2003).

The temperature dependence of activity was examined from 10 to 70 ºC wherein a maximum at 50 ºC was observed with 90% relative activity being retained from 40 to 50 ºC

33

(Figure 9B). This is in agreement with studies showing that most fungal chitinases have their temperature optimum between 40-50 ºC (Karthik et al., 2014).
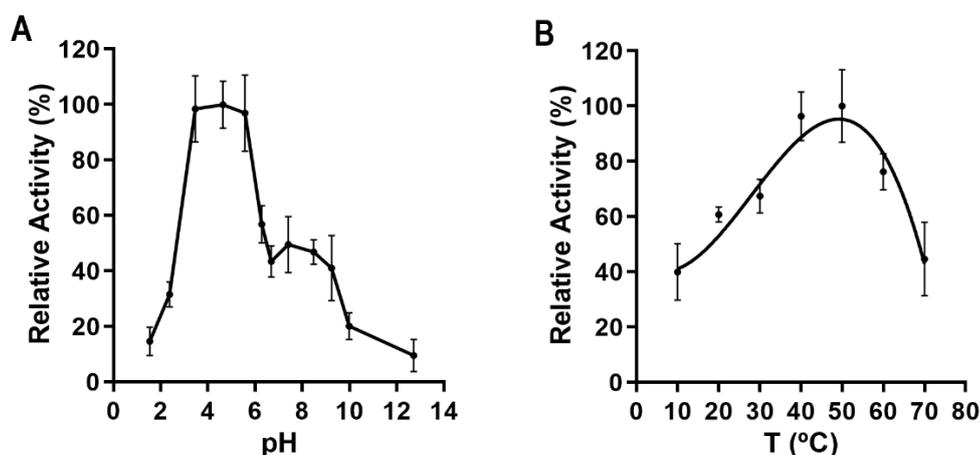

Figure 9. A. pH dependence of Cht3p activity. B. Temperature dependence of Cht3p activity.

The kinetic parameters were calculated utilizing the optimum conditions previously defined (pH 4.5 and 50 ºC) in assays of 5 min with the substrate CC at concentrations from 2.5 to 40 mg/mL. The K_m, V_{max}, k_{cat}, and k_{cat}/k_m values for the chitinolytic activity of the purified protein were 37.5 ± 19 mg/mL, 900 μM.min^{-1}, 7.5 s^{-1}, and 0.2 s/mg/mL, respectively. This K_m value can be considered high when compared with GH-18 chitinases from several fungi such as *Trichoderma harzianum* (0.65 mg/mL) (Deng et al., 2019) and *Trichoderma viride* (6.66 mg/ml) (Abu-Tahon and Isaac, 2020), thereby suggesting a weaker affinity for CC, but is also similar to those of other GH-18 chitinases such as those from *S. marcescens* GPS5 (47.92 mg/mL) (Vaikuntapu et al., 2016) and *Serratia proteamaculans* (35.1 mg/mL) (Madhuprakash et al., 2015). The k_{cat}, value of 7.5 s^{-1} is high on the range of values shown by other chitinases, indicating that our enzyme had a slightly better turnover efficiency than the average chitinase, such as those from *Penicillium ochrochloron* (2.37 s^{-1}) and *Rhizomucor miehei* (0.53 ± 0.02 s^{-1}) (Jiang et al., 2021; Patil et al., 2013).

Substrate specificity studies were carried out with the macrosubstrates displayed in Figure 10. Cht3p showed no cellulase, xylanase, nor pectinase activities, while also being inactive on the cell wall of *M. lysodeikticus*, indicating that this enzyme does not possess lysozyme activity (EC 3.2.1.17), an activity that has been reported for some other GH-18 enzymes. The enzyme hydrolyzed colloidal chitin (prepared from shrimp cells) and, to a lesser extent, powdered chitin (~5%) (prepared from shrimp cells), as well as the deacetylated chitin derivate chitosan (~63%). It also displayed a lower chitinolytic activity on water soluble CC

extracts (~40%), likely due to this being majorly composed of low molecular weight COS (GlcNAc, (GlcNAc)$_2$, and (GlcNAc$_3$)) upon which Cht3p is inactive. The low activity on chitin powder is probably due to poor accessibility related to the insoluble and calcified nature of this. Indeed, this substrate has already been reported to poorly induce chitinolytic activity (Stoykov et al., 2014). Finally the reduced activity on chitosan is likely due to its high deacetylation percentage (70%) that has been shown to have a negative effect on chitinase activity (Ohtakara et al., 1988).
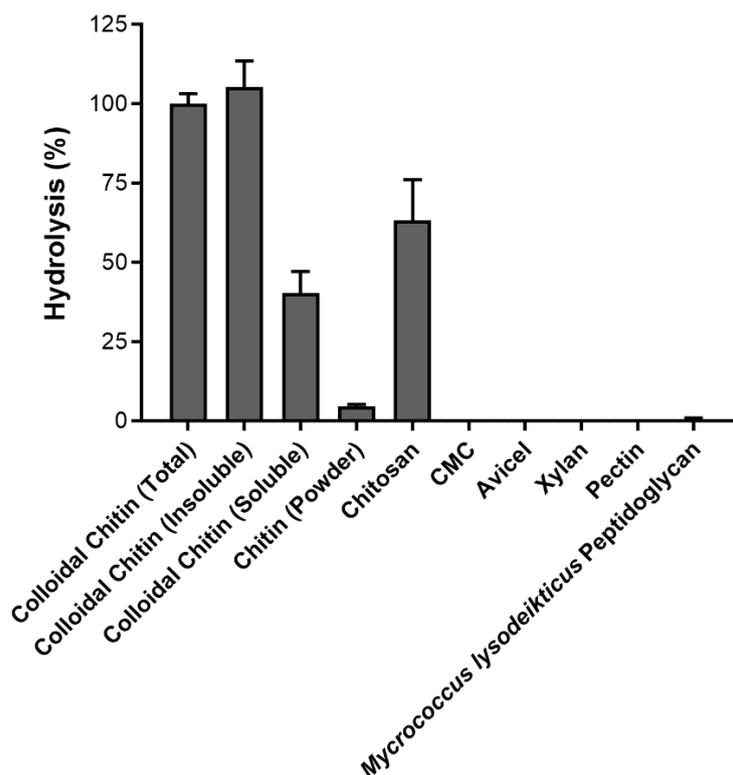

Figure 10. Substrate specificity for the Cht3p hydrolysis of macrosubstrates. 100% corresponds to Colloidal Chitin hydrolysis.

As shown in Table 2, studies of Cht3p activity on chito-oligosaccharides showed that this does not hydrolyze (GlcNAc)$_2$ nor (GlcNAc)$_3$ but does hydrolyze the larger chito-oligomers studied (GlcNAc)$_{4-6}$. (GlcNAc)$_4$ was completely hydrolyzed to (GlcNAc)$_2$; whereas (GlcNAc)$_5$ was hydrolyzed to (GlcNAc + (GlcNAc)$_4$) and ((GlcNAc)$_2$ + (GlcNAc)$_3$); and (GlcNAc)$_6$ was hydrolyzed to ((GlcNAc)$_2$ + (GlcNAc)$_4$) and ((GlcNAc)$_3$ + (GlcNAc)$_3$), with any remaining (GlcNAc)$_4$ being fully hydrolyzed to (GlcNAc)$_2$ (Figure 11).

Table 2. Substrate specificity for Cht3p's hydrolysis of chito-oligosaccharides.

Chito-oligomer	Products
$(GlcNAc)_2$	No hydrolysis
$(GlcNAc)_3$	No hydrolysis
$(GlcNAc)_4$	$(GlcNAc)_2 + (GlcNAc)_2$
$(GlcNAc)_5$	$(GlcNAc)_3 + (GlcNAc)_2$, $(GlcNAc)_1 +$ $(GlcNAc)_4$ and $(GlcNAc)_2 + (GlcNAc)_2$
$(GlcNAc)_6$	$(GlcNAc)_3 + (GlcNAc)_3$, $(GlcNAc)_4 +$ $(GlcNAc)_2$ and $(GlcNAc)_2 + (GlcNAc)_2$

Of the substrates tested, the enzyme was most active on chitohexaose, being 40-fold more active than on chitotetraose and 5-fold more active on chitopentaose. Such an increase in activity with substrate size is indicative of a substrate binding cleft with at least six subsites. Chitinases are generally placed in two major categories, being considered endo or exochitinases, as mentioned in the introduction. Endochitinases are defined as chitinases that cleave chitin $(GlcNAc)_n$ to generate $(GlcNAc)_x$ and $(GlcNAc)_{n-x}$ $(x \geq 2)$, while exochitinases are considered to cleave $(GlcNAc)_n$ to GlcNAc and $(GlcNAc)_{n-1}$ (Xie et al., 2021). Therefore, the release of various chito-oligosaccharides $((GlcNAc)_2$ and $(GlcNAc)_3)$ and the enzyme's incapability to digest $(GlcNAc)_3$ (Figure 11) suggests that Cht3p is a non-processive endochitinase.
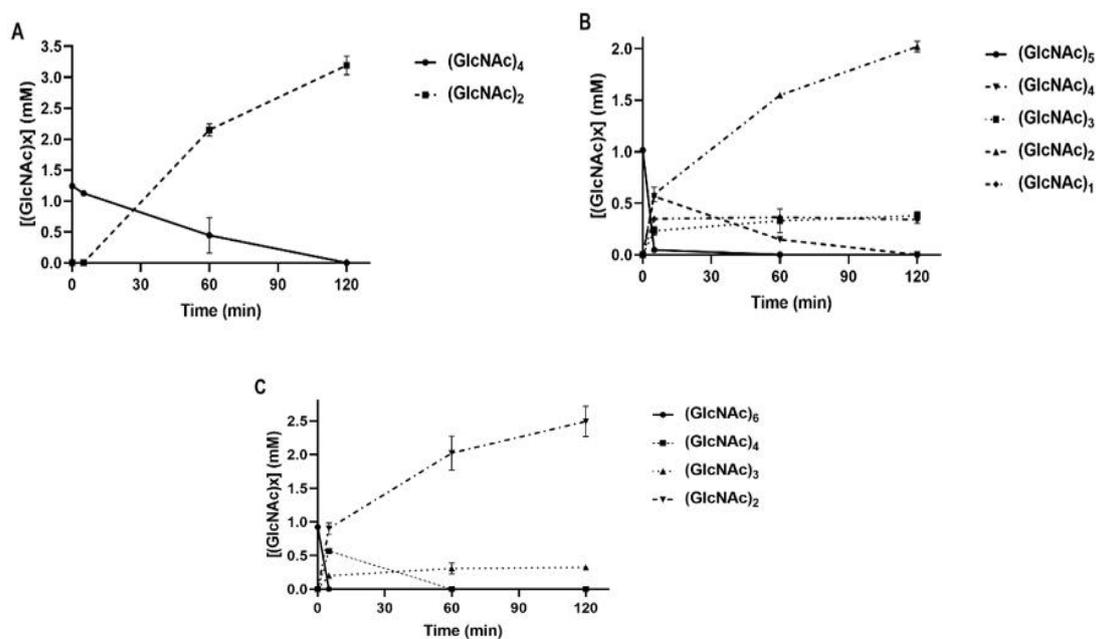
Figure 11. Cht3p hydrolysis of various chito-oligosacharides and products produced over time. Substrates: A. chitotetraose, B. chitopentaose and, C. chitohexaose.

CHAPTER 5. CONCLUSIONS AND FUTURE PERSPECTIVES

Candida albicans is an opportunistic pathogen and one of the most common causes of fungal infections worldwide. It causes superficial infections such as vulvovaginitis, chronic atrophic stomatitis, and chronic mucocutaneous candidiasis, but can also lead to life-threatening candidemia upon infection of internal organs and/or the blood stream. Currently, only a limited number of therapies are available for treatment of infection by this organism, these being mainly based on the use of anti-fungal agents, with, in addition, the development of resistance mechanisms in this fungi leading to limitations in the effectiveness of these therapies (Rodloff et al., 2011). Therefore, the development of novel therapies and strategies for treatment and prevention are called for, with the use of therapeutic vaccines gaining much recent interest. In this sense, a previous study investigating *C. albicans* cell wall surface proteins as potential immunoprotective agents, showed chitinase 3 (Cht3p) to induce a protective immune response in treated mice (Carneiro et al., 2015). To further investigate and develop this enzyme for this application as a vaccine antigen, or indeed for any applications, high levels of pure protein are required, in addition to an understanding of its physicochemical and functional characteristics. The present project was focused on this, on the heterologous expression of Cht3 in *Pichia pastoris*, on the development of an efficient and effective purification protocol, and on enhancing our understanding of the activity, stability and function of this enzyme.

Cht3p was successfully produced in *P. pastoris* GS115 using a previously developed protocol (Barbosa et al. 2017) and enabled a productivity of ~100 mg per liter of production culture. This productivity is within the range commonly reported when using batch production approaches with this host (Díaz-Jiménez et al., 2012; Duan et al., 2015; Mansur et al., 2005), and can be increased to the g/L levels by use of high-cell-density fed-batch production approaches. In addition, recombinant protein production with this host can be scaled up to industrial scale systems, thereby enabling for the potential industrial scale production and commercial use of this enzyme if required. The recombinant protein was produced in the extracellular environment, and while already being highly pure, as is commonly reported for *P. pastoris* extracellular produced proteins, was found to be characterized by trace protein contaminants and the presence of pigments. The pigments not only reduced protein purity, but also interfered with Cht3p matrix binding in subsequent chromatographic steps, leading to reduced yields, and due to the necessity for harsh cleaning procedures to remove the pigments, lead to reduced matrix lifespan. We developed a 4-step purification protocol with a Cht3p yield of ~50% for the efficient removal of the observed contaminants. Activated carbon treatment strongly reduced the pigments content and enabled higher protein yields and less column wear in subsequent chromatographic steps. Importantly, this treatment in combination with

hydrophobic interaction chromatography, ammonium sulphate precipitation and gel filtration chromatography enabled complete removal of pigments, as determined by absorption readings at 400 nm, and resulted in purified Cht3p as determined by SDS-PAGE analysis.

Stability and activity analyses of the purified protein indicated a stable protein active over a broad range of conditions. It was shown to be stable at pHs as low as pH 3 and as high as pH 9, and at temperatures up to ~50-60 ºC, with activity maxima at pH 3.5-5.5 and 40-50 ºC, and with retention of high activity outside these optima. Such characteristics are positive attributes for commercialization of the enzyme, enabling a wide range of storage and process conditions. In addition, analysis of the kinetic parameter indicated values within the range reported for previously reported homologous chitinases but with the turnover number of Cht3p being at the high end of this range. This high rate, in addition to the specificity of the enzyme for chitin and chitosan as well as its endochitinase mode of action point to a potential for this enzyme as an anti-fungal agent or pesticide.

Future studies should focus on an in-depth investigation and development of Cht3 for use as a vaccine antigen, via studies with animal models. The availability of an effective and efficient production and purification protocol as developed in the present project will provide for such studies. It provides for relatively high quantities of pure protein, sufficient for such studies, making use of a production system that can be fine-tuned for increased productivity and scaled-up to industrial scale levels. Furthermore, the increased understanding obtained in the present project of the enzymes physicochemical conditions will enable for an improved ability in designing the experimental conditions for analysis, as well as the vaccine formulations for use, and the enzyme storage conditions. Finally, the functional studies carried out herein indicate a potential for use in applications other than in vaccines, and future studies should investigate these.

CHAPTER 6. BIBLIOGRAPHY

Abu-Tahon, M. A., & Isaac, G. S. (2020). Anticancer and antifungal efficiencies of purified chitinase produced from *Trichoderma viride* under submerged fermentation. *The Journal of General and Applied Microbiology*, *66*(1), 32–40. https://doi.org/10.2323/jgam.2019.04.006

Adams, D. J. (2004). Fungal cell wall chitinases and glucanases. *Microbiology*, *150*(7), 2029–2035. https://doi.org/https://doi.org/10.1099/mic.0.26980-0

Adrangi, S., & Faramarzi, M. A. (2013). From bacteria to human: A journey into the world of chitinases. *Biotechnology Advances*, *31*(8), 1786–1795. https://doi.org/https://doi.org/10.1016/j.biotechadv.2013.09.012

Akakuru, O. U., Louis, H., Amos, P. I., Akakuru, O., Nosike, E. I., & Ef, O. (2018). The Chemistry of Chitin and Chitosan Justifying their Nanomedical Utilities. *Biochemical Pharmacology*, *7*, 1–6.

Al-Degs, Y. S., El-Barghouthi, M. I., El-Sheikh, A. H., & Walker, G. M. (2008). Effect of solution pH, ionic strength, and temperature on adsorption behavior of reactive dyes on activated carbon. *Dyes and Pigments*, *77*(1), 16–23. https://doi.org/https://doi.org/10.1016/j.dyepig.2007.03.001

Ali, A., & Ahmed, S. (2018). A review on chitosan and its nanocomposites in drug delivery. *International Journal of Biological Macromolecules*, *109*, 273–286. https://doi.org/https://doi.org/10.1016/j.ijbiomac.2017.12.078

Allonsius, C. N., Vandenheuvel, D., Oerlemans, E. F. M., Petrova, M. I., Donders, G. G. G., Cos, P., … Lebeer, S. (2019). Inhibition of Candida albicans morphogenesis by chitinase from Lactobacillus rhamnosus GG. *Scientific Reports*, *9*(1), 2900. https://doi.org/10.1038/s41598-019-39625-0

Andersen, M. D., Jensen, A., Robertus, J. D., Leah, R., & Skriver, K. (1997). Heterologous expression and characterization of wild-type and mutant forms of a 26 kDa endochitinase from barley (Hordeum vulgare L.). *Biochemical Journal*, *322*(3), 815–822. https://doi.org/10.1042/bj3220815

Aw, R., & Polizzi, K. M. (2013). Can too many copies spoil the broth? *Microbial Cell Factories*, *12*(1), 128. https://doi.org/10.1186/1475-2859-12-128

Barbosa, A., Collins, J., & Carvalho, A. (2017). *Development of a novel recombinant subunit antigen as a vaccination target against candidiasis : Cht3 from Candida albicans*.

Barboza-Corona, J. E., Nieto-Mazzocco, E., Velázquez-Robledo, R., Salcedo-Hernandez, R., Bautista, M., Jiménez, B., & Ibarra, J. E. (2003). Cloning, Sequencing, and Expression of the Chitinase Gene chiA74 from Bacillus thuringiensis. *Applied and Environmental Microbiology*, *69*(2), 1023–1029. https://doi.org/10.1128/AEM.69.2.1023-1029.2003

Barik, B., Nayak, P. S., Achary, L. S. K., Kumar, A., & Dash, P. (2020). Synthesis of alumina-based cross-linked chitosan–HPMC biocomposite film: an efficient and user-friendly adsorbent for multipurpose water purification. *New Journal of Chemistry*, *44*(2), 322–337. https://doi.org/10.1039/C9NJ03945G

Barr, K. A., Hopkins, S. A., & Sreekrishna, K. (1992). Protocol for efficient secretion of HSA developed from Pichia pastoris. *Pharm. Eng.*, *12*, 48–51.

Barroca, M., Santos, G., Johansson, B., Gillotin, F., Feller, G., & Collins, T. (2017). Deciphering the factors defining the pH-dependence of a commercial glycoside hydrolase family 8 enzyme. *Enzyme and Microbial Technology*, *96*, 163–169. https://doi.org/https://doi.org/10.1016/j.enzmictec.2016.10.011

Benhabiles, M. S., Salah, R., Lounici, H., Drouiche, N., Goosen, M. F. A., & Mameri, N. (2012). Antibacterial activity of chitin, chitosan and its oligomers prepared from shrimp shell waste. *Food Hydrocolloids*, *29*(1), 48–56. https://doi.org/https://doi.org/10.1016/j.foodhyd.2012.02.013

Berini, F., Katz, C., Gruzdev, N., Casartelli, M., Tettamanti, G., & Marinelli, F. (2018).

Microbial and viral chitinases: Attractive biopesticides for integrated pest management. *Biotechnology Advances*, *36*(3), 818–838. https://doi.org/https://doi.org/10.1016/j.biotechadv.2018.01.002

Bernfeld, P. B. T.-M. in E. (1955). *[17] Amylases, α and β*. https://doi.org/https://doi.org/10.1016/0076-6879(55)01021-5

Biotech, C. (2021). Chibio Biotech-Manufacturer of Vegetal Chitosan, Fungal Chitosan, Natural Ingredients. Retrieved from Chibio Biotech-Manuf. Veg. Chitosan Fungal Chitosan Nat. Ingred. website: https://www.fungalchitosan.com

Bokma, E., van Koningsveld, G. A., Jeronimus-Stratingh, M., & Beintema, J. J. (1997). Hevamine, a chitinase from the rubber tree Hevea brasiliensis, cleaves peptidoglycan between the C-1 of N-acetylglucosamine and C-4 of N-acetylmuramic acid and therefore is not a lysozyme. *FEBS Letters*, *411*(2), 161–163. https://doi.org/https://doi.org/10.1016/S0014-5793(97)00682-0

Bonanomi, G., Lorito, M., Vinale, F., & Woo, S. L. (2018). Organic Amendments, Beneficial Microbes, and Soil Microbiota: Toward a Unified Framework for Disease Suppression. *Annual Review of Phytopathology*, *56*(1), 1–20. https://doi.org/10.1146/annurev-phyto-080615-100046

Brameld, K. A., & Goddard, W. A. (1998). Substrate Distortion to a Boat Conformation at Subsite −1 Is Critical in the Mechanism of Family 18 Chitinases. *Journal of the American Chemical Society*, *120*(15), 3571–3580. https://doi.org/10.1021/ja972282h

Busilacchi, A., Gigante, A., Mattioli-Belmonte, M., Manzotti, S., & Muzzarelli, R. A. A. (2013). Chitosan stabilizes platelet growth factors and modulates stem cell differentiation toward tissue regeneration. *Carbohydrate Polymers*, *98*(1), 665–676. https://doi.org/https://doi.org/10.1016/j.carbpol.2013.06.044

Cao, S., Liu, Y., Shi, L., Zhu, W., & Wang, H. (2022). Correction: N-Acetylglucosamine as a platform chemical produced from renewable resources: opportunity, challenge, and future prospects. *Green Chemistry*, *24*(6), 2638. https://doi.org/10.1039/D2GC90017C

Carmona-Ribeiro, M. A. (2003). Bilayer-Forming Synthetic Lipids: Drugs or Carriers? *Current Medicinal Chemistry*, Vol. 10, pp. 2425–2446. https://doi.org/http://dx.doi.org/10.2174/0929867033456611

Carneiro, C., Correia, A., Collins, T., Vilanova, M., Pais, C., Gomes, A. C., … Sampaio, P. (2015). DODAB:monoolein liposomes containing Candida albicans cell wall surface proteins: A novel adjuvant and delivery system. *European Journal of Pharmaceutics and Biopharmaceutics*, *89*, 190–200. https://doi.org/https://doi.org/10.1016/j.ejpb.2014.11.028

Castillo, B. M., Dunn, M. F., Navarro, K. G., Meléndez, F. H., Ortiz, M. H., Guevara, S. E., & Palacios, G. H. (2016). Antifungal performance of extracellular chitinases and culture supernatants of Streptomyces galilaeus CFFSUR-B12 against Mycosphaerella fijiensis Morelet. *World Journal of Microbiology and Biotechnology*, *32*(3), 44. https://doi.org/10.1007/s11274-015-1993-0

Cereghino, J. L., & Cregg, J. M. (2000). Heterologous protein expression in the methylotrophic yeast Pichia pastoris. *FEMS Microbiology Reviews*, *24*(1), 45–66. https://doi.org/10.1111/j.1574-6976.2000.tb00532.x

Chen, W., Jiang, X., & Yang, Q. (2020). Glycoside hydrolase family 18 chitinases: The known and the unknown. *Biotechnology Advances*, *43*, 107553. https://doi.org/https://doi.org/10.1016/j.biotechadv.2020.107553

Chen, X., Yang, H., & Yan, N. (2016). Shell Biorefinery: Dream or Reality? *Chemistry – A European Journal*, *22*(38), 13402–13421. https://doi.org/https://doi.org/10.1002/chem.201602389

Cregg, J. M., Cereghino, J. L., Shi, J., & Higgins, D. R. (2000). Recombinant protein

expression in Pichia pastoris. *Molecular Biotechnology*, *16*(1), 23–52. https://doi.org/10.1385/MB:16:1:23

Crini, G. (2019). *Historical Landmarks in the Discovery of Chitin BT - Sustainable Agriculture Reviews 35: Chitin and Chitosan: History, Fundamentals and Innovations* (G. Crini & E. Lichtfouse, Eds.). https://doi.org/10.1007/978-3-030-16538-3_1

Daly, R., & Hearn, M. T. W. (2005). Expression of heterologous proteins in Pichia pastoris: a useful experimental tool in protein engineering and production. *Journal of Molecular Recognition*, *18*(2), 119–138. https://doi.org/https://doi.org/10.1002/jmr.687

Davidsen, J., Rosenkrands, I., Christensen, D., Vangala, A., Kirby, D., Perrie, Y., … Andersen, P. (2005). Characterization of cationic liposomes based on dimethyldioctadecylammonium and synthetic cord factor from M. tuberculosis (trehalose 6,6′-dibehenate)—A novel adjuvant inducing both strong CMI and antibody responses. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, *1718*(1), 22–31. https://doi.org/https://doi.org/10.1016/j.bbamem.2005.10.011

Davis, D. (2003). Adaptation to environmental pH in Candida albicans and its relation to pathogenesis. *Current Genetics*, *44*(1), 1–7. https://doi.org/10.1007/s00294-003-0415-2

de Amorim Araújo, J., Ferreira, T. C., Rubini, M. R., Duran, A. G. G., De Marco, J. L., de Moraes, L. M. P., & Torres, F. A. G. (2015). Coexpression of cellulases in Pichia pastoris as a self-processing protein fusion. *AMB Express*, *5*(1), 84. https://doi.org/10.1186/s13568-015-0170-z

Demirbas, A. (2009). Agricultural based activated carbons for the removal of dyes from aqueous solutions: A review. *Journal of Hazardous Materials*, *167*(1), 1–9. https://doi.org/https://doi.org/10.1016/j.jhazmat.2008.12.114

Deng, J.-J., Shi, D., Mao, H., Li, Z., Liang, S., Ke, Y., & Luo, X. (2019). Heterologous expression and characterization of an antifungal chitinase (Chit46) from Trichoderma harzianum GIM 3.442 and its application in colloidal chitin conversion. *International Journal of Biological Macromolecules*, *134*, 113–121. https://doi.org/https://doi.org/10.1016/j.ijbiomac.2019.04.177

Di Rosa, M., & Malaguarnera, L. (2016). Chitinase 3 Like-1: An Emerging Molecule Involved in Diabetes and Diabetic Complications. *Pathobiology*, *83*(5), 228–242. https://doi.org/10.1159/000444855

Díaz-Jiménez, D. F., Mora-Montes, H. M., Hernández-Cervantes, A., Luna-Arias, J. P., Gow, N. A. R., & Flores-Carreón, A. (2012). Biochemical characterization of recombinant Candida albicans mannosyltransferases Mnt1, Mnt2 and Mnt5 reveals new functions in O- and N-mannan biosynthesis. *Biochemical and Biophysical Research Communications*, *419*(1), 77–82. https://doi.org/https://doi.org/10.1016/j.bbrc.2012.01.131

Djenane, Z., Nateche, F., Amziane, M., Gomis-Cebolla, J., El-Aichar, F., Khorf, H., & Ferré, J. (2017). Assessment of the Antimicrobial Activity and the Entomocidal Potential of Bacillus thuringiensis Isolates from Algeria. *Toxins* , Vol. 9. https://doi.org/10.3390/toxins9040139

Drula, E., Garron, M.-L., Dogan, S., Lombard, V., Henrissat, B., & Terrapon, N. (2022). The carbohydrate-active enzyme database: functions and literature. *Nucleic Acids Research*, *50*(D1), D571–D577. https://doi.org/10.1093/nar/gkab1045

Duan, H., Wang, H., Ma, B., Jiang, P., Tu, P., Ni, Z., … Li, M. (2015). Codon optimization and expression of irisin in Pichia pastoris GS115. *International Journal of Biological Macromolecules*, *79*, 21–26. https://doi.org/https://doi.org/10.1016/j.ijbiomac.2015.04.030

Dünkler, A., Walther, A., Specht, C. A., & Wendland, J. (2005). Candida albicans CHT3 encodes the functional homolog of the Cts1 chitinase of Saccharomyces cerevisiae.

Fungal Genetics and Biology, *42*(11), 935–947.
https://doi.org/https://doi.org/10.1016/j.fgb.2005.08.001

Dutta, P., Dutta, J., & Tripathi, V. (2003). Chitin and chitosan: Chemistry, properties and applications. *J Sci Indus Res*, *63*.

El-Battari, A., Prorok, M., Angata, K., Mathieu, S., Zerfaoui, M., Ong, E., … Fukuda, M. (2003). Different glycosyltransferases are differentially processed for secretion, dimerization, and autoglycosylation. *Glycobiology*, *13*(12), 941–953. https://doi.org/10.1093/glycob/cwg117

Elieh-Ali-Komi, D., & Hamblin, M. R. (2016). Chitin and Chitosan: Production and Application of Versatile Biomedical Nanomaterials. *International Journal of Advanced Research*, *4*(3), 411–427. Retrieved from https://pubmed.ncbi.nlm.nih.gov/27819009

Foo, K. Y., & Hameed, B. H. (2010). An overview of dye removal via activated carbon adsorption process. *Desalination and Water Treatment*, *19*(1–3), 255–274. https://doi.org/10.5004/dwt.2010.1214

Fortis, F., Guerrier, L., Righetti, P. G., Antonioli, P., & Boschetti, E. (2006). A new approach for the removal of protein impurities from purified biologicals using combinatorial solid-phase ligand libraries. *ELECTROPHORESIS*, *27*(15), 3018–3027. https://doi.org/https://doi.org/10.1002/elps.200500847

Freier, T., Koh, H. S., Kazazian, K., & Shoichet, M. S. (2005). Controlling cell adhesion and degradation of chitosan films by N-acetylation. *Biomaterials*, *26*(29), 5872–5878. https://doi.org/https://doi.org/10.1016/j.biomaterials.2005.02.033

Frindy, S., Primo, A., Ennajih, H., el kacem Qaiss, A., Bouhfid, R., Lahcini, M., … El Kadib, A. (2017). Chitosan–graphene oxide films and CO2-dried porous aerogel microspheres: Interfacial interplay and stability. *Carbohydrate Polymers*, *167*, 297–305. https://doi.org/https://doi.org/10.1016/j.carbpol.2017.03.034

Fujita, K., Shimomura, K., Yamamoto, K., Yamashita, T., & Suzuki, K. (2006). A chitinase structurally related to the glycoside hydrolase family 48 is indispensable for the hormonally induced diapause termination in a beetle. *Biochemical and Biophysical Research Communications*, *345*(1), 502–507. https://doi.org/https://doi.org/10.1016/j.bbrc.2006.04.126

Fukamizo, F. (2000). Chitinolytic Enzymes: Catalysis, Substrate Binding, and their Application. *Current Protein & Peptide Science*, Vol. 1, pp. 105–124. https://doi.org/http://dx.doi.org/10.2174/1389203003381450

Fukamizo, T., Koga, D., & Goto, S. (1995). Comparative Biochemistry of Chitinases—Anomeric Form of the Reaction Products. *Bioscience, Biotechnology, and Biochemistry*, *59*(2), 311–313. https://doi.org/10.1271/bbb.59.311

Furqan, B., & Akhmaloka, A. (2019). Heterologous expression and characterization of thermostable lipase (Lk1) in Pichia pastoris GS115. *Biocatalysis and Agricultural Biotechnology*, *23*, 101448. https://doi.org/10.1016/j.bcab.2019.101448

Gan, Z., Yang, J., Tao, N., Liang, L., Mi, Q., Li, J., & Zhang, K.-Q. (2007). Cloning of the gene Lecanicillium psalliotae chitinase Lpchi1 and identification of its potential role in the biocontrol of root-knot nematode Meloidogyne incognita. *Applied Microbiology and Biotechnology*, *76*(6), 1309–1317. https://doi.org/10.1007/s00253-007-1111-9

García, A., & De Sanctis, J. B. (2014). An overview of adjuvant formulations and delivery systems. *APMIS*, *122*(4), 257–267. https://doi.org/https://doi.org/10.1111/apm.12143

Goldman, D., & Vicencio, A. (2012). The Chitin Connection. *MBio*, *3*. https://doi.org/10.1128/mBio.00056-12

Gregory, A., Williamson, D., & Titball, R. (2013). Vaccine delivery using nanoparticles . *Frontiers in Cellular and Infection Microbiology* , Vol. 3. Retrieved from https://www.frontiersin.org/article/10.3389/fcimb.2013.00013

Hakala, B. E., White, C., & Recklies, A. D. (1993). Human cartilage gp-39, a major secretory product of articular chondrocytes and synovial cells, is a mammalian member of a chitinase protein family. *Journal of Biological Chemistry*, *268*(34), 25803–25810. https://doi.org/10.1016/S0021-9258(19)74461-5

Halbedel, S., Prager, R., Banerji, S., Kleta, S., Trost, E., Nishanth, G., … Flieger, A. (2019). A Listeria monocytogenes ST2 clone lacking chitinase ChiB from an outbreak of non-invasive gastroenteritis. *Emerging Microbes & Infections*, *8*(1), 17–28. https://doi.org/10.1080/22221751.2018.1558960

Halder, S. K., Pal, S., & Mondal, K. C. (2019). *Biosynthesis of Fungal Chitinolytic Enzymes and Their Potent Biotechnological Appliances BT - Recent Advancement in White Biotechnology Through Fungi: Volume 1: Diversity and Enzymes Perspectives* (A. N. Yadav, S. Mishra, S. Singh, & A. Gupta, Eds.). https://doi.org/10.1007/978-3-030-10480-1_8

Hamel, F., Boivin, R., Tremblay, C., & Bellemare, G. (1997). Structural and Evolutionary Relationships Among Chitinases of Flowering Plants. *Journal of Molecular Evolution*, *44*(6), 614–624. https://doi.org/10.1007/PL00006184

Hamid, R., Khan, M. A., Ahmad, M., Ahmad, M. M., Abdin, M. Z., Musarrat, J., & Javed, S. (2013). Chitinases: An update. *Journal of Pharmacy & Bioallied Sciences*, *5*(1), 21–29. https://doi.org/10.4103/0975-7406.106559

Hao, J., Xu, L., He, H., Du, X., & Jia, L. (2013). High-level expression of Staphylococcal Protein A in Pichia pastoris and purification and characterization of the recombinant protein. *Protein Expression and Purification*, *90*(2), 178–185. https://doi.org/https://doi.org/10.1016/j.pep.2013.06.005

Hart, P. J., Monzingo, A. F., Ready, M. P., Ernst, S. R., & Robertus, J. D. (1993). Crystal Structure of an Endochitinase from Hordeum vulgare L. Seeds. *Journal of Molecular Biology*, *229*(1), 189–193. https://doi.org/https://doi.org/10.1006/jmbi.1993.1017

Hartl, L., Zach, S., & Seidl-Seiboth, V. (2012). Fungal chitinases: diversity, mechanistic properties and biotechnological potential. *Applied Microbiology and Biotechnology*, *93*(2), 533–543. https://doi.org/10.1007/s00253-011-3723-3

Hawtin, R. E., Zarkowska, T., Arnold, K., Thomas, C. J., Gooday, G. W., King, L. A., … Possee, R. D. (1997). Liquefaction ofAutographa californicaNucleopolyhedrovirus-Infected Insects Is Dependent on the Integrity of Virus-Encoded Chitinase and Cathepsin Genes. *Virology*, *238*(2), 243–253. https://doi.org/https://doi.org/10.1006/viro.1997.8816

Heggset, E. B., Hoell, I. A., Kristoffersen, M., Eijsink, V. G. H., & Vårum, K. M. (2009). Degradation of Chitosans with Chitinase G from Streptomyces coelicolor A3(2): Production of Chito-oligosaccharides and Insight into Subsite Specificities. *Biomacromolecules*, *10*(4), 892–899. https://doi.org/10.1021/bm801418p

Helland, R., Larsen, R. L., Finstad, S., Kyomuhendo, P., & Larsen, A. N. (2009). Crystal structures of g-type lysozyme from Atlantic cod shed new light on substrate binding and the catalytic mechanism. *Cellular and Molecular Life Sciences*, *66*(15), 2585–2598. https://doi.org/10.1007/s00018-009-0063-x

Hirakawa, H., Ochi, A., Kawahara, Y., Kawamura, S., Torikata, T., & Kuhara, S. (2008). Catalytic Reaction Mechanism of Goose Egg-white Lysozyme by Molecular Modelling of Enzyme–Substrate Complex. *The Journal of Biochemistry*, *144*(6), 753–761. https://doi.org/10.1093/jb/mvn133

Hoell, I. A., Dalhus, B., Heggset, E. B., Aspmo, S. I., & Eijsink, V. G. H. (2006). Crystal structure and enzymatic properties of a bacterial family 19 chitinase reveal differences from plant enzymes. *The FEBS Journal*, *273*(21), 4889–4900. https://doi.org/https://doi.org/10.1111/j.1742-4658.2006.05487.x

Horn, S. J., Sørbotten, A., Synstad, B., Sikorski, P., Sørlie, M., Vårum, K. M., & Eijsink, V.

G. H. (2006). Endo/exo mechanism and processivity of family 18 chitinases produced by Serratia marcescens. *The FEBS Journal*, *273*(3), 491–503. https://doi.org/https://doi.org/10.1111/j.1742-4658.2005.05079.x

Hsu, S. C., & Lockwood, J. L. (1975). Powdered Chitin Agar as a Selective Medium for Enumeration of Actinomycetes in Water and Soil. *Applied Microbiology*, *29*(3), 422–426. https://doi.org/10.1128/am.29.3.422-426.1975

Huet, J., Rucktooa, P., Clantin, B., Azarkan, M., Looze, Y., Villeret, V., & Wintjens, R. (2008). X-ray Structure of Papaya Chitinase Reveals the Substrate Binding Mode of Glycosyl Hydrolase Family 19 Chitinases. *Biochemistry*, *47*(32), 8283–8291. https://doi.org/10.1021/bi800655u

Ingvarsson, P. T., Schmidt, S. T., Christensen, D., Larsen, N. B., Hinrichs, W. L. J., Andersen, P., … Foged, C. (2013). Designing CAF-adjuvanted dry powder vaccines: Spray drying preserves the adjuvant activity of CAF01. *Journal of Controlled Release*, *167*(3), 256–264. https://doi.org/https://doi.org/10.1016/j.jconrel.2013.01.031

Iseli, B., Armand, S., Boller, T., Neuhaus, J.-M., & Henrissat, B. (1996). Plant chitinases use two different hydrolytic mechanisms. *FEBS Letters*, *382*(1), 186–188. https://doi.org/https://doi.org/10.1016/0014-5793(96)00174-3

Jang, M.-K., Kong, B.-G., Jeong, Y.-I., Lee, C. H., & Nah, J.-W. (2004). Physicochemical characterization of α-chitin, β-chitin, and γ-chitin separated from natural resources. *Journal of Polymer Science Part A: Polymer Chemistry*, *42*(14), 3423–3432. https://doi.org/https://doi.org/10.1002/pola.20176

Javanmard, A. S., Matin, M. M., & Bahrami, A. R. (2021). Polycistronic cellulase gene expression in Pichia pastoris. *Biomass Conversion and Biorefinery*. https://doi.org/10.1007/s13399-021-01765-7

Jiang, Z., Hu, S., Ma, J., Liu, Y., Qiao, Z., Yan, Q., … Yang, S. (2021). Crystal structure of a chitinase (RmChiA) from the thermophilic fungus Rhizomucor miehei with a real active site tunnel. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics*, *1869*(11), 140709. https://doi.org/https://doi.org/10.1016/j.bbapap.2021.140709

Joshi, V. B., Geary, S. M., & Salem, A. K. (2013). Biodegradable Particles as Vaccine Delivery Systems: Size Matters. *The AAPS Journal*, *15*(1), 85–94. https://doi.org/10.1208/s12248-012-9418-6

Jozala, A. F., Geraldes, D. C., Tundisi, L. L., Feitosa, V. de A., Breyer, C. A., Cardoso, S. L., … Pessoa, A. (2016). Biopharmaceuticals from microorganisms: from production to purification. *Brazilian Journal of Microbiology*, *47*, 51–63. https://doi.org/https://doi.org/10.1016/j.bjm.2016.10.007

Jung, W.-J., & Park, R.-D. (2014). Bioproduction of Chitooligosaccharides: Present and Perspectives. *Marine Drugs* , Vol. 12. https://doi.org/10.3390/md12115328

Karthik, N., Akanksha, K., & Pandey, A. (2014). Production, purification and properties of fungal chitinases--a review. *Indian Journal of Experimental Biology*, *52 11*, 1025–1035.

Kavroulakis, N., Ntougias, S., Besi, M. I., Katsou, P., Damaskinou, A., Ehaliotis, C., … Papadopoulou, K. K. (2010). Antagonistic bacteria of composted agro-industrial residues exhibit antibiosis against soil-borne fungal plant pathogens and protection of tomato plants from Fusarium oxysporum f.sp. radicis-lycopersici. *Plant and Soil*, *333*(1), 233–247. https://doi.org/10.1007/s11104-010-0338-x

Kaya, M., Mujtaba, M., Ehrlich, H., Salaberria, A. M., Baran, T., Amemiya, C. T., … Labidi, J. (2017). On chemistry of γ-chitin. *Carbohydrate Polymers*, *176*, 177–186. https://doi.org/https://doi.org/10.1016/j.carbpol.2017.08.076

Khan, F. I., Rahman, S., Queen, A., Ahamad, S., Ali, S., Kim, J., & Hassan, M. I. (2017). Implications of molecular diversity of chitin and its derivatives. *Applied Microbiology and Biotechnology*, *101*(9), 3513–3536. https://doi.org/10.1007/s00253-017-8229-1

KitoZyme. (2021). Leader in fungal and vegetal chitosan for consumer healthcare. Retrieved from https://www.kitozyme.com

Klis, F., & Brul, S. (2015). Adaptations of the Secretome of Candida albicans in Response to Host-Related Environmental Conditions. *Eukaryotic Cell*, *14*. https://doi.org/10.1128/EC.00142-15

Koga, D., Sasaki, Y., Uchiumi, Y., Hirai, N., Arakane, Y., & Nagamatsu, Y. (1997). Purification and characterization of Bombyx mori chitinases. *Insect Biochemistry and Molecular Biology*, *27*(8), 757–767. https://doi.org/https://doi.org/10.1016/S0965-1748(97)00058-1

Kong, X. F., Zhou, X. L., Lian, G. Q., Blachier, F., Liu, G., Tan, B. E., … Yin, Y. L. (2014). Dietary supplementation with chitooligosaccharides alters gut microbiota and modifies intestinal luminal metabolites in weaned Huanjiang mini-piglets. *Livestock Science*, *160*, 97–101. https://doi.org/https://doi.org/10.1016/j.livsci.2013.11.023

Kurland, C., & Gallant, J. (1996). Errors of heterologous protein expression. *Current Opinion in Biotechnology*, *7*(5), 489–493. https://doi.org/https://doi.org/10.1016/S0958-1669(96)80050-4

Ladet, S., David, L., & Domard, A. (2008). Multi-membrane hydrogels. *Nature*, *452*(7183), 76–79. https://doi.org/10.1038/nature06619

Langner, T., & Göhre, V. (2016). Fungal chitinases: function, regulation, and potential roles in plant/pathogen interactions. *Current Genetics*, *62*(2), 243–254. https://doi.org/10.1007/s00294-015-0530-x

Laukens, B., De Wachter, C., & Callewaert, N. (2015). *Engineering the Pichia pastoris N-Glycosylation Pathway Using the GlycoSwitch Technology BT - Glyco-Engineering: Methods and Protocols* (A. Castilho, Ed.). https://doi.org/10.1007/978-1-4939-2760-9_8

Lekjing, S. (2016). A chitosan-based coating with or without clove oil extends the shelf life of cooked pork sausages in refrigerated storage. *Meat Science*, *111*, 192–197. https://doi.org/https://doi.org/10.1016/j.meatsci.2015.10.003

Liang, T.-W., Chen, Y.-J., Yen, Y.-H., & Wang, S.-L. (2007). The antitumor activity of the hydrolysates of chitinous materials hydrolyzed by crude enzyme from Bacillus amyloliquefaciens V656. *Process Biochemistry*, *42*(4), 527–534. https://doi.org/https://doi.org/10.1016/j.procbio.2006.10.005

Lincopan, N., Espíndola, N. M., Vaz, A. J., Costa, M. H. B. da, Faquim-Mauro, E., & Carmona-Ribeiro, A. M. (2009). Novel immunoadjuvants based on cationic lipid: Preparation, characterization and activity in vivo. *Vaccine*, *27*(42), 5760–5771. https://doi.org/https://doi.org/10.1016/j.vaccine.2009.07.066

Liu, J., NanGong, Z., Zhang, J., Song, P., Tang, Y., Gao, Y., & Wang, Q. (2019). Expression and characterization of two chitinases with synergistic effect and antifungal activity from Xenorhabdus nematophila. *World Journal of Microbiology and Biotechnology*, *35*(7), 106. https://doi.org/10.1007/s11274-019-2670-5

Liu, Z., Tyo, K. E. J., Martínez, J. L., Petranovic, D., & Nielsen, J. (2012). Different expression systems for production of recombinant proteins in Saccharomyces cerevisiae. *Biotechnology and Bioengineering*, *109*(5), 1259–1268. https://doi.org/https://doi.org/10.1002/bit.24409

Lorito, M., Harman, G. E., Hayes, C. K., Broadway, R. M., Tronsmo, A., Woo, S. L., & Pietro, A. Di. (1993). Chitinolytic enzymes produced by trichoderma harzianum; antifungal activity of purified endochitinase and chitobiosidase. *Phytopathology*, *83*, 302–307.

Lu, Y., Wang, N., He, J., Li, Y., Gao, X., Huang, L., & Yan, X. (2018). Expression and characterization of a novel chitinase with antifungal activity from a rare actinomycete, Saccharothrix yanglingensis Hhs.015. *Protein Expression and Purification*, *143*, 45–51.

https://doi.org/https://doi.org/10.1016/j.pep.2017.10.013

Madhuprakash, J., El Gueddari, N. E., Moerschbacher, B. M., & Podile, A. R. (2015). Catalytic Efficiency of Chitinase-D on Insoluble Chitinous Substrates Was Improved by Fusing Auxiliary Domains. *PLOS ONE*, *10*(1), e0116823. Retrieved from https://doi.org/10.1371/journal.pone.0116823

Malik, A. (2019). Purification and properties of plant chitinases: A review. *Journal of Food Biochemistry*, *43*(3), e12762. https://doi.org/https://doi.org/10.1111/jfbc.12762

Malinowska-Pańczyk, E., Kołodziejska, I., Murawska, D., & Wołosewicz, G. (2009). The Combined Effect of Moderate Pressure and Chitosan on Escherichia coli and Staphylococcus aureus Cells Suspended in a Buffer and on Natural Microflora of Apple Juice and Minced Pork. *Food Technology and Biotechnology*, *47*, 202–209.

Mansur, M., Cabello, C., Hernández, L., País, J., Varas, L., Valdés, J., … Martínez, E. (2005). Multiple gene copy number enhances insulin precursor secretion in the yeast Pichia pastoris. *Biotechnology Letters*, *27*(5), 339–345. https://doi.org/10.1007/s10529-005-1007-7

Mat Amin, A., & Kang, W. C. (2013). Effect of chitosan on gelling properties, lipid oxidation, and microbial load of surimi gel made from African catfish (Clarias gariepinus). *International Food Research Journal*, *20*, 1585–1594.

Mathew, G. M., Madhavan, A., Arun, K. B., Sindhu, R., Binod, P., Singhania, R. R., … Pandey, A. (2021). Thermophilic Chitinases: Structural, Functional and Engineering Attributes for Industrial Applications. *Applied Biochemistry and Biotechnology*, *193*(1), 142–164. https://doi.org/10.1007/s12010-020-03416-5

Mattaveewong, T., Wongkrasant, P., Chanchai, S., Pichyangkura, R., Chatsudthipong, V., & Muanprasat, C. (2016). Chitosan oligosaccharide suppresses tumor progression in a mouse model of colitis-associated colorectal cancer through AMPK activation and suppression of NF-κB and mTOR signaling. *Carbohydrate Polymers*, *145*, 30–36. https://doi.org/https://doi.org/10.1016/j.carbpol.2016.02.077

Mazur, M., Zielińska, A., Grzybowski, M. M., Olczak, J., & Fichna, J. (2021). Chitinases and Chitinase-Like Proteins as Therapeutic Targets in Inflammatory Diseases, with a Special Focus on Inflammatory Bowel Diseases. *International Journal of Molecular Sciences*, Vol. 22. https://doi.org/10.3390/ijms22136966

McCreath, K. J., Specht, C. A., & Robbins, P. W. (1995). Molecular cloning and characterization of chitinase genes from Candida albicans. *Proceedings of the National Academy of Sciences*, *92*(7), 2544–2548. https://doi.org/10.1073/pnas.92.7.2544

Minke, R., & Blackwell, J. (1978). The structure of α-chitin. *Journal of Molecular Biology*, *120*(2), 167–181. https://doi.org/https://doi.org/10.1016/0022-2836(78)90063-3

Minyasab, S. A., Dhamane, S. P., Hazra, P., & Iyer, H. (2010). A method of purifying human growth hormone and purified growth hormone thereof. *Pub. No: WO/2010/134084. International Application No: PCT/IN2009/000380. Google Patents*.

Mohammed, A. R., Bramwell, V. W., Kirby, D. J., McNeil, S. E., & Perrie, Y. (2010). Increased potential of a cationic liposome-based delivery system: Enhancing stability and sustained immunological activity in pre-clinical development. *European Journal of Pharmaceutics and Biopharmaceutics*, *76*(3), 404–412. https://doi.org/https://doi.org/10.1016/j.ejpb.2010.09.008

Morganti, P., Tishchenko, G., Palombo, M., Kelnar, I., Brozová, L., Špírková, M., … Francesco, C. (2013). Chitin nanofibrils for biomimetic products: nanoparticles and nanocomposite chitosan films in health care. *Marine Biomaterials. Characterization, Isolation and Application*, 673–680.

Morin-Crini, N., Lichtfouse, E., Torri, G., & Crini, G. (2019). Applications of chitosan in food, pharmaceuticals, medicine, cosmetics, agriculture, textiles, pulp and paper,

biotechnology, and environmental chemistry. *Environmental Chemistry Letters*, *17*(4), 1667–1692. https://doi.org/10.1007/s10311-019-00904-x

Mukarram, M., Khan, M. M. A., Choudhary, S., Zehra, A., Naeem, M., & Aftab, T. (2021). *Natural Polysaccharides: Novel Plant Growth Regulators BT - Plant Growth Regulators: Signalling under Stress Conditions* (T. Aftab & K. R. Hakeem, Eds.). https://doi.org/10.1007/978-3-030-61153-8_15

Muzzarelli, R. A. A., Greco, F., Busilacchi, A., Sollazzo, V., & Gigante, A. (2012). Chitosan, hyaluronan and chondroitin sulfate in tissue engineering for cartilage regeneration: A review. *Carbohydrate Polymers*, *89*(3), 723–739. https://doi.org/https://doi.org/10.1016/j.carbpol.2012.04.057

Nagpure, A., Choudhary, B., & Gupta, R. K. (2014). Chitinases: in agriculture and human healthcare. *Critical Reviews in Biotechnology*, *34*(3), 215–232. https://doi.org/10.3109/07388551.2013.790874

Nasseri, A. T., Sara, R.-A., Mohammad Hossei, M., & Ghasemi, Y. (2011). Single Cell Protein: Production and Process. *American Journal of Food Technology*, *6*. https://doi.org/10.3923/ajft.2011.103.116

Ogris, M., Steinlein, P., Kursa, M., Mechtler, K., Kircheis, R., & Wagner, E. (1998). The size of DNA/transferrin-PEI complexes is an important factor for gene expression in cultured cells. *Gene Therapy*, *5*(10), 1425–1433. https://doi.org/10.1038/sj.gt.3300745

Ohtakara, A., Izume, M., & Mitsutomi, M. (1988). Action of Microbial Chitinases on Chitosan with Different Degrees of Deacetylation. *Agricultural and Biological Chemistry*, *52*(12), 3181–3182. https://doi.org/10.1080/00021369.1988.10869204

Oliveira, A. C. N., Martens, T. F., Raemdonck, K., Adati, R. D., Feitosa, E., Botelho, C., … Real Oliveira, M. E. C. D. (2014). Dioctadecyldimethylammonium:Monoolein Nanocarriers for Efficient in Vitro Gene Silencing. *ACS Applied Materials & Interfaces*, *6*(9), 6977–6989. https://doi.org/10.1021/am500793y

Öztopuz, Ö., Pekin, G., Park, R. D., & Eltem, R. (2018). Isolation and Evaluation of New Antagonist Bacillus Strains for the Control of Pathogenic and Mycotoxigenic Fungi of Fig Orchards. *Applied Biochemistry and Biotechnology*, *186*(3), 692–711. https://doi.org/10.1007/s12010-018-2764-9

Pan, X. Q., Shih, C. H. U. C., & Harday, J. (2005). Chitinase Induces Lysis of MCF-7 Cells in Culture and of Human Breast Cancer Xenograft B11-2 in SCID Mice. *Anticancer Research*, *25*(5), 3167 LP – 3172. Retrieved from http://ar.iiarjournals.org/content/25/5/3167.abstract

Paoletti, M. G., Norberto, L., Damini, R., & Musumeci, S. (2007). Human Gastric Juice Contains Chitinase That Can Degrade Chitin. *Annals of Nutrition and Metabolism*, *51*(3), 244–251. https://doi.org/10.1159/000104144

Park, B. K., & Kim, M.-M. (2010). Applications of Chitin and Its Derivatives in Biological Medicine. *International Journal of Molecular Sciences* , Vol. 11. https://doi.org/10.3390/ijms11125152

Park, J. K., Chung, M. J., Choi, H. N., & Park, Y. Il. (2011). Effects of the Molecular Weight and the Degree of Deacetylation of Chitosan Oligosaccharides on Antitumor Activity. *International Journal of Molecular Sciences* , Vol. 12. https://doi.org/10.3390/ijms12010266

Parsiegla, G., Reverbel, C., Tardif, C., Driguez, H., & Haser, R. (2008). Structures of Mutants of Cellulase Cel48F of Clostridium cellulolyticum in Complex with Long Hemithiocellooligosaccharides Give Rise to a New View of the Substrate Pathway during Processive Action. *Journal of Molecular Biology*, *375*(2), 499–510. https://doi.org/https://doi.org/10.1016/j.jmb.2007.10.039

Patel, S., & Goyal, A. (2011). Functional oligosaccharides: production, properties and

applications. *World Journal of Microbiology and Biotechnology*, *27*(5), 1119–1128. https://doi.org/10.1007/s11274-010-0558-5

Patil, N. S., Waghmare, S. R., & Jadhav, J. P. (2013). Purification and characterization of an extracellular antifungal chitinase from Penicillium ochrochloron MTCC 517 and its application in protoplast formation. *Process Biochemistry*, *48*(1), 176–183. https://doi.org/https://doi.org/10.1016/j.procbio.2012.11.017

Petsch, D., & Anspach, F. B. (2000). Endotoxin removal from protein solutions. *Journal of Biotechnology*, *76*(2), 97–119. https://doi.org/https://doi.org/10.1016/S0168-1656(99)00185-6

Rathore, A., & Gupta, R. (2015). Chitinases from Bacteria to Human: Properties, Applications, and Future Perspectives. *Enzyme Research*, *2015*, 1–8. https://doi.org/10.1155/2015/791907

Renkema, G. H., Boot, R. G., Muijsers, A. O., Donker-Koopman, W. E., & Aerts, J. M. F. G. (1995). Purification and Characterization of Human Chitotriosidase, a Novel Member of the Chitinase Family of Proteins (∗). *Journal of Biological Chemistry*, *270*(5), 2198–2202. https://doi.org/https://doi.org/10.1074/jbc.270.5.2198

Rodloff, A. C., Koch, D., & Schaumann, R. (2011). Epidemiology and antifungal resistance in invasive candidiasis. *European Journal of Medical Research*, *16*(4), 187. https://doi.org/10.1186/2047-783X-16-4-187

Rosano, G. L., & Ceccarelli, E. A. (2014). Recombinant protein expression in Escherichia coli: advances and challenges   . *Frontiers in Microbiology*   , Vol. 5. Retrieved from https://www.frontiersin.org/article/10.3389/fmicb.2014.00172

Roy, J. C., Salaün, F., Giraud, S., Ferri, A., Chen, G., & Guan, J. (2017). Solubility of chitin: solvents, solution behaviors and their related mechanisms. *Solubility of Polysaccharides*, *3*, 20–60.

Sadfi, N., Chérif, M., Fliss, I., Boudabbous, A., & Antoun, H. (2001). Evaluation of bacterila isolates from salty soils and bacillus thuringiensis strains for the biocontrol of fusarium dry rot of potato tubers. *Journal of Plant Pathology*, *83*(2), 101–117. Retrieved from http://www.jstor.org/stable/41998046

Sahai, A. S., & Manocha, M. S. (1993). Chitinases of fungi and plants: their involvement in morphogenesis and host—parasite interaction. *FEMS Microbiology Reviews*, *11*(4), 317–338. https://doi.org/10.1111/j.1574-6976.1993.tb00004.x

Sasaki, C., Vårum, K. M., Itoh, Y., Tamoi, M., & Fukamizo, T. (2006). Rice chitinases: sugar recognition specificities of the individual subsites. *Glycobiology*, *16*(12), 1242–1250. https://doi.org/10.1093/glycob/cwl043

Schwendener, R. A. (2014). Liposomes as vaccine delivery systems: a review of the recent advances. *Therapeutic Advances in Vaccines*, *2*(6), 159–182. https://doi.org/10.1177/2051013614541440

Seidl, V. (2008). Chitinases of filamentous fungi: a large group of diverse proteins with multiple physiological functions. *Fungal Biology Reviews*, *22*(1), 36–42. https://doi.org/https://doi.org/10.1016/j.fbr.2008.03.002

Selenius, O., Korpela, J., Salminen, S., & Gómez-Gallego, C. (2018). Effect of chitin and chitooligosaccharide on in vitro growth of Lactobacillus rhamnosus GG and Escherichia coli TG. *Applied Food Biotechnology*, *5*, 163–172. https://doi.org/10.22037/afb.v%vi%i.20468

Selvaggini, S., Munro, C., Paschoud, S., Sanglard, D., & Gow, N. (2004). Independent regulation of chitin synthase and chitinase activity in Candida albicans and Saccharomyces cerevisiae. *Microbiology (Reading, England)*, *150*, 921–928. https://doi.org/10.1099/mic.0.26661-0

Shamshina, J. L., Oldham (Konak), T., & Rogers, R. D. (2019). *Applications of Chitin in*

Agriculture BT - Sustainable Agriculture Reviews 36: Chitin and Chitosan: Applications in Food, Agriculture, Pharmacy, Medicine and Wastewater Treatment (G. Crini & E. Lichtfouse, Eds.). https://doi.org/10.1007/978-3-030-16581-9_4

Sharma, C. K., Vishnoi, V. K., Dubey, R. C., & Maheshwari, D. K. (2018). A twin rhizospheric bacterial consortium induces systemic resistance to a phytopathogen Macrophomina phaseolina in mung bean. *Rhizosphere*, *5*, 71–75. https://doi.org/https://doi.org/10.1016/j.rhisph.2018.01.001

Sharma, R. R., Singh, D., & Singh, R. (2009). Biological control of postharvest diseases of fruits and vegetables by microbial antagonists: A review. *Biological Control*, *50*(3), 205–221. https://doi.org/https://doi.org/10.1016/j.biocontrol.2009.05.001

Shimono, K., Matsuda, H., & Kawamukai, M. (2002). Functional Expression of Chitinase and Chitosanase, and Their Effects on Morphologies in the Yeast Schizosaccharomyces pombe. *Bioscience, Biotechnology, and Biochemistry*, *66*(5), 1143–1147. https://doi.org/10.1271/bbb.66.1143

Shiro, M., Ueda, M., Kawaguchi, T., & Arai, M. (1996). Cloning of a cluster of chitinase genes from Aeromonas sp. No. 10S-24. *Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression*, *1305*(1), 44–48. https://doi.org/https://doi.org/10.1016/0167-4781(95)00213-8

Shrestha, A., Sultana, R., Chae, J.-C., Kim, K., & Lee, K.-J. (2015). Bacillus thuringiensis C25 which is rich in cell wall degrading enzymes efficiently controls lettuce drop caused by Sclerotinia minor. *European Journal of Plant Pathology*, *142*(3), 577–589. https://doi.org/10.1007/s10658-015-0636-5

Shuhui, L., Mok, Y.-K., & Wong, W. S. F. (2009). Role of Mammalian Chitinases in Asthma. *International Archives of Allergy and Immunology*, *149*(4), 369–377. https://doi.org/10.1159/000205583

Silva, J. P. N., Coutinho, P. J. G., & Oliveira, M. E. C. D. R. (2008). Characterization of Monoolein-Based Lipoplexes Using Fluorescence Spectroscopy. *Journal of Fluorescence*, *18*(2), 555–562. https://doi.org/10.1007/s10895-007-0299-5

Silva, J. P. N., Oliveira, A. C. N., Casal, M. P. P. A., Gomes, A. C., Coutinho, P. J. G., Coutinho, O. P., & Oliveira, M. E. C. D. R. (2011). DODAB:monoolein-based lipoplexes as non-viral vectors for transfection of mammalian cells. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, *1808*(10), 2440–2449. https://doi.org/https://doi.org/10.1016/j.bbamem.2011.07.002

Silva, J. P. N., Oliveira, I. M. S. C., Oliveira, A. C. N., Lúcio, M., Gomes, A. C., Coutinho, P. J. G., & Oliveira, M. E. C. D. R. (2014). Structural dynamics and physicochemical properties of pDNA/DODAB:MO lipoplexes: Effect of pH and anionic lipids in inverted non-lamellar phases versus lamellar phases. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, *1838*(10), 2555–2567. https://doi.org/https://doi.org/10.1016/j.bbamem.2014.06.014

Souza-Neto, J. A., Gusmão, D. S., & Lemos, F. J. A. (2003). Chitinolytic activities in the gut of Aedes aegypti (Diptera:Culicidae) larvae and their role in digestion of chitin-rich structures. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *136*(3), 717–724. https://doi.org/https://doi.org/10.1016/S1095-6433(03)00224-1

Stoykov, Y., Krastanov, A., & Pavlov, A. (2014). Optimization of cultural conditions for production of chitinase by bacterial soil isolate. *Industrial, Medical and Environmental Applications of Microorganisms: Current Status and Trends, Wageningen Academic Publishers, Spain*, 618–624.

Sudheesh Kumar, P. T., Lakshmanan, V.-K., Anilkumar, T. V, Ramya, C., Reshmi, P., Unnikrishnan, A. G., … Jayakumar, R. (2012). Flexible and Microporous Chitosan

Hydrogel/Nano ZnO Composite Bandages for Wound Dressing: In Vitro and In Vivo Evaluation. *ACS Applied Materials & Interfaces*, *4*(5), 2618–2629. https://doi.org/10.1021/am300292v

Suganthi, M., Senthilkumar, P., Arvinth, S., & Chandrashekara, K. N. (2017). Chitinase from *Pseudomonas fluorescens* and its insecticidal activity against *Helopeltis theivora*. *The Journal of General and Applied Microbiology*, *63*(4), 222–227. https://doi.org/10.2323/jgam.2016.11.001

Suginta, W., & Sritho, N. (2012). Multiple Roles of Asp313 in the Refined Catalytic Cycle of Chitin Degradation by Vibrio harveyi Chitinase A. *Bioscience, Biotechnology, and Biochemistry*, *76*(12), 2275–2281. https://doi.org/10.1271/bbb.120559

Tang, W. J., Fernandez, J. G., Sohn, J. J., & Amemiya, C. T. (2015). Chitin Is Endogenously Produced in Vertebrates. *Current Biology*, *25*(7), 897–900. https://doi.org/https://doi.org/10.1016/j.cub.2015.01.058

Terpe, K. (2003). Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems. *Applied Microbiology and Biotechnology*, *60*(5), 523–533. https://doi.org/10.1007/s00253-002-1158-6

Terwisscha van Scheltinga, A. C., Armand, S., Kalk, K. H., Isogai, A., Henrissat, B., & Dijkstra, B. W. (1995). Stereochemistry of chitin hydrolysis by a plant chitinase/lysozyme and x-ray structure of a complex with allosamidin evidence for substrate assisted catalysis. *Biochemistry*, *34*(48), 15619–15623. https://doi.org/10.1021/bi00048a003

Tews, I., Terwisscha van Scheltinga, A. C., Perrakis, A., Wilson, K. S., & Dijkstra, B. W. (1997). Substrate-Assisted Catalysis Unifies Two Families of Chitinolytic Enzymes. *Journal of the American Chemical Society*, *119*(34), 7954–7959. https://doi.org/10.1021/ja970674i

Ubhayasekera, W. (2011). Structure and function of chitinases from glycoside hydrolase family 19. *Polymer International*, *60*(6), 890–896. https://doi.org/https://doi.org/10.1002/pi.3028

Udaya Prakash, N. A., Jayanthi, M., Sabarinathan, R., Kangueane, P., Mathew, L., & Sekar, K. (2010). Evolution, Homology Conservation, and Identification of Unique Sequence Signatures in GH19 Family Chitinases. *Journal of Molecular Evolution*, *70*(5), 466–478. https://doi.org/10.1007/s00239-010-9345-z

Ueda, M., Ohata, K., Konishi, T., Sutrisno, A., Okada, H., Nakazawa, M., & Miyatake, K. (2009). A novel goose-type lysozyme gene with chitinolytic activity from the moderately thermophilic bacterium Ralstonia sp. A-471: cloning, sequencing, and expression. *Applied Microbiology and Biotechnology*, *81*(6), 1077–1085. https://doi.org/10.1007/s00253-008-1676-y

Vaikuntapu, P. R., Rambabu, S., Madhuprakash, J., & Podile, A. R. (2016). A new chitinase-D from a plant growth promoting Serratia marcescens GPS5 for enzymatic conversion of chitin. *Bioresource Technology*, *220*, 200–207. https://doi.org/https://doi.org/10.1016/j.biortech.2016.08.055

van Aalten, D., Komander, D., Synstad, B., Gaseidnes, S., Peter, M., & Eijsink, V. (2001). Structural Insights into the catalytic mechanism of a family 18 exochitinase. *Proceedings of the National Academy of Sciences of the United States of America*, *98*, 8979–8984. https://doi.org/10.1073/pnas.151103798

van Loon, L. C., Rep, M., & Pieterse, C. M. J. (2006). Significance of Inducible Defense-related Proteins in Infected Plants. *Annual Review of Phytopathology*, *44*(1), 135–162. https://doi.org/10.1146/annurev.phyto.44.070505.143425

Veenhuis, M., Dijken, J. P. Van, & Harder, W. (1983). *The Significance of Peroxisomes in the Metabolism of One-Carbon Compounds in Yeasts* (A. H. Rose, J. G. Morris, & D. W. B.

T.-A. in M. P. Tempest, Eds.). https://doi.org/https://doi.org/10.1016/S0065-2911(08)60384-7

Vegan, C. (2021). Fungal chitin and chitosan. Retrieved from https://chitosanlab.com/vegan/

Vision, T., Medical, H., Gmbh, C., & Bio, K. (2017). *Global Chitin Market to be worth US $ 2900 Mn by 2027 - Future Market Insights*.

Wang, S.-L., Liou, J.-Y., Liang, T.-W., & Liu, K.-C. (2009). Conversion of squid pen by using Serratia sp. TKU020 fermentation for the production of enzymes, antioxidants, and N-acetyl chitooligosaccharides. *Process Biochemistry*, *44*(8), 854–861. https://doi.org/https://doi.org/10.1016/j.procbio.2009.04.006

Wang, S. L., & Chang, W. T. (1997). Purification and characterization of two bifunctional chitinases/lysozymes extracellularly produced by Pseudomonas aeruginosa K-187 in a shrimp and crab shell powder medium. *Applied and Environmental Microbiology*, *63*(2), 380–386. https://doi.org/10.1128/aem.63.2.380-386.1997

Wegner, G. H. (1990). Emerging applications of the methylotrophic yeasts. *FEMS Microbiology Reviews*, *7*(3–4), 279–283. https://doi.org/10.1111/j.1574-6968.1990.tb04925.x

Whittaker, M. M., & Whittaker, J. W. (2002). Characterization of recombinant barley oxalate oxidase expressed by Pichiapastoris. *JBIC Journal of Biological Inorganic Chemistry*, *7*(1), 136–145. https://doi.org/10.1007/s007750100281

Wildt, S., & Gerngross, T. U. (2005). The humanization of N-glycosylation pathways in yeast. *Nature Reviews Microbiology*, *3*(2), 119–128. https://doi.org/10.1038/nrmicro1087

Wu, C.-T., & Bradford, K. J. (2003). Class I Chitinase and β-1,3-Glucanase Are Differentially Regulated by Wounding, Methyl Jasmonate, Ethylene, and Gibberellin in Tomato Seeds and Leaves. *Plant Physiology*, *133*(1), 263–273. https://doi.org/10.1104/pp.103.024687

Xie, H., Zhang, S., & Li, S. (2006). Chitin and chitosan dissolved in ionic liquids as reversible sorbents of CO2. *Green Chemistry*, *8*(7), 630–633. https://doi.org/10.1039/B517297G

Xie, X.-H., Fu, X., Yan, X.-Y., Peng, W.-F., & Kang, L.-X. (2021). A Broad-Specificity Chitinase from Penicillium oxalicum k10 Exhibits Antifungal Activity and Biodegradation Properties of Chitin. *Marine Drugs* , Vol. 19. https://doi.org/10.3390/md19070356

Yeo, I. J., Lee, C.-K., Han, S.-B., Yun, J., & Hong, J. T. (2019). Roles of chitinase 3-like 1 in the development of cancer, neurodegenerative diseases, and inflammatory diseases. *Pharmacology & Therapeutics*, *203*, 107394. https://doi.org/https://doi.org/10.1016/j.pharmthera.2019.107394

Yesilirmak, F., & Sayers, Z. (2009). Heterelogous Expression of Plant Genes. *International Journal of Plant Genomics*, *2009*, 296482. https://doi.org/10.1155/2009/296482

Younes, I., Hajji, S., Frachet, V., Rinaudo, M., Jellouli, K., & Nasri, M. (2014). Chitin extraction from shrimp shell using enzymatic treatment. Antitumor, antioxidant and antimicrobial activities of chitosan. *International Journal of Biological Macromolecules*, *69*, 489–498. https://doi.org/https://doi.org/10.1016/j.ijbiomac.2014.06.013

Zhang, H., Huang, X., Fukamizo, T., Muthukrishnan, S., & Kramer, K. J. (2002). Site-directed mutagenesis and functional analysis of an active site tryptophan of insect chitinase. *Insect Biochemistry and Molecular Biology*, *32*(11), 1477–1488. https://doi.org/https://doi.org/10.1016/S0965-1748(02)00068-1

Zhang, P., Liu, W., Peng, Y., Han, B., & Yang, Y. (2014). Toll like receptor 4 (TLR4) mediates the stimulating activities of chitosan oligosaccharide on macrophages. *International Immunopharmacology*, *23*(1), 254–261. https://doi.org/https://doi.org/10.1016/j.intimp.2014.09.007

Zhou, Y., Li, S., Li, D., Wang, S., Zhao, W., Lv, Z., … Han, Y. (2020). Enzymatic

preparation of chitooligosaccharides and their anti-obesity application. *Bioscience, Biotechnology, and Biochemistry*, *84*(7), 1460–1466. https://doi.org/10.1080/09168451.2020.1744110

CHAPTER 7. ANEXXES

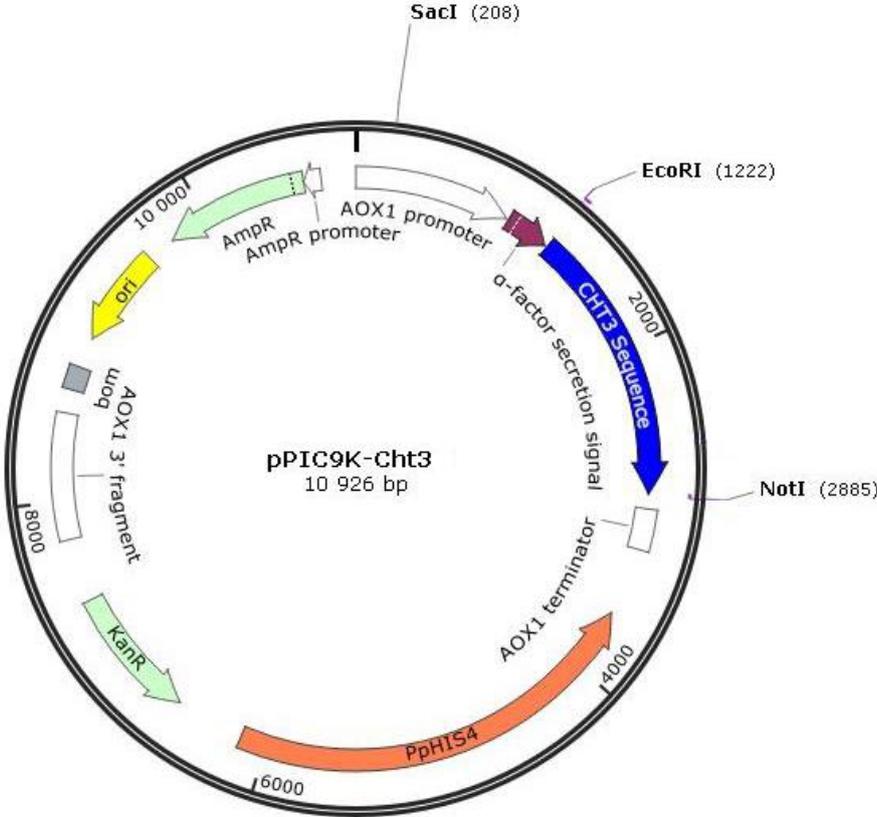# Annex I – Expression Vector pPIC9K-Cht3

Figure 12. Expression vector construct pPIC9K-Cht3 used for Cht3p production in *Pichia pastoris* GS115. The restriction digestion sites used for the *CHT3* gene cloning and plasmid linearization are indicated. Adapted from Barbosa et al., 2017.
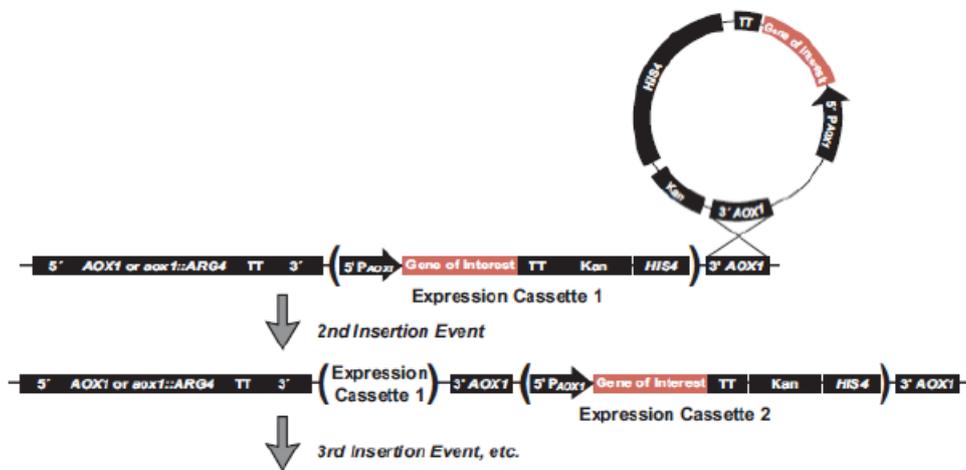
Annex II – Multiple Gene Insertion Events

Figure 13. Multiple gene insertion events with the linearized *P. pastoris* construct. Gene insertions can occur at the *AOX1*, *aox1::ARG4*, or *his4* loci. Taken from "Pichia Expression kit" manual.

Annex III – Standard Curve for Protein Concentration Determination
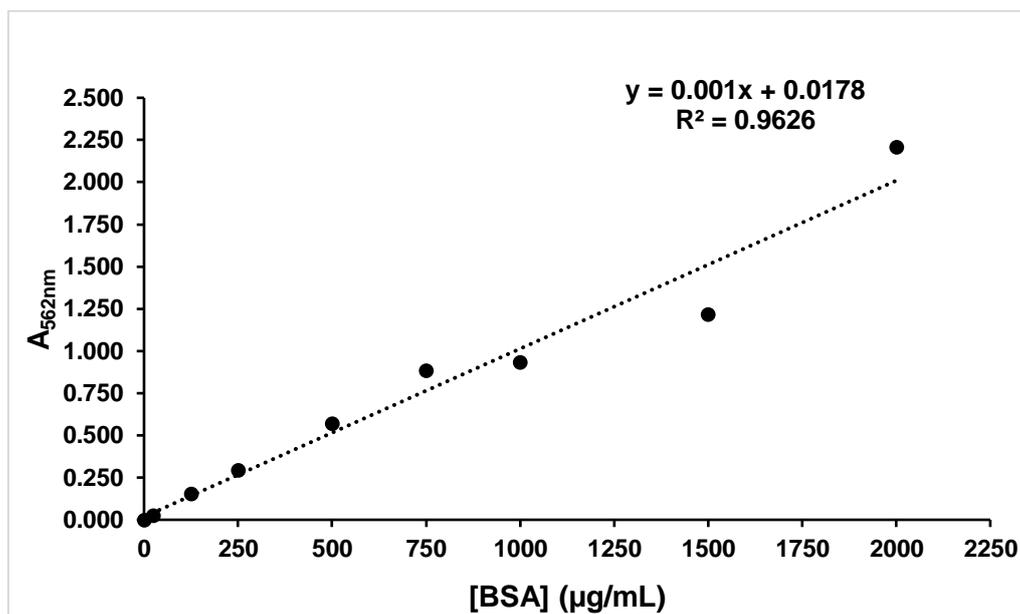
Figure 14. Standard curve for protein concentration determination with the Pierce™ BCA Protein Assay Kit (Thermo Scientific). Bovine serum albumin (BSA) was used as the protein standard.