

**Efficient production of *N*-acetylglucosamine  
with chitinolytic enzymes  
from *Myceliophthora thermophila* C1**

Małgorzata Kaisler

## **Thesis committee**

### **Promotor**

Prof. Dr G. Eggink  
Special Professor, Industrial Biotechnology  
Wageningen University & Research

### **Co-promotors**

Dr C.G. Boeriu,  
Senior scientist, Wageningen Food & Biobased Research  
Wageningen University & Research

Dr L.A.M. van den Broek  
Researcher and project leader, Wageningen Food & Biobased Research  
Wageningen University & Research

### **Other members**

Prof. Dr H.J. Wichers, Wageningen University & Research  
Dr M.A. Kabel, Wageningen University & Research  
Prof. Dr M.J.E.C. van der Maarel, University of Groningen, The Netherlands  
Dr D. Groeseneken, Citrique Belge, Belgium

This research was conducted under the auspices of the Graduate School VLAG (Advanced studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences)

# **Efficient production of *N*-acetylglucosamine with chitinolytic enzymes from *Myceliophthora thermophila* C1**

Małgorzata Kaisler

## **Thesis**

submitted in fulfilment of the requirements for the degree of doctor

at Wageningen University

by the authority of the Rector Magnificus,

Prof. Dr A. P. J. Mol,

in the presence of the

Thesis Committee appointed by the Academic Board

to be defended in public

on Wednesday February 6, 2019

at 1.30 p.m. in the Aula.

Małgorzata Kaisler

Efficient production of *N*-acetylglucosamine with chitinolytic enzymes from *Myceliophthora thermophila* C1

156 pages

PhD thesis, Wageningen University & Research, Wageningen, The Netherlands (2019).

With references, with summary in English.

ISBN 978-94-6343-577-2

DOI <https://doi.org/10.18174/467781>

# Contents

<b>Chapter 1</b>	Introduction	<b>7</b>
<b>Chapter 2</b>	Chitinase Chi1 from <i>Myceliophthora thermophila</i> C1, a thermostable enzyme for chitin and chitosan depolymerization	<b>27</b>
<b>Chapter 3</b>	$\beta$ - <i>N</i> -Acetylglucosaminidase <i>Mth</i> NAG from <i>Myceliophthora thermophila</i> C1, a thermostable enzyme for production of <i>N</i> -acetylglucosamine from chitin	<b>59</b>
<b>Chapter 4</b>	Biocatalytic cascade for the production of <i>N</i> -acetyl-D-glucosamine from chitin with Chitinase Chi1 and <i>N</i> -acetylglucosaminidase <i>Mth</i> NAG from <i>Myceliophthora thermophila</i> C1	<b>85</b>
<b>Chapter 5</b>	Chitinase Chi1 – a tool for fingerprint analysis of (modified)chitosan	<b>107</b>
<b>Chapter 6</b>	General Discussion	<b>119</b>
	<b>Summary</b>	<b>143</b>
	<b>Acknowledgments</b>	<b>147</b>
	<b>About the Author</b>	<b>151</b>



# Chapter 1

## General Introduction

Parts of this chapter are included in:

Kaisler, M., van den Broek, L.A.M., Boeriu, C.G., 2019. Chitosan as source for biobased building blocks. In: van den Broek, L.A.M. and Boeriu, C.G (eds.). Chitin and Chitosan: Properties and Applications. John Wiley & Sons Ltd, Chichester, UK





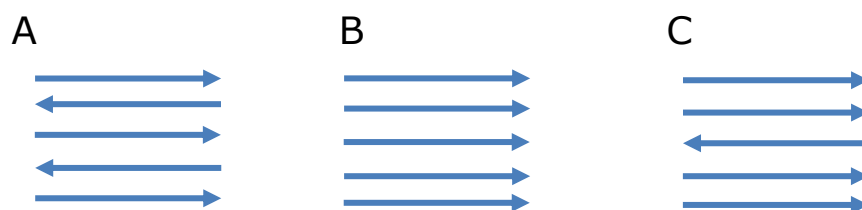
## **1.1. Chitin: from “fungine” to the world-wide used polymer**

### **1.1.1. Discovery of chitin**

In 1799, A. Hachett, an English scientist decalcified shells of crustaceans i.e. crabs, shrimps and prawns and discovered a “material particularly resistant to usual chemicals”. Twelve years later, Henri Braconnot, a French scientist, extracted this new material from mushrooms and named it “fungine”. In 1823, another French scientist, Odier, found “fungine” in exoskeletons of insects and named it “chitin”, after the Greek word for “tunic” (Muzarelli, 1977; Khoushab and Yamabhai, 2010). Further research on chitin led to the discovery by Lassaigue in 1843, that chitin contains nitrogen and by Ledderhose in 1878 that it is composed of glucosamine and acetic acid (Muzarelli, 1977). The term “chitin” survived to our times and refers to the one of the most abundant polymers, next to cellulose and lignin (Gao et al., 2016). Besides crustaceans, fungi and insects, chitin has been found throughout the world in numerous organisms including yeasts (Molano et al., 1980), sponges (Ehrlich et al., 2007), eggs of nematodes (Sharp, 2013), diatoms (Durkin et al., 2009), squid pen (Roberts, 1992), algae (Pearlmutter and Lembi, 1978), amoeba (Rudal and Kenchington, 1973), and in fish *Paralipophrys trigloides* (Wagner et al., 1993).

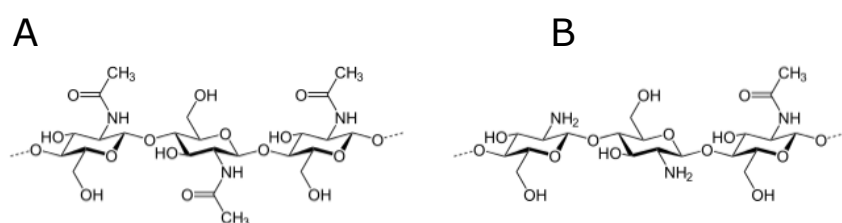
### **1.1.2. Structure of chitin and chitosan**

Depending on the source, chitin occurs in two allomorphs,  $\alpha$ - and  $\beta$ -forms (Rinaudo, 2006). In  $\alpha$ -chitin, the chains are arranged in anti-parallel manner (Figure 1.1.A), and they are densely packed with both inter- and intra-H bonds. Consequently,  $\alpha$ -chitin is unable to swell in water and is the most rigid chitin form (Minke and Blackwell, 1978). In  $\beta$ -chitin, chains are arranged in parallel manner (Figure 1.1.B) with a reduced number of intra-H bonds thus  $\beta$ -chitin is more susceptible to water swelling and has more flexible and softer structure (Gardner and Blackwell, 1975; Merzendorfer and Zimoch, 2003). The existence of a third form of chitin,  $\gamma$ -form (Figure 1.1.C), characterised by sets of two parallel chains and a single anti-parallel chain, was also proposed, but detailed analysis revealed, that it is a variant of  $\alpha$ -chitin (Atkins, 1985).  $\alpha$ -chitin is the most abundant form, which occurs in fungal and yeast cell walls, tendons and shells of crustaceans as well as in insect cuticle (Rinaudo, 2006), where it delivers mechanical strength and stability (Merzendorfer and Zimoch, 2003). Due to its abundance,  $\alpha$ -chitin is the major source of industrially available chitin.



**Figure 1.1.** Arrangement of the chains in  $\alpha$ -chitin (A),  $\beta$ -chitin (B), and  $\gamma$ -chitin (C).

From a chemical point of view, chitin is a linear polymer of *N*-acetylglucosamine (GlcNAc) and some glucosamine (GlcN) units linked with  $\beta$ -(1,4)- glycosidic bonds (Figure 1.2.) (Rinaudo, 2006). The common natural derivative of chitin is chitosan. Chitosan is produced after partial or full-deacetylation of chitin. Chitin and chitosan are distinguished by the degree of deacetylation (DDA). When DDA is below 50 %, then the polymer is entitled chitin and in the opposite, polymer with DDA above 50 % is entitled chitosan (Roberts, 1992). Both polymers can differ in the molecular weight (Mw). The average Mw of native chitin can exceed one million, whereas commercial chitosan products fall in the range from 100,000 to 1,200,000 (Struszczyk, 2002). Low Mw chitin and chitosan products are obtained by hydrolysis of the native polymer chain. DDA and Mw are important parameters influencing the physicochemical and biological properties of chitin and chitosan (Kumirska et al., 2011). The value of DDA and molecular weight determine solubility of the polymer in aqueous acid solutions and extent of swelling in water. The solubility increases with the increase of DDA and the decrease of Mw. Thus chitosan, in contrast to chitin, is soluble in most aqueous solutions of organic acids such as formic, acetic, lactic, and citric acid (Struszczyk, 2002).



**Figure 1.2.** Chemical structure of chitin (A) and chitosan (B).

### **1.1.3. Industrial application of chitin and chitosan**

In 1930s and 1940s, chitin and chitosan attracted considerable attention as materials with potential industrial applications. In that time, 50 patents describing the use of chitin and chitosan were published, e.g. for production of films, threads, sizing agents, tubes, straws, seamless sausage casings (Rigby et al., 1936; Thor, 1940) and water-proof paper (Arnold, 1936). However, lack of adequate manufacturing facilities and the strong competition from synthetic polymers restricted commercial development of these natural polymers. The revitalized interest in chitin appeared in 1970s, when chitin-containing seafood shells, i.e. crab, shrimp, and lobster, needed to be utilized. At that time, waste shells were dumped in landfill or the sea without any processing, that led to a serious environmental pollution of coastal areas (Ahmed and Ikram, 2017) and strict environmental regulations by USA banning the dumping of waste shells. Consequently, this waste stream was considered as a cheap resource of chitin, that with a production of approximately 6 to 8 million tons of chitin globally per year can be used almost without limitations (Gao, 2016). Therefore, utilization of chitin from waste shells is profitable from an environmental and an economic point of view. However, currently only a small part of this biopolymer is utilized. One of the reasons is the relatively laborious isolation process of chitin from crustacean shells. The isolation process comprises three steps: 1) demineralization (acid treatment mainly with HCl) which removes inorganic matters (mainly calcium carbonate), 2) deproteinization (alkaline treatment mainly with NaOH) for the removal of protein matter, 3) decolourization (bleaching by chemical reagents) to achieve colourless product (Pokhrel et al., 2015). The other reason is the robustness of chitin and its insolubility in water and common solvents (Pillai et al., 2009).

One of the most important industrial application of chitin is the production of chitosan. Materials obtained from chitosan have many beneficial biological properties, e.g. non-toxicity, biodegradability, biocompatibility, antioxidant and antimicrobial activities; therefore they have been used in tissue engineering and drug delivery in the medical and pharmaceutical fields (Kumirska et al., 2011). Chitosan has also high potential in many other sectors, e.g. in agriculture for improvement of crop yields by controlling fungal, viral and bacterial diseases (Sharp, 2013), and in environmental protection for removal of heavy metals (Zhang et al., 2016). In food technology chitosan can be used as a antimicrobial packaging material (Van den Broek et al., 2015), additives (clarification of fruits and beverages, emulsifier agent, thickening

and stabilizing agents, color stabilization), dietary fiber, for enzyme immobilization and encapsulation of nutraceuticals (Agullo et al., 2003).

The global industrial consumption of chitin and chitosan is steadily increasing. For example, the healthcare segment based on the use of chitin and chitosan was estimated at 264 million € in 2017 and is expected to reach a valuation of more than 0.85 billion € by the end of 2027 (<https://www.futuremarketinsights.com/reports/chitin-market>).

Furthermore, the development of new chitin- and chitosan-based materials with novel properties gained an extraordinary interest of scientist throughout the world. The number of publications on this subject is increasing and over 50,660 papers were published up to date (according to Scopus database in early 2018).

Despite being used in form of valuable biopolymers, chitin and chitosan may also be transformed into other useful products such as bioactive chitin- and chito-oligosaccharides and monomers.

## **1.2. Oligosaccharides and monomers from chitin and chitosan**

Glycosidic  $\beta$ -(1,4)-bonds in chitin and chitosan can be broken and the polymers are depolymerized to their oligosaccharides (CHOs) and monomers, GlcNAc and GlcN (Figure 1.3.).

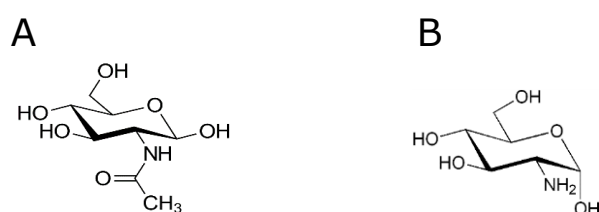
### **1.2.1. Oligosaccharides from chitin and chitosan**

CHOs can have different degree of polymerization (DP), pattern of acetylation (PA), and Mw which determine their chemical and biological properties (Aam et al., 2010). CHOs have been shown to have antimicrobial (No et al., 2002), antitumor and anti-inflammatory activities (Azuma et al., 2015) and they are still investigated for other potential applications. However, a medical therapy based on CHOs has not yet been developed. CHOs are mainly applied in research at the lab scale and are produced by manufacturers like Megazyme (Ireland) and Heppe Medical Chitosan GmbH (Germany).

### **1.2.2. Monomers from chitin and chitosan**

The monomers, GlcNAc and GlcN are biochemical precursors of amino sugars that serve a number of functions in cells and tissues located throughout the human body. They are important components of glycoproteins, proteoglycans and glycosaminoglycans (GAG), such

as hyaluronic acid, keratan sulfate and sulfated GAG, which are present in connective tissues e.g. cartilages (Igarashi et al., 2011). Consequently, GlcNAc, GlcN and their derivatives are believed to participate as a substrate in synthesizing GAG and proteoglycans in the damaged joint cartilage (Igarashi et al., 2011). For about three decades, GlcNAc and GlcN have been used as cheap and valuable pharmacological agents in prevention and treatment of osteoarthritis, the most common joint disease of knee and hip with symptoms of pain and progressive destruction of articular cartilage (Crolle and D'Este, 1980; Dalirfardouei et al., 2016).

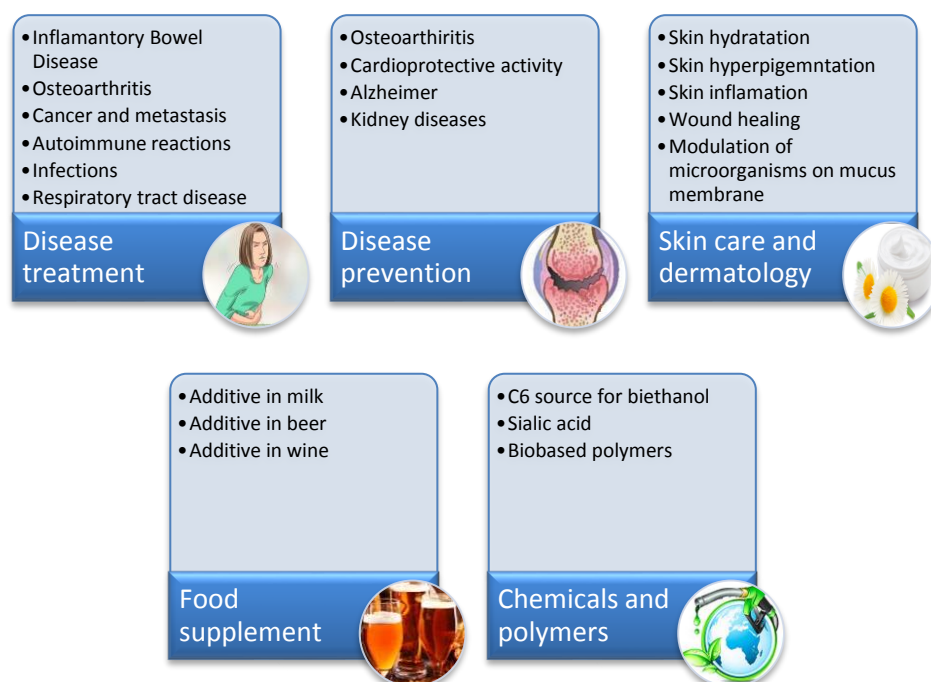


**Figure 1.3.** Chemical structure of *N*-acetylglucosamine (GlcNAc) (A) and glucosamine (GlcN) (B).

However, although many studies on GlcNAc and GlcN have showed a significant treatment effect, accompanied with remarkable safety, the clinical treatment of osteoarthritis with GlcNAc and GlcN is still a matter for debate. There exists an evident difference between European and the USA institutions regarding relative effectiveness of GlcNAc and its derivatives compared with placebo or other treatments. The European Society for Clinical and Economic Aspects of Osteoporosis and Osteoarthritis (ESCEO) recommends application of patented crystalline GlcN-sulfate and chondroitin sulfate as medication for osteoarthritis (Bruyère et al., 2016). However, in the USA GlcN and chondroitin are considered as dietary supplements and are not used for the therapy purposes. It is stated, that there is not enough evidence to prove positive influence of GlcN on restoring of cartilages (Júnior and Inácio, 2013; Vasiliadis and Tsikopoulos, 2017). Moreover, there are numerous studies with contradictory results on efficacy of GlcN on pain relief in osteoarthritis. The studies with very positive effects of GlcN were on the charge of being exaggerated and without a clinically relevant outcome. Furthermore, the positive results were obtained only for supplementation with GlcN sponsored by the pharmaceutical industry, what additionally decreased the trust in their correctness (Júnior and Inácio, 2013; Vasiliadis and Tsikopoulos, 2017).

GlcNAc is an important component of oligosaccharides in human milk (Kobata and Ginsburg, 1969; Miller et al., 1994). GlcNAc-containing oligosaccharides from human milk were shown to be essential for growth of bifidiobacteria like *Lactobacillus bifidus* var. *pensylvanicus* in infant colon, which are important for health functioning of the infant's organs (Gyorgy et al., 1974).

Due to their biological properties, GlcNAc and GlcN have been recommended in many other fields (Figure 1.4.) such as a therapeutic anti-inflammatory agent for treatment of chronic inflammatory diseases e.g. inflammatory bowel disease in children (Salvatore et al., 2000), and as an agent improving skin condition in skin care and dermatology (Kim et al., 2008; Bissett et al., 2007). As a food supplement, GlcNAc is applied as an additive in beer, wine and milk (Xu et al., 2004a, 2004b, 2004c). Furthermore, GlcNAc has been proposed as resource for chemicals (Inokuma et al., 2016) and as a potential building block for biobased polymers (Kobayashi et al., 2006; Galbis et al., 2015).



**Figure 1.4.** Application of *N*-acetylglucosamine (GlcNAc) and glucosamine (GlcN).

The versatile applications trigger a market demand for GlcNAc and its derivatives. The Global Glucosamine Market is expected to grow over the next decade and to reach approximately 0.88 billion € by 2025 (<https://www.prnewswire.com/news-releases/global-103-billion-glucosamine-market-analysis-trends-2016-industry-forecasts-to-2025-research-and-markets-300312509.html>). Therefore, development of an efficient process for production of GlcNAc is urgently needed by the industry.

### **1.3. Production processes for GlcNAc and GlcN**

GlcNAc can be produced by chemical or enzymatic hydrolysis of chitin or it can be synthesised during fermentation of suitable microorganisms (Table 1.1.).

#### **1.3.1. Chemical method**

Currently, chemical hydrolysis of chitin is the main method for production of GlcNAc. The process can be conducted in two ways. The first way is a direct hydrolysis of chitin performed with 15–36 % HCl at about 40–80 °C (Bohlman et al., 2004). The second way involves chitin hydrolysis to GlcN followed by *N*-acetylation of GlcN to GlcNAc. In this process chitin is treated with 20–37 % HCl at 100 °C (Mojarrad et al., 2007). Under such harsh conditions the acetyl group of GlcNAc is removed and the released GlcN is finally *N*-acetylated with acetic anhydride to regenerate the desired GlcNAc (Roseman and Ludowieg, 1953). The chemical production of GlcNAc from chitin is estimated to be economically feasible, but there are some drawbacks, including low yield (below 65 %), low specificity of chemical catalyst, high operational costs and generation of environmentally unfriendly acidic wastes (Chen et al., 2010).

#### **1.3.2. Biotransformation method**

GlcNAc and GlcN can be synthesised through fermentation of filamentous fungi, recombinant *Escherichia coli* or recombinant *Saccharomyces cerevisiae*. This method has fewer environmental issues than chemical method. However, low productivity and high production costs are the main disadvantages of this process, that weakens its economic competitiveness with the chemical method. After 7 days, incubation of filamentous fungi *Aspergillus* sp., *Monascus pilosus* and *Rhizopus oligosorus* resulted in GlcNAc production of 3.43 g L<sup>-1</sup>, 0.72 g L<sup>-1</sup>, and 0.31 g L<sup>-1</sup>, respectively (Hsieh et al., 2007). After 36 h incubation of *Aspergillus* sp. 14.36 g L<sup>-1</sup> GlcN was obtained by Zhang et al. (2012). Fermentation of recombinant *E. coli* was

more efficient and resulted in production of 110 g L<sup>-1</sup> (Deng et al., 2005), 70 g L<sup>-1</sup> (Chen et al., 2012a), and 73 g L<sup>-1</sup> (Chen et al., 2012b) of GlcNAc after 72, 16, and 18 hours, respectively. Production of 2 g L<sup>-1</sup> GlcNAc was obtained with a recombinant strain of *S. cerevisiae* (Lee and Oh, 2016). As a substrate, glucose has been commonly used.

**Table 1.1.** Different types of production methods for *N*-acetylglucosamine (GlcNAc).

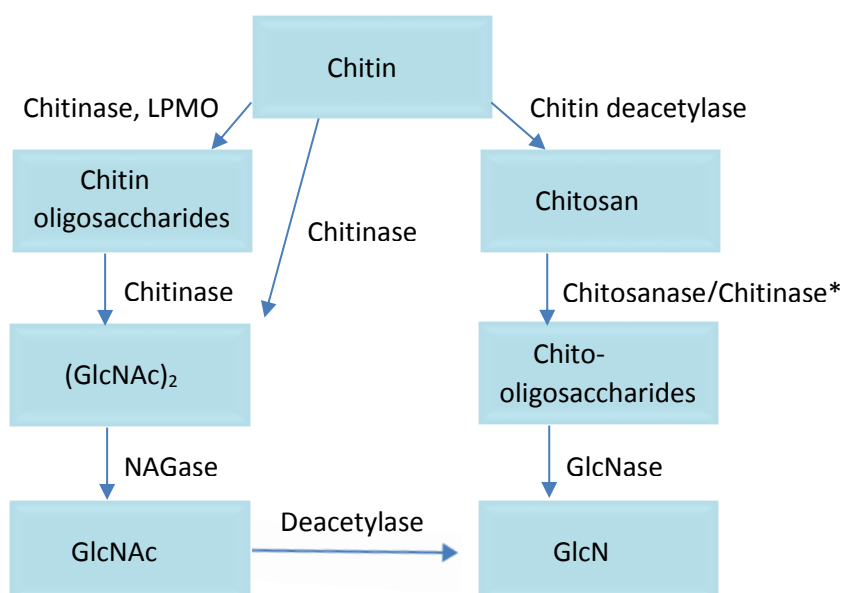
Type of production method	Process	Advantage	Disadvantage
<b>Chemical</b>			
1. Direct hydrolysis of chitin to GlcNAc	15-36 % HCl at 40–80 °C (Bohlman et al., 2004)	- short time of the reaction (7 h)	- acidic wastes, - low yield <65% - high energy costs - low selectivity of catalyst
2. Hydrolysis to GlcNAc and <i>N</i> -acetylation of GlcNAc	20–37 % HCl, 100 °C, (Mojarrad et al., 2007)  Solvent: 10 % methanol, <i>N</i> -acetylation with acetic anhydride (Roseman and Ludowieg, 1953) Solvent: Pyridine, purification, <i>N</i> -acetylation with acetic anhydride (Zhan, 2007)	- short time of the reaction (1–3 h)	- <i>N</i> -acetylation of GlcN needed - use of chemical solvents - low yield (43 %)
<b>Biotransformation</b>			
1. Production of GlcNAc from chitin	Chitin degrading-factors (CDFs) of bacterium <i>Chitinobacter tainanensis</i> (Chern et al., 2004; Chen et al., 2010)	- high yield (75 %) - high purity (>99 %)	- susceptible of phage contamination - high production costs
2. Synthesis of GlcNAc in the fermentation-based process	1) Intercellular synthesis in fungi e.g. <i>Saccharomyces cerevisiae</i> (Lee and Oh, 2016)  2) Intercellular synthesis in <i>Escherichia coli</i> (Deng, 2005)	- wide range of substrate - no allergy reaction  - high yield - high productivity - no allergy reaction	- low yield (≈10 %) - low productivity - high production costs  - susceptible of phage attack - high production costs
<b>Enzymatic</b>			
1. Chitinolytic enzymes	Hydrolysis of chitin with crude/purified enzymes	- high yield (41–≈100 %) - high purity (up to 100 %) - no chemical pollutions - reaction temperature lower than in chemical methods - high selectivity of catalyst	- low productivity - long-time incubation (up to 19 days) - chitin pre-treatment needed - high production costs of enzymes
2. Non-specific commercial enzymes	Hydrolysis of chitin with cellulase, hemicellulase, papain, pepsin, lipase, and pectinase (Sashiwa et al., 2001)	- high yield (76 %) - no chemical pollutions - reaction temperature lower than in chemical methods - commercially available enzymes	- long-time incubation (8 days) - enzyme mixture containing crude chitinolytic enzymes



### 1.3.3. Enzymatic method

Enzymatic hydrolysis of chitin catalysed by chitinolytic enzymes has been proposed as an environmental friendly alternative for chemical hydrolysis. Enzymes are biological catalysts that can replace chemical catalyst, HCl in this specific case (Fersht, 1985) and can be applied under milder conditions. Additionally, the application of enzymatic hydrolysis allows the better control of the production process, since enzymes have a higher substrate specificity and reaction selectivity than HCl.

In nature, chitin is degraded by chitinolytic enzymes via two major enzymatic pathways: chitinolytic and chitosanolytic, presented in Figure 1.5.



**Figure 1.5.** Enzymatic degradation of chitin via chitinolytic pathway (left) and chitosanolytic pathway (right). LPMO—lytic polysaccharide monooxygenase, NAGase—*N*-acetylglucosaminidase, GlcNase—glucosaminidase. \*Some chitinases are able to degrade chitosan.

The chitinolytic pathway involves initial enzymatic hydrolysis of the  $\beta$ -(1,4)-glycosidic bond in chitin chains (Gooday, 1990). There are three groups of chitin-hydrolysing enzymes: chitinases, *N*-acetylglucosaminidases (NAGase, also known as chitobiases) and lytic polysaccharide monooxygenases (LPMO) (Vaaje-Kolstad et al., 20110; Vaaje-Kolstad et al., 2013). According to the Carbohydrate-Active Enzymes Database (CAZy, <http://www.cazy.org/>), chitinases belong to glycoside hydrolase (GH) family 18 and 19, NAGases to GH 20 and 3 and LPMOs to the auxiliary activity (AA) family AA10 and AA11. Chitinases (EC 3.2.1.14) catalyse the hydrolysis of chitin chains into shorter oligosaccharides

and chitin dimer (GlcNAc)<sub>2</sub>. The products released by chitinases are further hydrolysed to GlcNAc by NAGases (EC 3.2.1.50). During chitin hydrolysis, chitinases and NAGases are supported by LPMOs (EC 1.14.99.53), which depolymerise chitin in oxidative way and introduce breaks in the chitin chains making chitin more accessible for chitinases and NAGases. The final product, GlcNAc, can be enzymatically deacetylated by *N*-acetylglucosamine deacetylase (EC 3.5.1.33) to GlcN.

The second pathway for chitin degradation involves deacetylation of chitin by chitin deacetylases (EC 3.5.1.41) to chitosan (Figure 1.5.). Chitosan is subsequently hydrolysed by chitosanases (EC 3.2.1.132) and exo-1,4-β-D-glucosaminidase (EC 3.2.1.165) which occur in GH 5, 7, 8, 46, 75, and 80. Some chitinases were also found to degrade chitosan and their activity was influenced by the DDA of chitosan, e.g. from *Streptomyces griseus* and *Serratia marcescens* (Ohtakara et al., 1990; Sørbotten et al., 2005).

The best known chitinolytic machinery has been described for bacterium *Serratia marcescens*, which produces three chitinases from GH 18 (ChiA, ChiB and ChiC), chitobiase (GH 20) and LPMOs (*Sm*LPMO10A, known as CBP21). These enzymes were shown to act synergistically in chitin degradation (Fuchs et al., 1986; Brurberg et al., 1996; Watanbe et al., 1997; Suzuki et al., 1998; Vaaje-Kolstad et al., 2005). Other well-known chitin-degrading microorganisms belong to genera *Trichoderma* (fungi) and *Streptomyces* (actinomycetes).

## **1.4. Crystallinity of chitin as a hurdle for the enzymatic process**

Despite large amounts of chitin produced in nature, chitin does not accumulate at the bottom of the oceans indicating, that chitin is efficiently degraded by organisms producing chitinolytic enzymes (Goody, 1990). However, the enzymatic depolymerisation of α-chitin in crustaceans is a very slow process (Sashiwa et al., 2001). The reason is the high crystallinity and insolubility of α-chitin, that hinders the accessibility of chitin to the enzymatic action. As the distance between chitin chains is reported to be about 0.47 nm (Rinaudo, 2006), it is not possible for hydrolytic enzymes to penetrate the crystalline structure of chitin particles (Jaworska and Roberts, 2016) and thus enzymes act only at amorphous parts of the chitin.

Therefore, the crystallinity of α-chitin is a relevant issue that has to be taken into account while developing an enzymatic process of production of GlcNAc from chitin. Already many

pre-treatment methods have been investigated for destruction of the crystallinity of chitin e.g. acid treatment (Binod et al., 2007), ionic liquids (Jaworska et al., 2012), ultrasonication and steam-explosion (Villa-Lerma et al., 2013),  $\gamma$ -radiation (Dziril et al., 2015), microwave irradiation (Roy et al., 2003), high pressure homogenization (Wei et al., 2017), mechanochemical grinding (Nakagawa et al., 2011), rapid depressurization from supercritical 1,1,1,2-tetrafluoroethane (Villa-Lerma et al., 2016) and ball mill grinding in dry mode (Chen et al., 2015), sub- and supercritical water pre-treatment (Osada et al., 2012). The pre-treatment of chitin is necessary for increasing the accessibility of chitin for enzymatic action. However, it is necessary to use an appropriate method that ensures the efficient destruction of the crystallinity of chitin but simultaneously protects the three-dimensional structure of chitin chains and protects acetyl groups of GlcNAc units. The acetyl groups of GlcNAc are crucial for the enzymatic action to occur since acetyl groups are involved in so called substrate-assisted catalysis conducted by GH 18 chitinases (Terwisscha van Scheltinga et al., 1995). Furthermore, the chosen method should be applicable at industrial scale i.e. the transition from the lab-scale to the industrial scale should be possible.

### **1.5. Thermostable enzymes from *Myceliophthora thermophila* C1 for chitin depolymerisation**

In accordance with the abundance of chitin, chitinolytic enzymes are also widely present in nature and are found in many types of organisms, predominantly in chitin-containing organisms, but also in organisms that do not generate chitin such as plants (Schlumbaum et al., 1986), mammals (Gooday, 1990) and humans (Hollak et al., 1994).

However, the largest potential as biocatalysts have microbial enzymes (Singh et al., 2016). Microbial enzymes can be easily produced extracellularly and purified from a culture broth. On a large scale, the enzymes produced by microbial hosts are cost-effective due to high production levels associated with standard expression, inexpensive culture media, ease of growth, and short fermentation cycles (Rigoldi et al., 2018). An additional advantage of microbial enzymes can be their thermostability. Thermostable enzymes tolerate high process temperatures, are resistant to denaturing agents, tolerate “harsh” purification and consequently last longer (Kristjanson, 1989). Thermostability is particularly advantageous for degradation of polysaccharide-rich biomass. Thermophilic enzymes were demonstrated to

degrade plant biomass faster than their mesophilic counterpart at elevated temperatures (Singh et al., 2016).

Thermophilic filamentous fungi are a potential reservoir of thermostable enzymes (Singh et al., 2016). In their natural habitats, they secrete various enzymes, e.g. proteases, (hemi-)cellulases, amylases, esterases, lipases and chitinases, and use them for degradation of large biopolymers such as proteins and polysaccharides (Visser et al., 2011). Chitinases have very diverse biological functions including a role in re-arranging of cell walls during growth, mycoparasitism, autolysis, and acquisition of chitin for nutritional purposes (Langner and Göhre, 2016).

*Myceliophthora thermophila* (syn. *Sporotrichum thermophile*) is a well-known thermophilic biomass degrading filamentous fungus (Apnis, 1963; Coutts and Smith, 1976; Singh, 2014). An isolate of *M. thermophila* C1 (previously known as *Chrysosporium lucknowense* C1; DuPont Industrial Biosciences) was subjected to a strain-development process, that lead to a development of a low protease/(hemi-) cellulase free *M. thermophila* C1-expression host (LC-strain), which is a mature enzyme production platform (Visser et al., 2011). The genome of *M. thermophila* C1 is rich in genes encoding for industrially relevant enzymes degrading biomass e.g. arabinohydrolase, xylanase, and recently discovered LPMO, of which many were overexpresses and characterised (Hinz et al., 2009; Kühnel et al., 2010; van Gool et al., 2013; Frommhagen et al., 2015).

*M. thermophila* C1 is a promising platform for production of thermostable chitinolytic enzymes. Visser et al. (2011) reported production of homologous chitinase of *M. thermophila* C1, designated as Chitinase Chi1. The secretion of Chitinase Chi1 indicates, that the fungus has extracellular chitinolytic machinery for the degradation of chitin. This chitinolytic machinery can be an important tool for the development of an efficient process for industrial production of chitin and chitosan-oligosaccharides and GlcNAc obtained from chitin and chitosan.

## 1.6. Aim of this thesis

The aim of this thesis was to:

- clone and overexpress genes encoding for Chitinase Chi1 and NAGase *MthNAG* from *M. thermophila* C1,
- characterise produced and purified Chitinase Chi1 and *MthNAG*,
- determine synergistic action of Chitinase Chi1 and *MthNAG* on chitin,
- determine the optimal process conditions for an efficient method to produce GlcNAc from chitin using Chitinase Chi1 and *MthNAG*,
- confirm the usefulness of Chitinase Chi1 as a suitable tool for fingerprint analysis of (modified)chitosan.

## 1.7. Thesis outline

Efficient degradation of chitin depends on the application of the appropriate enzymes. It is important to obtain enzymes with their natural structural and catalytic properties in order to understand their interactions with the substrate and to consider their applicability in the process. Enzymes produced homologously have the structure and properties, which are naturally created by the producing host. In **Chapter 2** we produced and characterised Chitinase Chi1 from *M. thermophila* C1. We homologously overexpressed the gene encoding for Chitinase Chi1 in a low protease/(hemi-) cellulase free *M. thermophila* C1-expression host (LC-strain). We purified the produced enzyme Chitinase Chi1 and analysed its mode of action towards chitin, different types of chitosan and chitin oligosaccharides. Furthermore, important was to find whether Chitinase Chi1 is a thermostable enzyme.

In **Chapter 3**, we investigated *N*-acetylglucosaminidase *MthNAG*, a second enzyme from the chitinolytic machinery of *M. thermophila* C1. We homologously overexpressed the gene encoding for *MthNAG* in *M. thermophila* C1 LC-strain. After production and purification of *MthNAG* protein, we analysed the structural and catalytic properties and the thermostability of the enzyme. Furthermore, we investigated, whether Chitinase Chi1 and *MthNAG* can synergistically degrade chitin.

The synergistic action of Chitinase Chi1 and *MthNAG* on degradation of chitin was studied in more detail in **Chapter 4**. In this chapter we focused on the design of the optimal conditions

for the production of GlcNAc from chitin. In order to improve the accessibility of chitin to the enzymes we pre-treated chitin by ball milling. The process was optimised with response surface methodology (RSM) with central composite design (CCD).

In **Chapter 5**, we present the practical application of Chitinase Chi1 for analysis of modified chitosan for determination of the modification effect on the chitosan structure. Chitinase Chi1 was used for hydrolysis of modified chitosan and the hydrolysis products could be measured with MALDI-TOF-MS. This finger print method was useful for drawing conclusions about the modification process of chitosan and structural properties of modified chitosan.

Finally, in **Chapter 6** we present the general discussion of the results obtained in this thesis. We summarize our results, place our findings into broader context of applications, discuss the main bottlenecks and propose a new strategy for production of GlcNAc from chitin based on the application of Chitinase Chi1 and *Mth*NAG to move beyond the current existing limitations in enzymatic production of GlcNAc from chitin.

## References

- Aam, B.B., Heggset, E.B., Norberg, A.L., Sørli, M., Vårum, K.M., Eijsink, V.G.H., 2010. Production of chitooligosaccharides and their potential applications in medicine. *Mar. Drugs* 8, 1482–1517
- Agullo, E., Rodriguez, M.S., Ramos, V., Albertengo, L., 2003. Present and future role of chitin and chitosan in food. *Macromol. Biosci.* 3, 521–530
- Ahmed, S., Ikram, S., 2017. Chitin and chitosan: history, composition and properties. In: Ahmed, S. and Ikram, S.(ed). *Chitosan: derivatives, composites and applications*. Scrivener Publishing LLC, Beverly, USA, pp. 1–24
- Apnis, A.E., 1963. Occurrence of thermophilic microfungi in certain alluvial soils near Nottingham. *Nova. Hed.* 5, 57–78
- Arnold, L.B., 1936. Fibrous product. US Patent NO. 2142986A
- Atkins E., 1985. Conformations in polysaccharides and complex carbohydrates. *Proc. Int. Symp. Biomol. Struct. Interactions, Supp. J. Biosci.* 8, 375–387
- Azuma, K., Osaki, T., Minami, S., Okamoto, Y., 2015. Anticancer and anti-inflammatory properties of chitin and chitosan oligosaccharides. *J. Funct. Biomater.* 6, 33–49
- Binod, P., Sandhya, C., Suma, P., Szakacs, G., Pandey, A., 2007. Fungal biosynthesis of endochitinase and chitinase in solid state fermentation and their application for the production of *N*-acetyl-D-glucosamine from colloidal chitin. *Bioresour. Technol.* 98, 2742–2748
- Bissett, D., Robinson, L.R., Raleigh, P.S., Miyamoto, K., Hakozi, T., Li, J., Klem, G.R., 2007. Reduction in the appearance of facial hyperpigmentation by topical *N*-Acetylglucosamine. *J. Cosmet. Dermatol.* 6, 20–26
- Bohlman, J.A., Schisler, D.O., Hwang, K.O., Henning J.P., Trinkle, J.R., Anderson, T.B., Steinke, J.D., Vanderhoff, A., 2004. *N*-acetyl-D-glucosamine and process for producing *N*-acetyl-D-glucosamine. US Patent NO. 6693188B2
- Brurberg, M.B., Nesl, I.F., Eijsink, V.G.H., 1996. Comparative studies of chitinases A and B from *Serratia marcescens*. *Microbiology* 142, 1581–1589
- Bruyère, O., Cooper, C., Pelletier, J.P., Maheu, E., Rannou, F., Branco, J., Luisa Brandi, M., Kanis, J.A., Altman, R.D., Hochberg, M.C., Martel-Pelletier, J., Reginster, J.Y., 2016. A consensus statement on the European Society for Clinical and Economic Aspects of Osteoporosis and Osteoarthritis (ESCEO) algorithm for the management of knee osteoarthritis-From evidence-based medicine to the real-life setting. *Semin. Arthritis Rheum.* 45(4 Suppl), S3–11
- Chen, J.-K., Shen, C.-R., Liu, C.-L., 2010. *N*-Acetylglucosamine: production and applications. *Mar. Drugs* 8, 2493–2516
- Chen, X., Liu, L., Li, J.H., Liu, J., Du, G.C., Chen, J., 2012a. Optimization of glucose feeding approaches for enhanced glucosamine and *N*-acetylglucosamine production by an engineered *Escherichia coli*. *J. Ind. Microbiol. Biotechnol.* 39, 359–365
- Chen, X., Liu, L., Li, J.H., Du, G.C., Chen, J., 2012b. Improved glucosamine and *N*-acetylglucosamine production by an engineered *Escherichia coli* via step-wise regulation of dissolved oxygen level. *Bioresour. Technol.* 110, 534–538
- Chen, X., Gao, Y., Wang, L., Chen, H., Yan, N., 2015. Effect of treatment methods on chitin structure and its transformation into nitrogen-containing chemicals. *Chempluschem* 80, 1565–1572
- Chern, L.-L., Stackebrandt, E., Lee, S.-F., Lee, F.-L., Chen, J.-K., Fu, H.-M., 2004. *Chitinibacter tainanensis* gen. nov., sp. nov., a chitin-degrading aerobe from soil in Taiwan. *Int. J. Syst. Evol. Microbiol.* 54, 1387–1391
- Coutts, A.D., Smith, R.E., 1976. Factors influencing the production of cellulases by *Sporotrichum thermophile*. *Appl. Environ. Microbiol.* 31, 819–826
- Crolle, G., D'Este, E., 1980. Glucosamine sulphate for the management of arthrosis: a controlled clinical investigation. *Curr. Med. Res. Opin.* 7, 104
- Dalirfardouei, R., Karimi, G., Jamialahmadi, K., 2016. Molecular mechanisms and biomedical applications of glucosamine as a potential multifunctional therapeutic agent. *Life Sci.* 152, 21–29
- Deng, M.D., Severson, D.K., Grund, A.D., Wassink, S.L., Burlingame, R.P., Berry, A., Running, J.A., Kunesh, C.A., Song, L., Jerrell, T.A., Rosson, R.A., 2005. Metabolic engineering of *Escherichia coli* for industrial production of glucosamine and *N*-acetylglucosamine. *Metab. Eng.* 7, 201–214
- Durkin, C.A., Mock, T., Armbrust E.V., 2009. Chitin in diatoms and its association with the cell wall. *Eukaryot. Cell* 8, 1038–1050
- Dziril, M., Grib, H., Laribi-Habchi, H., Drouiche, N., Abdi, N., Lounici, H., Pauss, A., Mameri N., 2015. Chitin oligomers and monomers production by coupling  $\gamma$ -radiation and enzymatic hydrolysis. *Ind. Eng. Chem.*

- Ehrlich, H., Krautter, M., Hanke, T., Simon, P., Knieb, C., Heinemann, S. Worch, H., 2007. First evidence of the presence of chitin in skeletons of marine sponges. Part II. Glass Sponges (Hexactinellida: Porifera). J. Exp. Zool. 308B, 473–483
- Fersht A., 1985. Enzyme structure and mechanism. W.H. Freeman, New York
- Frommhagen, M., Sforza, S., Westphal, A.H., Visser, J., Hinz, S.W.A., Koetsier, M.J., van Berkel, W.J.H., Gruppen, H., Kabel, M.A., 2015. Discovery of the combined oxidative cleavage of plant xylan and cellulose by a new fungal polysaccharide monooxygenase. Biotechnol. Biofuels 8, 101
- Fuchs, R.L., Mcpherson, S.A., Drahos, D.J., 1986. Cloning of a *Serratia marcescens* gene encoding chitinase. Appl. Environ. Microbiol. 51, 504–509
- Galbis, J.A., de Gracia García-Martín, M., de Paz, M.V., Galbis, E., 2015. Synthetic polymers from sugar-based monomers. Chem. Rev. 116, 1600–1636
- Gao, X., Chen, X., Zhang, J., Guo, W., Jin, F., Yan, N., 2016. Transformation of chitin and waste shrimp shells into acetic acid and pyrrole. ACS Sustain. Chem. Eng. 4, 3912–3920
- Gardner, K.H., Blackwell J., 1975. Refinement of the Structure of  $\beta$ -Chitin. Biopolymers 14, 1581–1595
- Gooday G.W., 1990. The ecology of chitin degradation. Adv. Microb. Ecol. 11, 387–430
- Gyorgy, P., Jeanloz, R.W., Von Nicolai, H., Zilliken, F., 1974. Undialyzable growth factors for *Lactobacillus bifidus* var. *pennsylvanicus*. Eur. J. Biochem. 43, 29–33
- Hinz, S.W.A., Pouvreau, L., Joosten, R., Bartels, J., Jonathan, M.C., Wery, J., Schols, H.A., 2009. Hemicellulase production in *Chrysosporium lucknowense* C1. J. Cereal. Sci. 50, 318–323
- Hollak, C.E.M., van Weely, S., van Oers, M.H.J., Aerts, J.M.F.G., 1994. Marked elevation of plasma chitotriosidase activity. J. Clin. Invest. 93, 1288–1292
- Hsieh, J.W., Wu, H.S., Wei, Y.H., Wang, S.S., 2007. Determination and kinetics of producing glucosamine using fungi. Biotechnol. Prog. 23, 1009–1016
- Igarashi, M., Kaga, I., Takamori, Y., Sakamoto, K., Miyazawa, K., Nagaoka I., 2011. Effects of glucosamine derivatives and uronic acids on the production of glycosaminoglycans by human synovial cells and chondrocytes. Int. J. Mol. Med. 27, 821–827
- Inokuma, K., Hasunuma, T., Kondo, A., 2016. Ethanol production from *N*-acetyl-D-glucosamine by *Scheffersomyces stipitis* strains. AMB Express 6, 83
- Jaworska, M.M., Kozlecki, T., Gorak A., 2012. Review of the application of ionic liquids as solvents for chitin. J. Polym. Eng. 32, 67–69
- Jaworska, M.M., Roberts, G.A.F., 2016. The influence of chitin structure on its enzymatic deacetylation. Chemical and Process Engineering 37, 261–267
- Júnior, O.V.L., Inácio A.M., 2013. Use of glucosamine and chondroitin to treat osteoarthritis: a review of the literature. Rev. Bras Ortop. 48, 300–306
- Khoushab, F., Yamabhai, M., 2010. Chitin Research Revisited. Mar. Drugs 8, 1988–2012
- Kim, S.-K., Ravichandran, Y.D., Khan, S.B., Kim, Y.T., 2008. Prospective of the cosmeceuticals derived from marine organisms. Biotechnol. Bioprocess Eng. 13, 511–523
- Kobata, A., Ginsburg V., 1969. Oligosaccharides of Human Milk: I. Isolation and characterization. Arch. Biochem. Biophys. 130, 509–513
- Kobayashi, S., Makino, A., Matsumoto, H., Kunii, S., Ohmae, M., Kiyosada, T., Makiguchi, K., Matsumoto, A., Horie, M., Shoda, S.-I., 2006. Enzymatic polymerization to novel polysaccharides having a glucose-*N*-acetylglucosamine repeating unit, a cellulose-chitin hybrid polysaccharide. Biomacromolecules 7, 1644–1656
- Kristjansson, J.K., 1989. Thermophilic organisms as sources of thermostable enzymes. TIBTECH 7, 349–353
- Kumirska, J., Weinhold, M. X., Thöming, J., Stepnowski, P., 2011. biomedical activity of chitin/chitosan based materials—influence of physicochemical properties apart from molecular weight and degree of *N*-acetylation. Polymers 3, 1875–1901
- Kühnel, S., Hinz, S.W.A., Pouvreau, L., Wery, J., Schols, H.A., Gruppen, H., 2010. *Chrysosporium lucknowense* arabinohydrolases effectively degrade sugar beet arabinan. Bioresour. Technol. 101, 8300–8307
- Langner, T., Göhre, V., 2016. Fungal chitinases: function, regulation, and potential roles in plant/pathogen interactions. Curr. Genet. 62, 243–254
- Lee, S.-W., Oh, M.-K., 2016. Improved production of *N*-acetylglucosamine in *Saccharomyces cerevisiae* by reducing glycolytic flux. Biotechnol. Bioeng. 113, 2524–2528
- Merzendorfer H., Zimoch L., 2003. Chitin metabolism in insects: structure, function and regulation of chitin synthases and chitinases. J. Exp. Biol. 206, 4393–4412

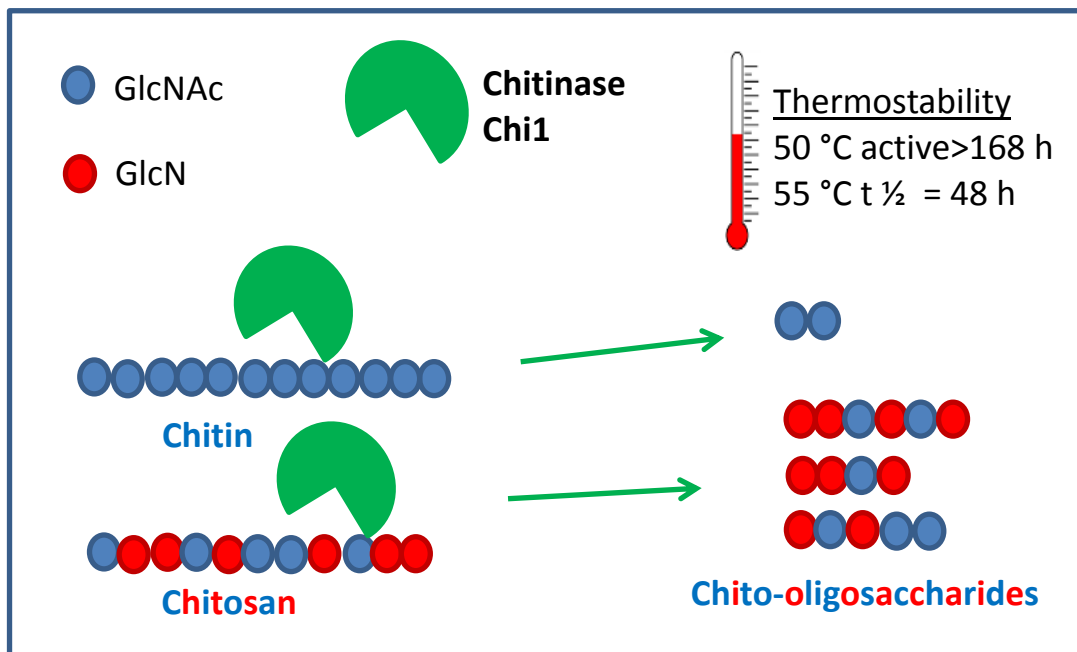


- Miller, J.B., Bull, S., Miller, J., McVeagh, P., 1994. The oligosaccharide composition of human milk: temporal and individual variations in monosaccharide components. *J. Pediatr. Gastroenterol. Nutr.* 19, 371–376
- Minke, R., Blackwell, J., 1978. The structure of alpha-chitin. *J. Mol. Biol.* 120, 167–181
- Mojarrad, J.S., Nemati, M., Valizadeh, H., Ansarin, M., Bourbour, S., 2007. Preparation of glucosamine from exoskeleton of shrimp and predicting production yield by response surface methodology. *J. Agric. Food Chem.* 55, 2246–2250
- Molano, J., Bowers, B., Cabib, E., 1980. Distribution of chitin in the yeast cell wall. An ultrastructural and chemical study. *J. Cell Biol.* 85, 199–212
- Muzarelli, R.A.A., 1977. Chitin. Pergamon Press: Oxford, UK
- Nakagawa, Y.S., Oyama, Y., Kona, N., Nikaido, M., Tanno, K., Kogawa, J., Inomata, S., Masuic, A., Yamamurac, A., Kawaguchi, M., Matahirac, Y., Totani, K., 2011. Development of innovative technologies to decrease the environmental burdens associated with using chitin as a biomass resource: mechanochemical grinding and enzymatic degradation. *Carbohydr. Polym.* 83, 1843–1849
- No, H.K., Young Park, N., Ho Lee, S., Meyers, S.P., 2002. Antibacterial activity of chitosans and chitosan oligomers with different molecular weights. *Int. J. Food Microbiol.* 74, 65–72
- Ohtakara, A., Matsunaga, H., Mitsutomi, M., 1990. Action pattern of *Streptomyces griseus* chitinase on partially N-acetylated chitosan. *Agric. Biol. Chem.* 54, 3191–3199
- Osada, M., Miura, C., Nakagawa, Y.S., Kaihara, M., Nikaido, M., Totani, K., 2012. Effect of sub- and supercritical water pretreatment on enzymatic degradation of chitin. *Carbohydr. Polym.* 88, 308–312
- Pearlmutter, N.L., Lembi C.A., 1978. Localization of chitin in algal and fungal cell walls by light and electron microscopy. *J. Histochem. Cytochem.* 26, 782–791
- Pillai, C.K.S. Willi, P., Sharma C.P., 2009. Chitin and chitosan polymers: chemistry, solubility and fiber formation. *Prog. Polym. Sci.* 34, 641–678
- Pokhrel, S., Nath Yadav, P., Adhikari R., 2015. Applications of chitin and chitosan in industry and medical science: a review. *Nepal J. Sci. Technol.* 16, 99–104
- Rigby, G.W., 1936. Substantially undegraded deacetylated chitin and process for producing the same. US Patent NO. 2040879A
- Rigoldi, F., Donini, S., Redaelli, A., Parisini, E., Gautieri, A., 2018. Review: Engineering of thermostable enzymes for industrial applications. *APL Bioeng.* 2, 011501
- Rinaudo, M., 2006. Chitin and chitosan: properties and applications. *Prog. Polym. Sci.* 31, 603–632
- Roberts, G.A.F., 1992. Structure of chitin and chitosan. In *Chitin Chemistry*; Roberts, G.A.E., Ed.; Palgrave Macmillan: London, UK; pp. 85–91
- Roseman, S., Ludowieg, J., 1953. N-acetylation of hexosamines. *J. Am. Chem. Soc.* 76, 301–302
- Roy, I., Mondal, K., Gupta, M.N., 2003. Accelerating enzymatic hydrolysis of chitin by microwave pretreatment. *Biotechnol. Prog.* 9, 1648–1653
- Rudall, K.M., Kenchington, W., 1973. The chitin system. *Biol. Rev.* 49, 597–636
- Sashiwa, H., Fujishima, S., Yamano, N., Kawasaki, N., Nakayama, A., Muraki, E., Aiba, S., 2001. Production of N-acetyl-D-glucosamine from  $\beta$ -chitin by enzymatic hydrolysis. *Chem. Lett.* 308–309
- Salvatore, S., Heuschkel, R., Tomlin, S., Davies, S.E., Edwards, S., Walker-Smith, J.A., French, I., Murch, S.H., 2000. A pilot study of N-acetylglucosamine, a nutritional substrate for glycosaminoglycan synthesis, in paediatric chronic inflammatory bowel disease. *Aliment. Pharmacol. Ther.* 14, 1567–1579
- Schlumberg, A., Mauch, F., Vögeli, U., Boller, T., 1986. Plant chitinases are potent inhibitors of fungal growth. *Nature* 324, 365–367
- Sharp, R.G., 2013. A review of the applications of chitin and its derivatives in agriculture to modify plant-microbial interactions and improve crop yields. *Agronomy* 3, 757–793
- Singh, B., 2014. *Myceliophthora thermophila* syn. *Sporotrichum thermophile*: a thermophilic mould of biotechnological potential. *Crit. Rev. Biotechnol.* 36, 59–69
- Singh, R., Kumar, M., Mittal, A., Mehta, P.K., 2016. Microbial enzymes: industrial progress in 21st century. *Biotech* 6, 174
- Sørbotten, A., Horn, S.J., Eijsink, V.G.H., Vårum, K.M., 2005. Degradation of chitosans with chitinase B from *Serratia marcescens*. *FEBS Journal* 272, 538–549
- Struszczyk, M.H., 2002. Chitin and Chitosan Part I. Properties and production. *Polimery* 47, 316–325
- Suzuki, K., Suzuki, M., Taiyogi, M., Nikaido, N., Watanabe, T., 1998. Chitin binding protein (CBP21) in the culture supernatant of *Serratia marcescens* 2170. *Biosci. Biotechnol. Biochem.* 62, 128–135
- 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, 15619–15623

- Thor, C.J.B., 1940, Process for producing articles of regenerated chitin and the resulting articles. US Patent NO. 2217823A
- Vaaje-Kolstad, G., Houston, D.R., Riemen, A.H., Eijsink, V.G.H., van Aalten, D.M., 2005. Crystal structure and binding properties of the *Serratia marcescens* chitin-binding protein CBP21. J. Biol. Chem. 280, 11313–11319
- Vaaje-Kolstad, G., Westereng, B., Horn, S.J., Liu, Z.L., Zhai, H., Sørli, M., Eijsink, V.G.H., 2010. An oxidative enzyme boosting the enzymatic conversion of recalcitrant polysaccharides. Science 330, 219–222
- Vaaje-Kolstad, G., Horn, S.J., Sørli, M., Eijsink, V.G.H., 2013. The chitinolytic machinery of *Serratia marcescens* – a model system for enzymatic degradation of recalcitrant polysaccharides. FEBS Journal 280, 3028–3049
- Van den Broek, L.A.M., Knoop, R.J.I., Kappen, F.H.J., Boeriu, C.G., 2015. Chitosan films and blends for packaging material. Carbohydr. Polym. 116, 237–242
- Van Gool, M.P., van Muiswinkel, G.C.J., Hinz, S.W.A., Schols, H.A., Sinitsyn, A.P., Gruppen, H., 2013. Two novel GH11 endo-xylanases from *Myceliophthora thermophila* C1 act differently toward soluble and insoluble xylans. Enzyme Microb. Technol. 53, 25–32
- Vasiliadis, H.S., Tsikopoulos K., 2017. Glucosamine and chondroitin for the treatment of osteoarthritis. World J. Orthop. 8, 1–11
- Villa-Lerma, G., González-Márquez, H., Gimeno, M., López-Luna, A., Bárzana, E., Shirai, K., 2013. Ultrasonication and steam-explosion as chitin pretreatments for chitin oligosaccharide production by chitinases of *Lecanicillium lecanii*. Bioresour. Technol. 146, 794–798
- Villa-Lerma, G., González-Márquez, H., Gimeno, M., Trombotto, S., David, L., Ifuku, S., Shirai, K., 2016. Enzymatic hydrolysis of chitin pretreated by rapid depressurization from supercritical 1,1,1,2-tetrafluoroethane toward highly acetylated oligosaccharides. Bioresour. Technol. 209, 180–186
- Visser, H., Joosten, V., Punt, P.J., Gusakov, A., Olson, P.T., Joosten, R., Bartels, J., Visser, J., Sinitsyn, A., Emalfarb, M., Verdoes, J., Wery, J., 2011. Development of a mature fungal technology and production platform for industrial enzymes based on a *Myceliophthora thermophila* isolate, previously known as *Chrysosporium lucknowense* C1. Ind. Biotechnol. 7, 214–223
- Wagner, G.P., Lo, J., Laine, R., Almeder, M., 1993. Chitin in the epidermal cuticle of a vertebrate (*Paralipophrys trigloides*, Blenniidae, Teleostei). Experientia 49, 317–319
- Watanabe, T., Kimura, K., Sumiya, T., Nikaidou, N., Suzuki, K., Suzuki, M., Taiyoji, M., Ferrer, S., Regue, M., 1997. Genetic analysis of the chitinase system of *Serratia marcescens* 2170. J. Bacteriol. 179, 7111–7117
- Wei, G., Zhang, A., Chen, K., Ouyang, P., 2017. Enzymatic production of *N*-acetyl-D-glucosamine from crayfish shellwastes pretreated via high pressure homogenization. Carbohydr. Polym. 171, 236–241
- Xu, Q., Liu, J., Yuan, Z., 2004a. The use of *N*-acetylglucosamine as additive in beer and beer comprising *N*-acetylglucosamine. WO Patent NO. 2004085603
- Xu, Q., Liu, J., Yuan, Z., 2004b. The use of *N*-acetylglucosamine as additive in wine and wine comprising *N*-acetyl Glucosamine. WO Patent NO. 2004085604
- Xu, Q., Liu, J., Yuan, Z., 2004c. The use of *N*-acetylglucosamine as additive in milk products. WO Patent NO. 2004093556
- Zhan, W.S., 2007. Process for preparing refined *N*-acetyl-D-aminoglucose. Patent CN1907993A
- Zhang, J.X., Liu, L., Li, J.H., Du, G.C., Chen, J., 2012. Enhanced glucosamine production by *Aspergillus* sp. BCRC 31742 based on the time-variant kinetics analysis of dissolved oxygen level. Bioresour. Technol. 111, 507–511
- Zhang, L., Zeng, Y., Cheng, Z., 2016. Removal of heavy metal ions using chitosan and modified chitosan: a review. J. Mol. Liq. 214, 175–191

# Chapter 2

**Chitinase Chi1 from *Myceliophthora thermophila* C1,  
a thermostable enzyme for chitin and chitosan depolymerization**



**Published as:**

**Krolicka, M., Hinz, S.W.A., Koetsier, M.J., Joosten, R., Eggink, G., van den Broek, L.A.M., Boeriu, C.G., 2018. J. Agric. Food Chem. 66, 1658–1669**

## ABSTRACT

A thermostable Chitinase Chi1 from *Myceliophthora thermophila* C1 was homologously produced and characterized. Chitinase Chi1 shows high thermostability at 40 °C (>140 h 90 % activity), 50 °C (>168 h 90 % activity), and 55 °C (half-life 48 h). Chitinase Chi1 has broad substrate specificity, and converts chitin, chitosan, modified chitosan and chitin oligosaccharides. The activity of Chitinase Chi1 is strongly affected by the degree of deacetylation (DDA), molecular weight (Mw) and side chain modification of chitosan. Chitinase Chi1 releases mainly (GlcNAc)<sub>2</sub> from insoluble chitin and chito-oligosaccharides with a polymerization degree (DP) ranging from 2 to 12 from chitosan, in a processive way. Chitinase Chi1 shows higher activity towards chitin oligosaccharides (GlcNAc)<sub>4-6</sub> than towards (GlcNAc)<sub>3</sub> and is inactive for (GlcNAc)<sub>2</sub>. During hydrolysis, oligosaccharides bind at subsites -2 to +2 in the enzyme's active site. Chitinase Chi1 can be used for chitin valorisation and for production of chitin and chito-oligosaccharides at industrial scale.

## 2.1. Introduction

Chitin consists of  $\beta$ -(1,4)-linked *N*-acetyl-D-glucosamine (GlcNAc) units and is one of the most abundant polymers in nature. Chitin is a main component found in shells of crabs, shrimps and lobsters which are popular types of seafood. Every year approximately 6 to 8 million tons of shell-waste are produced from the seafood industry globally (Gao et al., 2016). This waste stream represents a cheap and renewable resource of chitin, which can be used for production of value-added chemicals. Next to crustacean waste, chitin can also be isolated from insects and fungi. Products obtained from chitin and its deacetylated derivative chitosan can be used in medical applications, packaging, food and nutrition, biotechnology, agriculture and environmental protection (Kardas et al., 2012; van den Broek et al., 2015). In recent years, special interest has been paid to water-soluble chito-oligosaccharides, which can act as antimicrobial (No et al., 2002), antitumor and anti-inflammatory (Azuma et al., 2015) agents. Chitin and chito-oligosaccharides can be produced by chemical or enzymatic depolymerization of chitin and chitosan. Common procedures for the production of chito-oligosaccharides rely on acid catalysis, which is characterized by a low yield and high environmental impact. The use of enzyme catalysis for depolymerization of chitin and chitosan is a promising alternative to the chemical methods because it allows the production of specific chitin and chito-oligosaccharides in a controlled way and environmentally friendly process (Meena et al., 2014). Nevertheless, development of an efficient enzymatic process requires fundamental knowledge of the catalytic mechanisms of enzymes and understanding the interactions with their substrate.

In nature, chitin is degraded by three groups of enzymes: chitinases (EC 3.2.1.14), releasing water soluble chitin oligosaccharides from chitin, *N*-acetylglucosaminidases (EC 3.2.1.52), degrading products released by chitinases to monomers (Ike et al., 2005), and chitin-active lytic polysaccharide monooxygenases (LPMOs; EC 1.14.99.53) that cleave chitin crystalline chains in an oxidative way, yielding a lactone (C1-oxidation) and a ketoaldose (C4-oxidation) product (Vaaje-Kolstad et al., 2010; Vaaje-Kolstad et al., 2013; Forsberg et al., 2016). The copper-dependent LPMOs act in synergy with chitinases, and enhance the accessibility of chitin chains for chitinases and *N*-acetylglucosaminidases by disrupting the crystal structure of chitin and generation of more soluble polymer chains with increased susceptibility for enzymatic hydrolysis. Based on amino acid sequence, chitinases have been classified into the

glycoside hydrolase (GH) families 18 and 19, and *N*-acetylglucosaminidase into GH 20 and GH 3, according to the Carbohydrate-Active Enzymes Database (CAZy), (<http://www.cazy.org/>).

Chitinases are spread in nature and are involved in physiological processes of bacteria, archaea, fungi, animals, and plants (Adrangi et al., 2010; Hartl et al., 2011). In recent years special interest has been paid to thermostable chitinases from bacteria and fungi due to their potential application in bioconversion of chitin waste and in the industrial production of chitin oligosaccharides from chitin and chito-oligosaccharides from chitosan. The advantage of thermostable enzymes is that these enzymes do not lose their activity at higher temperatures which are implemented in bioconversion of waste and in industrial processes (Meena et al., 2014). A number of thermophilic chitinases have been described from bacteria including *Chitinophaga*, *Alcaligenes*, *Virgibacillus*, *Massilia*, *Paenibacillus*, *Streptomyces*, *Microbispora*, *Bacillus*, and *Brevibacillus* (Meena et al., 2014; Nawani et al., 2002). However, only a few thermophilic fungi have been explored for thermophilic chitinases like *Aspergillus fumigatus* (Xia et al., 2001), *Chaetomium thermophilum* (Li et al., 2010), *Gliocladium catenulatum* (Ma et al., 2012), *Rhizopus oryzae* (Chen et al., 2013), *Thermoascus aurantiacus* vs. *levisporus* (Li et al., 2010), *Thermomyces lanuginosus* (Zhang et al., 2015; Guo et al., 2008; Prasad and Palanivelu, 2012) *Trichoderma viridae* (Omumasaba et al., 2001).

The thermophilic filamentous fungus *Myceliophthora thermophila* C1 (previously known as *Chrysosporium lucknowense* C1; DuPont Biosciences) has been developed for homologous and heterologous protein expression (Visser et al., 2011). *M. thermophila* C1 has been used before for production of different cell wall degrading enzymes as described by Hinz and co-workers (Hinz et al., 2009; Kühnel et al., 2010). We reported before that *M. thermophila* C1 produces an endochitinase entitled Chitinase Chi1 (Visser et al., 2011). Dua et al. (2016) published recently an exochitinase rMtChit from *M. thermophila* BJA produced from the same gene sequence as Chitinase Chi1 by recombinant expression in *Pichia pastoris*, however, characteristics of exochitinase rMtChit differed significantly from our Chitinase Chi1. Since Chitinase Chi1 from *M. thermophila* C1 might have potential application in bioconversion of chitin waste sources and in industrial production of chitin and chito-oligosaccharides, a full characterization of the Chitinase Chi1 and understanding of the interactions of Chitinase Chi1 with its substrates is important. Here, we describe the production and detailed

characterization of Chitinase Chi1 with focus on thermostability, catalytic properties and mode of action on chitin, chitosan and chitin and chito-oligosaccharides.

## **2.2. Materials and methods**

### **2.2.1. Chemicals**

Chitin azure, chitin from shrimp shells, glycol chitosan, Schiff's reagent, 4-nitrophenyl-*N*-acetylglucosamine (GlcNAc-*p*NP), 4-nitrophenyl-*N,N'*-diacetyl- $\beta$ -D-chitobioside ((GlcNAc)<sub>2</sub>-*p*NP) and 4-nitrophenyl- $\beta$ -D-*N,N',N''*-triacetylchitotriose ((GlcNAc)<sub>3</sub>-*p*NP), were obtained from Sigma-Aldrich (St. Louis, USA). Oxidized chitosan (Mw 100 kDa, DDA 84 %, degree of oxidation 5 %, containing C6-aldehyde and carboxyl groups in a ratio of 20:1) was produced at Wageningen Food & Biobased Research (Wageningen, The Netherlands). Hydroxypropyl-chitosan was a kind gift from Nippon Suisan (Japan). Chitin oligosaccharides (GlcNAc)<sub>2-6</sub> were obtained from Megazyme (Co. Wicklow, Ireland). Chitosans were purchased from Heppe Medical Chitosan GmbH (Halle, Germany) and Nippon Suisan Kaisha LTD (Tokyo, Japan). The deacetylation degree (DDA in %) and molecular weight (Mw in kDa) are: chitosan 88 DDA/3000 and chitosan 90 DDA/100 (Nippon Suisan Kaisha LTD), chitosan 77 DDA/600, chitosan 78 DDA/600, chitosan 91 DDA/600 and chitosan 94 DDA/600 (Heppe Medical Chitosan GmbH). All other chemicals were of the highest purity available.

### **2.2.2. Swollen chitin preparation**

Swollen chitin was prepared according to Monreal and Reese (1969) with some modifications. Chitin from shrimp shells (1 g) was stirred in 25 mL 85 % (v/v) phosphoric acid and left at room temperature for 20 hours. Subsequently it was precipitated by pouring the gelatinous mixture into an excess of ice-cold water. The swollen chitin was separated by centrifugation at 3,000  $\times g$  and washed with demineralized water up to pH 6.0.

### **2.2.3. Overexpression of Chi1 gene in *M. thermophila* C1 and 2 L-scale**

#### **fermentation for production of Chitinase Chi1**

Overexpression of Chitinase Chi1 in *M. thermophila* C1 and the 2 L-scale fermentation for production of Chitinase Chi1 have been carried out based on the procedures previously reported: (i) the DNA sequence of the gene encoding (*Chi1*) for Chitinase Chi1 (GenBank accession number HI550986) was described in a patent (Verdoes et al., 2010), (ii) the

homologous overexpression of Chitinase Chi1 in *M. thermophila* C1 (previously known as *C. lucknowense* C1) was described in detail by Visser et al. (2011) and (iii) the preparation of a monocomponent strain of *M. thermophila* C1 producing Chitinase Chi1 and the detailed conditions of the fed-batch fermentation resulting in high production level of Chitinase Chi1 (7.5 g L<sup>-1</sup>) were also described by Visser et al. (2011). In short, the *Chi1* gene was amplified from genomic *M. thermophila* C1 DNA and cloned into a *M. thermophila* C1 expression vector. The expression cassette containing the *chi1* promoter, gene and the terminator obtained from the vector was transformed into a low protease/(hemi-)cellulase free *M. thermophila* C1-expression host. Ninety six transformants were grown in a microtiter plate (Verdoes et al., 2007) and screened for Chitinase Chi1 production levels in the culture broth using chitin azure as substrate. The transformant showing the highest level of chitinase activity was selected for fed-batch fermentation to produce Chitinase Chi1 at 2-L scale. The strain was grown aerobically in 2 L fermenters in mineral medium, containing glucose as carbon source, ammonium sulfate as nitrogen source and trace elements for the essential salts. The enzyme was produced at pH 6.0 and 32 °C (Verdoes et al., 2007). The supernatant containing Chitinase Chi1 was centrifuged at 20,000 × *g* for 20 minutes to remove the biomass and concentrated (4 fold) using a 5 kDa PES membrane (Vivacell® 70, Sartorius). The crude enzyme extract was subsequently dialyzed against 10 mM potassium phosphate buffer pH 6.0 and freeze-dried to obtain the crude enzyme preparation. Freeze-drying was used to prevent microbial decay of enzyme preparation and to avoid the use of preservatives.

#### **2.2.4. Sequence analysis of Chitinase Chi1**

Nucleotide and deduced amino acid sequences were analyzed using Clone Manager software. BLAST analysis of the deduced amino acid sequence of Chitinase Chi1 was performed at the NCBI server (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Analysis for conserved domains were performed using Conserved Domain Search and Conserved Domain Database (containing information from databases including Pfam, SMART, COG, PRK, TIGRFAM) (Marchlerbauer et al., 2015), at the NCBI server (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). The signal peptide was analyzed at the SignalP 4.0 server (<http://www.cbs.dtu.dk/services/SignalP/>) and theoretical isoelectric point (pI) was calculated with Compute pI/Mw tool on ExPASy server ([https://web.expasy.org/compute\\_pi/](https://web.expasy.org/compute_pi/)). Potential *N*-linked glycosylation sites and potential *O*-linked glycosylation sites were predicted



by NetOGlyc 4.0 Server and NetNGlyc 1.0 Server (<http://www.cbs.dtu.dk/services/>). To generate a 3D model of Chitinase Chi1 the deduced amino acid sequence of Chitinase Chi1 was submitted to the Phyre2 web portal for protein modelling (Kelley et al., 2015). The Phyre2 generated the protein model on the basis of its closest template.

### **2.2.5. Purification of Chitinase Chi1**

The freeze-dried crude enzyme preparation containing 142 mg total protein was dissolved in 50 mL 0.05 M Bis-Tris buffer pH 7.0 and purified by anion exchange chromatography using a HiPrep DEAE FF 16/10 column (GE Healthcare Bio-Science AB, Uppsala, Sweden) and an ÄKTA™ pure system (GE Healthcare Bio-Science AB, Uppsala, Sweden). The column was equilibrated with five column volumes (CV) 0.05 M Bis-Tris buffer pH 7.0 (buffer A). A sample of 50 mL was loaded onto the column and eluted using 0.05 M Bis-Tris buffer pH 7.0 followed by elution with 1 M NaCl in 0.05 M Bis-Tris buffer pH 7.0 (buffer B) as follows: 20 % buffer B for 10 CV and 45 % buffer B for 10 CV with a flow rate of 5 mL min<sup>-1</sup>. Fractions were collected and screened for chitinase and *N*-acetylglucosaminidase activity. The 10 mL fraction containing the highest chitinase activity was subjected to size exclusion chromatography and loaded onto a HiLoad 16/600 Superdex 75 pg column (GE Healthcare Bio-Science AB, Uppsala, Sweden). Proteins were eluted isocratic with 0.05 M Bis-Tris buffer pH 7.0 containing 0.15 M NaCl with a flow rate of 0.5 mL min<sup>-1</sup>. The absorbance was measured at 280 nm.

### **2.2.6. Enzyme assays and protein concentration**

During the purification process the activities of chitinase and *N*-acetylglucosaminidase were measured. Chitinase activity was determined using colloidal chitin azure (Shen et al., 2010) as substrate. The enzyme solution (0.05 mL) was incubated with 0.95 mL 5 % (w/v) colloidal chitin azure in 50 mM Bis-Tris buffer pH 7.0, and the mixture was incubated at 50 °C for 30 min. After incubation, the reaction was terminated by heating at 96 °C for 5 min, to inactivate the enzyme. The reaction mixture was centrifuged at 20,000 × *g* for 5 min and the absorbance of the supernatant was measured at 560 nm. An enzyme-free mixture was used as negative control, and each reported value was the average of duplicate tests. One enzyme unit was defined as a change in the absorbance of 0.01 min<sup>-1</sup> (Gómez Ramírez et al., 2004).

For *N*-acetylglucosaminidase activity, the reaction mixture contained 0.09 mL 2 mM GlcNAc-*p*NP in 0.1 M citrate-phosphate buffer pH 4 and 0.01 mL enzyme solution. After 10 min

incubation in a microtiter plate at 50 °C, 0.2 mL 0.25 M Tris/HCl buffer pH 8.8 was added to the mixtures and the absorbance at 405 nm was measured using a Tecan Safire plate reader (Grodig, Austria). One enzyme activity was defined as the amount of enzyme that liberated 1  $\mu$ mol of *p*NP per minute. The protein concentration was determined using the bicinchoninic acid assay (BCA) according to the recommendation of the supplier (Pierce) with bovine serum albumin as standard.

### **2.2.7. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), identification of glycosylated proteins and Isoelectric point determination**

Sodium dodecyl sulfate-polyacrylamide (10 % (w/v)) gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970). A NuPAGE Novex System (ThermoFisher Scientific, Bleiswijk, The Netherlands) with 10 % (w/v) Bis-Tris gels was used. Prior to electrophoresis, all samples were heated for 10 min at 70 °C in NuPAGE LDS Sample Buffer with NuPAGE Sample Reducing Agent, according to the instructions of the manufacturer. Gels were stained with SimplyBlue™ SafeStain according to the recommendation of the supplier (ThermoFisher Scientific). For detection of glycosylated proteins, the SDS-PAGE gel was stained using periodic acid-Schiff staining (PAS) (Zacharius et al., 1969). The gel was incubated subsequently for 1 h in 12.5 % (w/v) trichloroacetic acid, 1 h in 1 % (v/v) periodic acid/3 % (v/v) acetic acid, 1 h in 15 % (v/v) acetic acid (replaced every 10 min), and 1 h at 4 °C in the dark in Schiff's reagent (Sigma-Aldrich, Zwijndrecht, The Netherlands). Hereafter, the gel was washed two times for 5 min in 0.5 % (w/v) sodium bisulfite and destained in 7 % (v/v) acetic acid. The isoelectric point (pI) of Chitinase Chi1 was estimated by isoelectric focusing (IEF) using PhastGel™ IEF on a Pharmacia LKB Phast System (Pharmacia Biotech, Uppsala, Sweden) with a broad protein calibration kit (pH 3-10, GE Healthcare) as standard. Proteins were stained with Coomassie blue R-2.

### **2.2.8. Mass spectrometry**

The molecular weight of Chitinase Chi1 was determined by matrix assisted laser-desorption time-of-flight mass spectrometry (MALDI-TOF-MS). Samples were prepared by the dried droplet method on a 600  $\mu$ m AnchorChip target (Bruker), using 5 mg mL<sup>-1</sup> 2,5-dihydroxyacetophenone, 1.5 mg mL<sup>-1</sup> diammonium hydrogen citrate, 25 % (v/v) ethanol and

3 % (v/v) trifluoroacetic acid as matrix. Spectra were derived from ten 500-shots (1,000 Hz) acquisitions taken at non-overlapping locations across the sample. Measurements were made in the positive linear mode, with ion source 1, 25.0 kV; ion source 2, 23.3 kV; lens, 6.5 kV; pulsed ion extraction, 680 ns. Protein Calibration Standard II (Bruker) was used for external calibration.

### **2.2.9. Purity and identity of Chitinase Chi1**

To evaluate the identity and purity of Chitinase Chi1, a sample containing the purified Chitinase Chi1 was sent to The Scripps Research Institute, Proteomics Core (Jupiter, Florida, USA) for proteolytic digestion and HPLC-ESI-MS/MS analysis.

### **2.2.10. Influence of temperature and pH on activity and stability of Chitinase Chi1**

Influence of temperature on activity of Chitinase Chi1 was analyzed with swollen chitin as substrate in the range of 30–80 °C. The enzyme solution (0.05 mL) containing 1.6 µM Chitinase Chi1 was incubated with 0.95 mL of 1 % (w/v) swollen chitin in 0.1 M citrate-phosphate-borate buffer pH 6.0 at 50 °C while mixing at 800 rpm for 30 min. The reaction was terminated by heating at 96 °C for 5 min. The reaction mixture was centrifuged at 20,000 × *g* for 10 min. The produced reducing sugars in the supernatant were measured using the *p*-hydroxybenzoic acid hydrazide (PAHBAH) assay (Lever, 1973). An enzyme-free mixture was used as negative control, and each reported value was the average of duplicate tests. *N*-acetyl-D-glucosamine was used as a standard and one enzyme unit (U) was defined as the amount of enzyme that liberated 1 µmol reducing sugar per minute. The thermostability was determined by pre-incubating the purified Chitinase Chi1 (1.6 µM) at pH 6.0 (0.1 M citrate-phosphate-borate buffer) at various temperatures (40–60°C) for different time intervals up to 168 h. The influence of pH on activity of Chitinase Chi1 was determined by incubating Chitinase Chi1 (1.6 µM) at different pH levels (3.0–9.0) in 0.1 M citrate-phosphate-borate buffer using swollen chitin as substrate.

### **2.2.11. Determination of kinetic parameters**

The  $K_m$ ,  $V_{max}$  and  $k_{cat}$  values for swollen chitin and chitosan 90 DDA/100 kDa were calculated with GraphPad Prism software (GraphPad Software, USA). The reducing sugars produced in

the supernatant were measured using the *p*-hydroxybenzoic acid hydrazide (PAHBAH) assay (Lever, 1973).

#### **2.2.12. Depolymerization of chitosans with different Mw and DDA**

To elucidate the influence of Mw and DDA of chitosans on Chitinase Chi1 activity a wide range of different chitosans were tested including: glycol chitosan, hydroxypropyl chitosan, oxidized chitosan and chitosans with different DDA and Mw. Chitosans were used in a concentration of 0.1 % (w/v) with 40.7 nM of purified Chitinase Chi1 in 1 mL 0.05 M sodium phosphate buffer pH 6.0. The mixture was incubated at 50 °C while mixing at 800 rpm for 15 min. After incubation, the enzyme activity was terminated by heating at 96 °C for 5 min. The reaction mixture was centrifuged at  $20,000 \times g$  for 5 min. The reducing sugars produced in the supernatant were measured using the *p*-hydroxybenzoic acid hydrazide (PAHBAH) assay (Lever, 1973).

#### **2.2.13. Hydrolysis of swollen chitin and chitosan**

For the enzymatic hydrolysis of swollen chitin and chitosan 90 DDA/100 the reaction mixtures containing 1 mL of 0.45 % (w/v) substrate in 0.05 M sodium phosphate buffer pH 6.0 with 100 nM purified Chitinase Chi1 were incubated at 50 °C while mixing at 800 rpm. Aliquots were taken at different time intervals and the hydrolysis products were analyzed by High-Performance Anion-Exchange Chromatography (HPAEC) and MALDI-TOF-MS.

#### **2.2.14. Hydrolysis of chitin oligosaccharides and *p*NP-substrates**

Hydrolysis of chitin oligosaccharides (GlcNAc)<sub>2-6</sub> and *p*NP-substrates was followed in time. Incubations were performed with 25 nM purified Chitinase Chi1 in 0.5 mL reaction volume containing 2 mM substrate (GlcNAc)<sub>2-6</sub>, 1 mM (GlcNAc)<sub>2</sub>-*p*NP or (GlcNAc)<sub>3</sub>-*p*NP, and 50 mM sodium phosphate buffer pH 6.0. Samples were incubated at 50 °C and aliquots of 60 µL were taken at different time intervals. The reaction was terminated by heating at 96 °C for 5 min and the hydrolysis products were analyzed by HPAEC.

#### **2.2.15. High Performance Anion-Exchange Chromatography (HPAEC)**

An ICS-3000 Ion Chromatography HPLC system equipped with a CarboPac PA-1 column (2×250 mm) in combination with a CarboPac PA-guard column (2×25 mm) at 22 °C and a pulsed electrochemical detector (PAD) in pulsed amperometric detection mode (Dionex) at 30 °C was

used. A flow rate of 0.25 mL min<sup>-1</sup> was used and the column was equilibrated with water. The following gradient was used: 0–25 min H<sub>2</sub>O, 25–65 min at 0–0.045 M NaOH, 65–70 min at 0.045 M NaOH–1 M sodium acetate in 0.1 M NaOH, 70–75 min at 1 M sodium acetate in 0.1 M NaOH, 75–75.1 min 1 M sodium acetate in 0.1 M NaOH–0.1M NaOH, 75.1–80 min 0.1M NaOH, 80–95 min H<sub>2</sub>O. Post column addition was used for increasing the PAD signal by 0.5 M NaOH at a flow rate of 0.15 mL min<sup>-1</sup>.

### **2.2.16. Identification of chitin and chito-oligosaccharides by MALDI-TOF-MS**

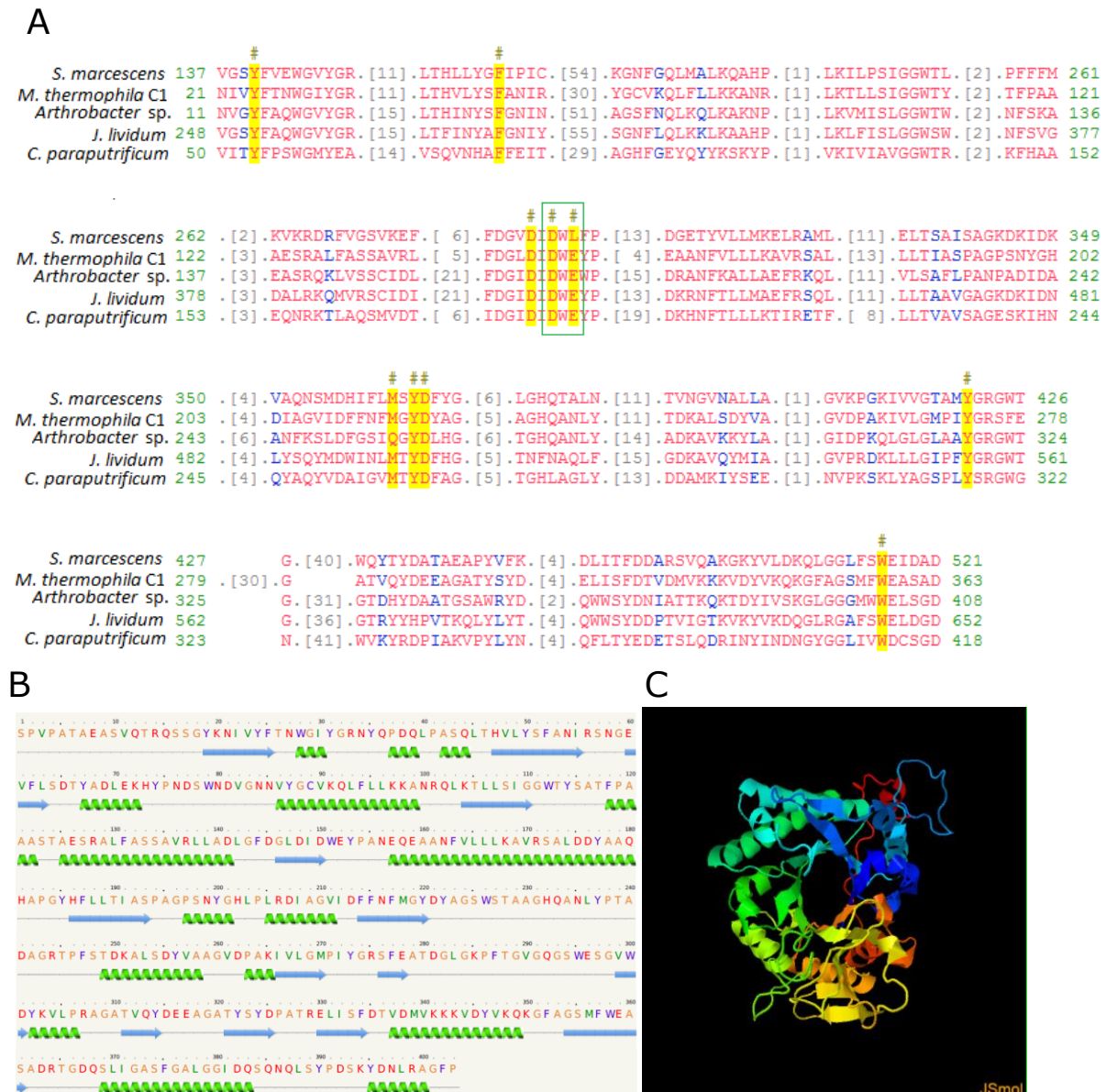
MALDI-TOF-MS was performed on a Bruker UltraFlextreme (Bruker Daltonics) in reflective mode and positive ions were examined. The instrument was calibrated for positive ions with a mixture of maltodextrin standards with known molecular masses. Samples were diluted in the matrix solution containing 10 mg mL<sup>-1</sup> 2,5-dihydroxybenzoic acid in 50 % (v/v) acetonitrile. For analysis, 1 µL of the mixture was transferred to the target plate and dried under a stream of dry air. The lowest laser intensity needed to obtain a good quality spectrum was applied and 10 times 50 laser shots randomly obtained from the sample, were accumulated.

## **2.3. Results**

### **2.3.1. Sequence analysis of Chitinase Chi1**

The putative gene *Chi1* encoding for Chitinase Chi1 had an ORF of 1,281-bp that encoded a protein with 426 amino acids, in which a signal peptide is predicted that consists of 23 amino acids in the *N*-terminal region of the protein. The deduced molecular weight of Chitinase Chi1 was 43.8 kDa and a theoretical *pI* was at pH 4.95. Four potential *O*-linked glycosylation sites, one potential *N*-linked glycosylation site, and 47 phosphorylation sites were found in the sequence. Multiple sequence alignment of the active site of the deduced protein sequence of Chitinase Chi1 (Figure 2.1.A) with Chitinase A from *Serratia marcescens* (1NH6\_A), Chitinase B from *Arthrobacter* sp. TAD20 (1KFW\_A), *Janthinobacterium lividum* (AAA83223), chitinase from *Clostridium paraputrificum* (BAD12045) revealed the presence of the conserved glycoside GH 18 domain in Chitinase Chi1. In total 10 conserved amino acids and one conserved active-site motif consisting of aspartate (D) and glutamate (E) residues forming the D-X-E motif were found in Chitinase Chi1. Modeling of the secondary structure of Chitinase Chi1 revealed that Chitinase Chi1 is composed of 16 α-helices and 14 β-sheets (Figure 2.1.B). 3D modelling of Chitinase Chi1 was based on similarity with chitinase from the fungus

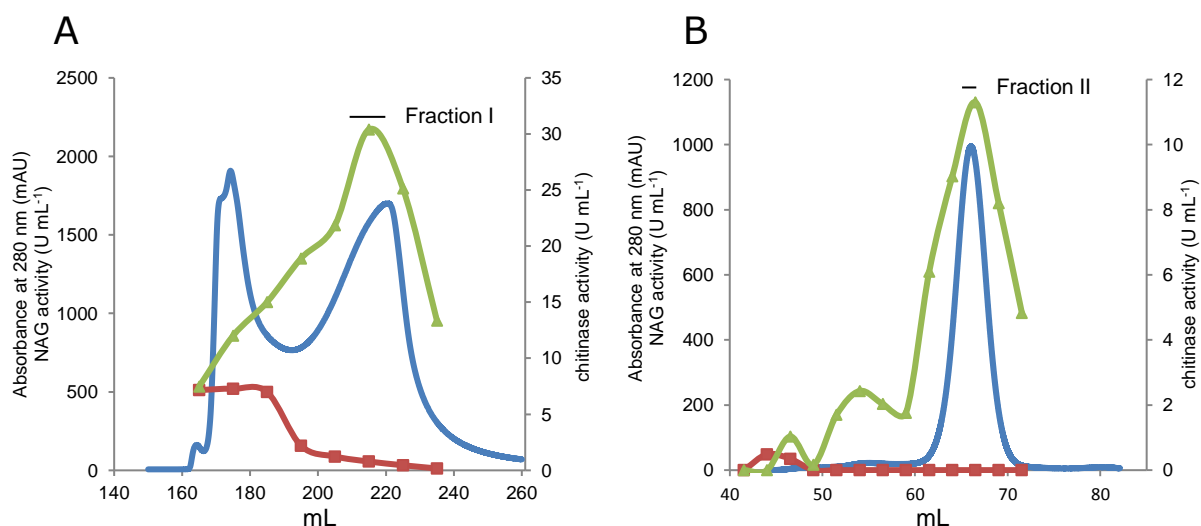
*Clonostachys rosea* belonging to GH 18 (template PDB entry: 3G6MA) (Figure 2.1.C). Using this sequence 386 amino acids residues (equal to 96 % of the whole amino acid sequence) have been modelled with 100 % confidence by the single highest scoring template. This modeling revealed the ( $\beta/\alpha$ )<sub>8</sub> barrel fold (TIM) of Chitinase Chi1.



**Figure 2.1.** Sequence analysis of Chitinase Chi1 from *Myceliophthora thermophila* C1. (A) Multiple sequence alignment of active site of Chitinase Chi1 from *Myceliophthora thermophila* C1 with active site of Chitinase A from *Serratia marcescens* (1NH6\_A), Chitinase B from *Arthrobacter* sp. TAD20 (1KFW\_A), *Janthinobacterium lividum* (AAA83223), and chitinase from *Clostridium parapatrificum* (BAD12045). Conserved residues are coloured in yellow and marked with (#) sign. Conserved D-X-E motif is shown in the green box. Chitinase Chi1 shares 10 amino acids with conserved hydrolases family 18 (GH 18) domain. Analysis was performed with Conserved Domain Search and Conserved Domain Database. (B) Secondary structure of Chitinase Chi1 predicted with Phyre2. (C) 3D modelling of Chitinase Chi1 was performed with Phyre2 software with chitinase from *Clonostachys rosea* belonging to GH 18 family (PDB entry 3G6MA) used as template.

### 2.3.2. Purification of Chitinase Chi1

The gene *Chi1* encoding for Chitinase Chi1 was successfully cloned into the *M. thermophila* C1-expression host. The transformant with the highest production was used for the production of high amounts of Chitinase Chi1, ( $7.5 \text{ g L}^{-1}$ ), in 2-L fermentation. From the culture broth, 15 g of protein containing about 60 % Chitinase Chi1 (based on SDS-PAGE, Figure 2.3.A) was obtained. The crude enzyme preparation was further subjected to a two-step purification process using anion exchange and size exclusion chromatography. The first purification step (Figure 2.2.A) resulted in the separation of the extract in two main protein peaks of which the peak eluting first showed mainly *N*-acetylglucosaminidase activity and the second peak chitinase activity. However, in the second peak some *N*-acetylglucosaminidase activity was detected. Therefore, in order to remove this activity, Fraction I from the first purification step was subjected to size exclusion chromatography using a Superdex 75 (Figure 2.2.B). This step enabled a clear separation between Chitinase Chi1 and the remaining *N*-acetylglucosaminidase. The specific activity of the purified Chitinase Chi1 was  $3.5 \text{ U mg}^{-1}$  for colloidal chitin azure (Table 2.1.).



**Figure 2.2.** Purification of Chitinase Chi1 from *Myceliophthora thermophila* C1 by ion exchange chromatography on DEAE-FF Sepharose (A) and size exclusion chromatography on Superdex 75 (B). Proteins (blue line) were detected at 280 nm. Chitinase activity (green line) was measured with chitin azure at pH 6.0, 50 °C. Activity of *N*-acetylglucosaminidase (red line) was assayed with GlcNAc-*p*NP at pH 4.0, 50 °C.

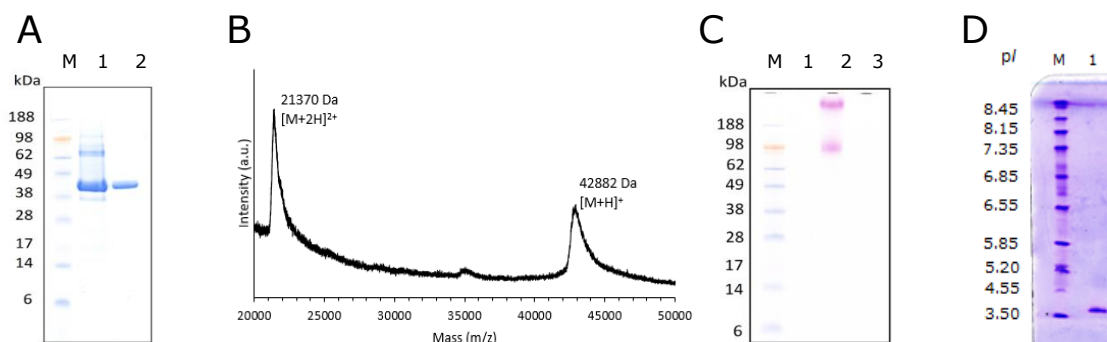
**Table 2.1.** Characterization of chitinase-containing fractions obtained during purification of Chitinase Chi1 from *Myceliophthora thermophila* C1.

Purification step	Volume (mL)	Total activity (U)	Total protein (mg)	Specific activity (U mg <sup>-1</sup> ) <sup>a</sup>
Crude extract	50	373	142	2.6
Anion exchange chromatography				
Fraction I	10	126	51	2.5
Size exclusion chromatography				
Fraction II	2.5	23	6.6	3.5

<sup>a</sup>Specific activity was assayed with 5 % (w/v) colloidal chitin azure at 50 °C and pH 7.0, and was calculated per mg protein.

### 2.3.3. Glycosylation, molecular weight and isoelectric point of Chitinase Chi1

Chitinase Chi1 was successfully purified to homogeneity as shown on SDS-PAGE as one single band (Figure 2.3.A) and was confirmed by HPLC-ESI-MS/MS analysis of the proteolytic digest (Figure 2.1.S1.), that identified only peptides originating from Chitinase Chi1. Chitinase Chi1 is a monomeric polypeptide and the molecular weight predicted from the protein sequence is 43.8 kDa. The SDS-PAGE revealed a molecular weight of 43 kDa (Figure 2.3.A). The molecular weight of the purified Chitinase Chi1 measured by MALDI-TOF-MS was shown to be 42.9 kDa (Figure 2.3.B). The MALDI-TOF-MS spectrum of Chitinase Chi1 showed an intense signal of the single charged protein  $[M+H]^+$  at  $m/z$  42,882 and the signal of the double-charged protein  $[M+2H]^{2+}$  at  $m/z$  21,370. Staining with periodic acid-Schiff (PAS) did not detect any glycosylation of Chitinase Chi1 (Figure 2.3.C). The isoelectric point of Chitinase Chi1 was found to be 3.95 (Figure 2.3.D).



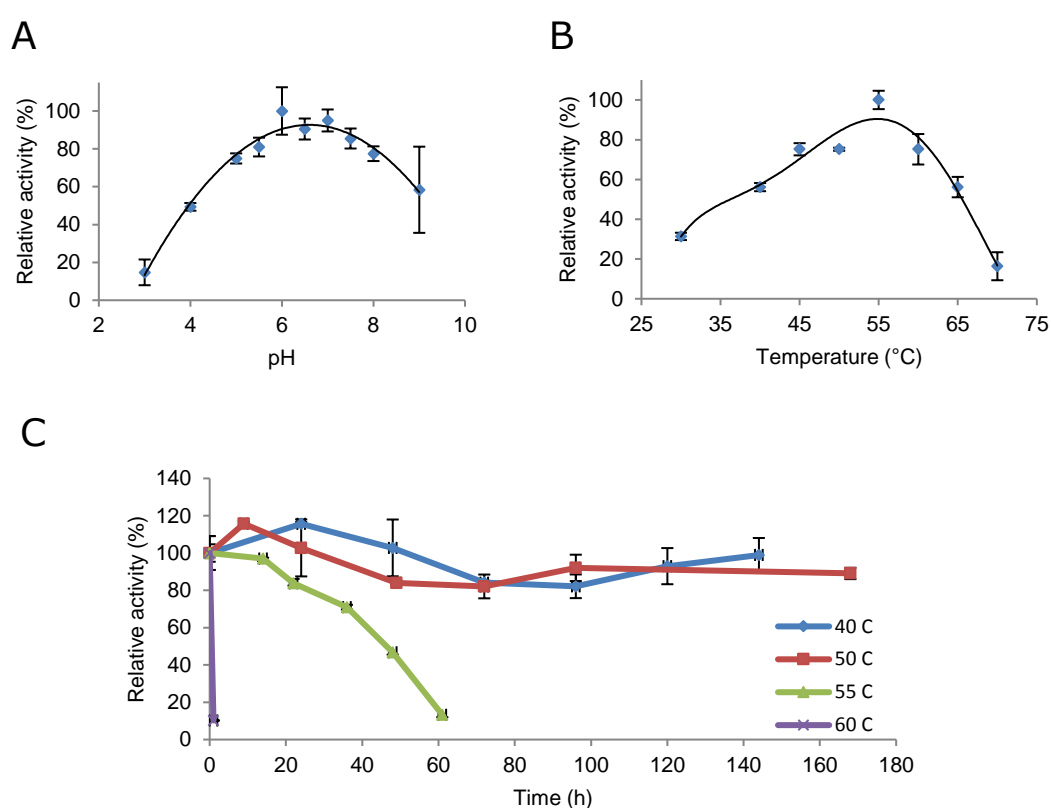
**Figure 2.3.** Analysis of purified Chitinase Chi1 from *Myceliophthora thermophila* C1. (A) molecular weight of Chitinase Chi1 determined by SDS-PAGE under reducing denaturing conditions. Lanes: M, standard protein molecular weight markers; 1, crude enzyme preparation of Chitinase Chi1; 2, purified Chitinase Chi1. (B) Molecular weight of Chitinase Chi1 determined by MALDI-TOF-MS. (C) Protein staining with periodic acid (PAS). Lanes: M, standard protein molecular weight markers; 1, purified Chitinase Chi1; 2, yeast invertase; 3, bovine serum albumin. (D) Isoelectric focusing (IEF). Lanes: M, pI marker; 1, purified Chitinase Chi1.



### 2.3.4. Influence of pH and temperature on activity and stability of

#### Chitinase Chi1

Chitinase Chi1 was active at pH 3.0 to 9.0 (Figure 2.4.A), and exhibited the highest activity at pH 6.0. Chitinase Chi1 showed activity from 30 to 70 °C with the highest activity at 55 °C (Figure 2.4.B) and it was remarkably stable at 40 °C (>140 h 90 % activity) and 50 °C (>168 h 90 % activity) (Figure 2.4.C). At 55 °C the enzyme reached its half-life after 48 h. Incubation at 60 °C resulted in fast inactivation of the enzyme, with a loss of 90 % of the initial catalytic activity after 1h incubation.



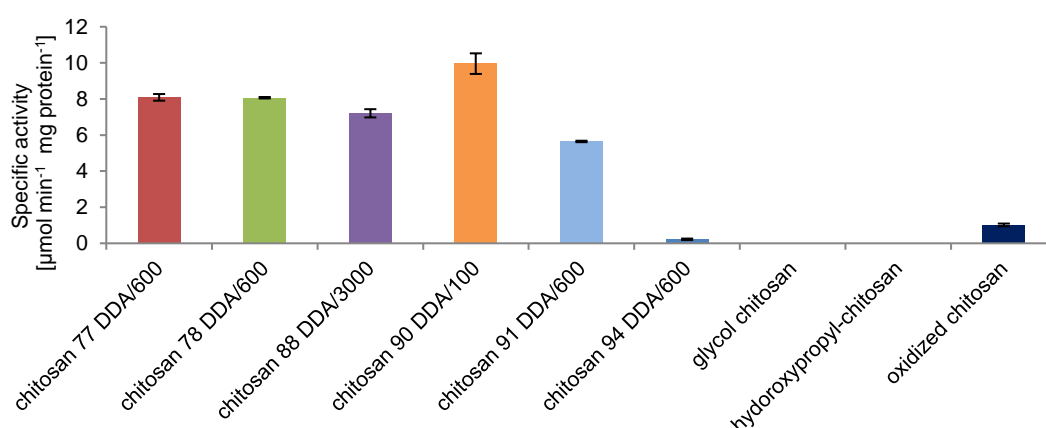
**Figure 2.4.** Enzyme characteristics of Chitinase Chi1. (A) Enzyme activities at various pH (pH 3.0–9.0) were measured at 50 °C in 0.1 M citrate-phosphate-borate buffer; (B) Enzyme activities at various temperatures (30–80 °C) were measured at pH 6.0 in 0.1 M citrate-phosphate-borate buffer; (C) Thermostability was measured by incubating Chitinase Chi1 at various temperatures (40–60 °C), and the residual activities were assayed at 50 °C. Reactions were performed with 1.6  $\mu$ M purified Chitinase Chi1 and 1 % (w/v) swollen chitin in 0.1 M citrate-phosphate-borate buffer for 30 min. The error bars represent the range of duplicate experiments.

### 2.3.5. Kinetic parameters for Chitinase Chi1

Kinetic parameters of Chitinase Chi1 were determined for swollen chitin and for chitosan 90 DDA/100. Activities were determined based on reducing sugars released during the reaction. For swollen chitin the specific activity was  $1.4 \pm 0.2 \text{ U mg}^{-1}$ ,  $V_{\max}$  was  $12.2 \pm 0.5 \mu\text{M min}^{-1}$ ,  $K_m$  was  $2.0 \pm 0.2 \text{ mg mL}^{-1}$ , and  $k_{\text{cat}}$  was  $0.11 \pm 0.0 \text{ s}^{-1}$ . For chitosan the specific activity was  $10.0 \pm 0.6 \text{ U mg}^{-1}$ ,  $V_{\max}$  was  $194 \pm 21.4 \mu\text{M min}^{-1}$ ,  $K_m$  was  $0.9 \pm 0.3 \text{ mg mL}^{-1}$ , and  $k_{\text{cat}}$  was  $1.9 \pm 0.2 \text{ s}^{-1}$ .

### 2.3.6. Influence of molecular weight and degree of deacetylation of chitosan on Chitinase Chi1 activity

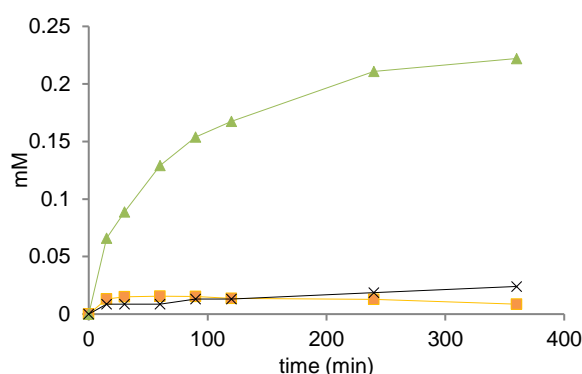
To evaluate the influence of the Mw (100, 600 and 3,000 kDa) and DDA (77, 78, 88, 90, 94) of chitosan on Chitinase Chi1 activity, a range of chitosans was tested (Figure 2.5.). In case of chitosans with the same Mw (600 kDa), Chitinase Chi1 showed decreased activity when DDA was increased. The highest activity was measured for chitosan with the lowest Mw (chitosan 90 DDA/100). For chitosan with similar DDA but different Mw (chitosan 88 DDA/3000 and chitosan 90 DDA/100), Chitinase Chi1 showed higher activity for lower Mw chitosan. Chitinase Chi1 degraded partially oxidized chitosan (i.e. chitosan with random oxidation at C6 positions), but showed 10 % of activity as measured for the untreated parent chitosan (chitosan 90 DDA/100). Chitinase Chi1 was not able to degrade glycol chitosan and hydroxypropyl-chitosan, which are both fully deacetylated.



**Figure 2.5.** Specific activity of Chitinase Chi1 on different types of chitosans. Chitosans were used in a concentration of 0.1 % (w/v) with 40.7 nM of purified Chitinase Chi1 in 1 mL 0.05 M sodium phosphate buffer pH 6.0. The reaction mixtures were incubated at 50 °C, for 15 min. The reducing sugars produced in the supernatant were measured using the *p*-hydroxybenzoic acid hydrazide (PAHBAH) assay. The results represent the average of duplicate experiments.

### 2.3.7. Degradation of chitin and chitosan by Chitinase Chi1

Degradation of chitin incubated with Chitinase Chi1 was followed in time and products released were analyzed by HPAEC. The main products formed were (GlcNAc)<sub>2</sub> next to small amount of (GlcNAc)<sub>3</sub> and GlcNAc (Figure 2.6.). After the first 30 min the rate of (GlcNAc)<sub>2</sub> release gradually decreased in time. The concentration of (GlcNAc)<sub>3</sub> and GlcNAc increased gradually up to 30 min of incubation and levelled off hereafter. At 90 min incubation the concentration of (GlcNAc)<sub>3</sub> started to decrease, when degradation into (GlcNAc)<sub>2</sub> and GlcNAc became the predominant reaction. The ratio (GlcNAc)<sub>2</sub> to GlcNAc at the end of the reaction was equal to 11. Chitin conversion expressed as the amount of (GlcNAc)<sub>2</sub> produced was 3.1 % after 6 h.



**Figure 2.6.** Release of chitin oligosaccharides during swollen chitin hydrolysis by Chitinase Chi1. Swollen chitin (0.45 % (w/v)) was incubated with purified Chitinase Chi1 (100 nM) at 50 °C in 1 mL 0.05 M sodium phosphate buffer pH 6.0. Aliquots were taken at different time intervals and the hydrolysis products were analyzed by High Performance Anion-Exchange Chromatography (HPAEC). Detected products were GlcNAc (cross), (GlcNAc)<sub>2</sub> (triangle), and (GlcNAc)<sub>3</sub> (square). Soft lines are only drawn as visual aids. The error bars represent the range of duplicate experiments.

Products released from chitosan 90 DDA/100 were analyzed by MALDI-TOF-MS. Chito-oligosaccharides were detected as potassium and/or sodium adducts and are summarized in Table 2.1.S1. Chitinase Chi1 was able to release a whole spectrum of hetero-oligosaccharides consisting of GlcNAc and GlcN units with a polymerization degree (DP) ranging from 2 to 12. The chito-oligosaccharide composition of the reaction mixture changed over time (Figure 2.1.S2.). At the early stages of the reaction, a large diversity of chito-oligosaccharides were formed, containing 2 to 6 GlcNAc units and 1 to 9 GlcN residues in different combinations and with a ratio GlcNAc/GlcN spanning the range between 0.5 to 4 (Table 2.1.S1., Figure 2.1.S2.). During the reaction, chito-oligosaccharides with more than three GlcNAc residues might be

further degraded by Chitinase Chi1, and new GlcN-enriched chito-oligosaccharides containing up to 10 GlcN residues and one or two GlcNAc residues are formed, that accumulate in time (Table 2.1.S1., Figure 2.1.S2.). Fully acetylated DP 2 ((GlcNAc)<sub>2</sub>, 447.2 m/z [M+Na]<sup>+</sup>) was identified from the early stages of the reaction and during the entire incubation. Fully acetylated DP 3 (650.3 m/z [M+Na]<sup>+</sup>) was detected only at 15 min incubation time, indicating that it was subsequently degraded by the enzyme. Accumulation of the heterologous dimer (DP 2) composed of GlcNAc and GlcN (405.2 m/z [M+Na]<sup>+</sup>) at longer reaction times, in the slow phase of the reaction, may indicate the ability of Chitinase Chi1 to cleave glycosidic linkages between GlcN and GlcNAc moieties in the chitosan chain, but only when GlcNAc is positioned in -1 subsite of the enzyme active site.

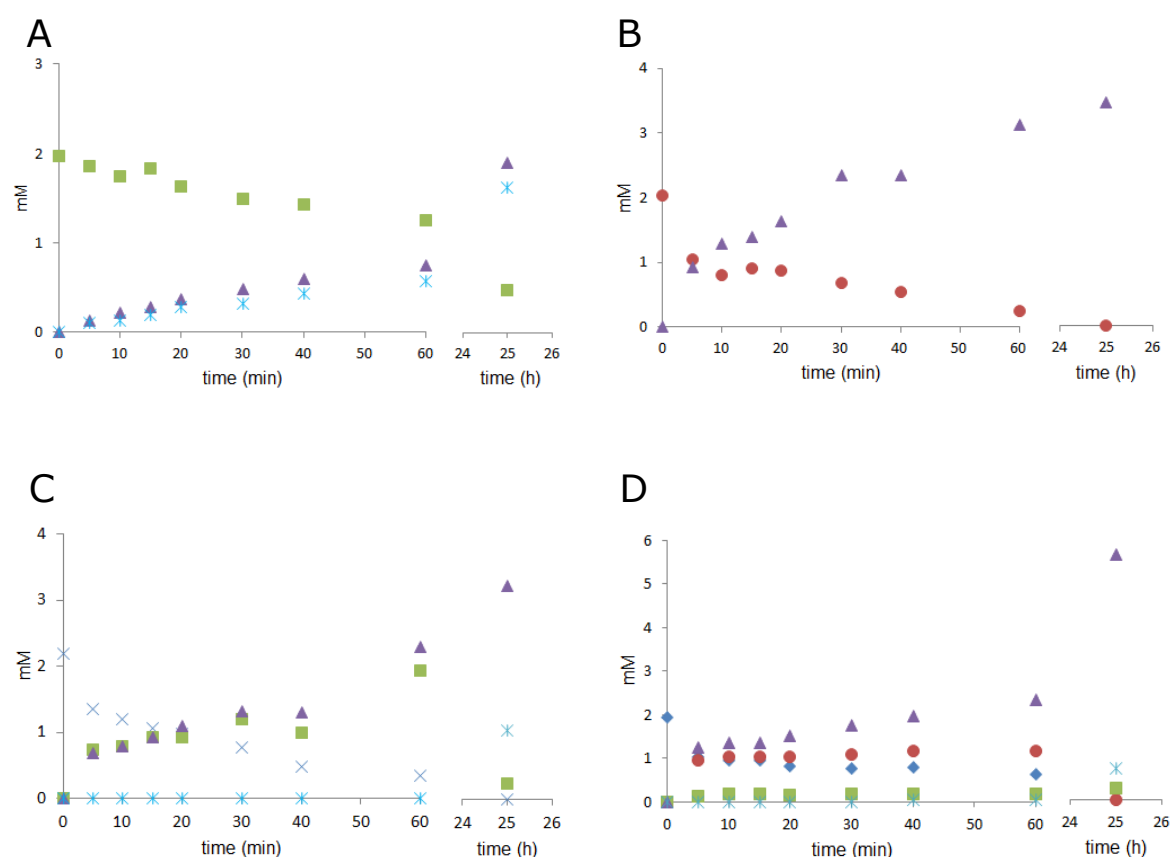
### **2.3.8. Degradation of chitin oligosaccharides and *p*NP-substrates by Chitinase Chi1**

In order to study the binding preferences and to determine the shortest possible substrate for Chitinase Chi1 the hydrolysis of chitin oligosaccharides (GlcNAc)<sub>2-6</sub> was followed in time and the hydrolysis products were analyzed by HPAEC (Figure 2.7.). (GlcNAc)<sub>2</sub> was not hydrolyzed by Chitinase Chi1 (data not shown), whereas (GlcNAc)<sub>3</sub> was cleaved to (GlcNAc)<sub>2</sub> and GlcNAc (Figure 2.7.A). (GlcNAc)<sub>4</sub> was split only to (GlcNAc)<sub>2</sub> (Figure 2.7.B). (GlcNAc)<sub>5</sub> was degraded to (GlcNAc)<sub>2</sub> and (GlcNAc)<sub>3</sub>, however, after 25 h (GlcNAc)<sub>3</sub> was degraded to GlcNAc and (GlcNAc)<sub>2</sub> (Figure 2.7.C). Depolymerization of (GlcNAc)<sub>6</sub> resulted in the initial release of (GlcNAc)<sub>4</sub> and (GlcNAc)<sub>2</sub> and small amounts of (GlcNAc)<sub>3</sub> (Figure 2.7.D). The released (GlcNAc)<sub>4</sub> was further degraded to (GlcNAc)<sub>2</sub> and (GlcNAc)<sub>3</sub> was cleaved to (GlcNAc)<sub>2</sub> and GlcNAc. The calculated rates for the degradation of chitin oligosaccharides were 0.18 mM min<sup>-1</sup> for (GlcNAc)<sub>6</sub>, 0.17 mM min<sup>-1</sup> for (GlcNAc)<sub>5</sub>, 0.20 mM min<sup>-1</sup> for (GlcNAc)<sub>4</sub>, and 0.02 mM min<sup>-1</sup> for (GlcNAc)<sub>3</sub>.

Among *p*NP-labelled chitin oligosaccharides, activity of Chitinase Chi1 was detected for (GlcNAc)<sub>3</sub>-*p*NP and (GlcNAc)<sub>2</sub>-*p*NP with the highest specific activity for (GlcNAc)<sub>2</sub>-*p*NP (Figure 2.1.S3.A). No activity was found for GlcNAc-*p*NP, which is in agreement that no activity was detected for (GlcNAc)<sub>2</sub>.

To investigate the reaction mechanism in more detail, degradation of labeled chitin oligosaccharides (GlcNAc)<sub>3</sub>-*p*NP and (GlcNAc)<sub>2</sub>-*p*NP was followed in time by HPAEC (Figure 2.1.S3.B). Hydrolysis of (GlcNAc)<sub>3</sub>-*p*NP yielded predominantly GlcNAc-*p*NP and (GlcNAc)<sub>2</sub>

(about 90 % end product) and low amounts of  $(\text{GlcNAc})_3$  (about 10 % end product). In time the released  $(\text{GlcNAc})_3$  was degraded further to  $(\text{GlcNAc})_2$  and  $\text{GlcNAc}$ . Products detected from the hydrolysis of  $(\text{GlcNAc})_2$ -pNP were  $(\text{GlcNAc})_2$ ,  $\text{GlcNAc}$ -pNP and small amounts of  $(\text{GlcNAc})_3$  (Figure 2.1.S4.B2). The substrate  $(\text{GlcNAc})_2$ -pNP used in this experiment did not contain  $(\text{GlcNAc})_3$  (Figure 2.1.S4.B1). Production of  $(\text{GlcNAc})_3$  can be explained by the transglycosylation catalyzed by Chitinase Chi1 and it is in agreement with results reported for chitinase MBP-CfcA from *Aspergillus niger* (van Munster et al., 2014).



**Figure 2.7.** Hydrolysis of chitin oligosaccharides  $(\text{GlcNAc})_3$  (A),  $(\text{GlcNAc})_4$  (B),  $(\text{GlcNAc})_5$  (C), and  $(\text{GlcNAc})_6$  (D) by Chitinase Chi1. Chitin oligosaccharides (2 mM) in 0.5 mL 0.05 M sodium phosphate buffer pH 6.0 were incubated with purified Chitinase Chi1 (25 nM). Aliquots were taken at different time intervals and the hydrolysis products were analyzed by High Performance Anion-Exchange Chromatography (HPAEC). Detected products were  $\text{GlcNAc}$  (star),  $(\text{GlcNAc})_2$  (triangle),  $(\text{GlcNAc})_3$  (square),  $(\text{GlcNAc})_4$  (circle),  $(\text{GlcNAc})_5$  (cross),  $(\text{GlcNAc})_6$  (diamond). Experimental work performed in duplicates and the standard deviation was less than 5 %.

## 2.4. Discussion

Analysis of the amino acid sequence of Chitinase Chi1 confirmed that Chitinase Chi1 is a real glycoside hydrolase from GH 18 family, which contains characteristic for this family D-X-E

motif. The 3D modelled structure revealed that Chitinase Chi1 has  $(\beta/\alpha)_8$  barrel fold (TIM) which is another characteristic for chitinases from GH 18.

Chitinase Chi1 was purified to homogeneity as confirmed by MALDI-TOF-MS and HPLC-MS/MS analysis of the proteolytic digests of the purified enzyme. The increase in specific activity after each purification step was not very extensive, and this might be due to the additive activity of the accompanying *N*-acetylglucosaminidase that was detected in the crude enzyme extract and which was still present in the fractions obtained after the first purification step using anion exchange chromatography. The ability of *N*-acetylglucosaminidase to act on the amorphous parts of chitin was reported for  $\beta$ -*N*-acetylhexosaminidase (LeHex20A) by Konno and co-workers (Konno et al., 2012). The second step of purification on size exclusion chromatography enabled a clear separation between Chitinase Chi1 and the remaining *N*-acetylglucosaminidase, resulting in the isolation of a pure enzyme Chitinase Chi1.

Molecular weight of Chitinase Chi1 measured with MALDI-TOF-MS was 43 kDa, which was different from a molecular weight calculated from the deduced protein sequence of 43.8 kDa. This difference shows that full-length Chitinase Chi1 undergoes post-translational proteolytic modification in the host *M. thermophila* C1. Detection of peptides from C-terminus end of Chitinase Chi1 with HPLC-MS/MS indicates that proteolytic removal takes place from N-terminus end of Chitinase Chi1. According to the calculated molecular weight, approximately 9 amino acids might be removed. Proteolytic processing has been previously described for chitinases from other microorganisms including chitinase ChiC from *Serratia marcescens* (Gal et al., 1998), chitinases from *Streptomyces olivaceoviridis* (Romaguera et al., 1992) and *Janthinobacterium lividum* (Gleave et al., 1995). Recently, Dua et al. (2016) reported the exochitinase rMtChit obtained by heterologous expression of the same gene sequence obtained from *M. thermophila* BJA in *Pichia pastoris*. This latter protein has a molecular weight of 48 kDa. The difference in molecular weight between Chitinase Chi1 and rMtChit can be explained by the fact that different cloning approaches and different hosts were used for enzyme production, i.e. Chitinase Chi1 was expressed homologously in *M. thermophila* while exochitinase rMtChit was expressed heterologously in the yeast *P. pastoris*. Furthermore, Chitinase Chi1 and exochitinase rMtChit differ clearly in the extent of glycosylation. Staining with PAS confirmed that Chitinase Chi1 was not or hardly glycosylated because no magenta colour formation was detected for Chitinase Chi1 at a concentration of 1 mg mL<sup>-1</sup>. In contrast,

exochitinase rMtChit gave an intense magenta colour, indicating glycosylation, as reported by Dua et al. (2016).

Chitinase Chi1 was found to have a pH optimum at pH 6.0 and a pI of 3.98, while the calculated pI from the amino acid sequence was 4.95. This difference between the theoretical pI predicted from the primary structure and the experimentally determined pI is common, since the pI of proteins is affected by several factors, including the solvent accessibility of amino acids. Some charged amino acids could be shielded by the folded structure of the enzyme and may not be exposed to the solvent, changing therefore the observed pI.

The highest activity of Chitinase Chi1 was detected at 55 °C which is in agreement with other thermophilic chitinases (Table 2.2.). Chitinase Chi1 showed excellent thermostability at 50 °C (>168 h, 90 % activity) and at 55 °C ( $t_{1/2}$  = 48 h). Reported thermostable fungal chitinases have also thermostability up to 50 °C, but all are less stable in time than Chitinase Chi1 (Table 2.2.). For example, the exochitinase rMtChit retained only 70 % of its activity when exposed to 45 °C for 5 h and showed a  $t_{1/2}$  = 113 minutes at 65 °C. During incubation at 50 °C (1 h) chitinase from *T. lanuginosus* retained 71 % of its activity whereas other chitinase from *T. lanuginosus* was able to preserve about 70 % of the enzyme activity after 6 h at 50 °C. In contrast, another chitinase from *T. lanuginosus* SY2 was 100 % active for 1h when incubating at 50 °C. Therefore, it can be concluded that Chitinase Chi1 can be classified as thermostable chitinase which shows excellent thermostability among other chitinases from thermophilic fungi.

Besides activity on chitin, Chitinase Chi1 showed also activity on the deacetylated chitin derivative – chitosan. Solubilized chitosan was more efficiently degraded by the enzyme than swollen chitin. This is in agreement with previous studies reporting that chitinases from GH 18 are able to cleave the glycosidic linkage of not only GlcNAc-GlcNAc but also GlcNAc-GlcN present in chitosan as long as a GlcNAc residue is bound at the -1 subsite (Ohtakara et al., 1990). Furthermore, higher activity on chitosan than on chitin indicates that substrate accessibility is an important parameter influencing chitinase activity as it was also observed for bacterial chitinase from *Ralstonia sp.* (Sutrisno et al., 2003).

**Table 2.2.** Properties of chitinases from thermophilic fungi.

Organism	Mw (kDa)	pH optimum	Temp. optimum (°C)	Temp. range (°C)	Thermostability (as % retained activity)	Reference
<i>Aspergillus fumigatus</i> YJ-407	46	5.0	50-60	45 to 60	100 % after 1 h at 45 °C; ≈70 % after 1 h at 55 °C; ≈20 % after 1 h at 60 °C;	Xia et al., 2001
<i>Chaetomium thermophilum</i>	47	5.5	60	40 to 70	100 % after 1 h at 50 °C; ≈90 % after 1 h at 60 °C;	Li et al., 2010
<i>Gliocladium catenulatum</i>	51	5.0-6.0	60	20 to 70	40 % after 20 min at 50 °C;	Ma et al., 2012
<i>Rhizopus oryzae</i>		5.5-6.0	60	50 to 70	100 % after 30 min at 50 °C; ≈40 % after 30 min at 60 °C;	Chen et al., 2013
<i>Myceliophthora thermophila</i> BJA	48	5.0	55	30 to 70	70 % after 5 h at 45°C; ≈50 % after 113 min at 65; ≈50 % after 48 min 75 °C;	Dua et al., 2016
<i>Thermoascus aurantiacus</i> vs. <i>levisporus</i>	48	8.0	50	40 to 60	≈90 % after 1 h at 50 °C; ≈30 % after 1 h at 60 °C;	Li et al., 2010
<i>Thermomyces langinensus</i>	44.1	5.0	50	30 to 60	50 % after 630 min at 50 °C; 50 % after 19 min at 60 °C	Zhang et al., 2015
<i>Thermomyces langinosus</i>	36.6	4.0	40	30 to 60	50 % at 40 °C after 578 min; 50 % at 50 °C after 14 min	Zhang et al., 2015
<i>Thermomyces langinosus</i> SY2	48	55	55	50 to 60	100 % after 1 h at 50 °C; ≈80 % after 1 h at 60 °C;	Guo et al., 2008
<i>Thermomyces langinosus</i>	42	60	60	30 to 70	70 % after 6 h at 50 °C;	Prasad and Palanivelu, 2012
<i>Trichoderma viride</i>	28	3.5	55 to 60		≈30 % after 3 h at 50 °C; ≈10 % after 3 h at 60 °C;	Omumasaba et al., 2001
<i>Myceliophthora thermophila</i> C1	43	6.0	55	30 to 70	≈90 % after >168 h at 50 °C; 50 % after 48 h at 55 °C 10 % after 10 min at 60 °C	This work

The activity of Chitinase Chi1 was strongly affected by DDA, Mw and presence of side groups (i.e. aldehyde, carboxyl) at the chitosan chain. In general, chitosan with a lower DDA (77 DDA) was degraded more efficiently than chitosan with high DDA (94 DDA). This result confirmed, that Chitinase Chi1 is a real GH 18 enzyme, which is dependent on the acetyl group of GlcNAc for catalysis. Thus, a decrease in the number of GlcNAc moieties present in the chitosan chain will result in less productive binding sites of Chitinase Chi1. Chitosan with low Mw (100 kDa) was degraded more efficiently than chitosan with high Mw (3,000 kDa) and the same DDA.



This result denotes, that chitosan with higher Mw, which also shows higher viscosity, is less accessible for the enzyme than the chitosan with a low Mw. Fully deacetylated modified chitosans with pending aliphatic side groups – such as glycol chitosan and hydroxypropyl-chitosan, were not degraded at all by the enzyme, showing that Chitinase Chi1 is a real chitinase, which is not able to cleave GlcN-GlcN bonds. Although steric hindrance due to side chains cannot be excluded, the results clearly suggest that the Chitinase Chi1 activity depends on the presence and the number of acetyl groups. It was shown for enzymes from GH 18, that the carbonyl oxygen from GlcNAc moiety act as a nucleophile during catalytic reaction of GH 18 enzymes. Similar activity on chitosan was reported for chitinase from *Streptomyces griseus* (Ohtakara et al., 1990).

Chitinase Chi1 released mainly (GlcNAc)<sub>2</sub> from chitin. Release of dimers was reported for other chitinases from GH 18 family (Ike et al., 2005; Horn et al., 2006a). In contrast, exochitinase rMtChit released only monomers from chitin (Dua et al., 2016). Although the amino acid sequence of Chitinase Chi1 and exochitinase rMtChit should be the same, differences in expression host and glycosylation influence the activity and mode of action of Chitinase Chi1 and exochitinase rMtChit.

Next to (GlcNAc)<sub>2</sub>, Chitinase Chi1 released small amount of GlcNAc and (GlcNAc)<sub>3</sub>, with a ratio of (GlcNAc)<sub>2</sub>/GlcNAc equal to 11. This ratio is commonly used for a rough assessment of enzyme processivity (Teeri et al., 1998; Hamre et al., 2015). Thus, Chitinase Chi1 may be considered as a processive chitinase. A decrease in the rate of chitin hydrolysis, which was observed after 30 min reaction is most likely due to the fact that Chitinase Chi1 enriched the recalcitrant regions of substrate. It was previously stated, that the activity of processive enzymes tends to decrease as the substrate is consumed and when the enzymes reach regions that hinder processive binding (Hamre et al., 2015). The release of (GlcNAc)<sub>2</sub> in higher molar amount than other chitin oligosaccharides from chitin was observed for processive and non-processive enzymes degrading recalcitrant polysaccharides, like for processive chitinases ChiA, ChiB and non-processive ChiC from *S. marcescens* (Horn et al., 2006a). Therefore, results obtained for Chitinase Chi1 with natural substrates indicate its processivity, but this conclusion is not indisputable. Important feature of processive exo-acting chitinases is so-called  $\alpha + \beta$  insertion domain that forms one 'wall' of the substrate binding cleft, which were found in ChiA and ChiB (Vaaje-Kolstad et al., 2013; Zees et al., 2009). This domain was not found in Chitinase

Chi1. However, the active site of Chitinase Chi1 is aligned with 60 amino acid residues, which may be important for interactions with the substrate and promote processivity of the enzyme. Aromatic residues in the active site of processive enzymes were shown to interact with the substrate during the processive mode of action (Hamre et al., 2015).

Processivity has been studied for other chitinases and also for cellulases (Wilson, 2012). It was shown, that processive enzymes slide with their active site on the single-polymer chain and stay closely associated with the substrate between subsequent hydrolytic reactions. During the processive degradation of chitin the enzymes release mainly dimers because the successive sugar units in the polymer are rotated by 180° and sliding of such polymers through the enzyme's active site will result in the productive binding only after every second sugar moiety. Rotation of the sugar units is particularly important for chitinases from family GH 18, since these enzymes require a correctly positioned *N*-acetyl group in their -1 subsite (Horn et al., 2006a).

It may be concluded, that Chitinase Chi1 is an endo-chitinase with high degree of processivity. It was stated that both endo- and exo-mechanisms can be combined with processive action (Zees et al., 2009) and that the most important difference between chitinases may be related to the ability of the enzymes to act in processive or non-processive way, rather than to their binding preferences (endo- or exo-manner) (Vaaje-Kolstad et al., 2013).

Chitinase Chi1 was able to release a broad spectrum of chitin and chito-oligosaccharides (DP2-DP12) from chitosan with 90 % DDA. The chito-oligosaccharide composition of the reaction mixture changed over time, indicating that release and further degradation of some chito-oligosaccharides might be simultaneously catalyzed by Chitinase Chi1, as it was shown in the experiment with chitin oligosaccharides (GlcNAc)<sub>2-6</sub>. The composition of chito-oligosaccharides and the accumulation of a (GlcNAc, GlcN) dimer at longer reaction time may indicate the ability of Chitinase Chi1 to cleave glycosidic linkages between GlcN and GlcNAc moieties in the chitosan chain, as it was reported for the chitinase G from *Streptomyces coelicolor* A3(2) from bacterial family GH19 chitinase (Heggset et al., 2009) and other bacterial chitinases (Jung and Park, 2014). Release of longer chito-oligosaccharides may indicate, that similarly to chitin degradation, Chitinase Chi1 can degrade chitosan in a processive way. Chitinases ChiA and ChiB from *S. marcescens* were shown to degrade chitosan in a processive

way (Horn et al., 2006b). In case of chitosan, processive enzymes stay attached to the substrate after productive (with a correctly positioned *N*-acetyl group in the sugar unit) or non-productive (with lack of a correctly positioned *N*-acetyl group in the sugar unit) initial binding to the substrate. Binding of the substrate will be followed by sliding of the substrate through the active site cleft by two sugar units at the time, until a new productive complex will be formed and an enzymatic reaction occurs (Horn et al., 2006a).

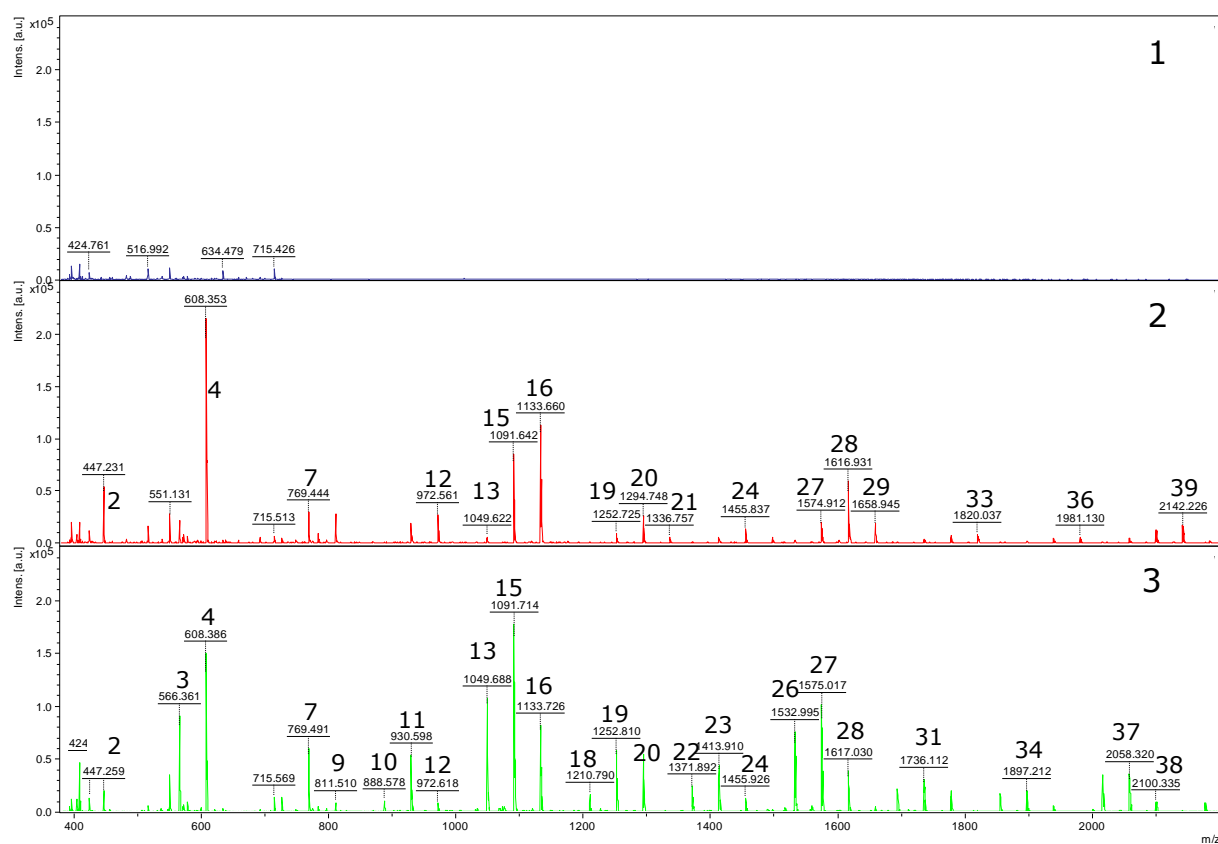
Chitinase Chi1 degraded chitin oligosaccharides with  $DP \geq 3$ . The absence of activity on  $(GlcNAc)_2$  ruled out the possibility that Chitinase Chi1 is an *N*-acetylglucosaminidase. All initial released chitin oligosaccharides with  $DP \geq 3$  were subject to further hydrolysis that yielded  $(GlcNAc)_2$  and GlcNAc as final products. Chitinase Chi1 showed increasing activities with increasing DP of chitin oligosaccharides. These data indicate that Chitinase Chi1 has a multi-subsite binding cleft and positioning of chitin oligosaccharides with  $DP \geq 4$  is more efficient than with DP 3, resulting in about 10 times faster conversion for longer-chain chitin oligosaccharides than for shorter ones. Additionally, experiments with  $(GlcNAc)_3$ -pNP and  $(GlcNAc)_2$ -pNP oligosaccharides bound to Chitinase Chi1 at subsites -2 to +2 in the active site during hydrolysis.

Overall, we showed here that homologously expressed Chitinase Chi1 releases mainly dimers from chitin and might use a processive mechanism. Depolymerization of chitosan resulted in the production of a wide range of chito-oligosaccharides. Chitin and chito-oligosaccharides are an emerging class of bioactive ingredients with potential biomedical, cosmetic and pharmaceutical applications. The need for green and biocompatible technologies for the production of chitin and chito-oligosaccharides reveals new perspectives for the application of biocatalysts. With its remarkable thermostability and activity in a wide range of pH, Chitinase Chi1 is a promising biocatalyst for bioconversion of chitin waste sources and production of chitin and chito-oligosaccharides from both chitin and chitosan at industrial scale.

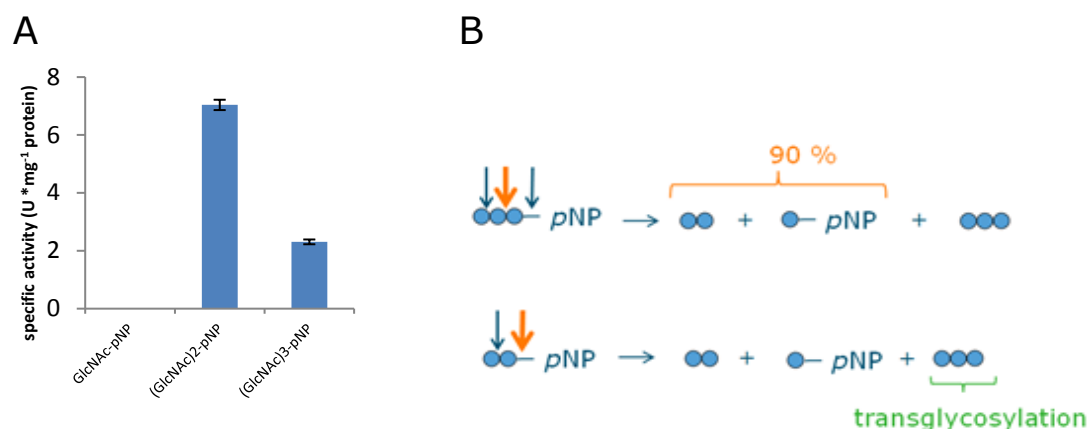
## 2.1. Supplementary Information

MVTPSLKKAA	LAALSFLPLL	SLASPVPTATA	EASVQTRQSS	GYKNIVYFTN	WGIYGRNYQP
DQLPASOLTH	VLYSFANIRS	NGEVFLSDTY	ADLEKHYPNP	SWNDVGNNVY	GCVKQLFLLK
KANRQLK <b>TLL</b>	<b>SIGGW</b> TYSAT	<b>FPAA</b> ASTAES	<b>RALFA</b> SSAVR	LLADLGFDGL	DIDWEYPANE
QEAA <sup>+</sup> NFVLL	KAVRSALDDY	AAQHAPGYHF	LLTIA <sup>+</sup> SPAGP	SNYGHLP <sup>+</sup> LRD	IAGVIDFFNF
MGYDYAGSWS	TAAGHQANLY	PTADAGR <b>TFF</b>	<b>STD</b> KALSDYV	<b>AAG</b> VDPAKIV	<b>LGM</b> PIYGRSF
<b>EAT</b> DGLGK <b>PF</b>	<b>TGV</b> GQGSWES	<b>GVW</b> DYKVLPR	<b>AGAT</b> VQYDEE	<b>AGAT</b> YSYDPA	<b>TREL</b> ISFDTV
DMVKKKVDYV	K <b>QKG</b> FAGSMF	<b>WEAS</b> ADRTGD	<b>QSLI</b> GASFGA	<b>LGGI</b> DQSQNQ	<b>LSYP</b> DSKYDN
<b>LRA</b> GFP					

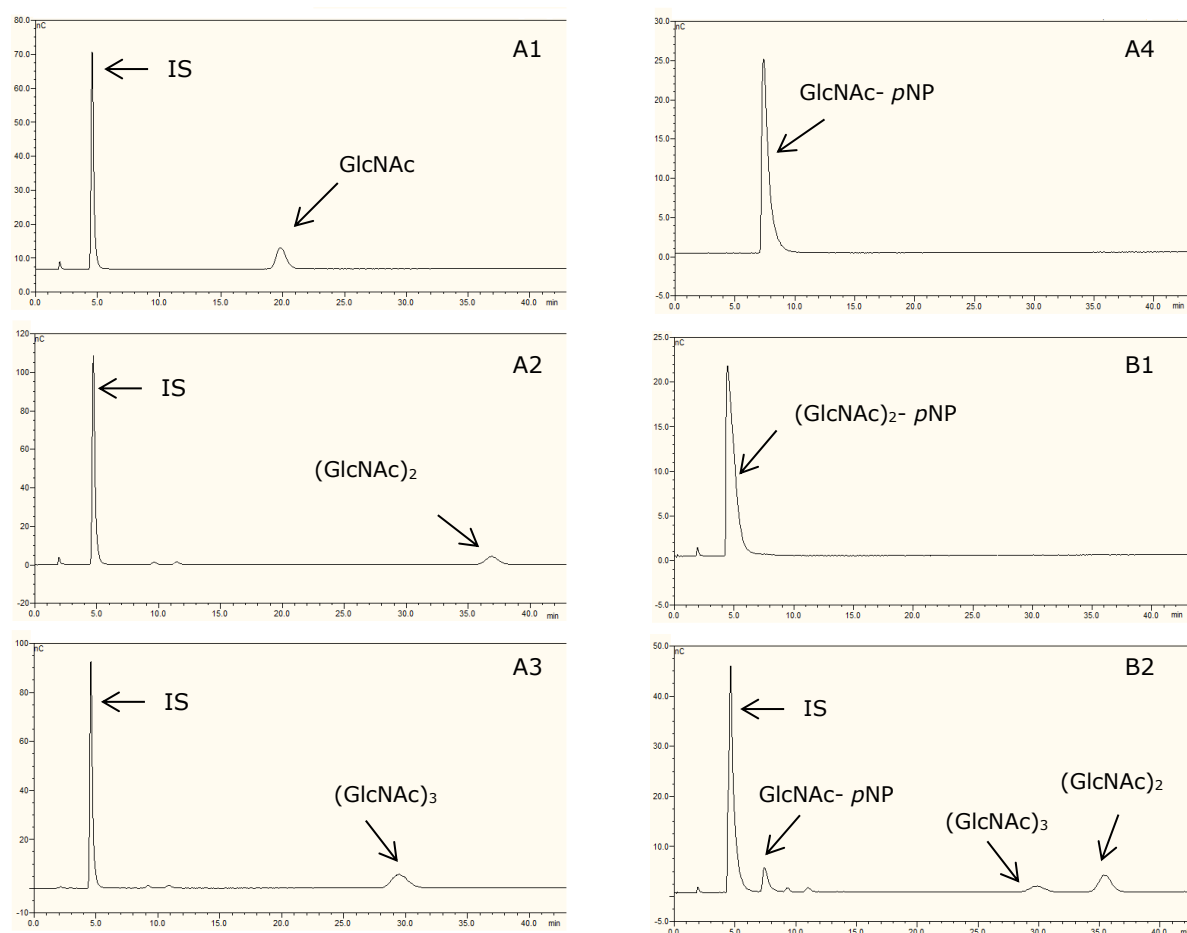
**Figure 2.1.S1.** Protein oligomers detected after proteolytic digestion of Chitinase Chi1 with HPLC-ESI-MS/MS analysis. Peptides (indicated in yellow) were only found matching the C-terminus of the enzyme.



**Figure 2.1.S2.** Spectra from matrix assisted laser-desorption time-of-flight mass spectrometry (MALDI-TOF-MS) obtained for chito-oligosaccharides released by Chitinase Chi1 from chitosan 90 DDA/100. Chitosan (0.45 % (w/v)) in 1 mL 0.05 M sodium phosphate buffer pH 6.0 was incubated with purified Chitinase Chi1 (100 nM) at 50 °C. Presented spectra are obtained for reaction at time 0 (1), 240 min incubation (2), and 1,440 min incubation (3). The type of chito-oligosaccharides and their corresponding masses are shown in Table 2.1.S1. The most predominant peaks were assigned.



**Figure 2.1.S3.** Specific activity of Chitinase Chi1 for GlcNAc-pNP, (GlcNAc)<sub>2</sub>-pNP and (GlcNAc)<sub>3</sub>-pNP (A) and mode of action of Chitinase Chi1 on (GlcNAc)<sub>3</sub>-pNP and (GlcNAc)<sub>2</sub>-pNP (B). Reactions with GlcNAc-pNP, (GlcNAc)<sub>2</sub>-pNP, and (GlcNAc)<sub>3</sub>-pNP (2 mM) in 0.5 mL 50 mM sodium phosphate buffer pH 6.0 were performed with purified Chitinase Chi1 (17 nM) at 50 °C for 15 min. Arrows indicate place of cleavage.



**Figure 2.1.S4.** High Performance Anion-Exchange Chromatography (HPAEC) elution profiles of standards of chito-oligosaccharides (GlcNAc)<sub>1-3</sub> and GlcNAc-pNP (A) and reaction products released after 60 min incubation of (GlcNAc)<sub>2</sub>-pNP with purified Chitinase Chi1 (B). Elution time recorded for: (A1) GlcNAc; (A2) (GlcNAc)<sub>2</sub>; (A3) (GlcNAc)<sub>3</sub>; (B1) substrate (GlcNAc)<sub>2</sub>-pNP; (B2) products released from (GlcNAc)<sub>2</sub>-pNP by Chitinase Chi1. Reaction with (GlcNAc)<sub>2</sub>-pNP (1 mM) in 0.5 mL 50 mM sodium phosphate buffer pH 6.0 was performed with purified Chitinase Chi1 (25 nM) at 50 °C for 60 min. For Figure A1, A2, A3 and B2, fucose was used as the internal standard (IS). For Figure A4 and B1 the IS was not used.

**Table 2.1.S1.** Chito-oligosaccharides released by Chitinase Chi1 from chitosan 90 DDA/100. Chitosan (0.45 % (w/v)) in 1 mL 0.05 M sodium phosphate buffer pH 6.0 was incubated with purified Chitinase Chi1 (100 nM) at 50 °C. Aliquots were taken at different time intervals and the hydrolysis products were analyzed by matrix assisted laser-desorption time-of-flight mass spectrometry (MALDI-TOF-MS). Sign (+) indicates the presence of chito-oligosaccharide in sample.

m/z	Composition	Peak Nr.	Type of adduct	Hydrolysis time (min)								
				0	15	30	60	90	120	240	360	1440
405.2	GlcNAc,GlcN	1	[M+Na] <sup>+</sup>						+	+	+	+
447.2	(GlcNAc) <sub>2</sub>	2	[M+Na] <sup>+</sup>		+	+	+	+	+	+	+	+
566.3	GlcNAc,(GlcN) <sub>2</sub>	3	[M+Na] <sup>+</sup>					+	+	+	+	+
608.3	(GlcNAc) <sub>2</sub> ,GlcN	4	[M+Na] <sup>+</sup>		+	+	+	+	+	+	+	+
650.3	(GlcNAc) <sub>3</sub>	5	[M+Na] <sup>+</sup>		+							
727.1	GlcNAc,(GlcN) <sub>3</sub>	6	[M+Na] <sup>+</sup>		+		+	+	+		+	+
769.3	(GlcNAc) <sub>2</sub> ,GlcN	7	[M+Na] <sup>+</sup>		+		+	+		+	+	+
784.0	(GlcNAc) <sub>2</sub> ,GlcN	8	[M+K] <sup>+</sup>					+	+	+	+	+
811.4	(GlcNAc) <sub>3</sub> ,GlcN	9	[M+Na] <sup>+</sup>		+	+	+	+	+	+	+	
888.6	GlcNAc,(GlcN) <sub>4</sub>	10	[M+Na] <sup>+</sup>									+
930.0	(GlcNAc) <sub>2</sub> ,GlcN	11	[M+Na] <sup>+</sup>						+	+	+	
972.4	(GlcNAc) <sub>3</sub> ,GlcN	12	[M+Na] <sup>+</sup>		+	+	+	+	+	+	+	
1014.4	(GlcNAc) <sub>4</sub> ,GlcN	13	[M+Na] <sup>+</sup>		+							
1049.7	GlcNAc,(GlcN) <sub>5</sub>	14	[M+Na] <sup>+</sup>									+
1091.5	(GlcNAc) <sub>2</sub> ,GlcN	15	[M+Na] <sup>+</sup>		+	+	+	+	+	+	+	+
1133.5	(GlcNAc) <sub>3</sub> ,GlcN	16	[M+Na] <sup>+</sup>		+	+	+	+	+	+	+	+
1175.5	(GlcNAc) <sub>4</sub> ,GlcN	17	[M+Na] <sup>+</sup>		+	+	+		+			
1210.7	GlcNAc,(GlcN) <sub>6</sub>	18	[M+Na] <sup>+</sup>									+
1252.0	(GlcNAc) <sub>2</sub> ,GlcN	19	[M+Na] <sup>+</sup>							+	+	+
1294.6	(GlcNAc) <sub>3</sub> ,GlcN	20	[M+Na] <sup>+</sup>		+	+	+	+	+	+	+	+
1336.6	(GlcNAc) <sub>4</sub> ,GlcN	21	[M+Na] <sup>+</sup>		+	+	+	+	+	+		
1371.9	GlcNAc,(GlcN) <sub>7</sub>	22	[M+Na] <sup>+</sup>									+
1413.0	(GlcNAc) <sub>2</sub> ,GlcN	23	[M+Na] <sup>+</sup>						+	+	+	+
1455.7	(GlcNAc) <sub>3</sub> ,GlcN	24	[M+Na] <sup>+</sup>		+		+	+	+	+	+	+
1497.7	(GlcNAc) <sub>4</sub> ,GlcN	25	[M+Na] <sup>+</sup>		+	+	+	+	+	+		
1532.9	GlcNAc,(GlcN) <sub>8</sub>	26	[M+Na] <sup>+</sup>									+
1575.0	(GlcNAc) <sub>2</sub> ,GlcN	27	[M+Na] <sup>+</sup>					+	+	+	+	+
1616.7	(GlcNAc) <sub>3</sub> ,GlcN	28	[M+Na] <sup>+</sup>		+	+	+	+	+	+	+	+
1658.8	(GlcNAc) <sub>4</sub> ,GlcN	29	[M+Na] <sup>+</sup>		+	+	+	+	+	+	+	
1700.8	(GlcNAc) <sub>5</sub> ,GlcN	30	[M+Na] <sup>+</sup>		+							
1736.1	(GlcNAc) <sub>2</sub> ,GlcN	31	[M+Na] <sup>+</sup>									+
1777.9	(GlcNAc) <sub>3</sub> ,GlcN	32	[M+Na] <sup>+</sup>						+	+	+	+
1820.0	(GlcNAc) <sub>4</sub> ,GlcN	33	[M+Na] <sup>+</sup>		+			+	+	+	+	
1897.2	(GlcNAc) <sub>2</sub> ,GlcN	34	[M+Na] <sup>+</sup>									+
1939.0	(GlcNAc) <sub>3</sub> ,GlcN	35	[M+K] <sup>+</sup>						+	+	+	+
1981.1	(GlcNAc) <sub>2</sub> ,GlcN	36	[M+Na] <sup>+</sup>		+				+	+	+	
2058.3	(GlcNAc) <sub>2</sub> ,GlcN	37	[M+Na] <sup>+</sup>									+
2100.0	(GlcNAc) <sub>3</sub> ,GlcN	38	[M+Na] <sup>+</sup>		+			+	+	+	+	+
2142.2	(GlcNAc) <sub>4</sub> ,GlcN	39	[M+Na] <sup>+</sup>		+		+	+	+	+	+	
2184.0	(GlcNAc) <sub>5</sub> ,GlcN	40	[M+Na] <sup>+</sup>		+	+	+	+	+			

## References

- Adrangi, S., Faramarzi, M.A., Shahverdi, A.R., Sepehrizadeh, Z., 2010. Purification and characterization of two extracellular endochitinases from *Massilia timonae*. *Carbohydr. Res.* 345, 402–407
- Azuma, K., Osaki, T., Minami, S., Okamoto, Y., 2015. Anticancer and anti-inflammatory properties of chitin and chitosan oligosaccharides. *J. Funct. Biomater.* 6, 33–49
- Chen, W.-M., Chen, C.-S., Jiang, S.-T., 2013. Purification and characterization of an extracellular chitinase from *Rhizopus Oryzae*. *J. Mar. Sci. Technol.* 21, 361–366
- Dua, A., Joshi, S., Satyanarayana, T., 2016. Recombinant exochitinase of the thermophilic mould *Myceliophthora thermophila* BJA: characteristics and utility in generating *N*-acetylglucosamine and in biocontrol of phytopathogenic fungi. *Biotechnol. Prog.* 33, 70–80
- Forsberg, Z., Nelson, C.E., Dalhus, B., Mekasha, S., Loose, J.S.M., Crouch, L.I., Røhr, Å.K., Gardner, J.G., Eijsink, V.G.H., Vaaje-Kolstad, G., 2016. Structural and functional analysis of a lytic polysaccharide monoxygenase important for efficient utilization of chitin in *Cellvibrio japonicus*. *J. Biol. Chem.* 291, 7300–7312
- Gal, S.W., Choi J.Y., Kim, C.Y., Cheong, Y. H., Choi, Y.J., Lee S.Y., Bahk J.D., Cho M.J., 1998. Cloning of the 52-kDa chitinase gene from *Serratia marcescens* KCTC2172 and its proteolytic cleavage into an active 35-kDa enzyme. *FEMS Microbiol. Lett.* 160, 151–158
- Gao, X., Chen, X., Zhang, J., Guo, W., Jin, F., Yan, N., 2016. Transformation of chitin and waste shrimp shells into acetic acid and pyrrole. *ACS Sustain. Chem. Eng.* 4, 3912–3920
- Gleave, A.P., Taylor, R.K., Morris B.A., Greenwood, D.R., 1995. Cloning and sequencing of a gene encoding the 69–kDa extracellular chitinase of *Janthinobacterium lividum*. *FEMS Microbiol. Lett.* 131, 279–288
- Gómez Ramírez, M., Rojas Avelizapa, L.I., Rojas Avelizapa, N.G., Cruz Camarillo, R., 2004. Colloidal chitin stained with Remazol Brilliant Blue R®, a useful substrate to select chitinolytic microorganisms and to evaluate chitinases. *J. Microbiol. Methods* 56, 213–219
- Guo, R.-F., Shi B.-S., Li, D.-C., Ma, W., Wei, Q., 2008. Purification and characterization of a novel thermostable chitinase from *Thermomyces lanuginosus* SY2 and cloning of its encoding gene. *Agric. Sci. China.* 7, 1458–1465
- Hamre, A.G., Jana, S., Reppert, N.K., Payne, C.M., Sørli, M., 2015. Processivity, substrate positioning, and binding: the role of polar residues in a family 18 Glycoside Hydrolase. *Biochemistry.* 54, 7292–7306
- Hartl, L., Zach, S., Seidl-Seiboth, V., 2011. Fungal chitinases: diversity, mechanistic properties and biotechnological potential. *Appl. Microbiol. Biotechnol.* 93, 533–543
- 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, 892–899
- Hinz, S.W.A., Pouvreau, L., Joosten, R., Bartels, J., Jonathan, M.C., Wery, J., Schols, H.A., 2009. Hemicellulase production in *Chrysosporium lucknowense* C1. *J. Cereal. Sci.* 50, 318–323
- Horn, S.J., Sørbotten, A., Synstad, B., Sikorski, P., Sørli, M., Vårum, K.M., Eijsink, V.G.H., 2006a. Endo/exo mechanism and processivity of family 18 chitinases produced by *Serratia marcescens*. *FEBS J.* 273, 491–503
- Horn, S.J., Sørli, M., Vaaje-Kolstad, G., Norberg, A.L., Synstad, B., Vårum, K.M., Eijsink, V.G.H., 2006b. Comparative studies of chitinases A, B and C from *Serratia marcescens*. *Biocatal. Biotransformation* 24, 39–53
- Ike, M., Nagamatsu, K., Shioya, A., Nogawa, M., Ogasawara, W., Okada, H., Morikawa, Y., 2005. Purification, characterization, and gene cloning of 46 kDa chitinase (Chi46) from *Trichoderma reesei* PC-3-7 and its expression in *Escherichia coli*. *Appl. Microbiol. Biotechnol.* 71, 294–303
- Jung, W.-J., Park, R.-D., 2014. Bioproduction of chito-oligosaccharides: present and perspectives. *Mar. Drugs* 12, 5328–5356
- Kardas, I., Struszczyk, M.H., Kucharska, M., van den Broek, L.A.M., van Dam, J.E.G., Ciechańska, D., 2012. Chitin and chitosan as functional biopolymers for industrial applications. In *The European Polysaccharide Network of Excellence (EPNOE)*. Navard, P., Ed.; Springer, Vienna, pp. 329–373
- Kelley, L.A., Mezulis, S., Yates, C.M., Wass, M.N., Sternberg, M.J.E., 2015. The Phyre2 web portal for protein modeling, prediction and analysis. *Nature Protocols.* 10, 845–858
- Konno, N., Takahashi, H., Nakajima, M., Takeda, T., Sakamoto, Y., 2012. Characterization of  $\beta$ -*N*-acetylhexosaminidase (LeHex20A), a member of glycoside hydrolase family 20, from *Lentinula edodes* (shiitake mushroom). *AMB Express.* 2, 29

- Kühnel, S., Hinz, S.W.A., Pouvreau, L., Wery, J., Schols, H.A., Gruppen, H., 2010. *Chrysosporium lucknowense* arabinohydrolases effectively degrade sugar beet arabinan. *Bioresour. Technol.* 101, 8300–8307
- Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685
- Lever, M., 1973. Colorimetric and fluorometric carbohydrate determination with *p*-hydroxybenzoic acid hydrazide. *Biochem. Med.* 7, 274–281
- Li, A.-N., Yu, K., Liu, H.-Q., Zhang, J., Li, H., Li D.-C., 2010. Two novel thermostable chitinase genes from thermophilic fungi: Cloning, expression and characterization. *Bioresour. Technol.* 101, 5546–5551
- Ma, G.-Z., Gao, H.-L., Zhang, Y.-H., Li, S.-D., Xie, B.-Y., Wu, S.-J., 2012. Purification and characterization of chitinase from *Gliocladium catenulatum* strain HL-1-1. *Afr. J. Microbiol. Res.* 6, 4377–4383
- Marchlerbauer, A., Derbyshire, M.K., Gonzales, N.R., Lu, S., Chitsaz, F., Geer, L.Y., Geer, R.C., He, J., Gwadz, M., Hurwitz, D. I., 2015. CDD: NCBI's conserved domain database. *Nucleic Acids Res.* 43, 222–226
- Meena, S., Gothwal, R.K., Mohan, M.K., Ghosh, P., 2014. Production and purification of a hyperthermostable chitinase from *Brevibacillus formosus* BISR-1 isolated from the Great Indian Desert soils. *Extremophiles* 18, 451–462
- Monreal, J., Reese, E.T., 1969. The chitinase of *Serratia marcescens*. *Can. J. Microbiol.* 15, 689–696
- Nawani, N.N., Kapadnis, B.P., Das, A.D., Rao, A.S., Mahajan, S.K., 2002. Purification and characterization of a thermophilic and acidophilic chitinase from *Microbispora* sp. V2. *J. Appl. Microbiol.* 93, 965–975
- No, H.K., Young Park, N., Ho Lee, S., Meyers, S.P., 2002. Antibacterial activity of chitosans and chitosan oligomers with different molecular weights. *Int. J. Food Microbiol.* 74, 65–72
- Ohtakara, A., Matsunaga, H., Mitsutomi, M., 1990. Action pattern of *Streptomyces griseus* chitinase on partially *N*-acetylated chitosan. *Agric. Biol. Chem.* 54, 3191–3199
- Omumasaba, C.A., Yoshida, N., Ogawa, K., 2001. Purification and characterization of a chitinase from *Trichoderma viride*. *J. Gen. Appl. Microbiol.* 47, 53–61
- Prasad, M., Palanivelu, P., 2012. Overexpression of a chitinase gene from the thermophilic fungus, *Thermomyces lanuginosus* in *Saccharomyces cerevisiae* and characterization of the recombinant chitinase. *J. Microb. Biochem. Technol.* 4, 86–91
- Romaguera, A., Menge, U., Breves, R., Diekmann, H., 1992. Chitinases of *Streptomyces olivaceoviridis* and significance of processing for multiplicity. *J. Bacteriol.* 174, 3450–3454
- Shen, C.-R., Chen, Y.-S., Yang, C.-J., Chen, J.-K., Liu, C.-L., 2010. Colloid chitin azure is a dispersible, low-cost substrate for chitinase measurements in a sensitive, fast, reproducible assay. *J. Biomol. Screen.* 15, 213–217
- Sutrisno, A., Ueda, M., Abe, Y., Nakazawa, M., Miyatake, K., 2003. A chitinase with high activity toward partially *N*-acetylated chitosan from a new, moderately thermophilic, chitin-degrading bacterium, *Ralstonia* sp. A-471. *Appl. Microbiol. Biotechnol.* 63, 398–406
- Teeri, T.T., Koivula, A., Linder, M., Wohlfahrt, G., Divne, C., Jonest, T.A., 1998. *Trichoderma reesei* cellobiohydrolases: why so efficient on crystalline cellulose? *Biochem. Soc. Trans.* 26, 173–178
- Vaaje-Kolstad, G., Westereng, B., Horn, S. J., Liu, Z. L., Zhai, H., Sørli, M., Eijsink, V.G.H., 2010. An oxidative enzyme boosting the enzymatic conversion of recalcitrant polysaccharides. *Science*. 330, 219–222
- Vaaje-Kolstad, G., Horn, S.J., Sørli, M., Eijsink, V.G.H., 2013. The chitinolytic machinery of *Serratia marcescens* – a model system for enzymatic degradation of recalcitrant polysaccharides. *FEBS J.* 280, 3028–3049
- Van den Broek, L.A.M., Knoop, R.J.I., Kappen, F.H.J., Boeriu, C.G., 2015. Chitosan films and blends for packaging material. *Carbohydr. Polym.* 116, 237–242
- Van Munster, J.M., Dobruchowska, J.M., Veloo, R., Dijkhuizen, L., van der Maarel, M.J.E.C., 2014. Characterization of the starvation-induced chitinase CfcA and  $\alpha$ -1,3-glucanase AgnB of *Aspergillus niger*. *Appl. Microbiol. Biotechnol.* 99, 2209–2223
- Verdoes, J.C., Punt, P.J., Burlingame, R.P., Pynnonen, C.M., Olson, P.T., Wery, J., Visser, J.H., Emalfarb, M.A.V.J., 2010. New fungal production system. *Int. Patent WO/2010/107303*
- Verdoes, J.C., Punt, P.J., Burlingame, R., Bartels, J., van Dijk, R., Slump, E., Meens, M., Joosten, R., Emalfarb, M. A., 2007. Dedicated vector for efficient library construction and high throughput screening in the hyphal fungus *Chrysosporium lucknowense*. *Ind. Biotechnol.* 3, 48–57
- Visser, H., Joosten, V., Punt, P.J., Gusakov, A., Olson, P.T., Joosten, R., Bartels, J., Visser, J., Sinitsyn, A., Emalfarb, M., Verdoes, J., Wery, J., 2011. Development of a mature fungal technology and production platform for industrial enzymes based on a *Myceliophthora thermophila* isolate, previously known as *Chrysosporium lucknowense* C1. *Ind. Biotechnol.* 7, 214–223
- Wilson, D.B., 2012. Processive and nonprocessive cellulases for biofuel production-lessons from bacterial genomes and structural analysis. *Appl. Microbiol. Biotechnol.* 93, 497–502

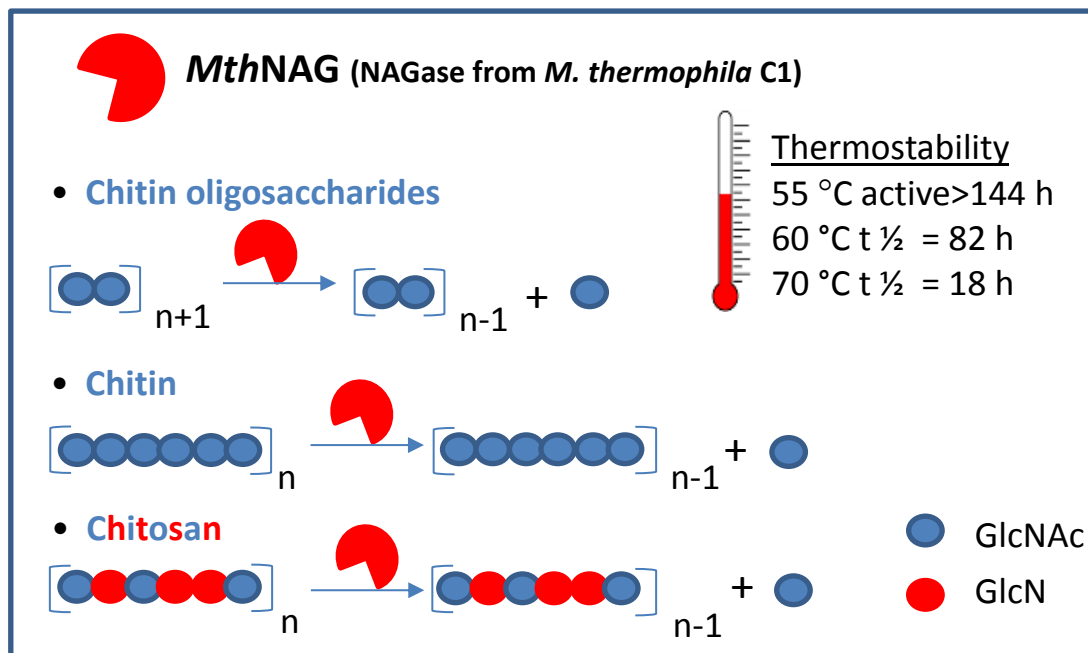


- Xia, G., Jin, C., Zhou, J., Yang, S., Zhang, S., Jin, C., 2001. A novel chitinase having a unique mode of action from *Aspergillus fumigatus* YJ-407. *Eur. J. Biochem.* 268, 4079–4085
- Zacharius, R.M., Zell, T.E., Morrison, J.H., Woodlock, J.J., 1969. Glycoprotein staining following electrophoresis on acrylamide gels. *Anal. Biochem.* 30, 148–152
- Zees, A.C., Pyrpasopoulos, S., Vorgias, C.E., 2009. Insights into the role of the ( $\alpha$ + $\beta$ ) insertion in the TIM-barrel catalytic domain, regarding the stability and the enzymatic activity of Chitinase A from *Serratia marcescens*. *BBA-Proteins Proteom.* 1794, 23–32
- Zhang, M., Puri, A.K., Govender, A., Wang, Z., Singh, S., Permaul, K., 2015. The multi-chitinolytic enzyme system of the compost-dwelling thermophilic fungus *Thermomyces lanuginosus*. *Process Biochem.* 50, 237–244



# Chapter 3

$\beta$ -N-Acetylglucosaminidase *Mth*NAG from *Myceliophthora thermophila* C1, a thermostable enzyme for production of N-acetylglucosamine from chitin



Published as:

Krolicka, M., Hinz, S.W.A., Koetsier, M.J., Eggink, G., van den Broek, L.A.M., Boeriu, C.G., 2018. Appl. Microbiol. Biotechnol. 102, 7441–7454

## ABSTRACT

Thermostable enzymes are a promising alternative for chemical catalysts currently used for the production of *N*-acetylglucosamine (GlcNAc) from chitin. In this study, a novel thermostable  $\beta$ -*N*-acetylglucosaminidase *Mth*NAG was cloned and purified from the thermophilic fungus *Myceliophthora thermophila* C1. *Mth*NAG is a protein with a molecular weight of 71 kDA as determined with MALDI-TOF-MS. *Mth*NAG has the highest activity at 50 °C and pH 4.5. The enzyme shows high thermostability above the optimum temperature: at 55 °C (144 h, 75 % activity), 60 °C (48 h, 85 % activity; half life 82 h) and 70 °C (24 h, 33 % activity; half life 18 h). *Mth*NAG releases GlcNAc from chitin oligosaccharides (GlcNAc)<sub>2-5</sub>, *p*-nitrophenol derivatives of chitin oligosaccharides (GlcNAc)<sub>1-3</sub>-*p*NP and the polymeric substrates swollen chitin and soluble chitosan. The highest activity was detected towards (GlcNAc)<sub>2</sub>. *Mth*NAG released GlcNAc from the non-reducing end of the substrate. We found that *Mth*NAG and Chitinase Chi1 from *M. thermophila* C1 synergistically degraded swollen chitin and released GlcNAc in concentration of approximately 130 times higher than when only *Mth*NAG was used. Therefore, Chitinase Chi1 and *Mth*NAG have great potential in the industrial production of GlcNAc.

### 3.1. Introduction

Large amounts of polysaccharides present in nature are an excellent source of valuable sugars, and chitin is one of them. Chitin is a vastly abundant polysaccharide and is used for the production of chitosan, chitin oligosaccharides and GlcNAc (Kardas et al., 2012). Generally, chitin consists of linearly  $\beta$ -(1,4)-linked *N*-acetylglucosamine molecules (GlcNAc). Chitosan, the deacetylated derivative of chitin, has wide application in many fields including medicine, pharmacology, environmental protection and biobased packaging (Elieh-Ali-Komi and Hamblin, 2016; Kardas et al., 2012; Van den Broek et al., 2015). Chitin oligosaccharides can be used as antimicrobial (No et al., 2002), antitumor and anti-inflammatory agents (Azuma et al., 2015). GlcNAc has gained great attention as a candidate for multiple applications (Chen et al., 2010). In medicine, GlcNAc is considered as an inexpensive and non-toxic treatment for numerous diseases including osteoarthritis, inflammatory bowel disease, viral or bacterial infections, intestinal diseases, and cancer and for proliferation of skin cells during wound healing (Dalirfardouei et al., 2016; Salvatore et al., 2000; Xu et al., 2006; Xu et al., 2007a; Xu et al., 2007b; Minami and Okamoto, 2007). In cosmetics, GlcNAc is a valuable ingredient for improving skin quality (Riordan, 1999; Bisset et al., 2007). In food industry, GlcNAc is used as an additive in beer, milk and wine (Xu et al. 2004a, 2004b, 2004c). Recently, GlcNAc was proposed as a biological C6 source for bioethanol production through fermentation. It was reported, that fermentation with *Mucor circinelloides* NBRC6746 and *Mucor ambiguous* NBRC8092 yielded approximately 18.6 and 16.9 g L<sup>-1</sup> ethanol from 50 g L<sup>-1</sup> GlcNAc, respectively (Inokuma et al., 2013).

Chitin is used for industrial production of GlcNAc and is obtained mainly from exoskeletons of crustaceans and to a lesser extent from insects and fungal cell walls (Rinaudo, 2006). Currently, degradation of chitin to GlcNAc from crustaceans is conducted with 15–36 % HCl at 40–80 °C (Chen et al., 2010). Another production process involves the use of concentrated HCl at boiling temperature. Such harsh conditions lead to degradation of chitin and deacetylation of the monomer to glucosamine (GlcN). The produced GlcN is subsequently *N*-acetylated with acetic anhydride. However, there appears to be several problems in producing GlcNAc by chemical depolymerization of chitin, including high operational costs, low yield (below 65 %) and acidic waste created by the use of HCl and acetic anhydride (Chen et al., 2010). A promising alternative for the chemical process is chitin depolymerization with enzymes. In

comparison to chemical process, enzymatic depolymerization is conducted under milder conditions, lower temperatures, and no hazardous wastes are released. In nature, chitin is degraded by three enzymes acting in concert: lytic polysaccharide monooxygenase (LPMO), chitinase and  $\beta$ -N-acetylglucosaminidase (NAGase). LPMOs (EC 1.14.99.53) act in an oxidative way on the surface of crystalline chitin, where they introduce chain breaks and generate oxidized chain ends, thus promoting further degradation by chitinases (Vaaje-Kolstad et al., 2010). Chitinases (EC 3.2.1.14) catalyze the cleavage of the glycosidic bond in chitin chains and release chitin oligosaccharides and chitin dimers as end products. Products released by chitinase are finally converted to GlcNAc by NAGases (Ike et al., 2005; Hartl et al., 2012). According to the Carbohydrate-Active Enzymes Database (CAZy) (<http://www.cazy.org/>) LPMOs are classified to the auxiliary activity (AA) family AA10 and AA11, chitinases to glycoside hydrolase (GH) family 18, and NAGases to GH20 and GH3.

Chitin degrading enzymes are naturally produced by fungi, bacteria, plants, yeasts, insects, and even vertebrates, among various (Bhattacharya et al., 2007; Keyhani and Roseman, 1996). However, enzymes potentially appropriate for GlcNAc production from chitin at industrial scale are thermostable enzymes obtained from fungi. Fungal thermostable enzymes are currently used in industry, e.g.  $\alpha$ -amylase in baking, because they tolerate high temperature, use shorter times to complete conversion and have long shelf life (Kristjansson, 1989; Østergaard and Olsen, 2010). Fungal thermophilic NAGases with a temperature optimum of 50–65 °C have been characterized from fungi. i.e. *Aspergillus niger* (Pera et al., 1997), *Beauveria bassiana* (Bidochka et al., 1993), *Lentinula edodes* (Konno et al., 2012), *Trichoderma harzianum* (Ulhoa and Peberdy, 1991; Lorito et al., 1994; Lisboa De Marco et al., 2004; Koga et al., 1991), *Trichoderma reesei* (Nogawa et al., 1998) and *Penicillium oxalicum* (Ryslavya et al., 2011). However, after prolonged incubations at optimal or higher temperatures their enzymatic activity diminished drastically or was lost. Therefore there is a need for more robust thermophilic NAGases with improved thermostability. Recently, we have reported the production and the characterization of the thermostable Chitinase Chi1, an endochitinase from the thermophilic filamentous fungus *Myceliophthora thermophila* C1 (Krolicka et al., 2018). In this study we present the cloning and the properties of NAGase *MthNAG*, a second enzyme in the chitinolytic machinery of *M. thermophila* C1.

## 3.2. Materials and methods

### 3.2.1. Substrates and chemicals

Chitin from shrimp shells, Schiff's reagent, 4-nitrophenyl-*N*-acetylglucosamine (GlcNAc-*p*NP), 4-nitrophenyl-*N,N'*-diacetyl- $\beta$ -D-chitobiose ((GlcNAc)<sub>2</sub>-*p*NP) and 4-nitrophenyl- $\beta$ -D-*N,N',N''*-triacetylchitotriose ((GlcNAc)<sub>3</sub>-*p*NP) and, 4-nitrophenyl-*N*-acetylgalactosamine (GalNAc-*p*NP) were obtained from Sigma-Aldrich (St. Louis, USA). GlcNAc was obtained from Sigma-Aldrich (St. Louis, USA). Chitin oligosaccharides (GlcNAc)<sub>2-6</sub> were obtained from Megazyme (Co. Wicklow, Ireland). Chitosan (90 % deacetylation degree (% DDA), 100 kDa molecular weight) was purchased from Nippon Suisan Kaisha LTD). Swollen chitin was prepared according to Monreal and Reese (Monreal and Reese, 1969) with some modifications as described by Krolicka et al. (2018). Swollen chitin had moisture content of 95.7 %. All other chemicals were of the highest purity available. Chitinase Chi1 from *M. thermophila* C1 was isolated and purified as described by Krolicka et al. (2018).

### 3.2.2. Sequence analysis of *MthNAG*

Nucleotide sequence of the gene encoding for *MthNAG* (*Mthnag*) and deduced amino acid sequence of *MthNAG* were analyzed using Clone Manager software. BLAST analysis was performed at the NCBI server (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Conserved domains were detected with Conserved Domain Search and Conserved Domain Database (Marchler-Bauer et al., 2015) at the NCBI server (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). The signal peptide was analyzed at the SignalP 4.0 server (<http://www.cbs.dtu.dk/services/SignalP/>) and the theoretical isoelectric point (pI) was calculated with Compute pI/Mw tool on ExPASy server ([https://web.expasy.org/compute\\_pi/](https://web.expasy.org/compute_pi/)). Potential *N*-linked and *O*-linked glycosylation sites were predicted by NetOGlyc 4.0 Server and NetNGlyc 1.0 Server (<http://www.cbs.dtu.dk/services/>). Prediction of the protein secondary structure and 3D was performed with the Phyre2 web portal ([www.sbg.bio.ic.ac.uk/phyre2](http://www.sbg.bio.ic.ac.uk/phyre2)). 3D modelling was performed on the basis of crystal structure of insect  $\beta$ -*N*-acetyl D-hexosaminidase of hex12 hexosaminidase (template ID: c3nsnA) which showed 90 % coverage with the *MthNAG* sequence.

### 3.2.3. Fungal strain

The ascomycetous fungus *Myceliophthora thermophila* C1 is listed in the All-Russian Collection of Microorganisms of the Russian Academy of Sciences with accession number: VKM F-3500D.

### 3.2.4. Gene cloning and protein production

The genome of *M. thermophila* C1 was screened for genes encoding for NAGases using a protein sequence of the fungal GH family 20, (XP\_003656648) published at NCBI. This sequence was blasted against the *M. thermophila* C1 genome data base (DuPont Biosciences) and only one gene sequence of a putative GH20 NAGase was found in the *M. thermophila* C1 genome data base and this gene sequence has previously been published in a patent (Verdoes et al., 2010). The native gene encoding for a putative GH20 NAGase found in the genome of *M. thermophila* C1 was designated *Mthnag*. *Mthnag* was chosen for overexpression and characterisation. The gene *Mthnag* was amplified from genomic *M. thermophila* C1 DNA with phusion DNA polymerase using the following designed primers (forward: GCTCGATTAAACATGTGGTCGCCG, reverse: GATGCGACCCGAATTCTCAAGCGACGA). The PCR program was as follows: 1 × 98 °C for 30 s, 35 × (98 °C for 10 s, 63.8 °C for 30 s, 72 °C for 30 s), 1 × 72 °C for 10 min. The amplified gene was cloned into a C1 expression vector and homologously overexpressed in *M. thermophila* C1 according to the method described by Visser et al. (Visser et al., 2011). In short, the expression cassette, containing the *chi1* promoter, the gene *Mthnag* and the terminator obtained from the expression vector, was transformed into a low protease/(hemi-) cellulase free *M. thermophila* C1-expression host. Ninety six randomly integrated transformants were grown in a microtiter plate (Verdoes et al., 2007) and screened for NAGase activity in the culture broth using GlcNAc-*p*NP as substrate. The transformant showing the highest activity of *MthNAG* was selected for a 2-L fed-batch fermentation as previously reported (Visser et al., 2011). The strain was grown aerobically in mineral medium, with glucose as carbon source, ammonium sulfate as nitrogen source and trace elements for essential salts (Verdoes et al., 2010). *MthNAG* was produced at pH 6.0 and 32 °C. The broth containing *MthNAG* was centrifuged at 20,000 × *g* for 20 minutes and the supernatant was filtered to remove cell biomass (filter 0.45 µm, Sartorius), concentrated 4-fold (5 kDa PES membrane, Vivace 70, Sartorius), dialyzed against 10 mM potassium phosphate buffer pH 6.0 and freeze-dried to obtain a crude enzyme preparation.



### 3.2.5. Purification of *Mth*NAG

The freeze-dried crude enzyme preparation (0.5 g) was dissolved in 50 mL 0.05 M Bis-Tris buffer pH 7.0 (Buffer A) and purified by anion exchange chromatography (IEX) and size exclusion chromatography (SEC) on an ÄKTA™ pure system. For IEX, the 50 mL sample was loaded onto a HiPrep DEAE FF 16/10 column (GE Healthcare Bio-Science AB, Uppsala, Sweden) and proteins were eluted with a gradient elution of 1 M NaCl in Buffer A as follows: 0–20 % for 10 column volumes (CV) and 20–45 % for 10 CV with a flow rate of 5 mL min<sup>-1</sup>. Fractions were collected and screened for NAGase activity. The fraction with the highest NAGase activity was loaded onto a HiLoad 16/600 Superdex 75 pg SEC column (GE Healthcare Bio-Science AB, Uppsala, Sweden). Proteins were eluted isocratic with Buffer A containing 0.15 M NaCl with a flow rate of 0.5 mL min<sup>-1</sup>. The absorbance was measured at 280 nm. Protein concentration was determined using the bicinchoninic acid assay (BCA) according to the instructions of the supplier (Pierce) with bovine serum albumin as standard. The evaluation of the purity and molecular weight of purified *Mth*NAG was performed by matrix assisted laser-desorption time-of-flight mass spectrometry (MALDI-TOF-MS) as described by Krolicka et al. (2018).

### 3.2.6. Enzyme activity assays and kinetic parameters

For the standard enzyme assay, the enzyme solution (0.01 mL) was incubated with GlcNAc-*p*NP (2 mM) in 0.09 mL citrate-phosphate buffer (0.1 M, pH 4) at 50 °C in a microtiter plate. After 10 min incubation 0.2 mL Tris/HCl buffer (0.25 M, pH 8.8) was added and the absorbance of released *p*-nitrophenol (*p*NP) was measured at 405 nm using a Tecan Safire plate reader (Grodig, Austria). One enzyme unit (U) was defined as the amount of enzyme required to release 1 μmol of *p*NP per minute. Activity on chitin oligosaccharide derivatives (*p*NP-derivatives) was assayed with (GlcNAc)<sub>1-3</sub>-*p*NP at standard enzyme conditions. Activity for chitin and chitosan was assayed with 0.8 μM *Mth*NAG incubated with 0.45 % (w/v) swollen chitin or soluble chitosan in 0.96 mL sodium acetate buffer (0.05 M, pH 4.5) at 50 °C for 10 min. Reactions were terminated by heating at 96 °C for 10 min and subsequently centrifuged at 20,000 × *g* for 5 min. The reducing sugars released from chitosan were analysed with the *p*-hydroxybenzoic acid hydrazide (PAHBAH) assay (Lever, 1973). For chitin, released GlcNAc was determined by High Performance Anion-Exchange Chromatography (HPAEC). One U was defined as the amount of enzyme that liberated 1 μmol GlcNAc per minute from chitin or chitosan. The *K*<sub>m</sub>, *V*<sub>max</sub>, *k*<sub>cat</sub> and *K*<sub>i</sub> of *Mth*NAG were determined for (GlcNAc)<sub>2</sub> and GlcNAc-*p*NP.

For GlcNAc-*p*NP, 2.1 nM *Mth*NAG was incubated with GlcNAc-*p*NP in the range of 0.05–4 mM under standard enzyme assay conditions. For (GlcNAc)<sub>2</sub>, 2.1 nM *Mth*NAG was incubated with (GlcNAc)<sub>2</sub> in the range of 0.05–3.5 mM in citrate-phosphate buffer (0.1 M, pH 4.5) and released GlcNAc was determined by HPAEC. The kinetic parameters were calculated with GraphPad Prism software v.7.0 (GraphPad Software Inc., San Diego, CA). For all experiments, each reported value was the average of duplicate tests.

### **3.2.7. Biochemical characterization**

The influence of pH on the activity of *Mth*NAG was determined by incubating 2.1 nM *Mth*NAG at different pH levels (3.0–7.0) in citrate-phosphate buffer (0.1 M) using the standard enzyme assay conditions. The influence of temperature on the activity of *Mth*NAG was analysed by incubating 2.1 nM *Mth*NAG at the temperature range of 30–80 °C using the standard enzyme assay conditions. Thermostability was determined by pre-incubating 2.1 nM *Mth*NAG at various temperatures (50–70 °C) in citrate-phosphate buffer (0.1 M, pH 4.5) for different time intervals and the remaining enzyme activity was determined by performing the standard enzyme assay. For all experiments, each reported value was the average of duplicate tests.

### **3.2.8. Electrophoresis and identification of glycosylated proteins**

Sodium dodecyl sulfate-polyacrylamide (10 % (w/v)) gel electrophoresis (SDS-PAGE) was performed under reducing conditions using a NuPAGE Novex System (ThermoFisher Scientific, Bleiswijk, The Netherlands) with 10 % (w/v) Bis-Tris gels. Gels were stained with SimplyBlue™ SafeStain according to the recommendation of the supplier (ThermoFisher Scientific). The isoelectric point (*pI*) of the enzyme was estimated by isoelectric focusing (IEF) using PhastGel™ IEF on a Pharmacia LKB Phast System (Pharmacia Biotech, Uppsala, Sweden) with a broad protein calibration kit (pH 3–10, GE Healthcare) as standard. Proteins were stained with Coomassie blue R-2. Glycosylation of proteins was detected by staining the SDS-PAGE gels with periodic acid-Schiff staining (PAS) (Zacharius et al., 1969).

### **3.2.9. Time-course of hydrolysis of chitin oligosaccharides and *p*NP-derivatives**

To analyse the mode of action the hydrolysis of chitin oligosaccharides (GlcNAc)<sub>2-5</sub> (0.18 mM) and (GlcNAc)<sub>2-3</sub>-*p*NP (0.18 mM), was performed with 2.1 nM *Mth*NAG in 0.33 mL citrate-phosphate buffer (0.1 M, pH 4.5) at 50 °C. Time-point samples were taken, heated at 96 °C for

5 min and centrifuged at  $20,000 \times g$  for 5 min. Hydrolysis products were analysed by HPAEC and each reported value was the average of duplicate tests. The quantification was based on calibration curves prepared for each chitin oligosaccharide.

### **3.2.10. Synergistic effect of *Mth*NAG and Chitinase Chi1 on chitin hydrolysis**

To study the synergistic effect of *Mth*NAG and Chitinase Chi1, chitin hydrolysis was performed with 0.5 % (w/v) swollen chitin in 1 mL citrate-phosphate buffer (0.1 M, pH 5.0) in two parallel runs: run I containing 0.8  $\mu$ M *Mth*NAG; run II containing 0.8  $\mu$ M *Mth*NAG and 1.2  $\mu$ M Chitinase Chi1. Samples were incubated at 50 °C with agitation at 800 rpm. In time samples were taken, heated at 96 °C for 10 min and centrifuged at  $20,000 \times g$  for 5 min. Hydrolysis products were analysed by HPAEC and each reported value was the average of duplicate test. The quantification was based on calibration curve for GlcNAc. The yields of GlcNAc production were calculated by comparing the amount of GlcNAc released to the maximal theoretical yield, which is equal to the initial substrate concentration considering that 1 mg of dry chitin could produce a maximum of 1.09 mg of GlcNAc.

### **3.2.11. High Performance Anion-Exchange Chromatography (HPAEC)**

HPAEC was performed on an ICS-3000 Ion Chromatography HPLC system equipped with a CarboPac PA-1 column (2 $\times$ 250 mm) in combination with a CarboPac PA-guard column (2 $\times$ 25 mm) at 22 °C and a pulsed electrochemical detector (PAD) in pulsed amperometric detection mode (Dionex) at 30 °C. The column was equilibrated with water. Sugars were eluted at a flow rate of 0.25 mL min<sup>-1</sup>. The gradient used was : 0–25 min H<sub>2</sub>O, 25–65 min at 0–0.045 M NaOH, 65–70 min at 0.045 M NaOH–1 M sodium acetate in 0.1 M NaOH, 70–75 min at 1 M sodium acetate in 0.1 M NaOH, 75–75.1 min 1 M sodium acetate in 0.1 M NaOH–0.1M NaOH, 75.1–80 min 0.1M NaOH, 80–95 min H<sub>2</sub>O. The PAD signal was increased by post column addition of 0.5 M NaOH at a flow rate of 0.15 mL min<sup>-1</sup>.

## **3.3. Results**

### **3.3.1. Sequence analysis of *Mth*NAG**

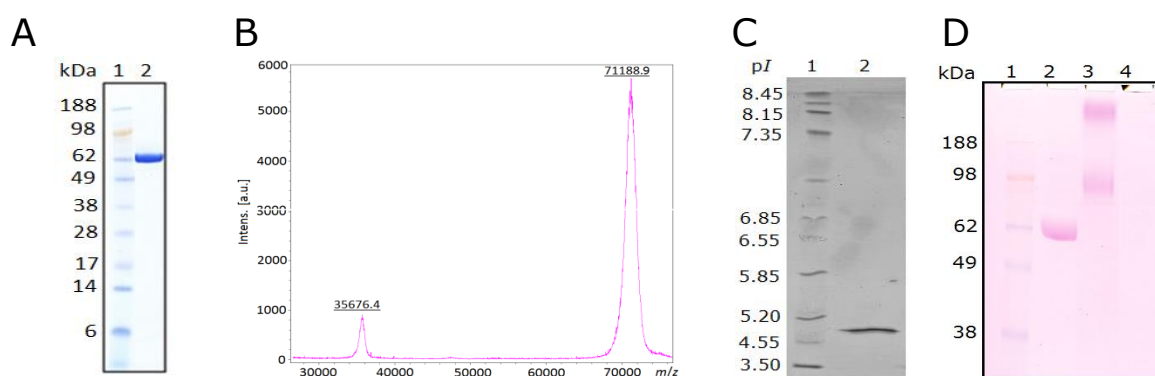
The putative gene (*Mthnag*) encoding for NAGase *Mth*NAG has an ORF of 2,005-bp. The deduced 582-amino acid sequence of *Mth*NAG was predicted to have a molecular weight of 62.6 kDa, a theoretical pI of 5.20, an N-terminal 21-amino acid signal peptide, three potential

*N*-linked glycosylation sites and two potential *O*-linked glycosylation sites. Multiple sequence alignment with other family GH20 hexosaminidases indicated that the protein sequence of *MthNAG* has the highest similarity to the GH family 20 protein from *M. thermophila* ATCC 42464 (accession: XP\_003658680). The protein sequence of *MthNAG* shared 80 % identity with the GH20 protein from *Thielavia terrestris* NRRL 8126 (accession: XP\_003656648) and  $\beta$ -hexosaminidase 2 from *Madurella mycetomatis* (accession: KXX82839), and more than 60 % identity was obtained for 79 GH20 proteins from fungal sources including *Fusarium* sp., *Colletotrichum* sp., *Trichoderma* sp., *Scedosporium* sp. and *Neurospora* sp. The protein sequence of *MthNAG* revealed three domains conserved in family GH-20 hexosaminidases: GH20\_HexA\_HexB-like domain (accession: cd06562), Glyco\_hydro\_20 domain (accession: pfam00728) and CHB\_HEX domain (accession: PF03173) (Figure 3.1.C) and a highly conserved pair of catalytic residues D-E preceded by a H-X-G-G motif (Figure 3.1.A). The modelled secondary structure of *MthNAG* consists of 16  $\alpha$ -helices and 21  $\beta$ -sheets (Figure 3.1.B) and modelled tertiary structure has a  $(\alpha/\beta)_8$  TIM-barrel structure with the active site lying at the centre of the barrel convex side (Figure 3.1.D).



### 3.3.2. Production and purification of *Mth*NAG

The gene encoding for *Mth*NAG (*Mthnag*) was amplified using the designed primers and cloned into the *M. thermophila* C1-expression host. The constructed *M. thermophila* C1 strain producing the highest activity of *Mth*NAG was chosen for the production of the enzyme in a 2-L fermenter. Protein concentration in the culture broth was 3.5 g L<sup>-1</sup>, of which the overexpressed *Mth*NAG represented 52 % of the total protein. After removal of biomass, the crude enzyme preparation was freeze dried and further subjected to a purification of *Mth*NAG using IEX and SEC. *Mth*NAG was purified to homogeneity as demonstrated by SDS-PAGE (Figure 3.2.A) and MALDI-TOF-MS (Figure 3.2.B). The specific activity of the purified *Mth*NAG was 432 U mg<sup>-1</sup>.



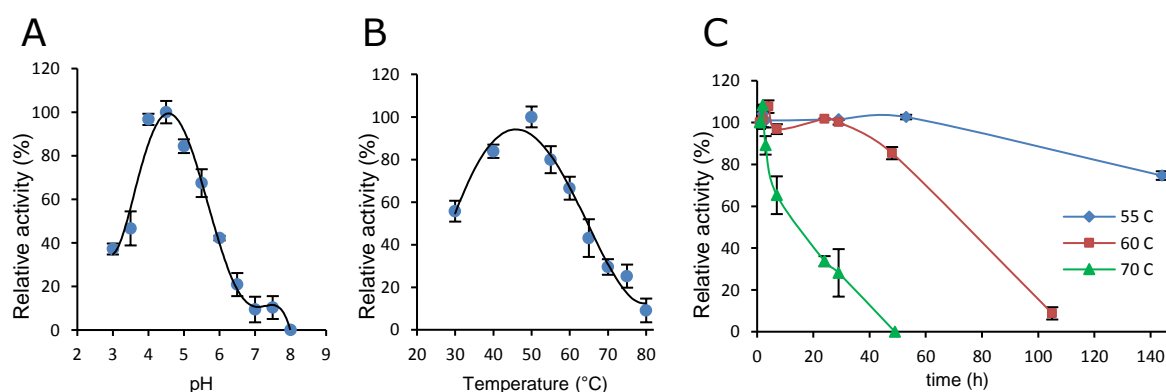
**Figure 3.2.** Molecular weight of *Mth*NAG from *Myceliophthora thermophila* C1 determined by (A) SDS-PAGE. Lanes: 1, protein marker; 2, purified *Mth*NAG after size exclusion chromatography (SEC); and molecular weight determined by (B) MALDI-TOF-MS. (C) Isoelectric focusing determined for *Mth*NAG. Lanes: 1, protein marker; 2, purified *Mth*NAG. (D) Staining with Schiff's reagent for glycosylated proteins. Lanes: 1, protein marker; 2, purified *Mth*NAG; 3, yeast invertase; 4, bovine serum albumin.

### 3.3.3. Molecular weight, Isoelectric point and glycosylation of *Mth*NAG

The molecular weight of *Mth*NAG measured by SDS-PAGE was 62 kDa (Figure 3.2.A) and by MALDI-TOF-MS was 71.2 kDa (Figure 3.2.B). In the MALDI-TOF-MS spectrum, the enzyme was identified as a monomeric protein at 71,188.9 [m/z] (single charged) and at 35,676.4 [m/z] (double charged). The measured isoelectric point of *Mth*NAG was detected at pH 4.9 (Figure 3.2.C). Staining of SDS-PAGE gel with PAS resulted in intense magenta color of the *Mth*NAG protein band (Figure 3.2.D), showing that the enzyme is glycosylated.

### 3.3.4. Biochemical properties of *Mth*NAG

The highest activity of *Mth*NAG was detected at pH 4.5 and 50 °C (Figure 3.3.A and 3.3.B). The activity measurement showed bell-shaped profiles in the pH range of 3 to 8 and between 30–80 °C. *Mth*NAG had a relatively high activity (>40 % of the maximum activity) in the range from 30 to 65 °C. At elevated temperatures, above the optimum, *Mth*NAG performed with 30, 25 and 10 % of relative activity at 70, 75 and 80 °C, respectively. *Mth*NAG was notably thermostable at 55 °C (75 % relative activity, at incubation time > 144 h), 60 °C (85 % relative activity, after 48 h incubation; half life 82 h) and 70 °C (33 % relative activity, after 24 h incubation; half life 18 h) (Figure 3.3.C).



**Figure 3.3.** Effects of pH and temperature on the activity of *Mth*NAG from *Myceliophthora thermophila* C1. (A) Optimum pH, (B) optimum temperature, and (C) thermostability.

### 3.3.5. GlcNAc and GalNAc release from diverse substrates with *Mth*NAG

The potential of *Mth*NAG to release GlcNAc was examined for the natural chitin dimer (GlcNAc)<sub>2</sub>, chromogenic chitin oligosaccharide derivatives (GlcNAc)<sub>1-3</sub>-pNP and polymeric substrates swollen chitin and soluble chitosan (Table 3.1.). Specific activity for (GlcNAc)<sub>2</sub> was expressed as the cleavage of two GlcNAc molecules and was 1077.8±0.4 U mg<sup>-1</sup>. Among chitin oligosaccharide pNP-derivatives, the enzyme showed the highest activity towards GlcNAc-pNP, which was about 200-fold higher than that towards (GlcNAc)<sub>2</sub>-pNP. Activity towards (GlcNAc)<sub>3</sub>-pNP was not detected. *Mth*NAG was able to release GalNAc from GalNAc-pNP but with activity lower than that for GlcNAc-pNP. Next to oligomeric substrates, *Mth*NAG was active towards polymeric chitin and chitosan. Activity towards chitin and chitosan was about 36,000-fold lower than that obtained for (GlcNAc)<sub>2</sub>. Kinetic parameters for *Mth*NAG were

determined for the natural substrate of the enzyme, (GlcNAc)<sub>2</sub> and its mimic, GlcNAc-*p*NP. For (GlcNAc)<sub>2</sub> the  $V_{\max}$  was  $37.3 \pm 5.4 \mu\text{M min}^{-1}$ ,  $K_m$  was  $0.25 \pm 0.05 \text{ mM}$ , and  $k_{\text{cat}}$  was  $293.7 \pm 42.3 \text{ s}^{-1}$ . For GlcNAc-*p*NP the  $V_{\max}$  was  $1.76 \pm 0.09 \mu\text{M min}^{-1}$ ,  $K_m$  was  $0.06 \pm 0.01 \text{ mM}$ , and  $k_{\text{cat}}$  was  $14.0 \pm 0.7 \text{ s}^{-1}$ . However,  $k_{\text{cat}}/K_m$  for GlcNAc-*p*NP ( $231.6 \text{ s}^{-1} \text{ mM}^{-1}$ ) was lower than  $k_{\text{cat}}/K_m$  for (GlcNAc)<sub>2</sub> ( $1174.8 \text{ s}^{-1} \text{ mM}^{-1}$ ). (GlcNAc)<sub>2</sub> and GlcNAc-*p*NP inhibited the activity of *Mth*NAG and their inhibition effect was detected at substrate concentration ( $K_i$ ) of  $0.50 \pm 0.11 \text{ mM}$  and  $1.96 \pm 0.26 \text{ mM}$ , respectively.

**Table 3.1.** Specific activities of *Mth*NAG from *Myceliophthora thermophila* C1.

Substrate	specific activity (U mg <sup>-1</sup> )
(GlcNAc) <sub>2</sub>	1077.8±0.4*
GlcNAc- <i>p</i> NP	432.0±0.3**
(GlcNAc) <sub>2</sub> - <i>p</i> NP	2.0±0.4**
(GlcNAc) <sub>3</sub> - <i>p</i> NP	ND****
GalNAc- <i>p</i> NP	345.7±3.3**
swollen chitin	0.03±0.0*
chitosan (91 % DDA)	0.02±0.0***

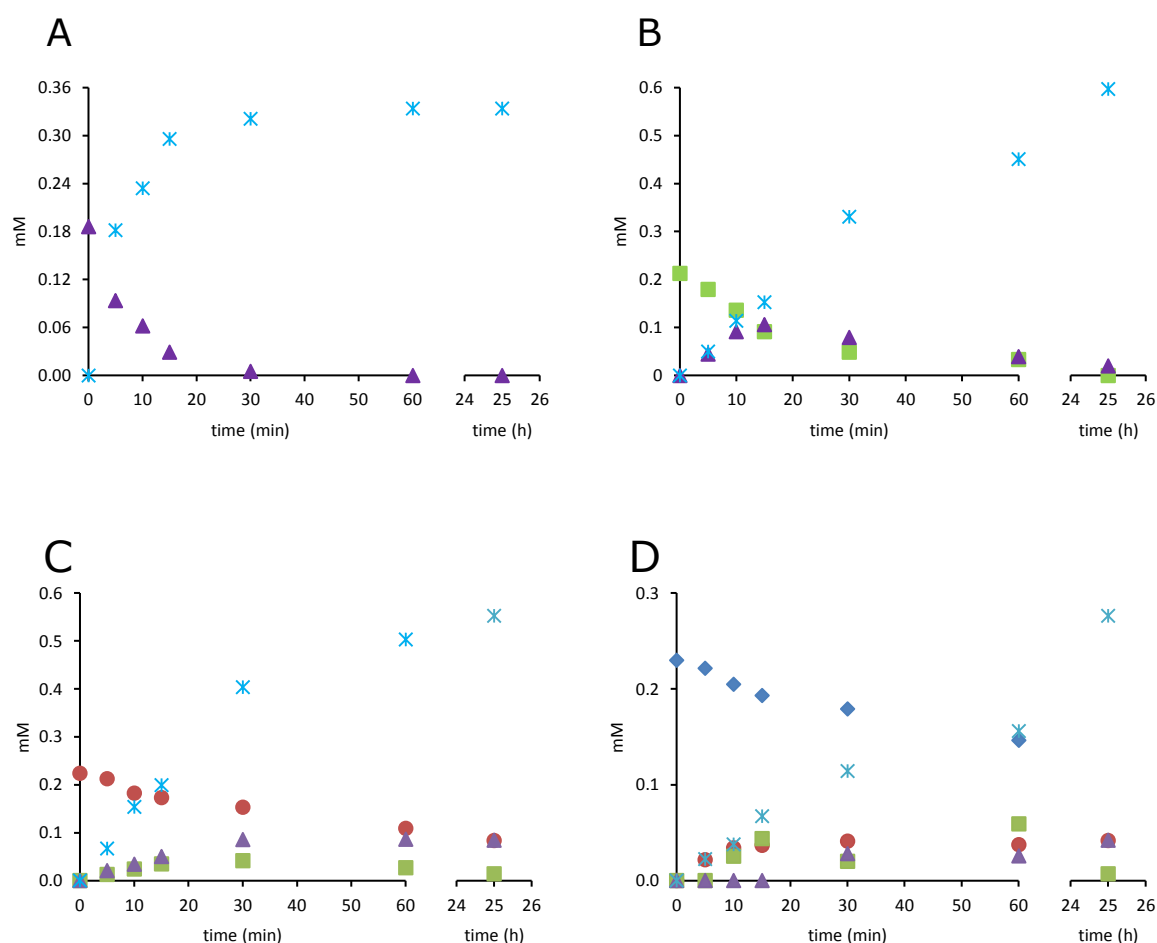
\* measured with High Performance Anion-Exchange Chromatography, \*\* with Tecan Safire, and \*\*\*reducing sugar assay, \*\*\*\*ND-not detected

### 3.3.6. Release of GlcNAc from chitin oligosaccharides and chitin oligosaccharide *p*NP-derivatives with *Mth*NAG

The release of GlcNAc from chitin oligosaccharides (GlcNAc)<sub>2-5</sub> catalysed by *Mth*NAG was followed in time and the reaction products were measured by HPAEC (Figure 3.4.). *Mth*NAG efficiently degraded (GlcNAc)<sub>2</sub> to two molecules GlcNAc and already after 30 min the substrate was fully degraded (Figure 3.4.A). All other oligosaccharides (GlcNAc)<sub>3-5</sub> were shortened by one GlcNAc molecule in time and the released intermediate products (GlcNAc)<sub>*n*-1</sub> were simultaneously degraded during the reaction. In the first 15 min, (GlcNAc)<sub>3</sub> was degraded to molar equivalent concentration of GlcNAc and (GlcNAc)<sub>2</sub> (Figure 3.4.B), and the released (GlcNAc)<sub>2</sub> dimers was degraded gradually hereafter. Hydrolysis of (GlcNAc)<sub>4</sub> and (GlcNAc)<sub>5</sub> resulted in the release of GlcNAc and the intermediate (GlcNAc)<sub>*n*-1</sub> oligosaccharides, which were further degraded (Figure 3.4.C and 3.4.D). The degradation rates in the first 5 min were

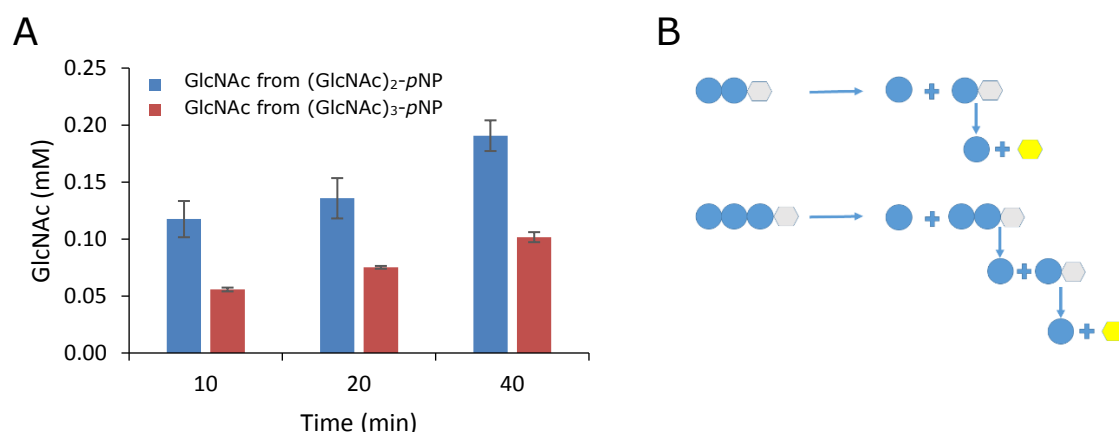


as follows:  $18.5 \mu\text{M min}^{-1}$  for  $(\text{GlcNAc})_2$ ,  $6.6 \mu\text{M min}^{-1}$  for  $(\text{GlcNAc})_3$ ,  $2.2 \mu\text{M min}^{-1}$  for  $(\text{GlcNAc})_4$ ,  $1.7 \mu\text{M min}^{-1}$  for  $(\text{GlcNAc})_5$ .



**Figure 3.4.** Hydrolysis of chitin oligosaccharides  $(\text{GlcNAc})_{2-5}$  by *MthNAG* from *Myceliophthora thermophila* C1. Reaction products obtained after incubation of *MthNAG* with  $(\text{GlcNAc})_2$  (A),  $(\text{GlcNAc})_3$  (B),  $(\text{GlcNAc})_4$  (C),  $(\text{GlcNAc})_5$  (D), identified by High Performance Anion-Exchange Chromatography (HPAEC). GlcNAc (star),  $(\text{GlcNAc})_2$  (triangle),  $(\text{GlcNAc})_3$  (square),  $(\text{GlcNAc})_4$  (circle),  $(\text{GlcNAc})_5$  (diamond).

To determine the mode of action of *MthNAG*, the enzyme was incubated with  $(\text{GlcNAc})_{2-3}$ -pNP and the release of the products was followed in time. Degradation of  $(\text{GlcNAc})_{2-3}$ -pNP progressed with the release of GlcNAc and intermediate  $(\text{GlcNAc})_{n-1}$ -pNP, which were subsequently hydrolysed by the enzyme. Similarly to chitin oligosaccharides, the enzyme showed preference towards shorter oligosaccharide substrate, since it degraded  $(\text{GlcNAc})_2$ -pNP two times faster than  $(\text{GlcNAc})_3$ -pNP (Figure 3.5.A). As shown in Figure 3.5.B, GlcNAc was cleaved by the enzyme from the non-reducing end of the substrate.



**Figure 3.5.** Cleavage of (GlcNAc)<sub>2</sub>-pNP and (GlcNAc)<sub>3</sub>-pNP with *MthNAG* from *Myceliophthora thermophila* C1. (A) Time course for degradation of (GlcNAc)<sub>2</sub>-pNP and (GlcNAc)<sub>3</sub>-pNP and (B) mode of action of *MthNAG*. GlcNAc depicted as blue circles and *p*-nitrophenol (pNP) as hexagon. Yellow colour indicates ionization of released pNP.

### 3.3.7. Effect of *MthNAG* and Chitinase Chi1 on the release of GlcNAc from swollen chitin

Chitinase Chi1 from *M. thermophila* C1 was previously shown to release mainly (GlcNAc)<sub>2</sub> and traces of (GlcNAc)<sub>3</sub> and GlcNAc from swollen chitin (Krolicka et al., 2018). In this work, Chitinase Chi1 and *MthNAG* were used to examine their synergistic effect on the release of GlcNAc from swollen chitin by performing two parallel runs and measuring the concentration of released GlcNAc in time. In run I, containing only *MthNAG*, the concentration of the released GlcNAc at the end of the reaction was  $3.1 \times 10^{-3}$  mM, while in run II, containing both enzymes, the concentration of GlcNAc was 0.39 mM at the end of incubation (Table 3.2.). *MthNAG* and Chitinase Chi1 synergistically released GlcNAc in concentration of approximately 130 times higher than when only *MthNAG* was used. GlcNAc yield obtained with the mixture of both enzymes was equal to 37.8 % which is about 13 times higher when only *MthNAG* was used (2.9 %).

**Table 3.2.** Release of GlcNAc during the hydrolysis of swollen chitin by *MthNAG* (Run I) and by the action of both *MthNAG* and Chitinase Chi1 (Run II) from *Myceliophthora thermophila* C1.

Time (min)	Run I ( <i>MthNAG</i> )		Run II ( <i>MthNAG</i> + Chitinase Chi 1)	
	GlcNAc concentration (mM)	GlcNAc Yield (%)	GlcNAc concentration (mM)	GlcNAc Yield (%)
15	$0.443 \times 10^{-3}$	0.4	0.12	4.9
30	$0.488 \times 10^{-3}$	0.5	0.14	5.9
60	$0.815 \times 10^{-3}$	0.8	0.19	7.9
90	$1.014 \times 10^{-3}$	0.9	0.22	9.0
120	$1.184 \times 10^{-3}$	1.2	0.24	9.7
1140	$3.078 \times 10^{-3}$	2.9	0.39	37.8

### 3.4. Discussion

The multiple sequence alignment of the protein sequence of *MthNAG* revealed the presence of conserved domains and catalytic residues typically found in GH 20 family hexosaminidases. The GH20\_HexA\_HexB-like domain is a representative domain of the active site of GH 20 hexosaminidases from microorganisms and higher organisms. The CHB\_HEX superfamily domain was suggested to be a carbohydrate binding domain since it resembles the crystallographic structure of a cellulose binding domain in cellulase from *Cellulomonas fimi* (Xu et al., 1995; Tews et al., 1996). Putative features for protein maturation were found in *MthNAG*, including a 21-amino acid signal peptide and five glycosylation motifs. Tertiary structure predicted with 3D modelling revealed an  $(\alpha/\beta)_8$  TIM-barrel conformation of *MthNAG*, which is typical for GH 20 hexosaminidases. According to all these features, *MthNAG* can be classified to GH 20 hexosaminidases.

The molecular weight of *MthNAG* measured with SDS-PAGE is in the range of molecular weights measured with SDS-PAGE for other fungal NAGases (Table 3.3).

**Table 3.3.** Properties of hexosaminidases from fungi.

[illegible]

**Table 3.3.** Properties of hexosaminidases from fungi. (Continuation)

Organism	Molecular mass (kDa)	Optimum pH	Optimum temperature (°C)	K <sub>m</sub> (mM)	pI	Activity (U mL <sup>-1</sup> )	Specific activity (U mg <sup>-1</sup> )	Substrate diversity	Reference
<i>Penicillium oxalicum</i>	141	3.0–4.5	—	0.48**	5.0	1,660** 2,450***	126** 186***	GlcNAc- <i>p</i> NP, GalNAc- <i>p</i> NP	Yamamoto et al., 1985
<i>Penicillium oxalicum</i>	160	3.0	50	0.14**	—	—	10.8**	GlcNAc- <i>p</i> NP, GalNAc- <i>p</i> NP, GlcNAc-4-MUF	Ryslava et al., 2011
<i>Phoma glomerata</i>	20	6.0–8.0	37	—	—	38.2**	1,020**	GlcNAc-4-MUF, GalNAc-4-MUF	Zhuravleva et al., 2004
<i>Pycnoporus cinnabarinus</i>	120	2.2	—	—	5.4	134** 149***	67** 75***	GlcNAc- <i>p</i> NP, GalNAc- <i>p</i> NP, (GlcNAc) <sub>2-4</sub>	Ohtakara et al., 1981
<i>Trichoderma harzianum</i> P1	72	5.0-5.5	60	—	4.6	17.4‡	11.8‡	(GlcNAc) <sub>1-2</sub> - <i>p</i> NP, Fungal cell walls	Lorito et al., 1994
<i>Trichoderma harzianum</i>	36	4.0	50–60	0.008**	—	—	—	GlcNAc- <i>p</i> NP, Fungal cell walls	Lisboa De Marco et al., 2004
<i>Trichoderma harzianum</i>	118	5.5	50	0.24*, 0.58**	—	7.3**	73**	GlcNAc- <i>p</i> NP	Ulhoa and Peberdy, 1991
<i>Trichoderma harzianum</i> AF 6-T8	150	5.2	50	—	—	960**	102**	GlcNAc- <i>p</i> NP, (GlcNAc) <sub>2-6</sub>	Koga et al., 1991
<i>Myceliophthora thermophila</i> C1	71.2	4.5	50	0.25*, 0.06 **	4.9	1,357* 544**	1,077.8* 432**	GlcNAc- <i>p</i> NP, GalNAc- <i>p</i> NP, (GlcNAc) <sub>2-3</sub> - <i>p</i> NP, (GlcNAc) <sub>2-6</sub> , swollen chitin, chitosan 91 % DDA	This paper

\* For (GlcNAc)<sub>2</sub>, \*\* For GlcNAc-*p*NP, \*\*\* For GalNAc-*p*NP, ¥ For colloidal chitin, § For mechanochemically ground chitin, † For (GlcNAc)<sub>2</sub> in µmol/h, # For GlcNAc-*p*NP in µmol/h, ‡ For GlcNAc-*p*NP in nkatal

However, the molecular weight of *MthNAG* measured with MALDI-TOF-MS (71.2 kDa) was 8.6 kDa higher than the molecular weight calculated from the protein sequence (62.6 kDa). This difference is most likely due to the post-translational glycosylation of *MthNAG* performed by *M. thermophila* C1, as the purified *MthNAG* gave a strong magenta signal upon Schiff's staining. Carbohydrates could potentially attach to the five glycosylation sites predicted in the *MthNAG* sequence. A similar increase in molecular weight of about 10 kDa caused by glycosylation was observed for NAGase from *T. harzianum* P1 (62.7 kDa) (Peterbauer et al., 1996). However, *MthNAG* and NAGase from *T. harzianum* P1 migrated differently in the SDS-PAGE. *MthNAG* appeared as a protein with lower molecular weight (62 kDa), while NAGase from *T. harzianum* P1 appeared as a protein with higher molecular weight (72 kDa). Anomalous behavior of glycoproteins has been described before and the presence of carbohydrates attached to the protein (Segrest et al., 1971; Matagne et al., 1991) and the tertiary structure of the protein (Rath et al., 2009; Pitt-Rivers and Ambesi Impiombato, 1968) were shown to influence this behavior. MALDI-TOF-MS is known to provide accurate molecular mass determination of proteins and glycoconjugates (Ledesma-Osuna et al., 2008; Yeboah and Yaylayan, 2001).

*MthNAG* was found to have a pH optimum at pH 4.5 and a *pI* of 4.9, while the calculated *pI* from the amino acid sequence was 5.2. This difference between the theoretical *pI* predicted from the primary structure and the experimentally determined *pI* is common, and is in the error limits of the method (Kozlowski, 2016).

*MthNAG* exhibits the highest catalytic activity at 50 °C, similar with other thermophilic fungal GH20 hexosaminidases (Table 3.3.). Interestingly, *MthNAG* showed a notable thermostability above its optimal temperature i.e. at 55 °C, 60 °C and 70 °C, that to our knowledge, has never been observed for other fungal NAGases. The in literature reported fungal thermophilic NAGases with temperature optimum  $\geq 50$  °C drastically lose their activities after prolonged incubation at optimal or higher temperatures. For example, NAGase 1 from *B. bassiana* has its temperature optimum at 57 °C but it lost 100 % of activity after 60 min incubation at that temperature (Bidochka et al., 1993). NAGase from *T. harzianum* with an optimum at 50–60 °C lost 50 % of its original activity within 1 h at 50 °C (Lisboa de Marco et al., 2004). At elevated temperatures *MthNAG* was more stable than LeHex20A from *L. edodes* and NAGase from *A. niger* 419. At 60 °C, *MthNAG* retained 85 % relative activity for 48 h, while LeHex20A from *L.*

*edodes* was inactivated within 30 min (Konno et al., 2012). NAGase from *A. niger* 419, which was most active at 65 °C, lost 30 % of activity after 15 min at 70 °C (Pera et al., 1997), while *Mth*NAG lost 35 % of activity after 5 hours at that temperature. The notable thermostability of *Mth*NAG is probably an evolutionary adaptation to preserve enzymatic function at elevated temperatures. This adaptation may include stabilizing interactions in folded protein, interactions between domains and presence of stable surface-exposed amino acids (Turner et al., 2007). Glycosylation of *Mth*NAG may have also a positive effect on the thermostability, as glycosylation was shown to stabilize the enzyme conformation of glucoamylase from *A. niger* (Jafari-Aghdam et al., 2005) and improved the pH stability of NAGase PoHEX from *Penicillium oxalicum* (Ryslavy et al., 2011).

*Mth*NAG was very active towards the dimeric substrate (GlcNAc)<sub>2</sub> and its mimic GlcNAc-*p*NP, which are typical substrates for *N*-acetylglucosaminidases. In addition, *Mth*NAG showed *N*-acetylgalactosaminidase activity as it released the GalNAc moiety from GalNAc-*p*NP. The activity ratio of 1.25 between *N*-acetylglucosaminidase and *N*-acetylgalactosaminidase activities indicates that the enzyme has more or less the same activity towards GalNAc and GlcNAc conjugates. *N*-acetylglucosaminidase and *N*-acetylgalactosaminidase activity are commonly found for members of the GH 20 hexosaminidase family.

*Mth*NAG showed activity for the trimeric chitin oligosaccharide derivative (GlcNAc)<sub>2</sub>-*p*NP but no activity was detected toward the tetrameric (GlcNAc)<sub>3</sub>-*p*NP (Table 3.1.). However, a time-course experiment with (GlcNAc)<sub>2-3</sub>-*p*NP revealed that GlcNAc was released from both substrates (Figure 3.5.) and that *Mth*NAG released GlcNAc from the non-reducing end of the substrate. In the case of enzymes acting from the non-reducing end, the release of *p*NP from chitin oligosaccharide *p*NP-derivatives depends on the length of the *p*NP-derivative and the longer the *p*NP-derivative, the more time the enzyme needs to reach the attached *p*NP and to release the dye. Therefore, it should be noted, that the spectrophotometric activity measurement with *p*NP-derivatives may give false results when the mode of action of the enzyme is not taken into account.

Studies of the kinetic parameters *Mth*NAG indicated, that (GlcNAc)<sub>2</sub> is a good substrate for the enzyme, but the enzyme has a lower affinity for the natural substrate (higher  $K_m$ ) than for the synthetic mimic GlcNAc-*p*NP (lower  $K_m$ ). However, the higher catalytic efficiency ( $k_{cat}$  and

$k_{cat}/K_m$ ) towards (GlcNAc)<sub>2</sub> indicates that (GlcNAc)<sub>2</sub> is a more preferred substrate than GlcNAc-*p*NP. The  $K_m$  for (GlcNAc)<sub>2</sub> was comparable with the one from LeHex20A from *L. edodes* (Konno et al., 2012), although it was higher than the reported values for NAGase from *T. harzianum* (Ulhoa and Peberdy, 1991) (Table 3.3.). Although, *Mth*NAG showed high enzymatic activity towards (GlcNAc)<sub>2</sub> and GlcNAc-*p*NP it was also inhibited by these substrates. The measured  $K_i$  for (GlcNAc)<sub>2</sub> was equal to two times of  $K_m$ , indicating that at  $K_i$  enzyme works at its  $V_{max}$ . For setting a process, it is important to work below the  $K_i$  for (GlcNAc)<sub>2</sub>, what can be achieved by slowly adding the substrate. Substrate inhibition is also reported for other NAGases. For example, GlcNAc-*p*NP at concentration of 0.4 mM inhibited fungal NAGase PoHEX from *P. oxalicum* (Ryslavy et al., 2011). Blind docking experiment conducted on PoHEX revealed that substrate inhibition was a result of the presence of a “secondary” binding site. Whether such an additional binding site is a reason of substrate inhibition by *Mth*NAG, additional blind docking experiment should be conducted for *Mth*NAG.

Next to hydrolysis of (GlcNAc)<sub>2</sub>, *Mth*NAG was capable of cleaving off GlcNAc moieties from chitin oligosaccharides (GlcNAc)<sub>3-5</sub>. The activity on chitin oligosaccharides has been reported for some other NAGases (Table 3.3.). The enzyme showed the highest preference towards (GlcNAc)<sub>2</sub>. In the first 5 min of incubation, (GlcNAc)<sub>2</sub> was converted approximately three times faster than (GlcNAc)<sub>3</sub> and almost 10 times faster than (GlcNAc)<sub>4</sub> and (GlcNAc)<sub>5</sub>. *Mth*NAG degraded chitin oligosaccharides (GlcNAc)<sub>3-5</sub> non-processively in a consecutive reaction, what means that oligosaccharides were shortened by GlcNAc in time and their intermediate (GlcNAc)<sub>n-1</sub> were simultaneously degraded by the enzyme. In this way, inhibition concentration of (GlcNAc)<sub>2</sub> is not reached and the reaction can proceed to completion.

Fungal NAGases differ in their substrate diversity and activity towards GlcNAc-*p*NP (Table 3.3.). They are in general known to degrade chitin dimers and oligosaccharides, but activity on polymeric chitin is not common for this group of enzymes. Activity towards chitin and chitosan makes *Mth*NAG a unique enzyme among NAGases. Only a few bacterial NAGases and the fungal LeHex20A from *L. edodes* and *M. anisopliae* were reported to degrade chitin to some extent (Suginta et al., 2010; Konno et al., 2012; St. Leger et al., 1991). Activity on chitosan has not been reported before. Keeping in mind that *Mth*NAG releases a single GlcNAc moiety from the non-reducing end, depolymerization of chitosan is only possible through the release of GlcNAc from the terminal site of chitosan chains. Furthermore, this result implies the



acetylation profile of the chitosan used in the experiment (90 % DDA), in which chitosan has numerous acetylated moieties at its non-reducing ends. The activity on polymeric substrates may indicate that *MthNAG* could be able to release GlcNAc moiety from glycopeptides, as it was reported for NAGase from *A. niger* (Bahl and Argawal, 1969).

However, the activity of *MthNAG* towards chitin and chitosan is relatively low and potential industrial production of GlcNAc from chitin cannot be based only on this enzyme. Therefore, chitin depolymerization was tested with addition of Chitinase Chi1, that was shown to release mainly (GlcNAc)<sub>2</sub> from chitin (Krolicka et al., 2018). *MthNAG* and Chitinase Chi1 were expected to work in synergy i.e. the (GlcNAc)<sub>2</sub> released from chitin by Chitinase Chi1 will be hydrolyzed by *MthNAG* to GlcNAc. The amount of GlcNAc released by *MthNAG* and Chitinase Chi1 was compared with the amount released by *MthNAG* only. It was observed, that when the enzymes worked together, they were able to release about 130 times more GlcNAc from swollen chitin than when only *MthNAG* was used. This result strongly indicates that these enzymes indeed work in synergy and can be used for the setup of an enzymatic production process of GlcNAc. The high thermostability of Chitinase Chi1 and *MthNAG* is an additional advantage for their potential industrial application.

In conclusion, a novel  $\beta$ -N-acetylglucosaminidase from the thermophilic fungus *M. thermophila* C1 was cloned and homologously overexpressed. The enzyme showed high specific activity toward (GlcNAc)<sub>2</sub>. *MthNAG* is notably thermostable and to the best of our knowledge, *MthNAG* is the first reported fungal NAGase with such high thermostability. Together with Chitinase Chi1, *MthNAG* released about 130 times more GlcNAc from swollen chitin than when used alone. Application of both *MthNAG* and Chitinase Chi1 as a two-enzyme catalyst is a promising tool for production of GlcNAc from chitin. Furthermore, *MthNAG* can be used for determination of deacetylation pattern of chitosan. In addition, due to the wide substrate specificity of *MthNAG*, this enzyme has potential to be used in combinations with other enzymes for deglycosylation of glycoconjugates such as glycans, glycoproteins, and glycolipids and for elucidation of their structure or changing their biological activity

## References

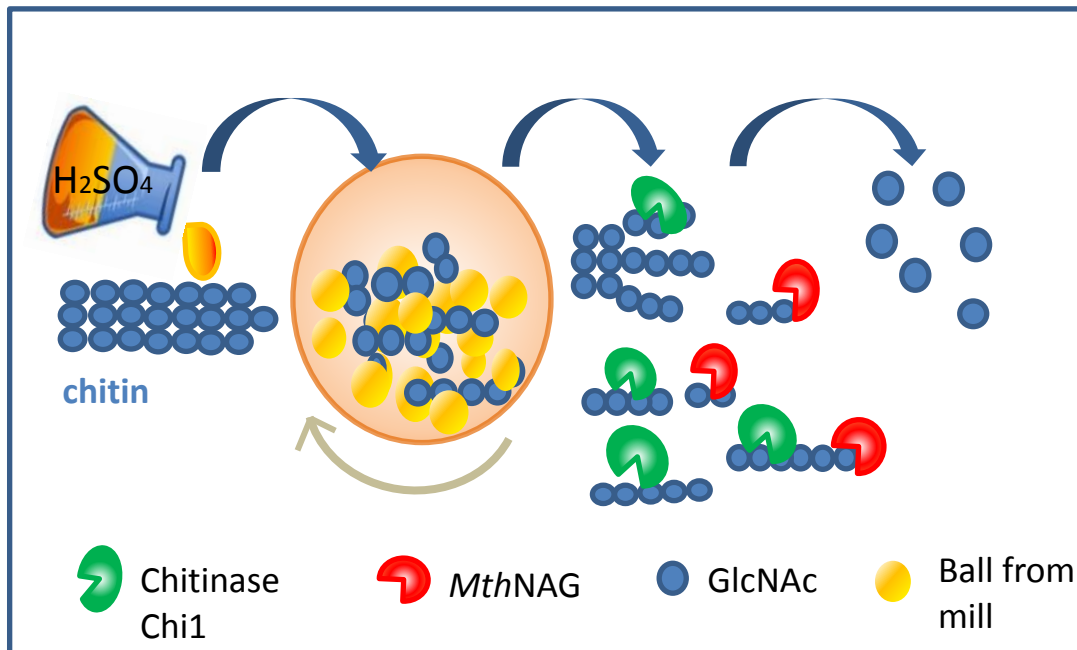
- Azuma, K., Osaki, T., Minami, S., Okamoto, Y., 2015. Anticancer and anti-inflammatory properties of chitin and chitosan oligosaccharides. *J. Funct. Biomater.* 6, 33–49
- Bahl, O.P., Agrawal, K.M.L., 1969. Glycosidases of *Aspergillus niger*: I. Purification and characterization of  $\alpha$ - and  $\beta$ -galactosidase and  $\beta$ -N-acetylglucosaminidase. *J. Biol. Chem.* 244, 2970–2978
- Bhattacharya, D., Nagpure, A., Gupta, R.K., 2007. Bacterial chitinases: properties and potential. *Crit. Rev. Biotechnol.* 27, 21–28
- Bidochka, M.J., Tong, K.I., Khachatourians, G.G., 1993. Partial purification and characterization of two extracellular N-acetyl-D-glucosaminidases produced by the entomopathogenic fungus *Beauveria bassiana*. *Can. J. Microbiol.* 39, 40–45
- Bissett, D.L., Robinson, L.R., Raleigh, P.S., Miyamoto, K., Hakozi, T., Li, J., Kelm, G.R., 2007. Reduction in the appearance of facial hyperpigmentation by topical N-acetylglucosamine. *J. Cosmet. Dermatol.* 6, 20–26
- Cannon, R.D., Niimi, K., Jenkinson, H.F., Shepherd, M.G., 1994. Molecular cloning and expression of the *Candida albicans*  $\beta$ -N-acetylglucosaminidase (HEX1). *Gene. J. Bacteriol.* 176, 2640–2647
- Chen, J.-K., Shen, C.-R., Liu, C.-L., 2010. N-Acetylglucosamine: production and applications. *Mar. Drugs.* 8, 2493–2516
- Diez, B., Rodriguez-Saiz, M., de la Fuente, J.L., Moreno, M.A., Barredo, J.L., 2005. The *nagA* gene of *Penicillium chrysogenum* encoding  $\beta$ -N-acetylglucosaminidase. *FEMS Microbiol. Lett.* 242, 257–264
- Dalirfardouei, R., Karimi, G., Jamialahmadi, K., 2016. Molecular mechanisms and biomedical applications of glucosamine as a potential multifunctional therapeutic agent. *Life Sciences* 152, 21–29
- Elieh-Ali-Komi, D., Hamblin, M.R., 2016. Chitin and chitosan: production and application of versatile biomedical nanomaterials. *Int. J. Adv. Res.* 4, 411–427
- Eriqez, L.A., Pisano, M.A., 1979. Purification and characterization of an extracellular  $\beta$ -N-acetylhexosaminidase from *Paecilomyces persicinus*. *J. Bacteriol.* 137, 620–626
- Gkargkas, K., Mamma, D., Nedev, G., Topakas, E., Christakopoulos, P., Kekos, D., Macris, B.J., 2004. Studies on a N-acetyl- $\beta$ -D-glucosaminidase produced by *Fusarium oxysporum* F3 grown in solid-state fermentation. *Process. Biochem.* 39, 1599–1605
- Hartl, L., Zach, S., Seidl-Seiboth, V., 2012. Fungal chitinases: diversity, mechanistic properties and biotechnological potential. *Appl. Microbiol. Biotechnol.* 93, 533–543
- Ike, M., Nagamatsu, K., Shioya, A., Nogawa, M., Ogasawara, W., Okada, H., Morikawa, Y., 2005. Purification, characterization, and gene cloning of 46 kDa chitinase (Chi46) from *Trichoderma reesei* PC-3-7 and its expression in *Escherichia coli*. *Appl. Microbiol. Biotechnol.* 71, 294–303
- Inokuma, K., Takano, M., Hoshino, K., 2013. Direct ethanol production from N-acetylglucosamine and chitin substrates by *Mucor* species. *Biochem. Eng. J.* 72, 24–32
- Jafari-Aghdam, J., Khajeh, K., Ranjbar, B., Nemat-Gorgani, M., 2005. Deglycosylation of glucoamylase from *Aspergillus niger*: effects on structure, activity and stability. *Biochim. Biophys. Acta* 1750, 61–68
- Jones, C.S., Kosman, D.J., 1980. Purification, properties, kinetics, and mechanism from  $\beta$ -N-acetylglucosaminidase from *Aspergillus niger*. *J. Biol. Chem.* 255, 11861–11869
- Kardas, I., Struszczyk, M.H., Kucharska, M., van den Broek, L.A.M., van Dam, J.E.G., Ciechańska D., 2012. Chitin and chitosan as functional biopolymers for industrial applications. In: Navard P(ed) The European Polysaccharide Network of Excellence (EPNOE). Springer, Vienna, pp. 329–373
- Keyhani, N.O., Roseman, S., 1996. The chitin catabolic cascade in the marine bacterium *Vibrio furnissii*. Molecular cloning, isolation, and characterization of a periplasmic  $\beta$ -N-acetylglucosaminidase. *J. Biol. Chem.* 271, 33425–33432
- Kim, S., Matsua, I., Ajiksaka, K., Nakajima H., Kitamoto, K., 2002. Cloning and characterization of the gene *nagA* that encodes  $\beta$ -N-acetylglucosaminidase from *Aspergillus nidulans* and its expression in *Aspergillus oryzae*. *Biosci. Biotechnol. Biochem.* 66, 2168–2175
- Koga, K., Iwamoto, Y., Sakamoto, H., Hatano, K., Sano, M., Kato, I., 1991. Purification and characterization of  $\beta$ -N-acetylhexosaminidase from *Trichoderma harzianum*. *Agric. Biol. Chem.* 55, 2817–2823
- Konno, N., Takahashi, H., Nakajima, M., Takeda, T., Sakamoto, Y., 2012. Characterization of  $\beta$ -N-acetylhexosaminidase (LeHex20A), a member of glycoside hydrolase family 20, from *Lentinula edodes* (shiitake mushroom). *AMB Express* 2, 29
- Kozłowski, L.P., 2016. IPC – Isoelectric Point Calculator. *Biol. Direct* 11, 55
- Kristjansson, J.K., 1989. Thermophilic organisms as sources of thermostable enzymes. *Trends Biotechnol.* 7, 349–353

- Krolicka, M., Hinz, S.W., Koetsier, M.J., Joosten, R., Eggink, G., van den Broek, L.A.M., Boeriu, C.G., 2018. Chitinase Chi1 from *Myceliophthora thermophila* C1, a thermostable enzyme for chitin and chitosan depolymerization. *J. Agric. Food. Chem.* 66, 1658–1669
- Ledesma-Osuna, A.I., Ramos-Clamont, G., Vázquez-Moreno, L., 2008. Characterization of bovine serum albumin glycated with glucose, galactose and lactose. *Acta Biochim. Pol.* 55, 491–497
- Lever, M., 1973. Colorimetric and fluorometric carbohydrate determination with *p*-hydroxybenzoic acid hydrazide. *Biochem. Med.* 7, 274–281
- Li, H.M., Crouch, J.A., Belanger, F.C., 2005. Fungal endophyte *N*-acetylglucosaminidase expression in the infected host grass. *Mycol. Res.* 109, 363–373
- Lisboa De Marco, J., Valadares-Inglis, M.C., Felix, C.R., 2004. Purification and characterization of an *N*-acetylglucosaminidase produced by a *Trichoderma harzianum* strain which controls *Crinipellis perniciosa*. *Appl Microbiol Biotechnol* 64, 70–75
- Lorito, M., Hayes, C.K., Di Pietro, A., Woo, S.L., Harman, G.E., 1994. Purification, characterization, and synergistic activity of a glucan 1,3- $\beta$ -glucosidase and an *N*-acetyl- $\beta$ -glucosaminidase from *Trichoderma harzianum*. *Mol. Plant. Pathol.* 84, 398–405
- Marchlerbauer, A., Derbyshire, M.K., Gonzales, N.R., Lu, S., Chitsaz, F., Geer, L.Y., Geer, R.C., He, J., Gwadz, M., Hurwitz, D.I., 2015. CDD: NCBI's conserved domain database. *Nucleic Acids Res.* 43, 222–226
- Matagne, A., Joris, B., Frere, J.-M., 1991. Anomalous behaviour of a protein during SDS/PAGE corrected by chemical modification of carboxylic groups. *Biochem. J.* 280, 553–556
- Matsuo, I., Kim, S., Yamamoto, Y., Ajsaka, K., Maruyama, J., Nakajima, H., Kitamoto, K., 2003. Cloning and overexpression of  $\beta$ -*N*-acetylglucosaminidase encoding gene *nagA* from *Aspergillus oryzae* and enzyme-catalyzed synthesis of human milk oligosaccharide. *Biosci. Biotechnol. Biochem.* 67, 646–650
- Mega, T., Ikenaka, T., Matsushima, Y., 1970. Studies on *N*-acetyl- $\beta$ -D-glucosaminidase of *Aspergillus oryzae* I. Purification and characterization of *N*-acetyl- $\beta$ -D-glucosaminidase obtained from Takadiastase. *J. Biochem.* 68, 109–117
- Minami, S., Okamoto, Y., 2007. Drug for remedy or treatment of wound. WO Patent NO.2005112948A1
- Monreal, J., Reese, E.T., 1969. The chitinase of *Serratia marcescens*. *Can. J. Microbiol.* 15, 689–696
- No, H.K., Young Park, N., Ho Lee, S., Meyers, S.P., 2002. Antibacterial activity of chitosans and chitosan oligomers with different molecular weights. *Int. J. Food. Microbiol.* 74, 65–72
- Nogawa, M., Takahashi, H., Kashiwagi, A., Ohshima, K., Okada, H., Morikawa, Y., 1998. Purification and characterization of exo- $\beta$ -D-glucosaminidase from a cellulolytic fungus, *Trichoderma reesei* PC-3-7. *Appl. Environ. Microbiol.* 64, 890–895
- Ohtakara, A., Yoshida, M., Murakami, M., Izumi, T., 1981. Purification and characterization of  $\beta$ -*N*-acetylhexosaminidase from *Pycnoporus cinnabarinus*. *Agric. Biol. Chem.* 45, 239–247
- Østergaard, L.H., Olsen, H.S., 2010. Industrial applications of fungal enzymes. In: M. Hofrichter (ed.) 2nd edn. Springer-Verlag Berlin Heidelberg, pp 269–290
- Pera, L.M., Infante Majolli, M.V., Baigori, M.D., 1997. Purification and characterization of a thermostable and highly specific  $\beta$ -*N*-acetyl-D-glucosaminidase from *Aspergillus niger* 419. *Biotechnol. Appl. Biochem.* 26, 183–187
- Peterbauer, C.K., Lorito, M., Hayes, C.K., Harman, G.E., Kubicek, C.P., 1996. Molecular cloning and expression of the *nag1* gene (*N*-acetyl- $\beta$ -D-glucosaminidase-encoding gene) from *Trichoderma harzianum* P1. *Curr. Genet.* 30, 325–331
- Pitt-Rivers, R., Ambesi Impiombato, F.S., 1968. The binding of sodium dodecyl sulphate to various proteins. *Biochem. J.* 109, 825–830
- Rath, A., Glibowicka, M., Nadeau, V.G., Chen, G., Deber, C.M., 2009. Detergent binding explains anomalous SDS-PAGE migration of membrane proteins. *PNAS* 106, 1760–1765
- Reyes, F., Calatayud, J., Vazquez, C., Jesfis Martinez, M., 1989.  $\beta$ -*N*-Acetylglucosaminidase from *Aspergillus nidulans* which degrades chitin oligomers during autolysis. *FEMS Microbiol. Lett.* 53, 83–87
- Rinaudo, M., 2006. Chitin and chitosan: properties and applications. *Prog. Polym. Sci.* 31, 603–632
- Riordan, N.H., 1999. Skin treatment system. US Patent NO. 005866142A
- Ryslava, H., Kalendova, A., Doubnerova, V., Skocdopol, P., Kumar, V., Kukacka, Z., Pompach, P., Vanek, O., Slamova, K., Bojarova, P., Kulik, N., Ettrich, R., Kren, V., Bezouska, K., 2011. Enzymatic characterization and molecular modeling of an evolutionarily interesting fungal  $\beta$ -*N*-acetylhexosaminidase. *FEBS Journal* 278, 2469–2484
- Salvatore, S., Heuschkel, R., Tomlin, S., Daviesà, S.E., Edwards, S., Walker-Smith, J.A., French, I., Murch, S.H., 2000. A pilot study of *N*-acetylglucosamine, a nutritional substrate for glycosaminoglycan synthesis, in paediatric chronic inflammatory bowel disease. *Aliment. Pharmacol. Ther.* 14, 1567–1579

- Segrest, J.P., Jackson, R.L., Andrews, E.P., Marchesi, V.T., 1971. Human erythrocyte membrane glycoprotein: a re-evaluation of the molecular weight as determined by SDS polyacrylamide gel electrophoresis. *Biochem. Biophys. Res. Commun.* 44, 390–395
- Suginta, W., Chuenark, D., Mizuhara, M., Fukamizo, T., 2010. Novel  $\beta$ -N-acetylglucosaminidases from *Vibrio harveyi* 650: Cloning, expression, enzymatic properties, and subsite identification. *BMC Biochemistry* 11, 40
- Sullivan, P.A., Mchugh, N.J., Romana, L.K., Shepherd, M.G., 1984. The secretion of N-acetylglucosaminidase during germ-tube formation in *Candida albicans*. *J. Gen. Microbiol.* 130, 13–22
- St. Leger, R.J., Cooper, R.M., Charnley, A.K., 1991. Characterization of chitinase and chitobiase produced by the entomopathogenic fungus *Metarhizium anisopliae*. *J. Invertebr. Pathol.* 58, 415–426
- Tews, I., Perrakis, A., Oppenheim, A., Dauter, Z., Wilson, K.S., Vorgias, C.E., 1996. Bacterial chitobiase structure provides insight into catalytic mechanism and the basis of Tay-Sachs disease. *Nat. Struct. Biol.* 3, 638–648
- Turner, P., Mamo, G., Nordberg Karlsson, E., 2007. Potential and utilization of thermophiles and thermostable enzymes in biorefining. *Microb. Cell. Fact.* 6, 9
- Ulhoa, C.J., Peberdy, J.F., 1991. Purification and characterization of an extracellular chitobiase from *Trichoderma harzianum*. *Curr. Microbiol.* 23, 285–289
- Vaaje-Kolstad, G., Westereng, B., Horn, S.J., Liu, Z.L., Zhai, H., Sørli, M., Eijsink, V.G.H., 2010. An oxidative enzyme boosting the enzymatic conversion of recalcitrant polysaccharides. *Science* 330, 219–222
- Van den Broek, L.A.M., Knoop, R.J.I., Kappen, F.H.J., Boeriu, C.G., 2015. Chitosan films and blends for packaging material. *Carbohydr. Polym.* 116, 237–242
- Verdoes, J.C., Punt, P.J., Burlingame, R., Bartels, J., van Dijk, R., Slump, E., Meens, M., Joosten, R., Emalfarb, M., 2007. A dedicated vector for efficient library construction and high throughput screening in the hyphal fungus *Chrysosporium lucknowense*. *Ind. Biotechnol.* 3, 48–57
- Verdoes, J.C., Punt, P.J., Burlingame, R.P., Pynnonen, C.M., Olson, P.T., Wery, J., Visser, J.H., Emalfarb, M.A.V.J., 2010. New fungal production system. *Int. Patent WO/2010/107303*
- Visser, H., Joosten, V., Punt, P.J., Gusakov, A., Olson, P.T., Joosten, R., Bartels, J., Visser, J., Sinitsyn, A., Emalfarb, M., Verdoes, J., Wery, J., 2011. Development of a mature fungal technology and production platform for industrial enzymes based on a *Myceliophthora thermophila* isolate, previously known as *Chrysosporium lucknowense* C1. *Ind. Biotechnol.* 7, 214–223
- Xu, Q., Liu, J., Yuan, Z., 2004a. The use of N-acetyl-D-glucosamine as additive in beer and beer comprising N-acetyl-D-glucosamine. *WO Patent NO.* 2004085603A1
- Xu, Q., Liu, J., Yuan, Z., 2004b. The use of N-acetyl-D-glucosamine as additive in milk products. *WO Patent NO.* 2004093556A1
- Xu, Q., Liu, J., Yuan, Z., 2004c. The use of N-acetyl-D-glucosamine as additive in wine and wine comprising N-acetyl-D-glucosamine. *WO Patent NO.* 2004085604A1
- Xu, Q., Liu, J., Yuan, Z., 2006. Use of N-acetyl-D-glucosamine in preparation of drugs for the treatment of cancer and metastasis. *EP Patent NO.* 1666046A1
- Xu, Q., Liu, J., Yuan, Z., 2007a. Use of N-acetyl-D-glucosamine in treatment of local lesions and systematic symptoms related to infections of virus or bacteria. *US Patent NO.* 20070042995A1
- Xu, Q., Liu, J., Yuan, Z., 2007ab. Compound antibacterial drugs comprising N-acetyl-D-glucosamine. *US Patent NO.* 20070191291A1
- Xu, G.-Y., Ong, E., Gilkes, N.R., Kilburn, D.G., Muhandiram, D.R., Harris-Brandts, M., Carver, J.P., Kay, L.E., Harvey, T.S., 1995. Solution structure of a cellulose-binding domain from *Cellulomonas fimi* by Nuclear Magnetic Resonance Spectroscopy. *Biochemistry* 34, 6993–7009
- Yamamoto, K., Lee, K.M., Kumagai, H., 1985. Purification and characterization of  $\beta$ -N-acetylhexosaminidase from *Penicillium oxalicum*. *Agric. Biol. Chem.* 49, 611–619
- Yeboah, F.K., Yaylayan, V.A., 2001. Analysis of glycosylated proteins by mass spectrometric techniques: qualitative and quantitative aspects. *Nahrung/Food* 45, 164–171
- Zacharius, R.M., Zell, T.E., Morrison, J.H., Woodlock, J.J., 1969. Glycoprotein staining following electrophoresis on acrylamide gels. *Anal. Biochem.* 30, 148–152
- Zhuravleva, N.V., Lukyanov, P.A., Pivkin, M.V., 2004. N-acetyl- $\beta$ -D-hexosaminidase secreted by the marine fungus *Phoma glomerata*. *Appl. Biochem. Microbiol.* 40, 448–453

# Chapter 4

Biocatalytic cascade for the production of *N*-acetyl-D-glucosamine from chitin with Chitinase Chi1 and *N*-acetylglucosaminidase *Mth*NAG from *Myceliophthora thermophila* C1



Submitted as:

Krolicka, M., Boeriu, C.G., Hinz, S.W.A., Koetsier, M.J., van den Broek, L.A.M., Boer, E.P.J., Eggink, G., 2018

## ABSTRACT

Enzymatic routes to *N*-acetylglucosamine (GlcNAc) from chitin are extensively studied as green alternative for current chemical methods. Here we report the optimization of GlcNAc production from ball-milled chitin with purified and well characterised Chitinase Chi1 and *N*-acetylglucosaminidase (NAGase) *Mth*NAG from *Myceliophthora thermophila* C1. Using central composite design (CCD) and response surface methodology (RSM), conditions were found that allow production of GlcNAc at high yield. After 6 h incubation at 50 °C, the yield of GlcNAc of 73.2 % from 1.6 % (w/v) ball-milled chitin treated with 25 µg Chitinase Chi1 and 20 µg *Mth*NAG was obtained. Chitin conversion to GlcNAc and short chain chitin oligosaccharides (DP3, DP4 and DP5) was 93.0 %. Similar yields were obtained at increased substrate concentration (5 % (w/v) and reaction volume. Crude enzymes produced by the host gave comparable results when used at the optimal process conditions for GlcNAc production from ball-milled chitin.

## 4.1. Introduction

*N*-Acetyl-D-glucosamine (GlcNAc) and its deacetylated derivative D-glucosamine (GlcN) have many important activities and are widely used as cosmetic ingredients (Kim et al., 2008), dietary supplements (Bak et al., 2014) and medical therapeutics (Tamai et al., 2003). GlcNAc and GlcN are reported to clinically alleviate knee osteoarthritis. GlcNAc was shown to have a potential effect on the cartilage metabolism at a lower dose (500–1,000 mg/day) compared with GlcN (1,500 mg/day) (Kubomura et al., 2017). GlcNAc has been also proposed as a potential C6 source for biofuel production (Inokuma et al., 2016). Currently, GlcNAc is produced from chitin, a polymer mainly found in shells of crustaceans and in fungal cell walls, or by microbial synthesis in a fermentation process.

The main route for producing GlcNAc from chitin is through chemical hydrolysis catalysed by a strong acid, such as HCl. Direct hydrolysis of chitin to GlcNAc can be performed with 15–36 % HCl at about 40–80 °C (Bohlman et al., 2004). An alternative process involves chitin degradation to GlcN and *N*-acetylation of GlcN to GlcNAc. GlcN is produced through dissolution of chitin in concentrated HCl in boiling water for 3 h and finally *N*-acetylated with acetic anhydride. The chemical production of GlcNAc from chitin is estimated to be economically feasible, but there are some drawbacks, including low yield (below 65 %), low specificity of chemical catalyst, high operational costs and generation of environmentally unfriendly acidic wastes (Chen et al., 2010). Microbial synthesis of GlcNAc through fermentation of filamentous fungi or recombinant *Escherichia coli* results in fewer environmental issues. However, this process is hampered by low productivity and high production costs, that weakens its economic competitiveness with the chemical hydrolysis of chitin (Liu et al., 2013).

Besides the chemical depolymerisation of chitin and fermentation, GlcNAc can be produced by depolymerisation of chitin performed by enzymes. Enzymes are biological catalysts that can depolymerise chitin under milder conditions than the reactions in the chemical process (Chen et al., 2010). Chitinolytic enzymes are widely found in nature in most living organisms including bacteria, fungi, yeast, plants and animals. The enzymes of microbial origin have been frequently used for hydrolysis of chitin (Chen et al., 2010). Enzymatic depolymerisation of chitin to GlcNAc is performed by synergistically acting chitinases, *N*-

acetylglucosaminidases (NAGase) and lytic polysaccharide monooxygenases (LPMOs). Chitinases (EC 3.2.1.14) cleave  $\beta$ -(1,4)-glycosidic linkages between GlcNAc moieties in chitin chains and release low molecular chitin oligosaccharides and dimers, which are finally degraded to GlcNAc by *N*-acetylglucosaminidase (NAGase) (EC 3.2.1.52) (Hartl et al., 2012). LPMOs (EC 1.14.99.53) open chitin chains using an oxidative mechanism (Vaaje-Kolstad et al., 2010), thereby accelerating the degradation of chitin due to the fact that the opened chitin chains are more accessible for chitinases. However, production of GlcNAc by enzymatic approaches at industrial scale still remains a big challenge. Although high yield and high purity of GlcNAc could be obtained, low productivity and long incubation times were the main drawbacks reported for this method. Most reports described maximum GlcNAc production yields of 41–85 % with incubation periods ranging between 1 and 19 days (Sashiwa et al., 2002; Sashiwa et al., 2003; Setthakaset, 2008).

The reason of low productivity of enzymatic reaction is a low accessibility of natural chitin due to its high crystallinity and insolubility. Chains in chitin are organized in sheets where they are tightly held by a number of intra-sheet hydrogen bonds, responsible for tight crystal network (Rinaudo, 2006). Therefore decreasing the crystallinity of chitin is necessary to make  $\beta$ -glycosidic linkages in chitin chains more accessible for enzymes. Various methods of chitin pre-treatment were investigated including acid treatment (Binod et al., 2007), ultrasonication and steam-explosion (Villa-Lerma et al., 2013),  $\gamma$ -radiation (Dziril et al., 2015), microwave irradiation (Roy et al., 2003), high pressure homogenization (Wei et al., 2017), mechanochemical grinding (Nakagawa et al., 2011), rapid depressurization from supercritical 1,1,1,2-tetrafluoroethane (Villa-Lerma et al., 2016) and ball mill grinding in dry mode (Chen et al., 2015). These methods showed a positive effect on the decrease of chitin crystallinity.

Another important parameter for an efficient enzymatic chitin degradation to GlcNAc is the composition of the enzyme cocktail. Commonly investigated were crude enzyme preparations without enzyme purification and enzyme characterisation (Pichyangkura et al., 2002; Jung et al., 2007; Setthakaset, 2008). To our best knowledge, a designed process for GlcNAc production based on the application of purified chitinolytic enzymes has not been reported.



In this work we report an optimized enzymatic process for GlcNAc production from chitin that was pre-treated with ball milling as described by Yabushita et al. (2015). Response surface methodology (RSM) with central composite design (CCD) was used for process optimization. The designed process was based on the synergetic degradation of ball-milled chitin by Chitinase Chi1 and *Mth*NAG obtained from *M. thermophila* C1 (Krolicka et al., 2018a, 2018b). Chitinase Chi1 and *Mth*NAG efficiently released GlcNAc from ball-milled chitin in short time (6 hours). Combination of ball-milled chitin and its enzymatic hydrolysis with Chitinase Chi1 and *Mth*NAG to GlcNAc is a promising process that can be used at industrial scale.

## **4.2. Materials and methods**

### **4.2.1. Chemicals**

Chitin powder grade and GlcNAc was obtained from Sigma-Aldrich (St. Louis, USA). Chitin oligosaccharides (GlcNAc)<sub>2-6</sub> were obtained from Megazyme (Co. Wicklow, Ireland). All other chemicals were of the highest purity available.

### **4.2.2. Preparation of ball-milled-H<sub>2</sub>SO<sub>4</sub>-chitin**

Prior ball-milling chitin was impregnated with H<sub>2</sub>SO<sub>4</sub> as described by Yabushita et al. (2015). Subsequently chitin was planetary ball-milled (Fritsch) at 500 rpm using zirconium oxide balls (10 mm, 17 balls) in a 50 mL zirconium oxide pot. The milling time was 6 hours with a 10 min interval after every 10 min of milling for cooling. Chitin oligosaccharides present in ball-milled-H<sub>2</sub>SO<sub>4</sub>-chitin were identified with matrix assisted laser-desorption time-of-flight mass spectrometry (MALDI-TOF-MS) and their concentrations were measured with High Performance Anion-Exchange Chromatography (HPAEC) as described before (Krolicka et al. 2018a).

### **4.2.3. Characterisation of ball-milled-H<sub>2</sub>SO<sub>4</sub> chitin with X-ray diffraction (XRD)**

X-ray diffraction (XRD) was used to determine the crystallinity of the chitin samples before and after ball milling. Wide angle X-ray scattering (WAXS) powder diffractograms were recorded on a Bruker D2 Phaser diffractometer in the angular range 5–40° (2θ), with a step size of 0.02° and an acquisition time of 2.0 s per step. The Cobalt Kα1 radiation (λ= 1.7902 Å) from the anode, generated at 30 kV and 10 mA, was monochromatized using a Ni filter.

#### 4.2.4. Enzymes for chitin hydrolysis

Chitinase Chi1 and *MthNAG* from *M. thermophila* C1 were purified and characterised as described before (Krolicka et al., 2018a, 2018b). Activity assay for Chitinase Chi1 and *MthNAG* towards ball-milled-H<sub>2</sub>SO<sub>4</sub>-chitin was conducted with 0.5 % (w/v) substrate, Chitinase Chi1 (5 µg) or *MthNAG* (5 µg) in 1 mL sodium acetate buffer (0.05 M, pH 5.0) at 50 °C with agitation at 800 rpm. Reactions were terminated after 15 min incubation by heating at 96 °C for 10 min. Samples were centrifuged at 20,000 × *g* for 5 min and the released reducing sugars in the supernatant were measured with the *p*-hydroxybenzoic acid hydrazide (PAHBAH) assay (Lever, 1973). *N*-acetyl-D-glucosamine was used as a standard and one enzyme unit (U) was defined as the amount of enzyme that liberated 1 µmol reducing sugar per minute.

#### 4.2.5. Experimental design

Optimization of GlcNAc production from ball-milled-H<sub>2</sub>SO<sub>4</sub>-chitin with Chitinase Chi1 and *MthNAG* was performed using response surface methodology (RSM) with central composite design (CCD). The experiments were designed using the software R (version 3.2.5). Standard 3-variable first order design with 8 corner points and 4 central points was created. The chosen optimization variables were: substrate concentration ( $x_1$ ), amount of Enzyme 1 (Chitinase Chi1) ( $x_2$ ), and amount of Enzyme 2 (*MthNAG*) ( $x_3$ ). The minimal (-1), central point (0) and the maximal (+1) values of variables are presented in Table 4.1. The values (-2) and (+2), which are so-called “star points”, were added to the design to create second-order CCD. This second-order CCD makes estimation of quadratic effects of variables possible. The settings of variables were determined from preliminary experiments with Chitinase Chi1 and *MthNAG*.

**Table 4.1.** Values of coded levels used in response surface methodology (RSM) with central composite design (CCD).

Parameter	Code		Level				
			-2	-1	0	+1	+2
Substrate (%)	$x_1$		1.3	1.5	2	2.5	2.7
Enzyme 1 (Chitinase Chi1)	$x_2$	Coded number	0.8	2	5	8	9.2
		( $\mu\text{g}$ enzyme)	(0.8 $\mu\text{g}$ )	(2 $\mu\text{g}$ )	(5 $\mu\text{g}$ )	(8 $\mu\text{g}$ )	(9.2 $\mu\text{g}$ )
Enzyme 2 ( <i>Mth</i> NAG)	$x_3$	Coded number	2.9	5	10	15	17.1
		( $\mu\text{g}$ enzyme)	(0.6 $\mu\text{g}$ )	(1 $\mu\text{g}$ )	(2 $\mu\text{g}$ )	(3 $\mu\text{g}$ )	(3.4 $\mu\text{g}$ )

All designed experiments were performed in random order to avoid bias. The response variables were the yields of total reducing sugars measured with the PAHBAH assay (Lever, 1973). The response variables are fitted by the following linear model (Equation 1):

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_1x_2 + b_5x_1x_3 + b_6x_2x_3 + b_7x_1^2 + b_8x_2^2 + b_9x_3^2 \quad (1)$$

where  $Y$  is the predicted response,  $b_0$  is an intercept (model constant),  $b_1 - b_9$  are the regression coefficients. The model selection has been done with Adjusted  $R^2$ . Visualisation of the response surface was done with R software.

#### 4.2.6. Chitin hydrolysis for experimental design

Amounts of Chitinase Chi1 and *Mth*NAG for hydrolysis of ball-milled- $\text{H}_2\text{SO}_4$ -chitin were used according to the CCD. Incubation conditions were chosen according to temperature and pH optimum reported before for Chitinase Chi1 and *Mth*NAG (Krolicka et al., 2018a, 2018b). Hydrolysis was performed in 1 mL sodium acetate buffer (0.05 M, pH 5.0) containing ball-milled- $\text{H}_2\text{SO}_4$ -chitin, Chitinase Chi1 and *Mth*NAG at 50 °C while mixing at 800 rpm for 6 hours. The reaction was terminated by heating at 96 °C for 10 min and samples were centrifuged at  $20,000 \times g$  for 5 min. Released reducing sugars in the supernatant were measured with the PAHBAH assay (Lever, 1973) with GlcNAc as standard. Observed and predicted product concentration was expressed in concentration (mM) of reducing sugars.

#### **4.2.7. Validation and optimisation of the model**

GlcNAc production from ball-milled-H<sub>2</sub>SO<sub>4</sub>-chitin obtained under conditions predicted with the model was further optimised with increased amounts of Chitinase Chi1 and *Mth*NAG in a designed setup (Setup1, Setup 2, and Setup 3). Hydrolysis of ball-milled-H<sub>2</sub>SO<sub>4</sub>-chitin with each setup was followed in time. The most promising setup was chosen for (1) production of GlcNAc in 10 mL reaction volume and for (2) 1 mL reaction with increased amount of substrate (5 % (w/v) ball-milled-H<sub>2</sub>SO<sub>4</sub>-chitin). Reaction (1) was performed in 25 mL flasks on a rotary carousel under conditions described above for chitin hydrolysis. Reaction (2) was performed in 1 mL volume under conditions described above for chitin hydrolysis. GlcNAc and chitin oligosaccharides released during chitin hydrolysis were measured with HPAEC. Chitin conversion was calculated as the percentage of released GlcNAc and chitin oligosaccharides weight (mg) to initial total ball-milled-H<sub>2</sub>SO<sub>4</sub>-chitin weight (mg). GlcNAc production yields were calculated by comparing the amount of GlcNAc released to the maximal theoretical yield, which is equal to the initial substrate concentration considering that 1 mg of chitin could produce a maximum of 1.08 mg of GlcNAc.

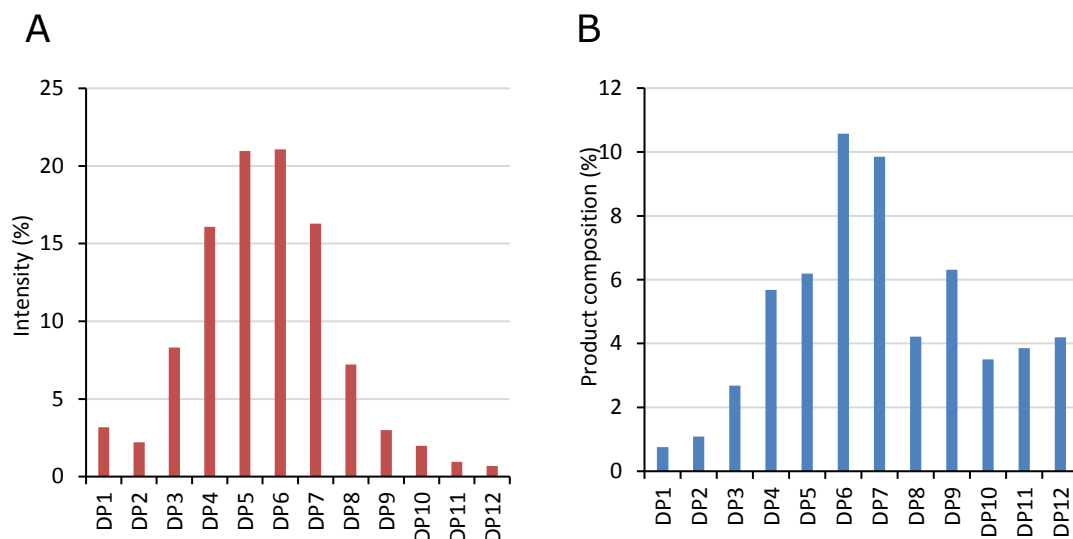
#### **4.2.8. Production of GlcNAc with crude enzyme preparations and increased substrate concentration**

Production of GlcNAc from ball-milled-H<sub>2</sub>SO<sub>4</sub>-chitin was tested with crude enzyme preparations of Chitinase Chi1 and *Mth*NAG. The amounts of the enzymes (in µg) were adjusted to the amounts used in the chosen setup in the previous experiment described above. Incubations were performed with the following enzyme mixtures: (1) purified Chitinase Chi1 (Chi1) and purified *Mth*NAG (*Mth*NAG), (2) crude preparation of Chitinase Chi1 (CR Chi1) and purified *Mth*NAG (*Mth*NAG), and (3) crude preparation of Chitinase Chi1 (CR Chi1) and crude preparation of *Mth*NAG (CR *Mth*NAG). Enzyme mixtures were used for hydrolysis of 1.7 and 5 % (w/v) ball-milled-H<sub>2</sub>SO<sub>4</sub>-chitin under chitin hydrolysis conditions described above.

## 4.3. Results and discussion

### 4.3.1. Properties of ball-milled-H<sub>2</sub>SO<sub>4</sub>-chitin and enzyme activities

Due to the high crystallinity and strong hydrogen bonding network, chitin is poorly accessible for enzymes. Therefore, efficiency of an enzymatic production of GlcNAc from chitin can be enhanced by applying a proper pre-treatment of chitin prior to enzymatic reaction. In this work chitin was impregnated with H<sub>2</sub>SO<sub>4</sub> and subsequently ball milled (ball-milled-H<sub>2</sub>SO<sub>4</sub>-chitin). According to the XRD measurements (Figure 4.1.S1.), the crystallinity of chitin has been drastically changed upon ball-milling pre-treatment. The diffraction pattern of untreated chitin with peaks observed at  $2\theta = 10.6^\circ$  and  $22.0^\circ$  is a typical pattern for crystalline chitin given in angle form, characterised by strong reflections at  $2\theta$  around  $9\text{--}10^\circ$  and  $2\theta$  of  $20\text{--}21^\circ$  and minor reflections at higher  $2\theta$  values, e.g., at  $26.4^\circ$  and higher (Kumirska et al., 2010). These peaks drastically decreased in ball-milled-H<sub>2</sub>SO<sub>4</sub>-chitin and only smooth peaks were detected after chitin pre-treatment. As a result, chitin with crystallinity of 79.8 % was converted to a fully amorphous form after the pre-treatment. After ball milling, soluble chitin oligosaccharides with DP1 to DP12 were detected with MALDI-TOF-MS (Figure 4.1.A). According to the HPAEC measurement, the total amount of DP1-DP6 was 27 % (w/w chitin) (Figure 4.1.B). The most abundant was DP6 (10.6 %), while the amount of GlcNAc (DP1) comprised only 0.8 % of the pre-treated chitin. The quantification of longer oligosaccharides DP7–DP12 was made on assumption, that the chromatographic behaviour of these oligosaccharides is similar to the chromatographic behaviour of DP6 and the calibration for DP6 was used for calculations. In ball-milled-H<sub>2</sub>SO<sub>4</sub>-chitin insoluble medium-high molecular weight chitin oligosaccharides are present seen as a chitin suspension before adding enzyme and starting the reaction (Figure 4.1.S2.). Specific enzymatic activities measured for Chitinase Chi1 and *Mth*NAG towards ball-milled-H<sub>2</sub>SO<sub>4</sub>-chitin were higher than activities previously reported towards chitin pre-treated with phosphoric acid (swollen chitin) (Krolicka et al., 2018a, 2018b). The specific activity of Chitinase Chi1 increased 3.8 fold from 1.4 U/mg (swollen chitin) to 5.3 U/mg (ball-milled-H<sub>2</sub>SO<sub>4</sub>-chitin) and that of *Mth*NAG increased 700 fold, from 0.03 U/mg (swollen chitin) to 21.6 U/mg (ball-milled-H<sub>2</sub>SO<sub>4</sub>-chitin). Ball-milling has been previously described as an effective pre-treatment method for chitin processing due to a high decrease in crystallinity (Yabushita et al., 2015; Nakagawa et al., 2011).



**Figure 4.1.** Water-soluble chitin oligosaccharides present in ball-milled- $\text{H}_2\text{SO}_4$ -chitin detected with matrix assisted laser-desorption time-of-flight mass spectrometry (MALDI-TOF-MS) (A) and their weight composition (%) based on measurement on High Performance Anion-Exchange Chromatography (HPAEC) (B). For HPAEC: the quantification of DP7-DP12 was based on the calibration curve for DP6 and the presented numbers are an assumption.

#### 4.3.2. Parameters optimization for GlcNAc production from ball-milled- $\text{H}_2\text{SO}_4$ -chitin

Ball-milled- $\text{H}_2\text{SO}_4$ -chitin was used as substrate for enzymatic production of GlcNAc with Chitinase Chi1 and *Mth*NAG. We previously reported that Chitinase Chi1 degrades polymeric chitin and chitin oligosaccharides with  $\text{DP} \geq 3$  and releases predominantly  $(\text{GlcNAc})_2$ , with small amounts of  $(\text{GlcNAc})_3$  and GlcNAc (Krolicka et al., 2018a). The second enzyme, *Mth*NAG, was characterised as NAGase, that showed the highest hydrolytic efficiency for  $(\text{GlcNAc})_2$  (Krolicka et al., 2018b). Hence, Chitinase Chi1 was expected to degrade chitin polymer and chitin oligosaccharides of ball-milled- $\text{H}_2\text{SO}_4$ -chitin to  $(\text{GlcNAc})_2$ , which would be converted by *Mth*NAG to GlcNAc. The reaction parameters (variables) that stimulate high conversion of ball-milled- $\text{H}_2\text{SO}_4$ -chitin to GlcNAc with Chitinase Chi1 and *Mth*NAG were investigated with RSM and experiments were designed with CCD. The CCD method was chosen since it is especially useful for determining the optimal level of significant variables and their potential interactions. In this study, values of the variables used and the values of the response variables obtained are summarized in Table 4.2.

**Table 4.2.** Central composite design (CCD) and experimental data for response surface analysis for GlcNAc production from ball-milled-H<sub>2</sub>SO<sub>4</sub>-chitin using Chitinase Chi1 and *Mth*NAG from *Myceliophthora thermophila* C1.

Sample number	Substrate (%)	Enzyme 1* (chitinase Chi1)	Enzyme 2* ( <i>Mth</i> NAG)	Observed product concentration (mM)	Predicted product concentration (mM)
1	1.5	2	5	20	21.7
2	2	5	10	26.1	26.5
3	2	5	10	25.7	26.5
4	1.5	8	15	28.6	29.1
5	2.5	8	15	22.2	23.5
6	2.5	8	5	23.2	22.0
7	2	5	10	26.9	26.5
8	1.5	2	15	22.4	23.1
9	1.5	8	5	27.1	27.6
10	2.5	2	5	17.8	18.2
11	2	5	10	25.6	26.5
12	2.5	2	15	18.7	19.7
13	2	0.8	10	24.8	23.1
14	2	5	17.1	27	25.8
15	2.7	5	10	19	18.4
16	2	9.2	10	29.8	29.9
17	2	5	2.9	25	23.8
18	1.3	5	10	26.6	24.7

\* Enzyme amounts are presented in coded number (amounts in µg and are presented in Table 4.1.)

Obtained *p*-values (*p* < 0.05) indicated that all variables have significant effect on product formation (Table 4.3.). Estimated regression coefficients of variables were fitted in a model equation (2) as follows:

$$Y = -17.5 + 37.71 * \text{Substrate} + 1.55 * \text{Enzyme 1} + 0.82 * \text{Enzyme 2} - 10.09 * (\text{Substrate})^2 - 0.03 * (\text{Enzyme 2})^2 - 0.37 * \text{Substrate} * \text{Enzyme 1} \quad (2)$$

The adjusted *R*<sup>2</sup> was equal to 0.8783 and the multiple *R*<sup>2</sup> was equal to 0.9212 (Table 4.3.). This result suggests that there is a good agreement between the experimental and predicted values of product formation. More in detail, 88% of the total variation in values of the ratio (*Y*) can be explained by 3 variables (substrate, Enzyme 1 and Enzyme 2) with 2

squares of these variables (substrate and Enzyme 2) and one interaction (substrate and Enzyme 1).

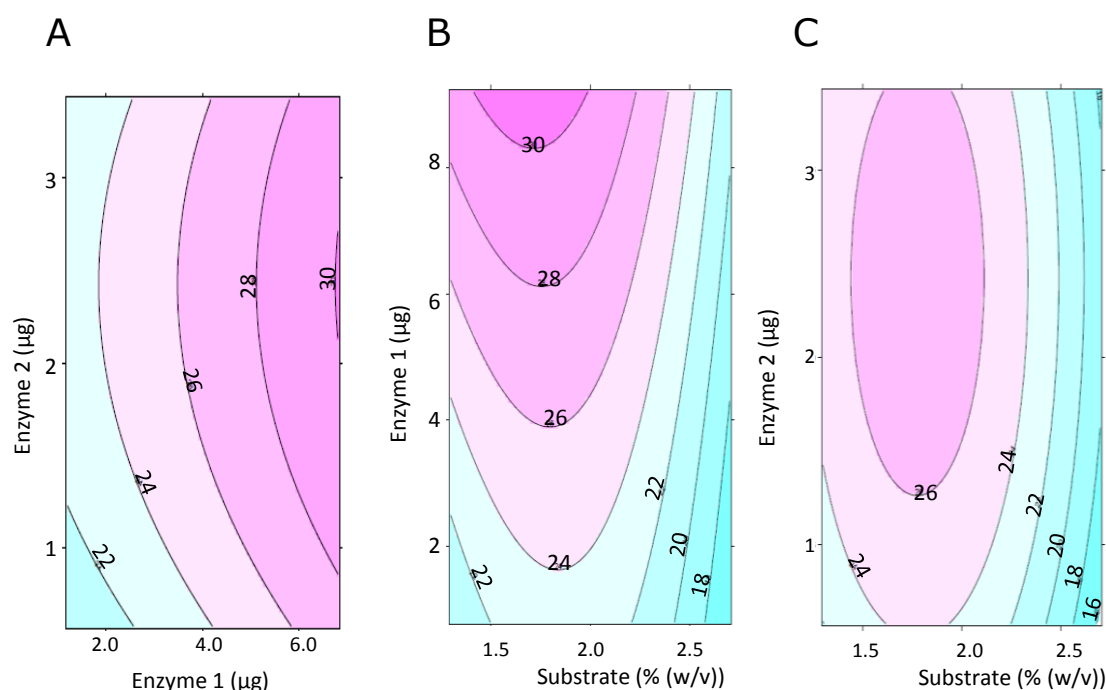
**Table 4.3.** Estimated regression coefficients and adjusted  $R^2$  obtained for central composite design (CCD).

Parameter	Estimated regression coefficients	$p$ -value
$b_0$ : Intercept	-17.46124	0.044839
$x_1$ : Substrate	37.70669	0.000298
$x_2$ : Enzyme 1 (Chi1)	1.54989	0.024322
$x_3$ : Enzyme 2 ( <i>Mth</i> NAG)	0.82243	0.040612
$x_1^2$ : (Substrate) <sup>2</sup>	-10.09418	0.000137
$x_3^2$ : (Enzyme 2) <sup>2</sup>	-0.03394	0.076555
$x_1 x_2$ : Substrate* Enzyme 1	-0.36667	0.233798
Multiple $R^2$	0.9212	
Adjusted $R^2$	0.8783	

Interactions between variables were visualised by constructing response contour plots, which explain the interaction between the response and the experimental data as function of the levels of two variables with the third variable at its central value (Figure 4.2.). The highest product concentration (30 mM) was reached when the highest amount of Enzyme 1 (Chitinase Chi1) (5  $\mu$ g) and moderate amount of Enzyme 2 (*Mth*NAG) (2  $\mu$ g) was used (Figure 4.2.A). Interaction between substrate concentration and amount of Enzyme 1 (Chitinase Chi1) showed, that the amount of enzyme at its “star point” caused the highest yield of product (30 mM) when the substrate concentration was in the range of 1.5 % (w/v) to 2 % (w/v) (Figure 4.2.B). The yield of product formation was hardly influenced by increasing the amount of Enzyme 2 (*Mth*NAG) even when the highest amount of Enzyme 2 at its “star point” was used (Figure 4.2.C). It was concluded that the substrate concentration and Enzyme 1 (Chitinase Chi1) were the most important variables influencing the yield of GlcNAc. The predicted optimum conditions for maximum GlcNAc production are 1.6 % (w/v) ball-milled- $H_2SO_4$ -chitin, 5  $\mu$ g Chitinase Chi1 and 2  $\mu$ g *Mth*NAG. RSM is a well-known method for optimising production processes and is extensively used in the food industry (Yolmeh and Jafari, 2017). In chitinase research, RSM has been used for statistical optimization of medium components and conditions for improved chitinase production (Singh et al., 2009; Hao et al., 2012; Meriem and Mahmoud, 2017; Mishra et al., 2012). RSM was also used for biodegradation of chitin bio-waste and concomitant production of



chitinase and GlcNAc by *Vibrio* sp. CFR173 (Suresh, 2012) and by chitinase from *Penicillium monverticillium* (Suresh et al., 2011) in solid state fermentation (SSF). However, the enzyme composition in SSF was not investigated. To the best of our knowledge the present study is the first reported process optimization with RSM for production of GlcNAc from ball-milled-H<sub>2</sub>SO<sub>4</sub>-chitin with well characterised enzyme composition.



**Figure 4.2.** Contour plots from response surface modelling (RSM). (A) Enzyme 2 vs Enzyme 1 when substrate was 2.0 % (w/v), (B) Enzyme 1 vs Substrate when Enzyme 2 was 2 μg, (C) Enzyme 2 vs Substrate when Enzyme 1 was 5 μg. Contours are depicted with colour scale. Enzyme 1 (Chitinase Chi1) and Enzyme 2 (*Mth*NAG) were used for hydrolysis of the substrate (ball-milled-H<sub>2</sub>SO<sub>4</sub>-chitin). Response is defined in concentration of reducing sugars (mM) produced during enzymatic hydrolysis of ball-milled-H<sub>2</sub>SO<sub>4</sub>-chitin.

### 4.3.3. Model validation and optimization

Optimization of GlcNAc production with Chitinase Chi1 and *Mth*NAG from *M. thermophila* C1 based on modelling with RSM and CCD revealed, that the concentration of ball-milled-H<sub>2</sub>SO<sub>4</sub>-chitin and amount of Chitinase Chi1 are the main parameters influencing chitin conversion to GlcNAc. Therefore, the next step was to further optimise the process with increased amounts of enzymes. Three enzyme setups containing Chitinase Chi1 and *Mth*NAG were prepared: Setup 1 (5 μg Chitinase Chi1 and 2 μg *Mth*NAG, as modelled with RSM), Setup 2 (25 μg Chitinase Chi1 and 20 μg *Mth*NAG), and Setup 3 (50 μg Chitinase Chi1 and 40 μg *Mth*NAG). The amount of *Mth*NAG in Setup 2 and Setup 3 was increased in order

to ensure efficient release of GlcNAc. Hydrolysis of ball-milled-H<sub>2</sub>SO<sub>4</sub>-chitin (1.6 % (w/v)) with each setup was monitored for 24 hours (Table 4.4.). It was observed, that the setups differed significantly in productivity. The highest GlcNAc concentration obtained with Setup 1 was 45.8 mM after 24 hours, while similar concentration of GlcNAc was obtained already after 2 hours with Setup 2. Setup 3 was most efficient, since it produced 50.8 mM of GlcNAc after 2 hours. The most noticeable difference between setups was observed after 6 hours incubation. The concentration of GlcNAc for Setup 2 and Setup 3 reached almost the maximum, while Setup 1 reached only approximately 70 % of the concentration obtained with other setups. The produced GlcNAc was of high purity at every time point of the incubation (Table 4.4.). After 6 hours, a GlcNAc purity of 96.4, 94.9 and 94.2 % was obtained for Setup 1, Setup 2 and Setup 3, respectively. Other products were traces of chitin oligosaccharides, particularly (GlcNAc)<sub>3</sub>, (GlcNAc)<sub>4</sub> and (GlcNAc)<sub>5</sub> as shown in Table 4.4. For further investigation, Setup 2 was chosen as an effective enzyme procedure for GlcNAc production at short time and with an optimised enzyme loading.

**Table 4.4.** Products released from ball-milled-H<sub>2</sub>SO<sub>4</sub>-chitin incubated with Chitinase Chi1 and *Mth*NAG from *Myceliophthora thermophila* C1 in three different setups: Setup 1 (5 µg Chitinase Chi1 and 2 µg *Mth*NAG), Setup 2 (25 µg Chitinase Chi1 and 20 µg *Mth*NAG), and Setup 3 (50 µg Chitinase Chi1 and 40 µg *Mth*NAG). Amounts of GlcNAc and chitin oligosaccharides expressed in concentration (mM), the yield of GlcNAc in percent (%), the molar product composition in percent (%) and chitin conversion in percent (%).

Setup/time	GlcNAc concentration (mM)	GlcNAc yield (%)	Molar product composition (%)				Chitin conversion (%)
			GlcNAc	(GlcNAc) <sub>3</sub>	(GlcNAc) <sub>4</sub>	(GlcNAc) <sub>5</sub>	
<b>Setup 1</b>							
time (h)							
2	31.0±0.9	43.3	95.6	1.6	2.3	0.5	54.1
6	35.6±1.3	49.7	96.4	1.5	1.9	0.2	60.5
8	38.2±0.9	53.3	95.5	1.7	2.5	0.3	66.8
24	45.8±0.5	63.9	94.7	2.1	3.0	0.2	82.1
<b>Setup 2</b>							
time (h)							
2	44.3±0.3	61.9	95.3	1.9	2.7	0.2	78.1
6	52.5±1.2	73.2	94.9	2.5	2.2	0.4	93.0
8	52.9±0.6	73.9	93.9	2.9	2.9	0.3	97.1
24	54.0±0.3	75.4	94.0	3.3	2.5	0.2	98.5
<b>Setup 3</b>							
time (h)							
2	50.8±0.2	70.9	94.8	2.3	2.4	0.4	90.5
6	56.0±1.3	78.1	94.2	3.2	2.2	0.4	100.0
8	54.8±0.4	76.5	93.7	3.5	2.6	0.2	100.0
24	55.5±0.2	77.4	93.5	3.7	2.6	0.2	100.0

#### 4.3.4. Production of GlcNAc from ball-milled-H<sub>2</sub>SO<sub>4</sub>-chitin in 10 mL reaction

Setup 2 with the optimised amount of Chitinase Chi1 and *Mth*NAG showed high efficiency in the production of GlcNAc from ball-milled-H<sub>2</sub>SO<sub>4</sub>-chitin. This setup was used for production of GlcNAc in 10 mL reaction (Figure 4.1.S2.). After enzymatic reaction, all insoluble medium-high molecular weight chitin oligosaccharides were hydrolysed to water soluble oligosaccharides. The concentration and the yield of GlcNAc (50.8 mM, 69.7 %) obtained after 6 hours incubation (Table 4.5.) were comparable to that obtained in a 1 mL-scale (52.5 mM, 73.2 %) (Table 4.4.).

**Table 4.5.** Production of GlcNAc from ball-milled-H<sub>2</sub>SO<sub>4</sub>-chitin incubated with Chitinase Chi1 and *Mth*NAG from *Myceliophthora thermophila* C1 in 10 mL reaction. The amounts of GlcNAc and chitin oligosaccharides are expressed as concentration (mM) and molar product composition (%). The yield of GlcNAc and chitin conversion are expressed in percent (%).

Time (h)	GlcNAc concentration (mM)	GlcNAc yield (%)	Molar product composition (%)			Chitin conversion (%)
			GlcNAc	(GlcNAc) <sub>3</sub>	(GlcNAc) <sub>4</sub>	
4	48.1±0.6	65.9	95.2	2.4	2.4	82.5
6	50.8±0.3	69.7	95.9	2.6	1.5	85.7
8	51.2±0.8	70.2	93.9	2.6	3.5	87.9
21	51.6±0.8	70.8	95.2	3.1	1.7	88.5

Even though the conversion of chitin was not complete (85 %), the productivity obtained in this study is higher than the productivity reported for other enzymatic processes in relation to enzyme loading and incubation time (Table 4.6.).

**Table 4.6.** Efficiency in enzymatic GlcNAc production with microbial chitinolytic enzymes.

Organism	Chitin	Enzyme	Enzyme load	GlcNAc yield (%)	Time	Reference
<i>Aeromonas</i> sp. PTCC1691	colloidal	crude enzymes	2 U/1*	79 %	24 h	Jamialahmadi et al., 2011
<i>Aeromonas hydrophila</i> H-2330	1) flake 2) powder	crude enzymes	36 %**	1) 66-77 % 2) 64-66 %	10 days	Sashiwa et al., 2002
<i>Aeromonas</i> sp. GJ-18	Swollen	crude enzymes	10 U/100*	1) 83 % 2) 94.9 %	1) 5 days 2) 9 days	Kuk et al., 2005
<i>Aspergillus</i> sp.	milled	crude enzymes	22 U/1000*	65 %	2 days	Setthakaset, 2008
<i>Bacillus licheniformis</i> SK-1	powder	crude enzymes	1 U/10*	41 %	6 days	Pichyangkura et al., 2002
<i>Burkholderia cepacia</i> TU09	powder	crude enzymes	1 U/33*	85 %	7 days	Pichyangkura et al., 2002
<i>Chitinolyticbacter meiyuanensis</i> SYBC-H1	crude	partial purified	10.2 U/40*	≈100 %	4 days	Zhang et al., 2016
<i>Paniebacillus illinoisensis</i> KJA-424	swollen	crude enzymes	0.66U/5*	62.2 %	24 h	Jung et al., 2007
<i>Paniebacillus barengoltzii</i> and <i>Rhizomucor miehei</i>	colloidal	purified enzymes	5.U chitinase, 1 U NAGase	92.6 %****	24h	Fu et al., 2014
<i>Streptomyces coelicolor</i> A3	powder	crude enzymes	concentrated enzyme extracts***	90 %	8 h	Nguyen-Thi and Doucet, 2016
<i>Trichoderma</i> sp. G and <i>Bacillus</i> sp.	powder	crude enzymes	1 mU/mg*	32 %	8 days	Kudan et al., 2010
<i>Myceliophthora thermophila</i> C1	ball-milled	purified enzymes	Chi1=0.2 %**, MthNAG=0.1%**	73.2 %	6 h	This paper

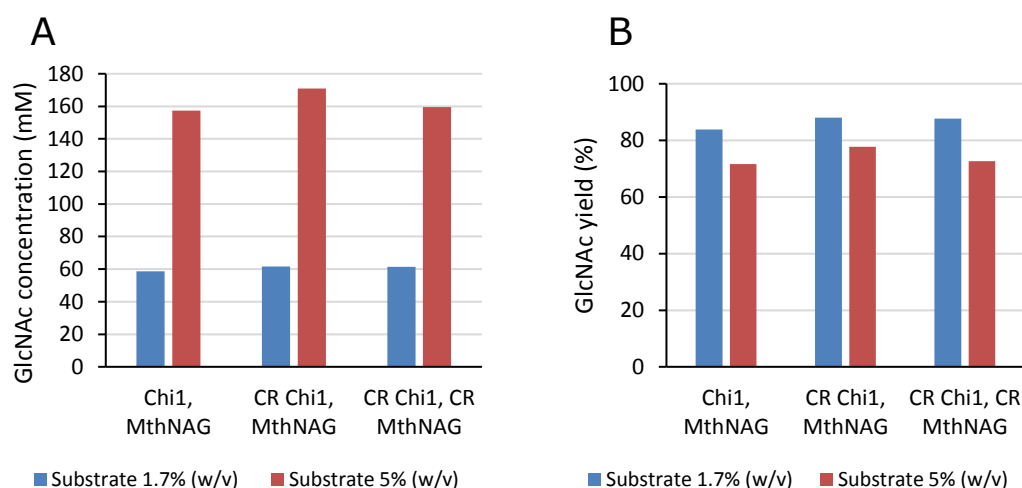
\* Unit of enzymatic activity per mg substrate, \*\*enzyme/substrate ratio, \*\*\* crude enzyme extracts were 10-fold concentrated and added to 10mg/mL chitin, \*\*\*\*expressed as conversion ratio, calculated as percentage of released GlcNAc weight (mg) to initial total colloidal chitin weight (mg). Chi1- Chitinase Chi1

For example, almost 100 % of chitin conversion to GlcNAc from chitin powder was obtained with chitinase from *Chitinolyticbacter meiyuanensis* SYBC-H1, but the incubation lasted 4 days (Zhang et al., 2016). A more efficient process was reported for *Streptomyces coelicolor* A3(2) (Nguyen-Thi and Doucet, 2016). The authors obtained a GlcNAc yield of 95 % after 8 hours incubation of chitin powder with 10 times concentrated crude enzyme preparation of chitinase ScChiC (10 g L<sup>-1</sup>) and NAGase ScHEX (7 g L<sup>-1</sup>). This enzyme loading was much higher than that used in our study (400 times higher for chitinase loading and 350 times higher for NAGase loading). Lately, after the discovery of LPMOs it appeared very attractive to convert crystalline chitin to GlcNAc using only an enzyme cocktail based on LPMOs and

hydrolytic chitinases and thus avoiding chitin pre-treatment. However, yields obtained from synergistically working LPMOs and chitinases are still very low. In the experiment with *Sg*LPMO10F from *Streptomyces griseus* and chitinase ChiA from *Serratia marcescens* a total yield of 8 % (as a yield for both GlcNAc and (GlcNAc)<sub>2</sub>) was obtained from crystalline chitin after 24 h incubation (Nakagawa et al., 2013). To conclude, the process presented in this study is more efficient than many other enzymatic processes. It can be used for production of GlcNAc at increased scale and at short time (6 hours), which is advantageous for saving time and energy.

#### **4.3.5. Production of GlcNAc from 5 % (w/v) ball-milled-H<sub>2</sub>SO<sub>4</sub>-chitin with purified enzymes and with crude enzyme preparations**

Production of GlcNAc was further investigated at increased substrate concentration (5 % (w/v) ball-milled-H<sub>2</sub>SO<sub>4</sub>-chitin) with three enzyme mixtures: (1) purified Chitinase Chi1 and purified *Mth*NAG according to Setup 2, (2) crude Chitinase Chi1 (CR Chi1) and purified *Mth*NAG, (3) crude Chitinase Chi1 (CR Chi1) and crude *Mth*NAG (CR *Mth*NAG). For all enzyme mixtures, the concentrations of GlcNAc obtained with 5 % (w/v) substrate were almost three times higher as compared with concentrations of GlcNAc released from the 1.7 % (w/v) substrate (Figure 4.3.). Furthermore, the mixture containing CR Chi1 and CR *Mth*NAG performed as efficient as the incubations with purified enzymes, indicating that crude enzyme preparations of Chitinase Chi1 and *Mth*NAG are appropriate for an efficient production of GlcNAc. The application of crude enzymes preparations can be beneficial at industrial scale especially for avoiding the laborious enzyme purification and thereby lowering the production costs. Additionally, the combination of crude enzyme preparations with more concentrated substrates can be advantageous for an effective usage of the production space.

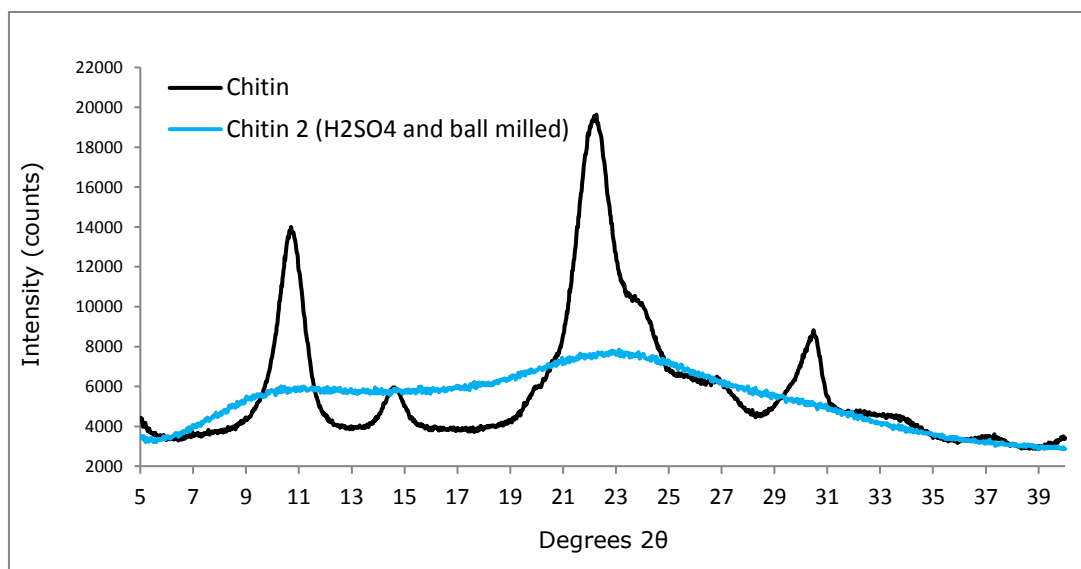


**Figure 4.3.** GlcNAc production from ball-milled- $\text{H}_2\text{SO}_4$ -chitin incubated with Chitinase Chi1 and *MthNAG* from *Myceliophthora thermophila* C1. GlcNAc concentration (A) and GlcNAc yield (B) obtained from 1.7 and 5.0 % (w/v) substrate incubated with three enzyme mixtures containing purified chitinase Chi1 (Chi1) and purified *MthNAG* (*MthNAG*), crude Chitinase Chi1 (CR Chi1) and purified *MthNAG*, and crude Chitinase Chi1 (CR Chi1) and crude *MthNAG* (CR *MthNAG*). Samples were incubated for 6 hours at 50 °C.

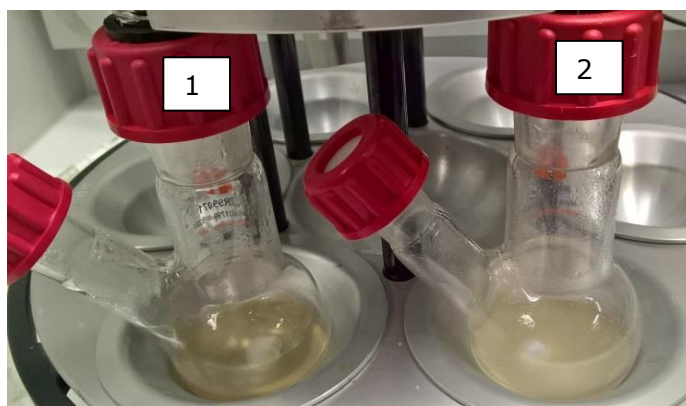
#### 4.4. Conclusions

Experimental design (CCD) with RSM is an adequate approach for optimizing the enzymatic production of GlcNAc from ball-milled- $\text{H}_2\text{SO}_4$ -chitin under mild conditions. CCD with RSM used for process modelling revealed that the substrate concentration and the amount of Chitinase Chi1 are the most important parameters determining the process efficiency. High GlcNAc yields and high chitin conversions have been obtained with purified Chitinase Chi1 and *MthNAG* and with their crude preparations. Enzymatic reactions conducted at increased substrate concentration and increased volumes revealed the possibility of upscaling the production process of GlcNAc based on Chitinase Chi1 and *MthNAG*.

## 4.1. Supplementary Information



**Figure 4.1.S1.** X-ray diffraction (XRD) diffractogram of untreated chitin (black) and ball-milled- $\text{H}_2\text{SO}_4$ -chitin (blue).



**Figure 4.1.S2.** Setup of a 10-mL scale for production of GlcNAc from ball-milled- $\text{H}_2\text{SO}_4$ -chitin incubated with Chitinase Chi1 and *MthNAG* from *Myceliophthora thermophila* C1. Sample 1 contains hydrolytic products after 21 hours enzymatic incubation, and Sample 2 contains substrate before enzymatic incubation.

## References

- Bak, Y.-K., Lampe, J.W., Sung, M.-K., 2014. Effects of dietary supplementation of glucosamine sulfate on intestinal inflammation in a mouse model of experimental colitis. *J. Gastroenterol. Hepatol.* 29, 957–963
- Binod, P., Sandhya, C., Suma, P., Szakacs, G., Pandey, A., 2007. Fungal biosynthesis of endochitinase and chitobiase in solid state fermentation and their application for the production of *N*-acetyl-D-glucosamine from colloidal chitin. *Bioresour. Technol.* 98, 2742–2748
- Bohlman, J.A., Schisler, D.O., Hwang, K.O., Henning J.P., Trinkle, J.R., Anderson, T.B., Steinke, J.D., Vanderhoff, A., 2004. *N*-acetyl-D-glucosamine and process for producing *N*-acetyl-D-glucosamine. US Patent NO. 6693188B2
- Chen, X., Gao, Y., Wang, L., Chen, H., Yan, N., 2015. Effect of treatment methods on chitin structure and its transformation into nitrogen-containing chemicals. *Chempluschem* 80, 1565–1572
- Chen, J.-K., Shen, C.-R., Liu, C.-L., 2010. *N*-Acetylglucosamine: production and applications. *Mar. Drugs* 8, 2493–251
- Dziril, M., Grib, H., Laribi-Habchi, H., Drouiche, N., Abdi, N., Lounici, H., Pauss, A., Mameri N., 2015. Chitin oligomers and monomers production by coupling  $\gamma$ -radiation and enzymatic hydrolysis. *Ind. Eng. Chem. Res.* 26, 396–401
- Fu, X., Yan, Q., Yang, S., Yang, X., Guo, Y., Jiang, Z., 2014. An acidic, thermostable exochitinase with  $\beta$ -*N*-acetylglucosaminidase activity from *Paenibacillus barengoltzii* converting chitin to *N*-acetylglucosamine. *Biotechnol. Biofuels* 7, 174
- Hao, Z., Cai, Y., Liao X., Zhang, X., Fang, Z., Zhang, D., 2012. Optimization of nutrition factors on chitinase production from a newly isolated *Chitolyticbacter meiyuanensis* SYBC-H1. *Braz. J. Microbiol.* 43, 177–186
- Hartl, L., Zach, S., Seidl-Seiboth, V., 2012. Fungal chitinases: diversity, mechanistic properties and biotechnological potential. *Appl. Microbiol. Biotechnol.* 93, 533–543
- Inokuma, K., Hasunuma, T., Kondo, A., 2016. Ethanol production from *N*-acetyl-D-glucosamine by *Scheffersomyces stipitis* strains. *AMB Express* 6, 83
- Jamialahmadi, K., Behravan, J., Fathi Najafi, M., Tabatabaei Yazdi, M., Shaverdi, A.R., Faramarzi, M.A., 2011. Enzymatic production of *N*-acetyl-D-glucosamine from chitin using crude enzyme preparation of *Aeromonas* sp. PTCC1691. *Biotechnology* 10, 292–297
- Jung, W.-J., Souleimanov A., Park, R.-D., Smith, D.L., 2007. Enzymatic production of *N*-acetyl chitooligosaccharides by crude enzyme derived from *Paenibacillus illioisensis* KJA-424. *Carbohydr. Polym.* 67, 256–259
- Kim, S.-K., Ravichandran, Y.D., Khan, S.B., Kim, Y.T., 2008. Prospective of the cosmeceuticals derived from marine organisms. *Biotechnol. Bioprocess Eng.* 13, 511–523
- Krolicka, M., Hinz, S.W.A., Koetsier, M.J., Joosten, R., Eggink, G., van den Broek, L.A.M., Boeriu C.G., 2018a. Chitinase Chi1 from *Myceliophthora thermophila* C1, a thermostable enzyme for chitin and chitosan depolymerization. *J. Agric. Food. Chem.* 66, 1658–1669
- Krolicka, M., Hinz, S.W.A., Koetsier, M.J., Eggink, G., van den Broek, L.A.M., Boeriu C.G., 2018b.  $\beta$ -*N*-Acetylglucosaminidase *MthNAG* from *Myceliophthora thermophila* C1, a thermostable enzyme for production of *N*-acetylglucosamine from chitin. *Appl. Microbiol. Biotechnol.*, 7441–7454
- Kubomura, D., Ueno, T., Yamada, M., Tomonaga, A., Nagaoka, I., 2017. Effect of *N*-acetylglucosamine administration on cartilage metabolism and safety in healthy subjects without symptoms of arthritis: A case report. *Exp. Ther. Med.* 13, 1614–1621
- Kudan, S., Eksittikul, T., Pichyangkura, R., Park, R.D., 2010. Preparation of *N*-acetyl-D-glucosamine and *N,N*-diacetylchitobiose by enzymatic hydrolysis of chitin with crude chitinases. *J. Biotechnol.* 150S, S1–S576
- Kuk, J.H., Jung, W.J., Jo, G.H., Kim, Y.C., Kim, K.Y., Park, R.D., 2005. Production of *N*-acetyl- $\beta$ -D-glucosamine from chitin by *Aeromonas* sp. GJ-18 crude enzyme. *Appl. Microbiol. Biotechnol.* 68, 384–389
- Kumirska, J., Czerwicka, M., Kaczyński, Z., Bychowska, A., Brzozowski, K., Thöming, J., Stepnowski, P., 2010. Application of spectroscopic methods for structural analysis of chitin and chitosan. *Mar. Drugs* 8, 1567–1636
- Lever, M., 1973. Colorimetric and fluorometric carbohydrate determination with *p*-hydroxybenzoic acid hydrazide. *Biochem. Med.* 7, 274–281
- Liu, L., Liu, Y., Shin, H., Chen, R., Li, J., Du, G., Chen, J., 2013. Microbial production of glucosamine and *N*-acetylglucosamine: advances and perspectives. *Appl. Microbiol. Biotechnol.* 97, 6149–6158

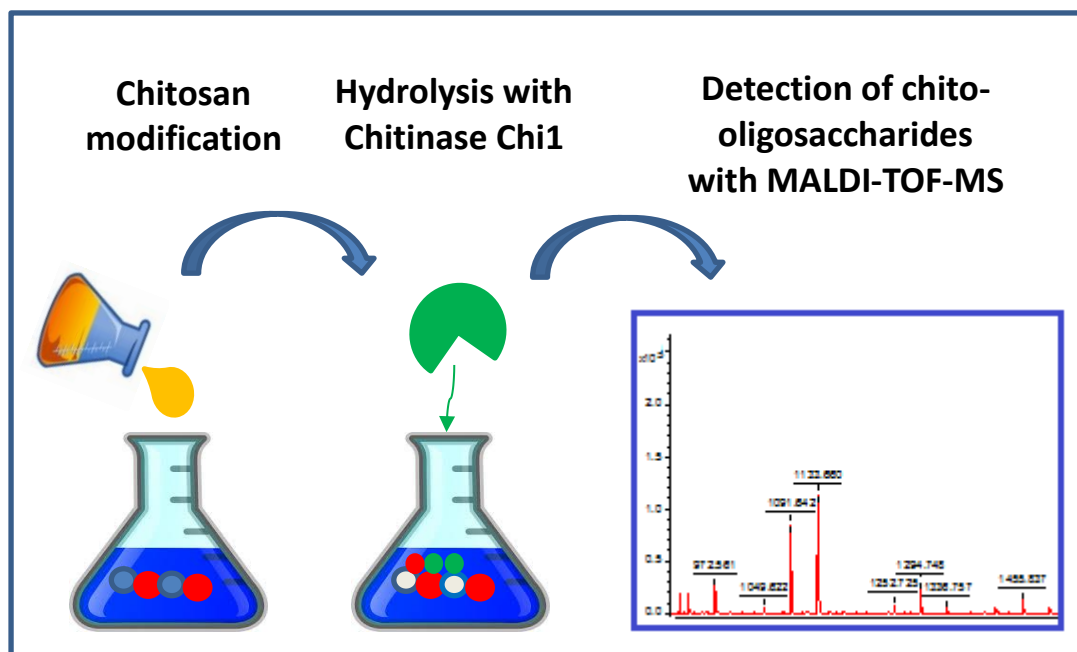


- Meriem, G., Mahmoud, K., 2017. Optimization of chitinase production by a new *Streptomyces griseorubens* C9 isolate using response surface methodology. *Ann. Microbiol.* 67, 175–183
- Mishra, P., Kshirsagar, P.R., Nilegaonkar, S.S., Singh S.K., 2012. Statistical optimization of medium components for production of extracellular chitinase by *Basidiobolus ranarum*: a novel biocontrol agent against plant pathogenic fungi. *J. Basic Microbiol.* 52, 539–548
- Nakagawa, Y.S., Oyama, Y., Kon, N., Nikaido, M., Tanno, K., Kogawa, J., Inomata, S., Masui, A., Yamamura, A., Kawaguchi, M., Matahara, Y., Totani, K., 2011. Development of innovative technologies to decrease the environmental burdens associated with using chitin as a biomass resource: Mechanochemical grinding and enzymatic degradation. *Carbohydr. Polym.* 83, 1843–1849
- Nakagawa, Y.S., Eijsink, V.G.H., Totani, K., Vaaje-Kolstad, G., 2013. Conversion of  $\alpha$ -chitin substrates with varying particle size and crystallinity reveals substrate preferences of the chitinases and Lytic Polysaccharide Monooxygenase of *Serratia marcescens*. *J. Agric. Food Chem.* 61, 11061–11066
- Nguyen-Thi, N., Doucet, N., 2016. Combining chitinase C and *N*-acetylhexosaminidase from *Streptomyces coelicolor* A3(2) provides an efficient way to synthesize *N*-acetylglucosamine from crystalline chitin. *J. Biotechnol.* 220, 25–32
- Pichyangkura, R., Kudan, S., Kuttiyawong, K., Sukwattanasinitt, M., Aiba, S., 2002. Quantitative production of 2-acetamido-2-deoxy-D-glucose from crystalline chitin by bacterial chitinase. *Carbohydr. Res.* 337, 557–559
- Rinaudo, M., 2006. Chitin and chitosan: properties and applications. *Prog. Polym. Sci.* 31, 603–632
- Roy, I., Mondal, K., Gupta, M.N., 2003. Accelerating enzymatic hydrolysis of chitin by microwave pretreatment. *Biotechnol. Prog.* 9, 1648–1653
- Sashiwa, H., Fujishima, S., Yamano, N., Kawasaki, N., Nakayama, A., Muraki, E., Hiraga, K., Oda, K., Aiba, S., 2002. Production of *N*-acetyl-D-glucosamine from  $\alpha$ -chitin by crude enzymes from *Aeromonas hydrophila* H-2330. *Carbohydr. Res.* 337, 761–763
- Sashiwa, H., Fujishima, S., Yamano, N., Kawasaki, N., Nakayama, A., Muraki, E., Sukwattanasinitt, M., Pichyangkura, R., Aiba, S., 2003. Enzymatic production of *N*-acetyl-D-glucosamine from chitin. Degradation study of *N*-acetylchitooligosaccharide and the effect of mixing of crude enzymes. *Carbohydr. Polym.* 51, 391–395
- Setthakaset, P., Pichyangkura, R., Ajavakom, A., Sukwattanasinitt, M., 2008. Preparation of *N*-acetyl-D-glucosamine using enzyme from *Aspergillus* sp. JMMM 18, 53–57
- Singh, A.K., Mehta, G., Chhatpar, H.S., 2009. Optimization of medium constituents for improved chitinase production by *Paenibacillus* sp. D1 using statistical approach. *Lett. Appl. Microbiol.* 49, 708–714
- Suresh, P.V., Anil Kumar, P.K., Sachindra, N.M., 2011. Thermoactive  $\beta$ -*N*-acetylhexosaminidase production by a soil isolate of *Penicillium monoverticillium* CFR 2 under solid state fermentation: parameter optimization and application for *N*-acetyl chitooligosaccharides preparation from chitin. *World J. Microbiol. Biotechnol.* 27, 1435–1447
- Suresh, P.V., 2012. Biodegradation of shrimp processing bio-waste and concomitant production of chitinase enzyme and *N*-acetyl-D-glucosamine by marine bacteria: production and process optimization. *World J. Microbiol. Biotechnol.* 28, 2945–2962
- Tamai, Y., Miyatake, K., Okamoto, Y., Takamori, Y., Sakamoto, K., Minami, S., 2003. Enhanced healing of cartilaginous injuries by *N*-acetyl-D-glucosamine and glucuronic acid. *Carbohydr. Polym.* 54, 251–262
- Vaaje-Kolstad, G., Westereng, B., Horn, S.J., Liu, Z.L., Zhai, H., Sørli, M., Eijsink, V.G.H., 2010. An oxidative enzyme boosting the enzymatic conversion of recalcitrant polysaccharides. *Science* 330, 219–222
- Villa-Lerma, G., González-Márquez, H., Gimeno, M., Trombotto, S., David, L., Ifuku, S., Shirai, K., 2016. Enzymatic hydrolysis of chitin pretreated by rapid depressurization from supercritical 1,1,1,2-tetrafluoroethane toward highly acetylated oligosaccharides. *Bioresour. Technol.* 209, 180–186
- Villa-Lerma, G., González-Márquez, H., Gimeno, M., López-Luna, A., Bárzana, E., Shirai, K., 2013. Ultrasonication and steam-explosion as chitin pretreatments for chitin oligosaccharide production by chitinases of *Lecanicillium lecanii*. *Bioresour. Technol.* 146, 794–798
- Wei, G., Zhang, A., Chen, K., Ouyang P., 2017. Enzymatic production of *N*-acetyl-D-glucosamine from crayfish shellwastes pretreated via high pressure homogenization. *Carbohydr. Polym.* 171, 236–241
- Yabushita, M., Kobayashi, H., Kuroki, K., Ito, S., Fukuoka, A., 2015. Catalytic depolymerization of chitin with retention of *N*-acetyl group. *ChemSuschem* 8, 3760–3763
- Yolmeh, M., Jafari, S.M., 2017. Applications of response surface methodology in the food industry processes. *Food Bioprocess. Technol.* 10, 413–433
- Zhang, A., Gao, C., Wang, J., Chen, K., Ouyang, P., 2016. An efficient enzymatic production of *N*-acetyl-D-glucosamine from crude chitin powders. *Green Chem.* 18, 2147–2154



# Chapter 5

Chitinase Chi1 – a tool for fingerprint analysis of (modified)chitosan



This chapter is a part of:

da Silva, S.B., Krolicka, M., van den Broek, L.A.M., Frissen, A.E., Boeriu, C.G., 2018. Carbohydr. Polym. 186, 299–309

## ABSTRACT

Nowadays, the production of tailored chitosan derivatives is gaining much attention. Chitosans can be modified with different methods like oxidation to acquire new properties in comparison to their native form resulting in new applications. The elucidation of the structure of these chitosan derivatives is important to understand the behaviour (structure-function relation) of the polymer. Therefore, the analysis of the structure at molecular scale (fingerprinting) can bring insights in this understanding. Here, we present a method based on the application of the fungal thermostable Chitinase Chi1 from *Myceliophthora thermophila* C1 and matrix assisted laser-desorption time-of-flight mass spectrometry (MALDI-TOF-MS). Chitinase Chi1 hydrolysed oxidised chitosan derivatives and the low molecular enzyme-resistant oligosaccharides were identified with MALDI-TOF-MS. Depending on the degree of oxidation (OD), different oligosaccharides were identified. For the chitosan with the lowest OD only short oligosaccharides (degree of polymerization of DP2 and DP3) with low carbonyl/carboxyl ratio were detected. For chitosans with higher OD larger oligosaccharides (DP2–DP5) with different carbonyl/carboxyl ratio were identified.

## 5.1. Introduction

Chitin and chitosan are polysaccharides abundantly present in nature. They are copolymers of *N*-acetylglucosamine (GlcNAc) and glucosamine (GlcN), and in chitin the number of *N*-acetylated units is higher than in chitosan. Commonly, the polysaccharide with a degree of deacetylation (DDA) lower than 50 % is entitled chitin, while the DDA above 50 % is entitled chitosan. Chitin and chitosan are also distinguished by solubility in 0.1 M acetic acid. Chitin is not-soluble, while chitosan is soluble in this solution (Weinhold et al., 2009).

Chitin is found mainly in the exoskeleton of crustaceans, insects, fungi and algae (Kardas et al., 2012) and chitosan is found in some fungi e.g. belonging to *Mucorales* (Bartnicki-Garcia and Nickerson, 1962). Industrially, chitosan is produced by partial or fully deacetylation of chitin by base treatment and it is considered as natural biopolymer (Rinaudo, 2006). Chitosan is a positively charged polymer at neutral pH and there are immense possibilities for chemical and mechanical modifications to generate materials with novel properties, functions and applications in many fields such as biomedicine, pharmaceuticals, metal chelation, and food additives (Kardas et al., 2012). As a consequence, numerous controlled chemical modification reactions are being explored (Mourya and Inamdar, 2008). The application of chitosan derivatives relies on their structure thus special emphasis has been paid to perform detailed structural analysis of chitosan and get insights into the structure-function relationships of chitosans and its derivatives. The understanding of the structure-function relationship can also help to predict its effect on the natural environment and/or synthetic products (Kumirska et al., 2009a, 2009b). Important parameters, which were found to influence the physicochemical properties of chitosan are the molecular weight (Mw), degree of polymerisation (DP), DDA, and pattern of acetylation (P<sub>A</sub>) (Weinhold et al., 2009; Cord-Landwehr et al., 2016). The characterisation can be performed with numerous analytical methods, such as Fourier transform infrared (FT-IR) spectroscopy, nuclear magnetic resonance (NMR) spectroscopy, and X-ray spectroscopy (Rinaudo, 2006). Another option is an enzymatic fingerprinting, which is based on partial depolymerisation of chitosan with an specific enzyme followed by detection of hydrolysis products with mass spectrometry (MS) (Niehues et al., 2017). Such a method has been widely applied for analysis of other polymers like pectin with endopolgalacturonase from *Kluyveromyces fragilis* (Daas et al., 1999). The advantage of applying enzymes is their high

cleavage specificity compared to chemical or physical methods. For example, chemical depolymerisation of chitosan with strong acids can lead to the complete depolymerisation of chitosan to its monomers.

In this paper we describe an analytic fingerprint method for analysis of modified chitosans. The method is based on the hydrolysis of chitosans with the thermostable fungal Chitinase Chi1 from *Myceliophthora thermophila* C1 (Krolicka et al., 2018) and the detection of released products by MALDI-TOF-MS. Chitinase Chi1 has been shown to hydrolyse a wide range of chitosans with different Mw, DDA and chain modification (Krolicka et al., 2018). Here we use Chitinase Chi1 to hydrolyse chitosans modified by oxidation of C6 in their GlcNAc and GlcN units. The method for chitosan oxidation was developed in our lab and was based on a 2,2,6,6-tetramethylpiperidinoxyl radical (TEMPO)-laccase catalytic redox system, in which TEMPO was regenerated with laccase from *Trametes versicolor* (da Silva et al., 2018). In total, four new oxidized chitosan derivatives were obtained (Product 1, 2, 3, 4). The main aim of the fingerprint analysis was to elucidate the P<sub>A</sub> and get insights into the structure-function relationship of oxidized chitosans.

## **5.2. Materials and methods**

### **5.2.1. Native and modified chitosans**

Native chitosan (Flonac C) with a Mw of 100 kDa and DDA of 82.8 % was obtained from Nippon Suisan Kaisha, Ltd. (Japan). This chitosan was used for production of modified chitosans as described by da Silva et al. (2018). Shortly, native chitosan was dissolved overnight in 0.1 M hydrochloric acid or acetic acid. Dissolved chitosans were oxidised using a TEMPO-laccase catalytic redox system. The TEMPO was regenerated with laccase from *Trametes versicolor*. In total, four new types of chitosan derivatives (products) with different OD were obtained and are shown in Table 5.1.

**Table 5.1.** Characteristics of modified chitosans.

Product	Acid <sup>a</sup>	Degree of deacetylation (% DDA)	Degree of oxidation (% OD)	Aldehyde content (mmol kg <sup>-1</sup> chitosan)	Carboxylate content (mmol kg <sup>-1</sup> chitosan)
Product 1	HAc <sup>b</sup>	83.6	1.1	50.9	13.5
Product 2	HAc <sup>b</sup>	79.8	7.0	385	28.0
Product 3	HAc <sup>b</sup>	83.4	4.6	264	10.4
Product 4	HCl	82.7	4.0	227	10.8

<sup>a</sup>0.1 M acid diluted solution used to dissolve chitosan prior to TEMPO-laccase oxidation, <sup>b</sup> HAc is acetic acid

### 5.2.2. Enzyme

Chitinase Chi1 from *Myceliophthora thermophila* C1 was purified and characterized as described by Krolicka et al. (2018).

### 5.2.3. Enzymatic hydrolysis of native and modified chitosans

Enzymatic hydrolysis of native and modified chitosans was performed with Chitinase Chi1 from *M. thermophila* C1 (Krolicka et al., 2018). Samples containing chitosans and enzyme were incubated at 50 °C for 24 h under gentle stirring. The reaction was terminated by heating at 96 °C for 10 minutes. The soluble low molecular mass fragments (oligosaccharides) obtained after the hydrolysis of native and modified chitosans were identified using MALDI-TOF-MS.

### 5.2.4. Mass spectrometry

MALDI-TOF-MS mass spectra of (oxidized) chito-oligosaccharides were recorded on a Bruker UltraFlextreme (Bruker Daltonics, Germany) in the reflective mode and positive ions were examined. The instrument was calibrated using maltodextrins with known molecular masses and the matrix solution consisted of 10 mg mL<sup>-1</sup> dihydroxy benzoic acid (DHB) in 50 % (v/v) acetonitrile in milliQ water. Prior to analysis, samples were desalted by adding a small amount of Dowex AG50W-X8 resin in the hydrogen form (Bio-Rad, Hercules, CA, USA) to 50 µL sample solution. Hereafter, the suspension was briefly centrifuged and 1 µL of the supernatant was transferred to 9 µL DHB matrix solution. Finally, 0.5 µL of the mixture was added to the matrix plate and dried under a stream of dry air. The lowest laser intensity required to obtain a good quality spectrum was used and 10 times 50 laser shots randomly obtained from the sample were accumulated. Measurements were performed in the m/z 300–3,000 range.

### 5.3. Results and discussion

Fingerprinting methods have been developed as specialized analytic methods delivering information about a unique pattern and indicating the presence of particular molecules in bipolymers. For example, this technique was used to determine the non-esterified distribution in pectin with endopolygalacturonase (Daas et al., 1999). For chitosan, it was reported that fingerprinting based on the application of a specific chitosanase can be used for structural analysis of chitosans with similar DP and different  $P_A$  (Niehues et al., 2017). It was assumed, that the  $P_A$  should have significant impact on biological activity (enzyme recognition) and on physiochemical activity (charge density) for the chitosans with exactly the same  $M_w$  and DDA but different  $P_A$  (Weinhold et al., 2009). This assumption can be also valid for modified chitosans, e.g. modified by oxidation. Introduction of additional CHO and COOH groups in the chitosan chains in a certain pattern of substitution ( $P_S$ ) could influence the characteristics of the chitosan polymer and the activity of the enzymes degrading chitosan (decrease or even diminish enzyme activity).

As we showed before, a thermostable Chitinase Chi1 from *M. thermophila* C1 was able to hydrolyse an oxidized chitosan with 5 % OD (DDA of 84 %,  $M_w$  of 100 kDa) (Krolicka et al., 2018). Moreover, the enzyme showed broad activity flexibility in degrading a wide range of different chitosans with DDA up to 94 % and  $M_w$  up to 3,000 kDa. Chitinase Chi1 was able to cleave the glycosidic linkages between GlcNAc-GlcNAc and GlcNAc-GlcN in chitin and chitosan. The activity on oxidised chitosan was measured with reducing sugars assay (Krolicka et al., 2018). However, the detailed analysis of the composition and the type of the hydrolysis products was not performed. Here we show the detailed analysis of the soluble degradation products of four different types of oxidized chitosan (Product 1, 2, 3, 4). The oxidation of the native chitosan was performed for improvement of its solubility. In general, Product 1 was produced at low TEMPO concentration while Product 2, 3, 4 were produced in the reaction with high TEMPO concentration (10 % wt. vs chitosan). Product 1, 2 and 3 showed improved solubility, while Product 4 became a gel as a result of its dissolution in HCl before performing oxidation. The amount of TEMPO used in oxidation directly influenced the OD of produced chitosans. The analysis of the OD and DDA after oxidation process was performed as described by da Silva et al. (2018) and the results are presented in Table 5.1. In general, Product 1 was



characterised by a low OD, while Product 2,3 and 4 were characterised by a high OD. All the products were separately incubated with Chitinase Chi1 at the optimum temperature (50 °C) of the enzyme to ensure the optimum conditions for the enzymatic reaction. However, chitosan was not fully degraded to soluble oligosaccharides and part of each chitosan Product stayed in a polymeric form. Therefore after the enzymatic reaction, the polymers remaining in the samples were precipitated with acid and the water soluble chitosan oligosaccharides were separated. The water soluble chitosan oligosaccharides released by Chitinase Chi1 were analysed by MALDI-TOF-MS and their composition is summarised in Table 5.2.

**Table 5.2.** Fragments obtained after enzymatic hydrolysis of native and modified chitosans with Chitinase Chi1 from *Myceliophthora thermophila* C1. Fragments were identified with MALDI-TOF-MS.

Ion composition	Type of adducts	Native chitosan	Product 1	Product 2	Product 3	Product 4
(GlcN) <sub>2</sub>	[M+H] <sup>+</sup>	x	x		x	
GlcNAc,GlcN	[M+Na] <sup>+</sup>	x	x	x		x
(GlcNAc) <sub>2</sub>	[M+H] <sup>+</sup>				x	
(GlcNAc) <sub>2</sub>	[M+Na] <sup>+</sup>	x				
(GlcNAc) <sub>2</sub> ,GlcN	[M+Na] <sup>+</sup>	x	x	x		x
(GlcNAc) <sub>3</sub>	[M+K] <sup>+</sup>	x	x	x		
GlcNAc,GlcN	[M( <b>COOH</b> )+H] <sup>+</sup>		x			
GlcNAc,GlcN	[M( <b>COOH</b> ) <sub>2</sub> +K] <sup>+</sup>		x	x	x	x
(GlcNAc) <sub>2</sub>	[M( <b>CHO</b> ) <sub>2</sub> +H] <sup>+</sup>		x			
(GlcNAc) <sub>2</sub> ,GlcN	[M( <b>COOH</b> )+K] <sup>+</sup>		x			
GlcNAc,(GlcN) <sub>3</sub>	[M( <b>CHO</b> ) <sub>4</sub> +H] <sup>+</sup>		x			
(GlcN) <sub>2</sub>	[M( <b>CHO</b> )+H] <sup>+</sup>					x
(GlcN) <sub>2</sub>	[M( <b>CHO</b> )+Na] <sup>+</sup>			x		
(GlcN) <sub>2</sub>	[M( <b>CHO</b> ) <sub>2</sub> +K] <sup>+</sup>			x	x	
(GlcN) <sub>2</sub>	[M( <b>COOH</b> )+K] <sup>+</sup>			x		
(GlcN) <sub>2</sub>	[M( <b>COOH</b> ) <sub>2</sub> +Na] <sup>+</sup>				x	
(GlcN) <sub>2</sub>	[M( <b>COOH</b> ) <sub>2</sub> +K] <sup>+</sup>				x	
(GlcNAc) <sub>2</sub> ,GlcN	[M( <b>CHO</b> )+H] <sup>+</sup>				x	
(GlcNAc) <sub>2</sub> ,GlcN	[M( <b>CHO</b> )+K] <sup>+</sup>			x		
(GlcNAc) <sub>2</sub> ,GlcN	[M( <b>COOH</b> )+Na] <sup>+</sup>			x		
(GlcNAc) <sub>2</sub> ,GlcN	[M( <b>COOH</b> )+K] <sup>+</sup>			x		
GlcNAc,(GlcN) <sub>2</sub>	[M( <b>COOH</b> )+Na] <sup>+</sup>				x	
(GlcNAc) <sub>3</sub>	[M( <b>CHO</b> ) <sub>3</sub> +Na] <sup>+</sup>			x		
(GlcNAc) <sub>3</sub>	[M( <b>COOH</b> ) <sub>3</sub> +Na] <sup>+</sup>				x	
(GlcN) <sub>4</sub>	[M( <b>CHO</b> ) <sub>4</sub> +K] <sup>+</sup>					x
GlcNAc,(GlcN) <sub>4</sub>	[M( <b>COOH</b> )+Na] <sup>+</sup>				x	
GlcNAc,(GlcN) <sub>4</sub>	[M( <b>CHO</b> ) <sub>4</sub> +Na] <sup>+</sup>				x	

M(**COOH**), oxidised fragment: + 14 DA mass shift, indicating a carboxyl group

M(**CHO**), oxidised fragment: - 2 Da mass shift, indicating an aldehyde group

Due to its broad specificity, Chitinase Chi1 was able to hydrolyse all oxidized chitosan products, but the composition of the released oligosaccharides differed significantly depending on the chitosan product. For native chitosan, homo- and hetero-oligosaccharides consisting of GlcNAc and GlcN units with low DP, namely dimers (DP2) and trimers (DP3) were identified.

The main unsubstituted chitosan oligosaccharides identified are  $(\text{GlcN})_2$ ,  $(\text{GlcNAc})_2$ ,  $(\text{GlcNAc,GlcN})$ ,  $(\text{GlcNAc})_3$  and  $((\text{GlcNAc})_2,\text{GlcN})$ . For oxidised chitosans (Product 1, 2, 3, 4) a variety of additional oxidized and not-oxidized oligosaccharides with DP2–DP5 consisting of GlcNAc and GlcN units, with aldehyde and carboxyl substitution ranging from one to four were detected. Interestingly, all oxidized oligosaccharides contained either carbonyl or carboxyl groups and never both groups were present in the released oligosaccharides.

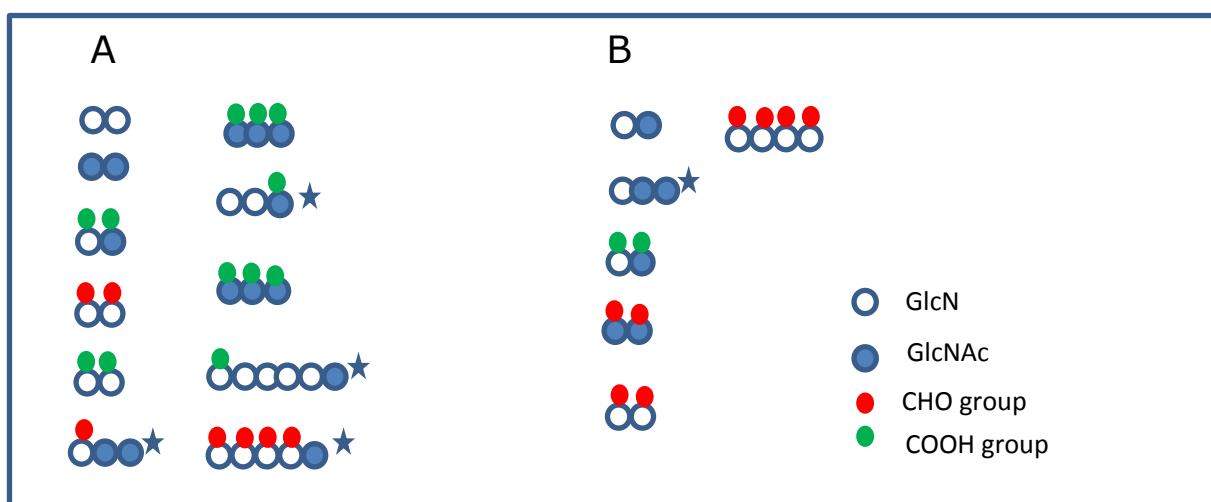
From Product 1, Chitinase Chi1 released oligosaccharides with DP2–DP4 and low carbonyl/carboxyl ratio, which is due to the low OD of this chitosan. The low OD is a result of the lesser amount of TEMPO and laccase used in the oxidation process than in case of the other products. Product 1 contains less GlcNAc/GlcN units substituted with carbonyl/carboxyl groups than other Products and can be easier degraded by Chitinase Chi1 to shorter oligosaccharides. In case of Product 2 and Product 3, Chitinase Chi1 released larger oligosaccharides with high carbonyl/carboxyl ratio. These Products have higher OD and thus their hydrolysis is more difficult for the enzyme. Chitinase Chi1 has to “search” for a not-substituted GlcNAc unit, which is needed for catalytic activity of the enzyme. Chitinases are dependent on the acetyl groups in the GlcNAc unit in their substrate, since they use the acetyl group in the substrate assisted catalysis. From Product 4, with high OD, the enzyme released only two types of not-oxidised oligosaccharides (DP1 and DP2), three types of oxidized oligosaccharides with DP2  $(\text{GlcNAc, GlcN})$ ,  $(\text{GlcNAc})_2$  and  $(\text{GlcN})_2$ , and an oligosaccharide composed of fully oxidized  $(\text{GlcN})_4$  bearing CHO group.

The release of specific oligosaccharides by hydrolytic action of Chitinase Chi1 gives information about the pattern of substitution, which can also be regarded to  $P_s$ . For Product 2 and Product 3, formation of larger oligosaccharides with one or multiple substitutions suggest a heterogeneous distribution of C6-carbonyl and C6-carboxyl groups along the polymer chain, and the random formation of aldehyde (CHO) and carboxyl (COOH) clusters, respectively. For Product 4 low molecular  $\text{COO}^-$ , CHO clusters and only one short  $\text{COO}^-$ -oxidized oligomer  $(\text{GlcNAc,GlcN})$  were identified, indicating a homogeneous distribution of COOH along the chain.

Product 3 and Product 4 are chemically similar (DDA and OD, Table 5.1.), but they significantly differ in their macroscopic properties and behaviour. Product 3 showed an increased solubility

in sodium phosphate buffer at pH 7.4 due to the increased carboxylate content. For Product 4, the oxidation process did not increase the solubility, in contrast, it produced a cross-linked structure capable of forming a hydrogel at pH 7.4. The difference between the Product 3 and Product 4 may be related to the dissolution of these Products in different acids before the oxidation reaction was performed. Acetic acid used for Product 3 and HCl used for product 4 (Table 5.1.). According to Thevarajah et al. (2016), the extent of chitosan dissolution in HCl solution is higher than in acetic acid solution. Moreover, HCl can induce additional deacetylation and a higher degree of hydrolysis than acetic acid. The protonation of  $\text{NH}_2$  is also higher with HCl than with acetic acid (Rinaudo et al., 1999). These effects added to the formation of chitosan acetate by dissolution in acetic acid are sufficient to induce different changes in the chain conformation depending on the used solvent. Differences in the chain conformation could directly influence the accessibility of TEMPO and thus pattern of oxidation and distribution of CHO and COOH groups. Based on Table 5.2., showing the released oligosaccharide structures, and the cleavage preference of the enzyme a schematic overview of oligosaccharide released from Product 3 and 4 was made. As previously mentioned, Chitinase Chi1 require for its reaction an unsubstituted GlcNAc unit positioned in (-1) subsite of its active site. Up to date, it was known that at subsite (+1) either a GlcNAc or GlcN unit can be positioned. However, regarding to the released products with substitution on both reducing and non-reducing end, it can be concluded that Chitinase Chi1 accepts also an oxidized GlcNAc/GlcN unit with either carbonyl or carboxyl group at its (+1) subsite of the active site. Furthermore, these currently discovered broad specificity of Chitinase Chi1 influences the release of specific oligosaccharides and gives information about the distribution of oxidation in chitosan. As shown in Table 5.1. and Figure 5.1., Chitinase Chi1 released a series of shorter oxidized and not-oxidized oligosaccharides (minimum ten possible oligosaccharides), indicating that the distribution of oxidation was more random. However, from Product 4, only six types of products were released with one characteristic chito-oligosaccharide composed of four fully oxidized GlcN units. Presence of this fully oxidised GlcN oligomers may indicate in a more block-wise distribution of CHO and COOH groups in the chitosan chains. These difference in Product 3 and Product 4 indicates, that indeed the  $P_s$  influences the characteristics of the chitosan and for understanding the structure-properties relationship of chitosans. This is of importance in characterisation of unknown chitosans and chitosans with similar chemical composition but different behaviour. However, this method

can only be used for chitosans, which contain sufficient number of non-oxidized GlcNAc units for maintaining the activity of the enzyme.



**Figure 5.1.** Schematic representation of the oligosaccharides released from two chitosan products: (A) Product 3 and (B) Product 4 by the action of Chitinase Chi1 from *Myceliphthora thermophila* C1. Products 3 and Product 4 were characterised with similar DDA and OD. The hydrolysis products were detected by MALDI-TOF-MS. A blue star indicates, that there are multiple products possible regarding sequence of the GlcNAc/GlcN units or the position of the CHO/COOH group in the oligosaccharide.

## 5.4. Conclusions

Here we show, that hydrolysis of oxidised chitosans can be used for elucidation of substrate specificity of chitinolytic enzymes and for analysis of the structure of modified chitosans (fingerprint analysis). Both approaches are based on hydrolysis of oxidized chitosans with an enzyme and subsequently detection of released products with MALDI-TOF-MS. In our experiments, we used thermostable Chitinase Chi1 and we demonstrated, that Chitinase Chi1 accepts oxidized GlcNAc/GlcN units at its (+1) subsite of its active site, while GlcNAc has to be positioned at (-1) subsite. Chitinase Chi1 was also used in the fingerprint analysis of oxidized chitosans. The composition of released oligosaccharides revealed that there is an important relationship between structure and properties of chitosan. Chitosans with higher OD were characterised with improved solubility than chitosans with lower OD. However, two chitosans with similar DDA and OD were found to differ in the solubility, and only with the fingerprint method described in this paper it was possible to explain the difference. The analysis of the oligosaccharides released by Chitinase Chi1, showed that these chitosans differed significantly in the distribution pattern of CHO and COOH groups. Therefore, the fingerprint analysis is of a great importance for chitosan characterisation and for better understanding of the

structure-properties relationship of chitosans. This method is especially useful for the determination of the pattern of the substitution ( $P_S$ ) in oxidized chitosans.

## References

- Bartnicki-Garcia, S., W.J. Nickerson, 1962. Nutrition, growth and morphogenesis of *Mucor rouxii*. J. Bacteriol. 84, 841–858
- Cord-Landwehr, S., Melcher, R.L.J., Kolkenbrock, S., Moerschbacher, B.M., 2016. A chitin deacetylase from the endophytic fungus *Pestalotiopsis* sp. efficiently inactivates the elicitor activity of chitin oligomers in rice cells. Scientific Repots. 6, 38018
- Daas, P.J.H., Meyer-Hansen, K., Schols, H.A., De Ruiter, G.A., Voragen, A.G.J., 1999. Investigation of the non-esterified galacturonic acid distribution in pectin with endopolygalacturonase. Carbohydr. Res. 318, 135–145
- da Silva, S.B., Krolicka, M., van den Broek, L.A.M., Frissen, A. E., Boeriu, C.G., 2018. Water-soluble chitosan derivatives and pH-responsive hydrogels by selective C-6 oxidation mediated by TEMPO-laccase redox system. Carbohydr. Polym. 186, 299–309
- Kardas, I., Struszczyk, M.H., Kucharska, M., van den Broek, L.A.M., van Dam, J.E.G., Ciechańska, D., 2012. Chitin and chitosan as functional biopolymers for industrial applications. In The European Polysaccharide Network of Excellence (EPNOE). Navard, P., Ed.; Springer, Vienna; pp. 329–373
- Krolicka, M., Hinz, S.W., Koetsier, M.J., Joosten, R., Eggink, G., van den Broek L.A.M., Boeriu, C.G., 2018. Chitinase Chi1 from *Myceliophthora thermophila* C1, a thermostable enzyme for chitin and chitosan depolymerization. J. Agric. Food Chem. 66, 1658–1669
- Kumirska, J., Weinhold, M., Sauvageau, J.C.M., Thöming, J., Kaczynski, Z., Stepnowski, P., 2009a. Determination of the pattern of acetylation of low-molecular-weight chitosan used in biomedical applications. J. Pharmaceut. Biomed. 50, 587–590
- Kumirska, J., Weinhold, M., Steudte, S., Thöming, J., Brzozowski, K., Stepnowski P., 2009b. Determination of the pattern of acetylation of chitosan samples: Comparison of evaluation methods and some validation parameters. Int. J. Biol. Macromol. 45, 56–60
- Mourya, V.K., Inamdar, N.N., 2008. Chitosan-modifications and applications: opportunities galore. React. Funct. Polym. 68, 1013–1051
- Niehues, A., Wattjes, J., Bénétteau, J., Rivera-Rodriguez, G.R., Moerschbacher, B.M., 2017. Chitosan analysis by enzymatic/mass spectrometric fingerprinting and in silico predictive modeling. Anal. Chem. 89, 12602–12608
- Rinaudo, M., 2006. Chitin and chitosan: properties and applications. Prog. Polym. Sci. 31, 603–632
- Rinaudo, M., Pavlov, G., Desbrières, J., 1999. Influence of acetic acid concentration on the solubilization of chitosan. Polymer. 40, 7029–7032
- Thevarajah, J.J., Bulanadi, J.C., Wagner, M., Gaborieau, M., Castignolles, P., 2016. Towards a less biased dissolution of chitosan. Anal. Chim. Acta 935, 258–268
- Weinhold, M., Sauvageau, J.C.M., Kumirska, J., Thöming, J., 2009. Studies on acetylation patterns of different chitosan preparations. Carbohydr. Polym. 78, 678–684

# Chapter 6

## General Discussion

**This chapter is the basis of the review:**

**Krolicka, M., Koetsier, M.J., van den Broek, L.A.M., Boeriu, C.G., 2018. Enzymatic production of *N*-acetylglucosamine: achievements and perspectives. Appl. Microbiol. Biotechnol., submitted**





## **6.1. Chitin/chitosan as biomass for monomers in the biobased economy**

The bioeconomy is an economy founded on biomass instead of fossil fuels. In the bioeconomy, renewable biological resources are used to replace fossil fuels as well as for production of food, feed and biobased products. Biobased products are the focus of the biobased economy (Mills, 2015). The vision of the bio-based economy is to obtain for example building blocks for materials and chemicals from renewable biological resources, such as plants, microorganisms and animals and to create the opportunity to reduce the emission of greenhouse gas and to reduce our dependence on coal, oil and gas (European Association for Bioindustries, 2011; McCormick and Kautto, 2013).

A biomass side stream (biowastes) generated by different industries is considered as an interesting renewable biological resource for building blocks. The recovery of valuable components as biopolymers, fats and fine chemicals, pigments and pharmaceuticals from biowastes decreases landfilling of wastes and protects the environment. Moreover, conversion of cheap biowastes to added-value products can bring additional economic benefit (Jardine and Sayed, 2016). Recovered biopolymers like proteins, lignin, starch and chitin are of high importance, since they can be used directly as feed or food or they can be applied as a resource of building blocks for production of non-food biobased products.

Chitin, one of the most abundant biopolymer on Earth, is generated in huge amounts as a by-product (biowaste) from the food and chemical industry. So far, the sea-food industry generates the biggest amounts of chitin-containing biomass (6-8 million tons/year produced globally) comprising the shells from crustaceans (e.g. prawns, shrimps, crabs, lobsters) (Halder and Mondal, 2018). Unfortunately, this biomass is considered as a waste and is often just dumped in the sea or landfilled without any processing (Yan and Chen, 2015). However, such a landfilling leads to a serious environmental pollution of coastal areas thus in 1970s USA introduced strict environmental regulations and banned the dumping of waste shells without any processing (Ahmed and Ikram, 2017). In some countries, e.g. in Australia, disposal of crustacean shells is costly and can be up to 130 € per tonne (Yan and Chen, 2015). The part of the crustacean shells from food industry is processed by drying and desizing (i.e. crushing and milling) and is sold as a low-value fertilizer with a price (44 €/tonne) much lower in comparison to the price of dried crustacean shells (85-102 €/tonne) (Yan and Chen, 2015).

However, chitin-containing biomass has a much higher potential than only being a low-value fertilizer. Currently, there is much effort being done to valorise chitin-containing biomass (Jardine and Sayed, 2016). Furthermore, the valorisation of chitin and its derivatives is an important aspect of the blue bioeconomy, which recognises the need to maximise the enormous economic potential presented by the ocean while preserving it (Day et al., 2016).

Besides chitin, chitin-containing biomass comprises proteins, fats, glucans and pigments (e.g. astaxanthin) (Figure 6.1.). All the components can be separated in a biorefinery concept using chemical or enzymatic extraction methods. Apart from food, refined components can be used as animal feed (e.g. proteins) and other parts can be further converted to valuable chemicals or materials. Lipids can be used in the production of high value oil, biodiesel and animal feed (Yan and Chen, 2015). Calcium carbonate is applied in pharmaceutical, agricultural, construction and paper industries (Yan and Chen, 2015). Astaxanthin, a carotenoid with antioxidant properties, is widely used in aquaculture feeds, food, cosmetic, pharmaceutical and medical applications (Prameela et al., 2017). Glucans recovered from fungi have potential application in food and feed, medicine and pharmacy (Zhu et al., 2016).

The first step of chitin valorisation is its extraction (Figure 6.2.). Traditionally, chitin is extracted by removal of proteins (deproteinization) with alkali treatment (e.g. with NaOH) and removal of calcium carbonate ( $\text{CaCO}_3$ ) (demineralization) with acidic treatment (e.g. HCl) (Arbia et al., 2013; Younes and Rinaudo, 2015). After extraction, chitin is often decoloured with acetone or an organic acid solvent mixture (Hamed et al., 2016).

The more valuable biopolymer chitosan is obtained through deacetylation of chitin, in the process where acetyl groups attached to GlcN are removed often under alkaline conditions (Mao et al., 2017). There are three characteristics, which make chitosan an interesting industrial polysaccharide: 1) chitosan behaves as a polyelectrolyte with positive charge density at low pH; 2) chitosan is the only known high molecular weight cationic polysaccharide, while other polysaccharides are generally either neutral or anionic; 3) chitosan is often claimed to be GRAS (Generally Recognized As Safe) and bioabsorbable (Bellich et al., 2016). These characteristics enable chitosan to have application in various fields. For example, chitosan is used in medicine (as antibacterial lining for the bandages and wound dressing), in agriculture (for coatings for seeds to enhance disease resistance), in food industry (as food preservative,





active edible packaging, dietary fibre) (Hamed et al., 2016; Van den Broek et al. 2015). Chitosan is also advertised as an dietary supplement for weight-loss, since it was shown to bind fat and hence chitosan should prevent fat digestion in human body (Bellich et al., 2016).

Chitosan prices depend on the quality of the product given mainly by chitosan's degree of deacetylation, Mw, water solubility, viscosity and residual protein, pigment and mineral (Gomez-Rios et al., 2017). The demand for chitosan is increasing every year. In 2010, the global market for chitosan was estimated at 0.1 million tons, while in 2015 0.2 million tons were produced (<http://2014.igem.org>). Global chitosan market size is expected to reach 2.2 million € by 2022 from 1.1 million € in 2015 (<https://www.alliedmarketresearch.com/>).

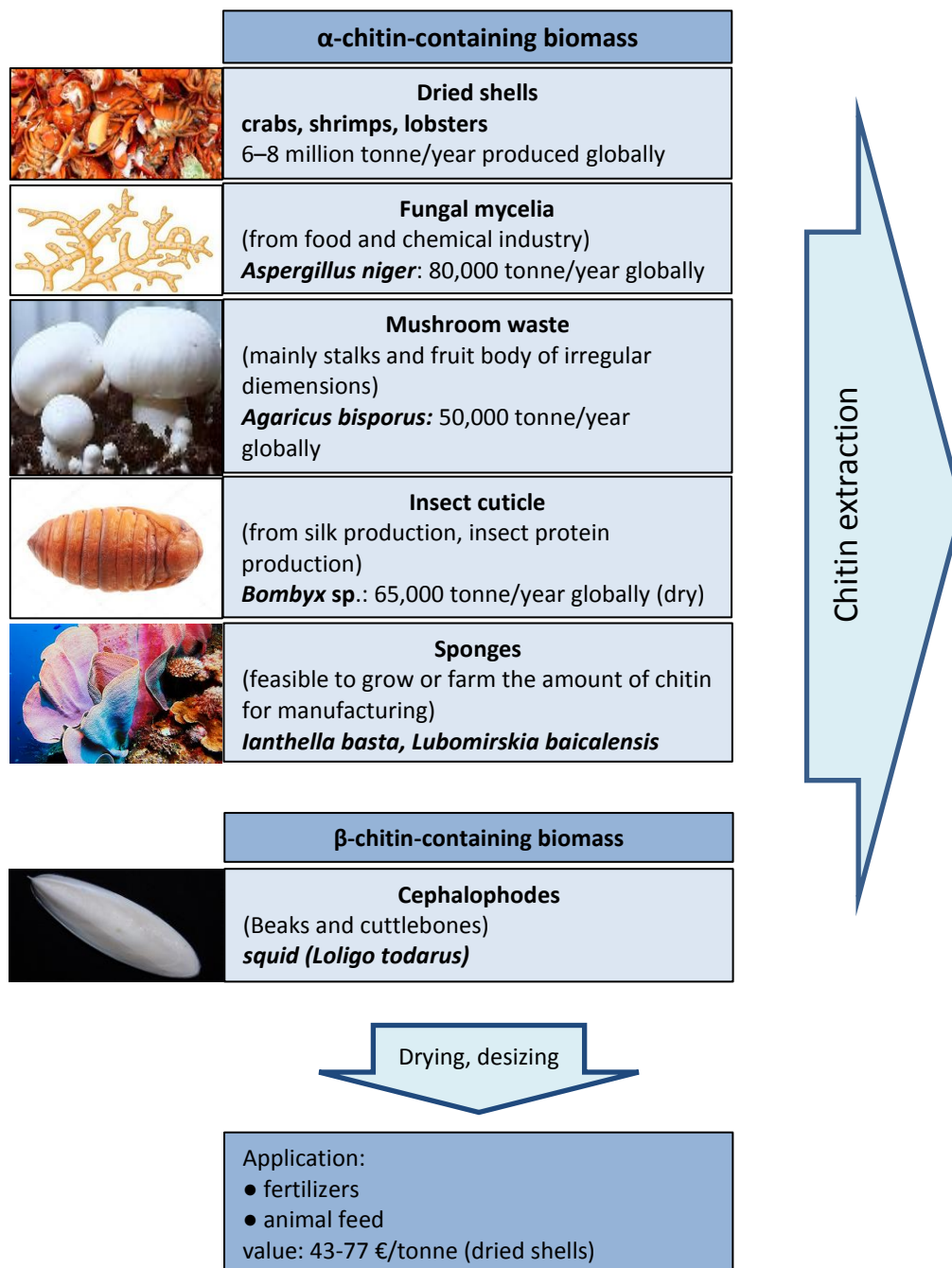
Both chitin and chitosan can be hydrolysed to their oligosaccharides (CHOs) and monomers (GlcNAc and GlcN) (Figure 6.2.). Hydrolysis is traditionally performed with chemical methods using a strong acid as catalyst (e.g. HCl) which breaks the glycosidic bonds in chitin and chitosan polymers (Hamed et al., 2016). CHOs have a polymerisation degree (DP) less than 20 and have an average molecular weight less than 3.9 kDa (Lodhi et al., 2014). GlcNAc and GlcN are produced by chemical hydrolysis by direct depolymerisation or by depolymerisation and de-acetylation of GlcNAc to GlcN and subsequent re-acetylation to GlcNAc with acetate anhydride (Bohlman et al., 2004; Mojarrad et al., 2007; Roseman and Ludowieg, 1954).

There is an increasing interest in CHOs, GlcNAc and GlcN as bioactive compounds (Liaqat and Eltem, 2018). They are more suitable for some industrial applications than chitin and chitosan due to their low viscosity, low molecular weight, short chain lengths and water solubility (Hamed et al., 2016; Liaqat and Eltem, 2018). CHOs are mostly used for research purposes, but they are also promising cholesterol lowering, anticancer, anti-bacterial, and immuno-enhancing agents (Liaqat and Eltem, 2018). CHOs are also used for enhancing plant growth (Smith and Habib, 2011) and as elicitors of plant defence (Liaqat and Eltem, 2018). GlcNAc and GlcN are widely used for treatment of joint diseases (e.g. osteoarthritis) and inflammatory bowel disease in children (Salvatore et al., 2000). GlcNAc was shown to have a potential effect on the cartilage metabolism at a lower dose (500–1,000 mg/day) compared with GlcN (1,500 mg/day) (Kubomura et al., 2017). The European Society for Clinical and Economic Aspects of Osteoporosis and Osteoarthritis (ESCEO) recommends a treatment of a chronic symptomatic low-acting drugs for osteoarthritis (SYSADOAs) based on glucosamine sulphate as a first-line

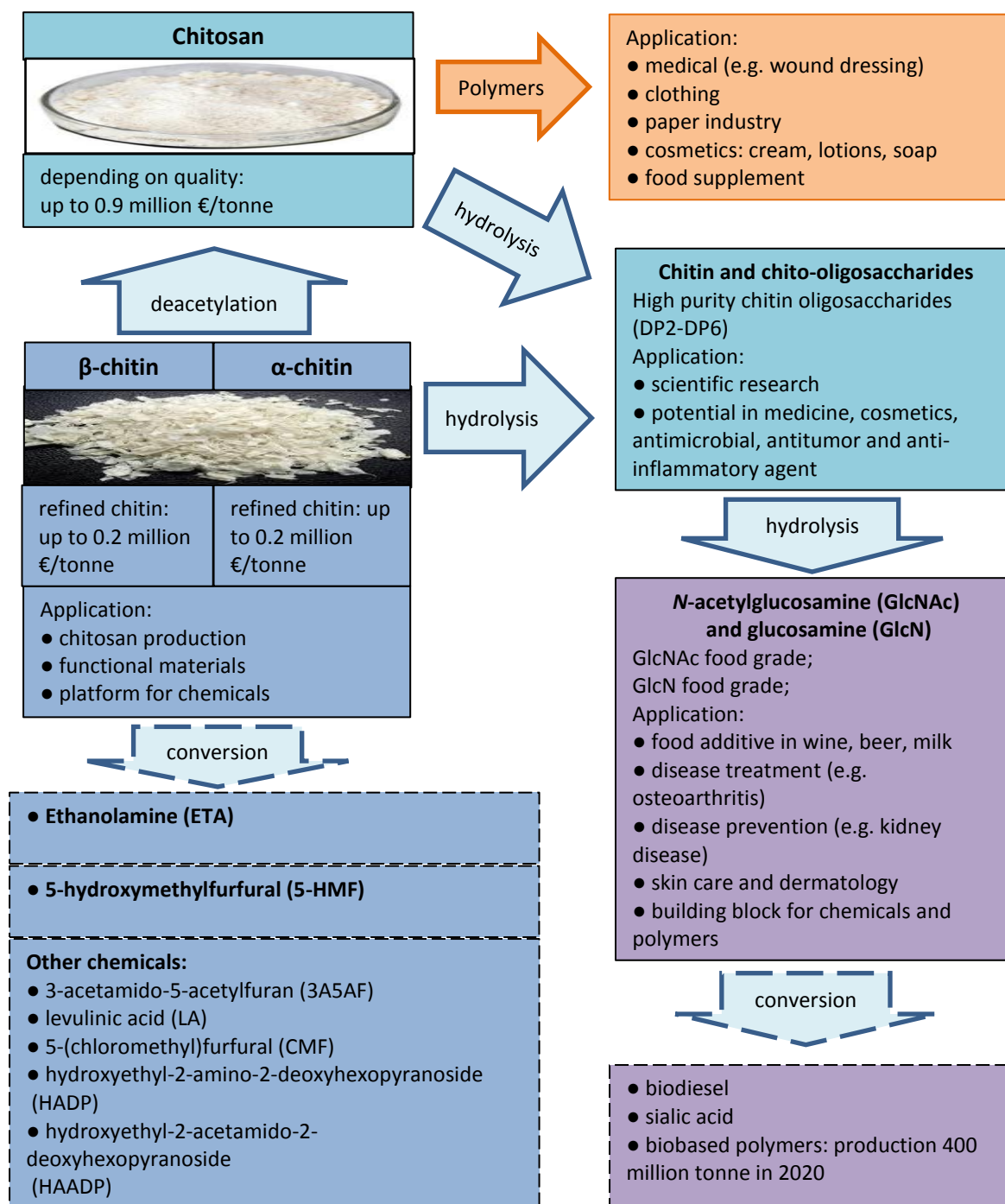
therapy (Bruyère et al., 2016). In cosmetics, GlcNAc is a valuable ingredient for improving skin quality (Riodan, 1999; Bisset et al., 2007). In food industry, GlcNAc is used as an additive in beer, milk and wine (Xu et al., 2004a, 2004b, 2004c). Recently, GlcNAc was proposed as a biological C6 source for bioethanol production through fermentation (Inokuma et al., 2013). Other promising valorisation of the monomers is their application as platform chemicals for synthesis of high-value chemicals like ethanolamine (ETA) and 5-hydroxymethylfurfural (5 HMF) among others (Figure 6.2.). ETA is used in power plants for CO<sub>2</sub> sequestration and in skin-friendly household cleaners, soaps and surfactants (Yan and Chen, 2015). HMF is an important platform chemical as it can be converted into numerous sustainable chemicals such as alkoxymethylfurfurals, levulinic acid, adipic acid, furan-based monomers etc. which can be used to make different polymers (Putten et al., 2013). Thus more sustainable and economical feasible routes are needed for the growing demand of GlcNAc and GlcN.

	 <b>Cephalopods</b>	 <b>Crustaceans</b>	 <b>Insects</b>	 <b>Fungi</b>
<b>Industrial main stream</b>	meat	meat	<ul style="list-style-type: none"> <li>• silk production</li> <li>• meat</li> </ul>	<ul style="list-style-type: none"> <li>• organic acids production</li> <li>• brewing and baking</li> <li>• mushroom cultivation</li> <li>• enzymes production</li> <li>• antibiotic production</li> </ul>
<b>Industrial side stream</b>	Beaks and cuttlebones	shells	cuticle	Mycelia, mushroom waste
<b>BIOMASS composition</b>	26-50 % d.w. chitin 40-64 % d.w. proteins	15-40 % d.w. chitin 20-40 % d.w. proteins 0.4 % d.w. pigments 20-50 % d.w. CaCO <sub>3</sub>	up to 60 % d.w. chitin up to 50 % d.w. proteins up to 5 % d.w. lipids	14-16 % d.w. chitin 15-35 % d.w. glucans 2-3 % d.w. protein
<b>PROCESS</b>	Chemical or enzymatic extraction of components (biorefinery)			
<b>CONVERSION and APPLICATION</b>	glucans pigment (Astaxanthin) chitin proteins lipids calcium carbonate	→ food, medicine, pharmacy → food/ feed , cosmetics → chitosan, CHOs, GlcNAc, GlcN, functional materials → animal feed, fertilizers → high value oil, biodiesel, animal feed → minerals for agriculture, pharmaceuticals, construction and paper industries		

**Figure 6.1.** Valorisation of chitin-containing biomass within the biobased economy.



**Figure 6.2.** Application of chitin. Dashed lines indicate potential applications.



**Figure 6.2.** Application of chitin. Dashed lines indicate potential applications. (Continuation)

## 6.2. Enzymes able to produce monomers

It was already predicted 50 years ago, that chitinolytic enzymes would play a key role in the depolymerisation of chitin into its building blocks, however, there are still no commercially available cocktails of chitinolytic enzymes industrially applied for the production of GlcNAc. In contrast, the modern biotechnological research on lignocellulose, another biomass form industrial side streams, is very advanced and strategies for the biorefinery of lignocellulose have been developed. The monomers, glucose and xylose, are enzymatically obtained from the biomass and converted to bioethanol during fermentation and the lignin rich residues are burned for heat and electricity. Since 2014, the bioethanol from the lignocellulosic has been produced on a commercial scale by the companies GranBio and Raizen (Brazil), Poet & DSM and Aboengoa (US), and M&G (Italy) (Johansen, 2016).

The carbohydrate structure of chitin is very similar to the structure of cellulose. Both polymers are highly crystalline with tight H-bonding network between individual polymer chains, therefore they are very rigid and difficult to access by hydrolytic enzymes (Eijsink et al., 2008). The limited accessibility to the substrate directly influences the efficiency of enzymes and the overall economy of the process. Years ago, the hydrolysis of cellulose to fermentable sugars required 40–100 times more enzyme per gallon of ethanol than hydrolysis of corn starch, which is easily accessible for enzymes (Merino and Cherry, 2007). As a consequence, the costs of enzymes was a major factor that limited the profitability of producing cellulose-based bioethanol (Eijsink et al., 2008). It was concluded, that the technologies that produce more efficient enzymes would have beneficial effects for the biofuel industry, not only because they lower enzyme cost per gallon of ethanol but also because they might result in faster processing times, which, in turn, reduce capital investment per gallon of production capacity (Eijsink, 2008). The successful decrease of about 20-fold in the enzyme use cost played an important role in the economic viability of a biotechnological process for converting lignocellulose into ethanol (Johansen, 2016). This achievement gives faith, that also chitin can be used for production of GlcNAc in a sustainable economic feasible process with enzymes as biocatalysts.

More than ten years ago, research showed that rigidity of chitin indeed arises problems in chitin conversion. Degradation experiments of crystalline chitin with crude chitinolytic enzyme preparations from bacteria and fungi e.g. *Aeromonas hydrophila* H-2330, *Bacillus licheniformis*

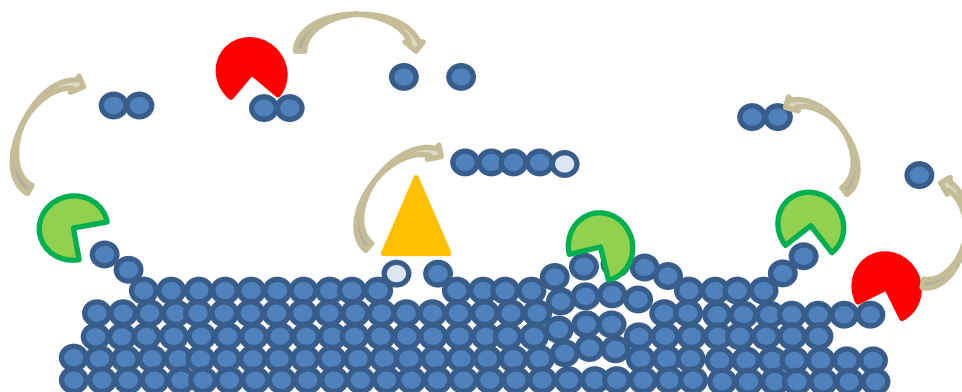


SK-1, *Burkholderia cepacia* TU09, *Aspergillus* sp. typically took few days and the yield of GlcNAc was still low (41–85 %) (Sashiwa et al., 2002; Pichyangkura et al, 2002; Setthakaset et al., 2008). The problem of chitin inaccessibility has been solved by development of pre-treatment methods, such as steam explosion or ball-milling, which considerably increased the accessibility of chitin and the hydrolytic efficiency of enzymes (Lee et al., 1996; Villa-Lerma et al., 2013).

The search of efficient enzymes is still ongoing. Commonly, enzymes are obtained by screening different organisms producing the enzymes of interest. Microbial enzymes have the largest potential as biocatalyst since, on a large scale, the enzymes produced by microbial hosts (e.g. bacteria and fungi) are cost-effective due to high production levels associated with standard expression, inexpensive culture media, ease of growth, and short fermentation cycles (Rigoldi et al., 2018).

For a long time, the accepted model for enzymatic degradation of chitin has been based on hydrolytic enzymes (glycosyde hydrolases, GH), where chitinases (EC 3.2.1.14) hydrolyse chitin into chitin oligosaccharides (mainly dimers) of GlcNAc, which are subsequently degraded by *N*-acetylglucosaminidases (NAGase; EC 3.2.1.52), producing GlcNAc monomers (Hoel et al., 2010). Some NAGases were also found to work on polymeric chitin (Konno et al., 2012) and chitinases to work on chitosan (Ohtakara et al., 1990). However, the conventional hydrolytic model has been challenged for the past few years, when in 2010, Vaaje-Kolstad et al., reported that the protein CBP21 from *Serratia marcescens* degrades crystalline chitin in an oxidative way introducing breaks in chitin chains and releases oxidized chitin oligosaccharides. Currently CBP21 is classified as a lytic polysaccharide monooxygenases *Sm*LPMO10A (LPMO; EC 1.14.99.53). The activity of LPMOs is very appreciated, since they do not only open up the crystalline structure and make it more accessible for chitinase and NAGase but also release chitin oligosaccharides, which can be further degraded by hydrolases (Figure 6.3.). LPMOs working on chitin have been showed to have a rescuing effect on hydrolytic enzymes which tend to get stuck on the substrate during the degradation process (Vaaje-Kolstad et al., 2005). The concerted work of chitinases, NAGases and LMPOs represents a whole picture of chitin degradation to GlcNAc. LPMOs were shown to decrease the enzyme load of hydrolases and due to their superior effects on polymer degradation, and therefore LPMOs are used as an

important component in commercial enzyme cocktails for production of bioethanol from lignocellulose.



**Figure 6.3.** Enzymatic degradation of chitin with chitinolytic enzymes. Chitinases (green enzymes), *N*-acetylglucosaminidases (NAGases) (red enzymes) and lytic polysaccharide monooxygenases (LPMO (yellow enzymes). Blue circle indicates GlcNAc unit. Light blue circle indicates oxidized GlcNAc unit.

Since the production of bioethanol from lignocellulose-rich biomass reached the industrial scale, it is worth to take few lessons from the approach used in the bioethanol production, and apply them in the production of GlcNAc from chitin. However, before the application of LPMOs for the production of GlcNAc from chitin, few things have to be considered. First of all, all enzymatic components should work in synergy. It was reported, that synergy between enzymes can differ depending on the structural form of chitin. For  $\beta$ -chitin, the presence of *Sm*LPMO10A (CBP21) had no or little effect on the endo-acting chitinase ChiC from *S. marcescens*, while *Sm*LPMO10A greatly enhanced the initial rate of exo-acting chitinases ChiA and ChiB (Hamre et al., 2015). On the other hand Nakagawa et al. noticed that *Sm*LPMO10A hardly influenced the activity of ChiA on  $\alpha$ -chitin (Nakagawa et al., 2013). Secondly, an important aspect of enzymatic synergy are conditions, which should be optimal for all enzymes i.e. hydrolases and LPMOs have to work optimal under the same conditions. It should be noted, that LPMOs work in an oxidative way and they release oxidized products (lactones), which have to be removed after the reaction. Furthermore, it was observed that in the absence of the substrate LPMOs release superoxide as a product of each redox cycle (Kjaergaard et al., 2014). Superoxide is spontaneously transformed into hydrogen peroxide ( $H_2O_2$ ) that reacts with transition metals such as iron and copper to form highly toxic hydroxyl radicals and other reactive oxygen species. These toxic radicals may be very harmful for other enzymes in the enzyme cocktail. Destructive oxidative side reactions may be reduced by improving the binding capability of enzyme to the substrate by an introduction of

carbohydrate binding modules (CBMs) to LPMO (Isaksen et al., 2014; Kittl et al., 2012). CBMs are a class of sugar-binding proteins typically found as a part of a larger multi-modular enzyme. The conventional role of a CBM is to bind to carbohydrate substrate and direct the catalytic machinery onto its substrate, thus enhancing the catalytic efficiency of the enzyme (Boraston et al., 2004). Another option to decrease the harmful effect of  $H_2O_2$  is the addition of the enzyme catalase to the reaction mixture, the enzyme which converts  $H_2O_2$  to water and oxygen (Scott et al., 2016). Therefore, the supplementation of LPMOs probably need an extra enzyme that has no direct role in chitin degradation. From a technical point of view, there will be an adjustment of bioreactors needed for good oxygen transfer, since LPMOs will suffer from the lack of oxygen (Ezeilo et al., 2017). Finally, the cost of the process might be decreased through the addition of LPMOs, when LPMOs would prove their effect on decreasing the overall enzyme load.

However, the research on LPMOs is still ongoing. Currently, nine from 24 known LPMOs showed activity on chitin, of which one enzyme AoLPMO11A originates from the fungus *Aspergillus oryzae* (Vaaje-Kolstad, 2017; Hemsworth et al., 2014). Many sequences of other fungal LPMOs working on chitin have been found, but experimental data is missing (Berrin et al., 2017).

A very important aspect of efficiency of the enzymes is their stability. The enzyme stability belongs to the “application-wise” characteristics, which are desirable for industrially applicable enzymes. Currently, more attention is paid to the increased thermostability rather than to “conventional” parameters such as temperature and pH profiles (Harris et al., 2014). In general, increased thermostability is needed for enzymes to survive industrial settings and for flexibility with respect to the process configurations. Such a flexibility allows improvement of the hydrolysis performance particularly of the hydrolysis of polymeric substrates as chitin. Therefore, the thermostable enzymes are an ideal option for hydrolysis of chitin to GlcNAc.

Thermostable enzymes can be obtained from bacteria and fungi. Thermophilic fungi have already been used for production of diverse thermophilic enzymes at industrial-scale for biomass degradation (Harris et al., 2014) and they might be the promising enzyme producers for chitinolytic enzymes.

Chitinolytic enzymes were characterised from many fungal strains, of which some are classified as thermophilic belonging to the genera *Aspergillus* sp. (Pera et al., 1997; Xia et al., 2001), *Chaetomium* sp. (Li et al., 2010), *Gliocladium* sp. (Ma et al., 2012), *Rhizopus* sp. (Chen et al., 2013), *Myceliophthora* sp. (Dua et al., 2016), *Thermoascus* sp. (Li et al., 2010), *Thermomyces* sp. (Guo et al., 2008; Prasad and Palanivelu, 2012; Zhang et al., 2015), *Trichoderma* sp. (Ulhoa and Peberdy, 1991; Lorito et al., 1994; Lisboa De Marco et al., 2004; Koga et al., 1991). Chitinolytic enzymes obtained from the industrially used thermophilic fungal strains like *Aspergillus* and *Trichoderma* lose their enzymatic activity drastically after incubations in the range of minutes to 1 hour at optimal or higher temperatures. Therefore, there is a need to search for enzymes with improved thermostability.

In this thesis a chitinase (Chitinase Chi1) and a NAGase (*MthNAG*) from *Myceliophthora thermophila* C1 were characterized. Both enzymes showed increased thermostability. In comparison to other fungal chitinolytic enzymes, Chitinase Chi1 and *MthNAG* are the only enzymes, which are stable up to 1 week at their optimum temperature at 50–55 °C up till now (**Chapter 2** and **Chapter 3**). The thermostability of Chitinase Chi1 and *MthNAG* can lead to the improvement of the overall economy of the production of GlcNAc from chitin as it was shown for thermostable enzymes used for lignocellulose hydrolysis (Viikari et al., 2007). The high specific activity of Chitinase Chi1 and *MthNAG* will decrease the amount of enzymes needed for chitin hydrolysis. Chitinase Chi1 and *MthNAG* were shown to be able to degrade polymeric chitin to GlcNAc in a synergistic way. This synergy is important for an efficient production process, where dimers released by Chitinase Chi1 are further degraded to two GlcNAc units.

Chitinase Chi1 and *MthNAG* are hydrolases and their efficiency relies on the accessibility of chitin and chitin oligosaccharides. These enzymes hardly access the crystalline structure of commercial chitin, even in the reduced and more or less uniform size like flakes and practical grade powder. In this form, enzymes can work only on the surface of the chitin particles but are not be able to penetrate the particles (Jaworska and Roberts, 2016). We showed that Chitinase Chi1 efficiently degraded chitin oligosaccharides and amorphous parts of swollen chitin (**Chapter 2**) and released (GlcNAc)<sub>2</sub> which were efficiently degraded by *MthNAG* (**Chapter 3**). Chitinase Chi1 and *MthNAG* synergistically hydrolysed swollen chitin, however, the yield of GlcNAc, 37.8 % after 24 h incubation, indicated that the swollen chitin (pre-treated

with phosphoric acid) is not a suitable substrate (**Chapter 3**). Furthermore, the use of excess amount of phosphoric acid is not an environmentally friendly approach.

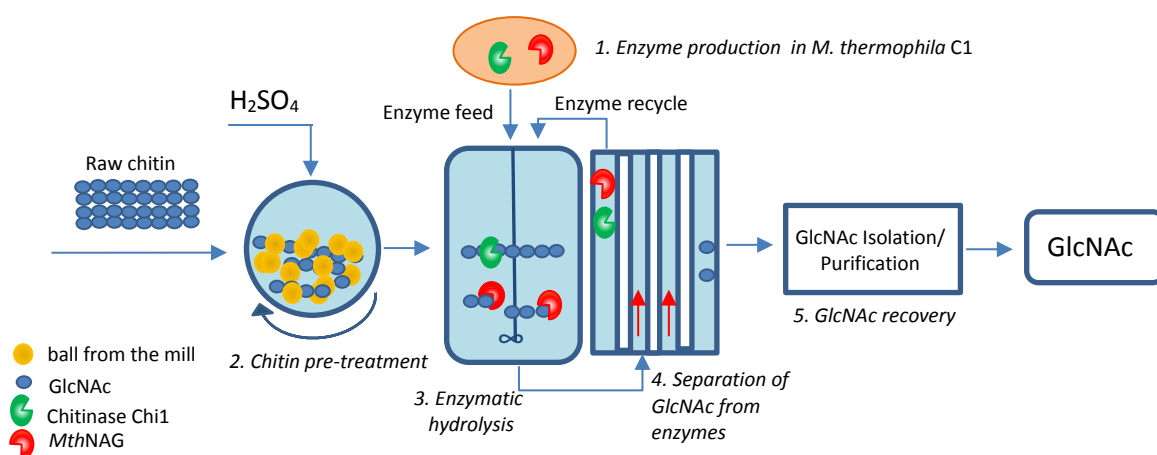
Different treatment methods to process chitin biomass have been reported e.g. ball mill grinding, steam explosion, alkaline treatment, phosphoric acid treatment and dissolution in ionic liquids (Chen et al., 2015), which endow chitin with substantially different structure changes and reactivity. Chen et al. (2015), showed that the ball milling is by far the most effective method to destroy the crystalline regions and the networks of the polymer chains in chitin.

In this thesis, we used a ball milling for amorphization and for conversion of chitin to soluble oligosaccharides as described by Yabushita et al. (2015). In this method the ball milling was combined with the impregnation of chitin with low amount of H<sub>2</sub>SO<sub>4</sub> (the molar amount of chitin based on GlcNAc units was 8.1 times higher than the molar amount of H<sub>2</sub>SO<sub>4</sub>) which resulted in the selective cleavage of glycosidic bonds between the GlcNAc units rather than amide bonds of the acetyl group at GlcNAc. The obtained amorphous chitin (ball-milled-H<sub>2</sub>SO<sub>4</sub>-chitin) had fully destroyed crystallinity and contained water soluble chitin oligosaccharides, with DP2 to DP12 (**Chapter 4**). A very low amount GlcNAc (DP1) was found in the treated chitin (0.8 % w/w) thus further enzymatic action of Chitinase Chi1 and *Mth*NAG were essential for production of high amounts of GlcNAc. We showed that ball-milled-H<sub>2</sub>SO<sub>4</sub>-chitin is efficiently hydrolysed to GlcNAc by Chitinase Chi1 and *Mth*NAG, with conversion as high as 73.2 % (**Chapter 4**). The highest yield of the GlcNAc production was obtained at optimal enzyme load and Chitinase Chi1/*Mth*NAG activity ratio that were determined by process optimization with Central Composite Design (CCD) and Response Surface Analysis (RSA). Further investigation showed, that the increase of the concentration of substrate (from 1.6 to 5.0 % w/w) leads to a higher production of GlcNAc in the process. Finally, the crude enzyme preparation of Chitinase Chi1 and *Mth*NAG hydrolysed ball-milled-H<sub>2</sub>SO<sub>4</sub>-chitin as efficient as purified enzymes, indicating that the crude preparations may be used in the process without purification.

### 6.3. A process design to enzymatically produce GlcNAc monomers on large scale

Here, we present a “proof-of-concept”, that an improved enzymatic method can be beneficial over chemical methods. The most important advantage of these enzymes instead of acids is their selectivity in cleaving glycosidic bonds between GlcNAc units without destroying the final products. Additionally, the enzymes work efficiently under milder conditions than that used for chemical process.

Here we propose a setup for the process based on hydrolysis of ball-milled- $\text{H}_2\text{SO}_4$ -chitin with Chitinase Chi1 and *Mth*NAG obtained from *M. thermophila* C1 (Figure 6.4.). The proposed process is an integration of five operation units: (1) production of the enzymes Chitinase Chi1 and *Mth*NAG in *M. thermophila* C1, (2) chitin pre-treatment, (3) enzymatic hydrolysis of chitin, (4) separation of GlcNAc from Chitinase Chi1 and *Mth*NAG, and (5) GlcNAc recovery.



**Figure 6.4.** Production process for GlcNAc based on hydrolysis of chitin with Chitinase Chi1 (green enzyme) and *Mth*NAG (red enzyme) from *Myceliophthora thermophila* C1. 1. Production of enzymes Chitinase Chi1 and *Mth*NAG in *M. thermophila* C1, 2. Chitin pre-treatment with  $\text{H}_2\text{SO}_4$  impregnation and ball-milling and preparation of ball-milled- $\text{H}_2\text{SO}_4$ -chitin, 3. Hydrolysis of ball-milled- $\text{H}_2\text{SO}_4$ -chitin with Chitinase Chi1 and *Mth*NAG in continuous stirred bioreactor, 4. Cross-flow filtration for separation of GlcNAc from Chitinase Chi1 and *Mth*NAG, 5. GlcNAc recovery with crystallization, filtration and drying.

#### (1) Production of Chitinase Chi1 and *Mth*NAG

*M. thermophila* C1 is used to produce chitinolytic enzymes. This filamentous fungus has been developed to a low protease/(hemi-) cellulase free *M. thermophila* C1-expression host (LC-strain) as a proprietary mature enzyme production system. It was reported, that the developed genetic tools for *M. thermophila* C1 enable overexpression of homologous and

heterologous proteins with high production levels up to 100 g L<sup>-1</sup> (Visser et al., 2011). Therefore, *M. thermophila* C1 is an appropriate enzyme producing host that can ensure a high production level of chitinolytic enzymes suitable for industrial processes. We successfully cloned and overexpressed homologous genes encoding for Chitinase Chi1 and *Mth*NAG (**Chapter 2** and **Chapter 3**) in new single-component *M. thermophila* C1 strains based on LC-strain. In the crude enzyme preparations Chitinase Chi1 and *Mth*NAG represented about 60 % and 52 % of the total protein, respectively. The removal of other enzymes and proteins through a purification process was needed for characterisation of Chitinase Chi1 and *Mth*NAG (**Chapter 2** and **Chapter 3**). Particularly the separation of Chitinase Chi1 from a NAGase present in the crude enzyme preparation of Chitinase Chi1 was crucial to determine the correct activity and mode of action (**Chapter 2**). Homologous overexpression of proteins, i.e. production of enzymes in the host of which the gene encoding the enzyme is obtained, ensures correct maturing of the enzyme regarding posttranslational modification including enzyme folding and glycosylation. Heterologous production of enzymes may influence the structure and activity of the produced enzyme. The gene encoding for Chitinase Chi1 has been heterologously produced in *Pichia pastoris* by Dua et al. (2016) and the protein obtained by Dua et al. was designated as exochitinase rMtChit. The mode of action, glycosylation level and thermostability of rMtChit differed from that of Chitinase Chi1 (more discussed in **Chapter 2**). Therefore, our approach with homologous enzyme production enabled us to produce Chitinase Chi1 and *Mth*NAG with their natural characteristics and in this case beneficial properties.

## (2) Chitin pre-treatment

The ball milling is commonly used at industrial scale for desizing of numerous types of materials, for example, cocoa products in the food industry. According to the supplier (Vekamaf Services B.V., Rotterdam, Netherlands), ball mills have many benefits including excellent product fineness and homogeneity, extremely low energy consumption, and consistent product quality. Therefore, we conclude that the pre-treatment of chitin with ball milling can be cost effective and time efficient pre-treatment method for chitin. The pre-treated chitin will be added to the bioreactor either continuously or semi-continuously to maintain an optimum chitin concentration in the bioreactor.

### (3) Enzymatic hydrolysis of chitin

The hydrolysis of ball-milled-H<sub>2</sub>SO<sub>4</sub>-chitin with Chitinase Chi1 and *Mth*NAG can be conducted in a continuous stirred bioreactor for 6 hours. Chitinase Chi1 was most active at pH 6.0 and 55 °C and *Mth*NAG was most active at pH 4.5 and 50 °C (**Chapter 2** and **Chapter 3**). Therefore, the bioreactor will be maintained at pH 5.0 and an isothermal bioreactor temperature between 50 and 55 °C with mixing 800 rpm. These conditions of enzymatic hydrolysis are much milder than that used for chemical hydrolysis. From the operational point of view, milder conditions of hydrolysis are not so corrosive to the bioreactor as concentrated acids. The application of relatively high temperatures has an additional advantage i.e. high temperature suppresses microbial-contamination of the resulting GlcNAc product with most mesophilic bacteria, fungi, and yeast (Haynes et al., 1999).

The use of more concentrated substrates is a good approach for an efficient use of the production space and increase of productivity. As shown in **Chapter 4**, the hydrolysis of the increased amount of substrate should be possible, since the sample containing 5 % (w/v) ball-milled-H<sub>2</sub>SO<sub>4</sub>-chitin was not viscous and should not result in problems from an operational point of view. However, in order to be economically feasible, the initial concentration of solids should be above 20 % dry matter. Therefore, additional experiments for the reaction mixture with such a high concentration should be performed.

### (4) Separation of GlcNAc from Chitinase Chi1 and *Mth*NAG

After the hydrolysis of ball-milled-H<sub>2</sub>SO<sub>4</sub>-chitin with Chitinase Chi1 and *Mth*NAG performed in the bioreactor, the GlcNAc product has to be separated from the enzymes. An appropriate mean for separation is a continuous cross-flow ultrafiltration system (cross-flow membrane filtration), since this technology has been used widely in industry for example for extraction of soluble antibiotics from fermentation liquors. In this type of ultrafiltration the reaction mixture travels tangentially across the surface of the filter (Koros et al., 1996). The advantage of ultrafiltration over the standard precipitation method is that the enzymes can be continuously recycled. In case of precipitation with e.g. ammonium sulphate, enzymes are separated through dead-end filtration and they need to be recovered, what could lead to the decrease of their activity. Using cross-flow membrane filtration, a good separation of GlcNAc from Chitinase Chi1 and *Mth*NAG can be obtained with a membrane with a 10 kDa Mw cut-off. Chitinase Chi1 has Mw of 42 kDa and *Mth*NAG has Mw of 71 kDa therefore during the



filtration both enzymes are retained by the filter in the retentate, while GlcNAc molecules penetrate the filter pores to the permeate. No protein contaminants are expected in the permeate when the enzymes have been purified. In the case of crude enzyme preparations, separation of enzymes/proteins from the produced GlcNAc could be performed by the traditional precipitation and dead-end filtration or centrifugation. Application of either purified or crude enzymes should be taken into account for calculations of the costs of the process to balance costs of purification and costs of separation. The separated enzymes can either be recycled to the bioreactor or recovered for later use. Due to the general good stability, Chitinase Chi1 and *Mth*NAG can be recycled numerous times. However, it has to be investigated how many times the enzymes (purified or in their crude extracts) can be recycled and used in the process. Recycled enzymes will be added to the bioreactor and eventually a portion of fresh enzyme will be added to compensate for enzyme deactivation with time. The permeate stream containing the GlcNAc product will be directed to a GlcNAc recovery unit.

#### (5) GlcNAc recovery

In the recovery unit, GlcNAc product can be separated from the solution using a crystallization method. GlcNAc present in the solution will be precipitated by addition of ethanol as an antisolvent. Precipitated GlcNAc will be separated from water by centrifugation and crystals of GlcNAc will be finally dried. Crystallization from solutions is a common method of separation and purification of products and is used for example, in the pharmaceutical industry for the isolation and synthesis of pure active pharmaceutical ingredients (Rohani, 2010) or in the food industry for production of glucose (Alves et al., 2007). Products obtained are characterised by high purity and uniform crystals (Alves et al., 2007).

### 6.4. Conclusions and perspectives

GlcNAc plays an important role in human lives as an component of human body and has numerous applications as food supplement and bioactive ingredient in pharmaceutical and medical applications. In the last years, the role of GlcNAc as a promising building block for biobased chemicals and materials, e.g. ETA, 5-HMF, polyamides, has been proven. Currently, GlcNAc is produced based on depolymerization of chitin with corrosive chemical catalysts (e.g. HCl). This process is environmentally unfriendly and studies are carried out to develop more efficient and suitable methods. A promising alternative is depolymerisation of chitin with

specific biological catalysts – enzymes. Chitinolytic enzymes work under milder conditions and are more specific than chemical catalysts, and do not cause environmental pollutions since they are biodegradable and catalyse a process with low waste.

However, application of an enzymatic method for depolymerization of chitin for GlcNAc production faces few challenges i.e. efficiency of enzymes, crystallinity and rigidity of chitin and process setup. Below we present a short summary of the preferences and considerations for each aspect:

- 1) Enzymes (preferably of microbial origin, thermostable) and their amounts (designed with process design tools)
- 2) Pre-treatment of  $\alpha$ -chitin from crustacean shells (suitable for amorphization; cost effective; possible to be scaled up) or use of  $\alpha$ -chitin with lower crystallinity (obtained from insects and fungi)
- 3) Process setup (economic calculations for application of process units e.g. bioreactors, filters; reuse reactants e.g. enzymes; application of an efficient separation unit for separation of produced GlcNAc from enzymes and unconverted material for high purity of the product)

In this thesis different aspects have been investigated for the enzymatic production of GlcNAc. Two chitinolytic enzymes Chitinase Chi1 and NAGase *MthNAG* obtained from *Myceliophthora thermophila* C1, a thermophilic fungus have been studied. Both enzymes showed high thermostability and specific mode of action. Chitinase Chi1 and *MthNAG* depolymerized chitin in a synergistic way i.e. the products released by Chitinase Chi1 were degraded by *MthNAG*. The combination of the thermostability and ability to a synergistic degradation of chitin make Chitinase Chi1 and *MthNAG* promising catalysts for production of GlcNAc from chitin at industrial scale.

## References

- Ahmed, S., Ikram, S., 2017. Chitin and chitosan: history, composition and properties. In Ahmed, S. and Ikram, S.(ed) Chitosan: Derivatives, Composites and Applications. Scrivener Publishing LLC, Beverly, USA, pp. 1–24
- Alves, L.A., Almeida e Silva, J.B., Giuliatti, M., 2007. Solubility of D-glucose in water and ethanol/water mixtures. J. Chem. Eng. Data 52, 2166–2170
- Arbia, W., Arbia, L., Adour, L., Amrane, A., 2013. Chitin extraction from crustacean shells using biological methods – a review. Food Technol. Biotechnol. 51, 12–25
- Bellich, B., D’Agostino, I., Semeraro, S., Gamini, A., Cesàro, A., 2016. “The Good, the Bad and the Ugly” of chitosans. Mar. Drugs 14, 99
- Berrin, J.-G., Rosso, M.-N., Hachem, M.A., 2017. Fungal secretomics to probe the biological functions of lytic polysaccharide monooxygenases. Carbohydr. Res. 448, 155–160
- Bissett, D.L., Robinson, L.R., Raleigh, P.S., Miyamoto, K., Hakozaiki, T., Li, J., Kelm, G.R., 2007. Reduction in the appearance of facial hyperpigmentation by topical N-acetylglucosamine. J. Cosmet. Dermatol. 6, 20–26
- Bohlman, J.A., Schisler, D.O., Hwang, K.O., Henning J.P., Trinkle, J.R., Anderson, T.B., Steinke, J.D., Vanderhoff, A., 2004. N-acetyl-D-glucosamine and process for producing N-acetyl-D-glucosamine. US Patent NO. 6693188B2
- Boraston, A.B., Bolam, D.N., Gilbert, H.J., Davies, G.J., 2004. Carbohydrate-binding modules: fine-tuning polysaccharide recognition. Biochem. J. 382, 769–781
- Bruyère, O., Cooper, C., Pelletier, J.P., Maheu, E., Rannou, F., Branco, J., Luisa Brandi, M., Kanis, J.A., Altman, R.D., Hochberg, M.C., Martel-Pelletier, J., Reginster, J.Y., 2016. A consensus statement on the European Society for Clinical and Economic Aspects of Osteoporosis and Osteoarthritis (ESCEO) algorithm for the management of knee osteoarthritis-from evidence-based medicine to the real-life setting. Semin. Arthritis Rheum. 45(4 Suppl), S3–11
- Chen, W.-M., Chen, C.-S., Jiang, S.-T., 2013. Purification and characterization of an extracellular chitinase from *Rhizopus oryzae*. J. Mar. Sci. Technol. 21, 361–366
- Chen, X., Gao, Y., Wang, L., Chen, H., Yan, N., 2015. Effect of treatment methods on chitin structure and its transformation into nitrogen-containing chemicals. ChemPlusChem 80, 1565–1572
- Day, J., Hughes, A., Greenhill, L., Stanley, M.S., 2016. Blue Biotechnology. Commonwealth Blue Economy Report Series, No. 5. Commonwealth Secretariat: London.
- Dua, A., Joshi, S., Satyanarayana, T., 2016. Recombinant exochitinase of the thermophilic mould *Myceliophthora thermophila* BJA : Characteristics and utility in generating N-acetylglucosamine and in biocontrol of phytopathogenic fungi. Biotechnol. Prog. 33, 70–80
- Eijsink, V.G.H., Vaaje-Kolstad, G., Vårum, K.M., Horn, S.J., 2008. Towards new enzymes for biofuels: lessons from chitinase research. Trends Biotechnol. 26, 228–235
- European Association for Bioindustries (EuropaBio). Building a Bio-based Economy for Europe in 2020; European Association for Bioindustries: Brussels, Belgium, 2011.
- Ezeilo, U.R., Zakaria, I.I., Huyop, F., Wahab, R.A., 2017. Enzymatic breakdown of lignocellulosic biomass: the role of glycosyl hydrolases and lytic polysaccharide monooxygenase. Biotechnol. Biotechnol. Equip. 31, 647–662
- Gómez-Ríos, D., Barrera-Zapata, R., Ríos-Estapa, R., 2017. Comparison of process technologies for chitosan production from shrimp shell waste: a techno-economic approach using Aspen Plus®. Food Bioprod. Process. 103, 49–57
- Guo, R.-F., Shi B.-S., Li, D.-C., Ma, W., Wei, Q., 2008. Purification and characterization of a novel thermostable chitinase from *Thermomyces lanuginosus* SY2 and cloning of its encoding gene. Agric. Sci. China. 7, 1458–1465
- Halder, S.K., Mondal, K.C., 2018. Microbial valorization of chitinous bioresources for chitin extraction and production of chito-oligomers and N-acetylglucosamine: trends, perspectives and prospects. In: J.K. Patra et al. (eds.), Microbial Biotechnology, pp. 69–107
- Hamed, I., Ozogul, F., Regenstein, J.M., 2016. Industrial applications of crustacean by-products (chitin, chitosan, and chitoooligosaccharides): a review. Trends Food Sci. Technol. 48, 40–50
- Hamre, A.G., Eide, K.B., Wold, H.H., Sørli, M., 2015. Activation of enzymatic chitin degradation by a lytic polysaccharide monooxygenase. Carbohydr. Res. 407, 166–169
- Harris, P.V., Xu, F., Kreel, N.E., Kang, C., Fukuyama, S., 2014. New enzyme insights drive advances in commercial ethanol production. Curr. Opin. Chem. Biol. 19, 162–170

- Haynes, C.A., Aloise, P., Creagh, A.L., 1999. Process for producing *N*-acetyl-D-glucosamine. US Patent NO 5998173A
- Hemsworth, G.R., Henrissat, B., Davies, G.J., Walton, P.H., 2014. Discovery and characterization of a new family of lytic polysaccharide mono-oxygenases. *Nat. Chem. Biol.* 10, 122–126
- Hoell, I.A., Vaaje-Kolstad, G., Eijsink, V.G.H., 2010. Structure and function of enzymes acting on chitin and chitosan. *Biotechnol. Genet. Eng. Rev.* 27, 331–366
- Inokuma, K., Takano, M., Hoshino, K., 2013. Direct ethanol production from *N*-acetylglucosamine and chitin substrates by *Mucor* species. *Biochem. Eng. J.* 72, 24–32
- Isaksen, T., Westereng, B., Aachmann, F.L., Agger, J.W., Kracher, D., Kittl, R., Ludwig, R., Haltrich, D., Eijsink, V.G.H., Horn, S.J., 2014. A C4-oxidizing lytic polysaccharide monooxygenase cleaving both cellulose and cello-oligosaccharides. *J. Biol. Chem.* 289, 2632–2642
- Jardine, A., Sayed S., 2016. Challenges in the valorisation of chitinous biomass within the biorefinery concept. *Curr. Opin. Green Sustain. Chem.* 2, 34–39
- Johansen, K.S., 2016. Discovery and industrial applications of lytic polysaccharide mono-oxygenases. *Biochem. Soc. Trans.* 44, 143–149
- Kittl, R., Kracher, D., Burgstaller, D., Haltrich, D., Ludwig, R., 2012. Production of four *Neurospora crassa* lytic polysaccharide monooxygenases in *Pichia pastoris* monitored by a fluorimetric assay. *Biotechnol. Biofuels* 5, 79
- Kjaergaard, C.H., Qayyum, M.F., Wong, S.D., Xu, F., Hemsworth, G.R., Walton, D.J., Young, N.A., Davies, G.J., Walton, P.H., Johansen, K.S., Hodgson, K.O., Hedman, B., Solomon, E.I., 2014. Spectroscopic and computational insight into the activation of O<sub>2</sub> by the mononuclear Cu center in polysaccharide monooxygenases. *Proc. Natl. Acad. Sci. USA* 111, 8797–8802
- Koga, K., Iwamoto, Y., Sakamoto, H., Hatano, K., Sano, M., Kato, I., 1991. Purification and characterization of  $\beta$ -*N*-acetylhexosaminidase from *Trichoderma harzianum*. *Agric. Biol. Chem.* 55, 2817–2823
- Konno, N., Takahashi, H., Nakajima, M., Takeda, T., Sakamoto, Y., 2012. Characterization of  $\beta$ -*N*-acetylhexosaminidase (LeHex20A), a member of glycoside hydrolase family 20, from *Lentinula edodes* (shiitake mushroom). *AMB Express.* 2, 29
- Koros, W.J., Ma, Y.H., Shimidzu, T., 1996. Terminology for membranes and membrane processes. *Pure & Appl. Chem.* 68, 1479–1489
- Kubomura, D., Ueno, T., Yamada, M., Tomonaga, A., Nagaoka, I., 2017. Effect of *N*-acetylglucosamine administration on cartilage metabolism and safety in healthy subjects without symptoms of arthritis: a case report. *Exp. Ther. Med.* 13, 1614–1621
- Lee, Y.-H., Bae, Y.-K., Jeong, E.-J., 1996. Enzymatic hydrolysis of crystalline chitin in agitated bead reaction system and its reaction characteristics. *J. Microbiol. Biotechnol.* 6, 432–438
- Li, A.-N., Yu, K., Liu, H.-Q., Zhang, J., Li, H., Li, D.-C., 2010. Two novel thermostable chitinase genes from thermophilic fungi: Cloning, expression and characterization. *Bioresour. Technol.* 101, 5546–5551
- Liaqat, F., Eltem, R., 2018. Chitooligosaccharides and their biological activities: a comprehensive review. *Carbohydr. Polym.* 184, 243–259
- Lisboa De Marco, J., Valadares-Inglis, M.C., Felix, C.R., 2004. Purification and characterization of an *N*-acetylglucosaminidase produced by a *Trichoderma harzianum* strain which controls *Crinipellis perniciosa*. *Appl. Microbiol. Biotechnol.* 64, 70–75
- Lodhi, G., Kim, Y.-S., Hwang, J.-W., Kim, S.-K., Jeon, Y.-J., Je, J.-Y., Ahn, C.-B., Moon, S.-H., Jeon, B.-T., Park, P.-J., 2014. Chitooligosaccharide and its derivatives: preparation and biological applications. *Biomed. Res. Int.* 1–13
- Lorito, M., Hayes, C.K., Di Pietro, A., Woo, S.L., Harman, G.E., 1994. Purification, characterization, and synergistic activity of a glucan 1,3- $\beta$ -glucosidase and an *N*-acetyl- $\beta$ -glucosaminidase from *Trichoderma harzianum*. *Mol. Plant Pathol.* 84, 398–405
- Ma, G.-Z., Gao, H.-L., Zhang, Y.-H., Li, S.-D., Xie, B.-Y., Wu, S.-J., 2012. Purification and characterization of chitinase from *Gliocladium catenulatum* strain HL-1-1. *Afr. J. Microbiol. Res.* 6, 4377–4383
- Mao, X., Guo, N., Sun, J., Xue, C., 2017. Comprehensive utilization of shrimp waste based on biotechnological methods: a review. *J. Clean Prod.* 143, 814–823
- McCormick, K., Kautto, N., 2013. The Bioeconomy in Europe: An Overview. *Sustainability* 5, 2589–2608
- Merino, S.T. and Cherry, J., 2007. Progress and challenges in enzyme development for biomass utilization. *Adv. Biochem. Eng. Biotechnol.* 108, 95–120
- Mills E., 2015. The Bioeconomy. *TNI and Hands on the Land.*

- Mojarrad, J.S., Nemati, M., Valizadeh, H., Ansarin, M., Bourbour, S., 2007. Preparation of glucosamine from exoskeleton of shrimp and predicting production yield by response surface methodology. *J. Agric. Food Chem.* 55, 2246–2250
- Nakagawa, Y. S., Eijsink, V.G.H., Totani, K., Vaaje-Kolstad, G., 2013. Conversion of  $\alpha$ -chitin substrates with varying particle size and crystallinity reveals substrate preferences of the chitinases and lytic polysaccharide monooxygenase of *Serratia marcescens*. *J. Agric. Food Chem.* 61, 11061–11066
- Ohtakara, A., Matsunaga, H., Mitsutomi, M., 1990. Action pattern of *Streptomyces griseus* chitinase on partially *N*-acetylated chitosan. *Agric. Biol. Chem.* 54, 3191–3199
- Pera, L.M., Infante Majolli, M.V., Baigori, M.D., 1997. Purification and characterization of a thermostable and highly specific  $\beta$ -*N*-acetyl-D-glucosaminidase from *Aspergillus niger* 419. *Biotechnol. Appl. Biochem.* 26, 183–187
- Pichyangkura, R., Kudan, S., Kuttiyawong, K., Sukwattanasinitt, M., Aibac S., 2002. Quantitative production of 2-acetamido-2-deoxy-D-glucose from crystalline chitin by bacterial chitinase. *Carbohydr. Res.* 337, 557–559
- Prameela, K. Venkatesh, Immandi, S.B., Kasturi, A.P.K., Krishna, Ch. R., Mohan, Ch.M., 2017. Next generation nutraceutical from shrimp waste: The convergence of applications with extraction methods. *Food Chem.* 237, 121–132
- Prasad, M., Palanivelu, P., 2012. Overexpression of a chitinase gene from the thermophilic fungus, *Thermomyces lanuginosus* in *Saccharomyces cerevisiae* and characterization of the recombinant chitinase. *J. Microb. Biochem. Technol.* 4, 86–91
- Putten, R., van der Waal, J., Jong, E., Rasrendra, C., Heeres, H., de Vries, J., 2013. Hydroxymethylfurfural, a versatile platform chemical made from renewable resources. *Chem. Rev.* 113, 1499–1597
- Rigoldi, F., Donini, S., Redaelli, A., Parisini, E., Gautieri, A., 2018. Review: Engineering of thermostable enzymes for industrial applications. *APL Bioeng.* 2, 011501
- Riordan, N.H., 1999. Skin treatment system. US Patent NO. 005866142A
- Rohani S., 2010. Applications of the crystallization process in the pharmaceutical industry. *Front. Chem. Eng. China* 4, 2–9
- Roseman, S., Ludowieg, J., 1954. *N*-Acetylation of hexosamines. *J. Am. Chem. Soc.* 76, 301–302
- Salvatore, S. Heuschkel, R., Tomlin, S., Davies, S.E., Edwards, S., Walker-Smith, J.A., French, I., Murch, S.H., 2000. A pilot study of *N*-acetylglucosamine, a nutritional substrate for glycosaminoglycan synthesis, in paediatric chronic inflammatory bowel disease. *Aliment. Pharmacol. Ther.* 14, 1567–1579
- Sashiwa, H., Fujishima, S., Yamano, N., Kawasaki, N., Nakayama, A., Muraki, E., Hiraga, K., Oda, K., Aiba, S., 2002. Production of *N*-acetyl-D-glucosamine from alpha-chitin by crude enzymes from *Aeromonas hydrophila* H-2330. *Carbohydr. Res.* 337, 761–763
- Setthakaset, P., Pichyangkura, R., Ajavakom, A., Sukwattanasinitt, M., 2008. Preparation of *N*-acetyl-D-glucosamine using enzyme from *Aspergillus* sp. *JMMM* 18, 53–57
- Scott, B.R., Huang, H.Z., Frickman, J., Halvorsen, R., Johansen, K.S., 2016. Catalase improves saccharification of lignocellulose by reducing lytic polysaccharide monooxygenase-associated enzyme inactivation. *Biotechnol. Lett.* 38, 425–434
- Smith, R.S., Habib, A., 2011. WO2013044208A2
- Ulhoa, C.J., Peberdy, J.F., 1991. Purification and characterization of an extracellular chitobiase from *Trichoderma harzianum*. *Curr. Microbiol.* 23, 285–289
- Vaaje-Kolstad, G., Horn, S.J., van Aalten, D.M., Synstad, B., Eijsink, V.G., 2005. The non-catalytic chitin-binding protein CBP21 from *Serratia marcescens* is essential for chitin degradation. *J. Biol. Chem.* 280, 28492–28497
- Vaaje-Kolstad, G., Westereng, B., Horn, S.J., Liu, Z.L., Zhai, H., Sørli, M., Eijsink, V.G.H., 2010. An oxidative enzyme boosting the enzymatic conversion of recalcitrant polysaccharides. *Science* 330, 219–222
- Vaaje-Kolstad, G., Forsberg, Z., Loose, J.S.M., Bissaro, B., Eijsink, V.G.H., 2017. Structural diversity of lytic polysaccharide monooxygenases. *Curr. Opin. Struct. Biol.* 44, 67–76
- Van den Broek, L.A.M., Knoop, R.J.I., Kappen, F.H.J., Boeriu, C.G. 2015. Chitosan films and blends for packaging material. *Carbohydr. Polym.* 116, 237–242
- Viikari, L., Alapuranen, M., Puranen, T., Vehmaanperä, J., Siika-aho, M., 2007. Thermostable enzymes in lignocellulose hydrolysis. *Adv. Biochem. Engin./Biotechnol.* 108, 121–145
- Villa-Lerma, G., González-Márquez, H., Gimeno, M., López-Luna, A., Bárzana, E., Shirai, K., 2013. Ultrasonication and steam-explosion as chitin pretreatments for chitin oligosaccharide production by chitinases of *Lecanicillium lecanii*. *Bioresour. Technol.* 146, 794–798
- Xia, G., Jin, C., Zhou, J., Yang, S., Zhang, S., Jin, C., 2001. A novel chitinase having a unique mode of action from *Aspergillus fumigatus* YJ-407. *Eur. J. Biochem.* 268, 4079–4085

- Xu, Q., J Liu, J., Yuan, Z., 2004a. The use of *N*-acetyl-D-glucosamine as additive in beer and beer comprising *N*-acetyl-D-glucosamine. WO Patent NO. 2004085603A1
- Xu, Q., J Liu, J., Yuan, Z., 2004b. The use of *N*-acetyl-D-glucosamine as additive in milk products. WO Patent NO. 2004093556A1
- Xu, Q., J Liu, J., Yuan, Z., 2004c. The use of *N*-acetyl-D-glucosamine as additive in wine and wine comprising *N*-acetyl-D-glucosamine. WO Patent NO. 2004085604A1
- Yan, N., Chen, X., 2015. Don't waste seafood waste. *Nature* 524, 155–157
- Yabushita, M., Kobayashi, H., Kuroki, K., Ito, S., Fukuoka, A., 2015. Catalytic depolymerization of chitin with retention of *N*-Acetyl group. *ChemSusChem* 8, 3760–3763
- Younes, I., Rinaudo M., 2015. Chitin and chitosan preparation from marine sources. structure, properties and applications. *Mar. Drugs* 13, 1133–1174
- Zhang, M., Puri, A.K., Govender, A., Wang, Z., Singh, S., Permaul. K., 2015. The multi-chitinolytic enzyme system of the compost-dwelling thermophilic fungus *Thermomyces lanuginosus*. *Process Biochem.* 50, 237–244
- Zhu, F., Du, B., Xu, B., 2016. A critical review on production and industrial applications of betaglucans. *Food Hydrocoll.* 52, 275–288

# Summary

Chitin is a natural biopolymer abundantly occurring in nature and is a main source for production of its monomer - *N*-acetylglucosamine (GlcNAc). GlcNAc is widely used in medical treatment of joints disorders e.g. osteoarthritis, in skin care and dermatology as moisture agent, in food as a food additive. In this thesis we present the development of an enzymatic process for production of GlcNAc from chitin with use of chitinolytic enzymes Chitinase Chi1 and *N*-acetylglucosaminidase (NAGase) *Mth*NAG from *Myceliophthora thermophila* C1.

In **Chapter 1** we describe the main current and future applications of GlcNAc. Also a general comparison of chemical and enzymatic production methods of GlcNAc is given. The market demand for GlcNAc is predicted to grow in coming years thus GlcNAc has to be produced in an efficient and environmentally friendly process. The enzymatic process seems a promising alternative for currently used chemical methods, if the problem of poor accessibility of chitin to the enzymatic action and the stability of the enzymes will be solved. The application of an efficient pre-treatment method of chitin and thermostable chitinolytic enzymes is a way to solve this problem.

In **Chapter 2 and Chapter 3** we describe the homologous overproduction of Chitinase Chi1 and *Mth*NAG in a low protease/(hemi-) cellulase free *M. thermophila* C1-expression host. Overproduced enzymes were purified and their mode of action, catalytic properties and thermostability were characterised. Both enzymes showed remarkable thermostability that has never been observed for this group of fungal enzymes. Chitinase Chi1 and *Mth*NAG synergistically hydrolysed chitin i.e. the products released from polymeric chitin by Chitinase Chi1 were hydrolysed by *Mth*NAG to a final product, GlcNAc. The synergistic action of Chitinase Chi1 and *Mth*NAG and their thermostability was fundamental for the development of an enzymatic production process of GlcNAc.

Due to the high crystallinity and strong hydrogen bonding network, chitin is poorly accessible for enzymes. Therefore, efficiency of an enzymatic production of GlcNAc from chitin can be enhanced by applying a proper pre-treatment of chitin prior to enzymatic reaction. In **Chapter 4** we investigated the production of GlcNAc from chitin impregnated with H<sub>2</sub>SO<sub>4</sub> and subsequently ball milled (ball-milled-H<sub>2</sub>SO<sub>4</sub>-chitin). According to the XRD measurements, the crystallinity of chitin has been drastically decreased upon ball-milling pre-treatment and the accessibility of chitin was considerably improved for the enzymatic action of Chitinase Chi1 and *Mth*NAG.



Subsequently, we optimized the production of GlcNAc from ball-milled-H<sub>2</sub>SO<sub>4</sub>-chitin based on a catalytic cascade catalysed by Chitinase Chi1 and *Mth*NAG. Using central composite design (CCD) and response surface methodology (RSM), we found conditions that allow production of GlcNAc at high yield. After 6 h incubation at 50 °C, the yield of GlcNAc of 73.2 % from 1.6 % (w/v) ball-milled chitin treated with 25 µg Chitinase Chi1 and 20 µg *Mth*NAG was obtained. The product contained approximately 93 % (mol) GlcNAc, next to short chitin oligosaccharides with DP 3, 4 and 5. Similar results were obtained in preparative experiments, at higher chitin load and reaction volume, proving the validity of the model. The high yield of GlcNAc obtained for Chitinase Chi1 and *Mth*NAG confirms the applicability of these enzymes in GlcNAc production from chitin.

In **Chapter 5** we describe the application of Chitinase Chi1 as a tool for a fingerprint analysis of (modified)chitosan. Since Chitinase Chi1 showed a wide substrate specificity (**Chapter 2**) and was active on chitosans with the degree of deacetylation (DDA) up to 94 %, the enzyme was used for hydrolysis of modified chitosans with different degree of oxidation (OD). The oligosaccharides released by Chitinase Chi1 were identified with MALDI-TOF-MS. Depending on the degree of oxidation (OD), different oligosaccharides were identified. The results were used for the elucidation of the structure of the oxidised chitosans and for understanding the behaviour (structure-function relation) of this polymers.

In **Chapter 6** we give an overview on chitin and chitosan as a biomass for monomers in the biobased economy. We showed the valorisation routes for chitin from different types of chitin-containing biomass and we gave an overview on current and potential applications of GlcNAc. Furthermore, we discussed the main drawbacks of the enzyme-based methods for the production of GlcNAc from chitin and we identified the possible solutions. Finally, we present a setup for the process based on hydrolysis of ball-milled-H<sub>2</sub>SO<sub>4</sub> chitin with Chitinase Chi1 and *Mth*NAG and give suggestions for the consideration of the important aspects of the enzymatic production of GlcNAc from chitin.



# Acknowledgments



At the end of my PhD-journey I would like to thank people who contributed and helped to the realisation of this thesis.

**Carmen, Ben and Gerrit**, thank you for being my supervisors. Carmen, thank you for giving me opportunity to start my PhD thesis working on the very interesting project. Thank you for your never ending willingness to help in every situation, especially in very difficult once. The door of your office was always open and you were always ready to give advice and share your experience. I have learnt a lot from you and you helped me to develop myself from a student to a scientist. **Ben**, I am very grateful for teaching me different measurement methods in the laboratory. I always appreciated your friendly way of teaching and patience that you showed towards me and other students. **Gerrit**, thank you for your feedback about my performance during our meetings. It helped me to realize that I was developing myself, even though I did not see it myself. Your “flying helicopter concept” helped me also to see things from a distance and to have a better overview on the situation. I tried to step in that helicopter from time to time during my PhD studies and it really helped.

**Sandra, Martijn and Jan Very**, I was very happy to have you as my supervisors at DuPont Biosciences (before, Dyadic NL). Thank you for your involvement in the project and for crucial comments. Also other people from “old Dyadic” **Mengying, Femke, Gonny, Cristina, Mary, Jurian, Rob, Theo, Jeffrey, Hans V., Hans K., Reijer, Krishna, Gudrun, Caroline, Robin, Aniek, Iris, Marianne, Jurgen, Robert, Petra, Sanaz, Laura, Wim**. I would like to thank for their help in the laboratory and for nice talks during coffee breaks.

During my PhD I was attending activities of two groups from Wageningen University & Research, namely **FBR** and **BPE**. For people from FBR, I am very thankful to **Guus, Marinella, Jan, Linda, Frits, Rajeesh, Shanmugam, Wouter T., Willem, Remco, Rutger, David** for your help in the laboratory and nice talks. Also, I would like to thank **Anamaria**, when you were at FBR for your research, it was great to have a friend in you! I would like to thank **Alniek** and **Yacinta** for your help with HPLC. **Emil, Jan Springer** and **Marc**, I would like to thank you for help with protein methods i.e. MALDI-TOF-MS and IEF. It was nice to be part of FBR and to attend Christmas Dinner with you!

I would like acknowledge the Master student **Syukur** that I supervised during my PhD. Thank you for your involvement!

Of course I cannot forget the activities like We Day, Labouitjes and Christmas Dinners with colleagues from BPE group **Vincent, Mitsue, Edgar, Jorijn, Ilse, Youri, Catalina, Jeroen, Enrico, Fabian, Gerard, Agi, Luci, Lenny, Aziz, Pauline, Kiira, Rafa, Stephanie, Iago, Giulia, Snezana, Fred, Sebastiaan, Wendy**. Thank you, **Richard**, for help with Akta system at BPE.

I would like to thank my friends from ICC for being great suppr to me **Esperanza, Gosia T., Gosia Ś., Hady, Henri, Jacek, John, Jorge, Laura, Marianne, Monica, Przemek, Sam, Stanislas, Tammy, Tomek, Wim**.

**Andrada** thank you for your support, sharing your knowledge with me, encouraging discussions and for many moments of fun!

**My family** for your support, encouragement and love. Thanks that you believed in me. **Mamo i Tato**, dziękuję Wam za wszystko co dla mnie zrobiliście, aby moje marzenie o doktoracie się spełniło.

**Aga i Witek**, dzięki, że jesteście moim wspólnym rodzeństwem. Zawsze doceniałam to, że w chwilach trudnych możemy na siebie polegać i trzymamy się razem.

Sonja Dankeschön, dass Du mich nach Wageningen gebracht hast. Du hast mich an meinem ersten Tag in Wageningen unterstützt und ich schätze das sehr.

**Heinz und Ingrid**, Dankeschön für Eure Unterstützung besonders in dem letzten Jahr und für warme Aufnahme in Eurer Familie.

Wielkie dzięki chciałabym złożyć **Agacie R., Moni L., Ani W.**, za wasze wsparcie dziewczyny. Dziękuję, że mogłam Wam się czasem pożalić i że zawsze dostałam słowa otuchy i wsparcia!

**Thomas**, Dankeschön für Deine Unterstützung und Glaube an mich. Während meines Studiums unsere Liebe hat sich schön entwickelt und wir haben geheiratet. Jetzt mit dem neuen Namen kann ich ein neues Kapitel in meinem (und unserem gemeinsamen) Leben anfangen. Ich freue mich, dass wir gemeinsam gehen!

# About the author





## Curriculum Vitae

**Małgorzata Kaisler** (Królicka) was born on 3 August 1985 in Biskupiec, Poland. In 2004 she obtained her high-school certificate from ZSOK in Lidzbark Warminski, Poland, in order to study Biotechnology in Environmental Protection at the University of Warmia and Mazury (UWM) in Olsztyn, Poland. During her master degree she was a member of a polish Academic Sport Association (AZS) and she was representing UWM at sport competitions running 200 and



400 meters. She was awarded with University Sport Scholarship from UWM for her sport achievements. In 2009, she wrote a Master thesis about bioinformatic analysis of regulation of genes engaged in the synthesis of natural polyesters polyhydroxyalkanoates (PHAs) in *Pseudomonas* species. The results of her research were published in Environmental Biotechnology. In 2009, she started Master studies at Internationales Hochschulinstitut (IHI) Zittau, Germany, currently part of TU Dresden, Germany. In her Master thesis she focused on characterisation of enzymes from fungus *Agarocybe aegerita* involved in the decomposition of a natural polymer – lignin. She finished her studies with Master degree in Biotechnology and Applied Ecology. In 2013, she started her PhD project at Wageningen University & Research.

## List of publications

**Kaisler, M.**, van den Broek, L.A.M., Boeriu, C.G., **2019**. Chitosan as source for biobased building blocks. In: van den Broek, L.A.M. and Boeriu, C.G (eds.). Chitin and Chitosan: Properties and Applications. John Wiley & Sons Ltd, Chichester, UK

da Silva, S.B., **Krolicka, M.**, van den Broek, L.A.M., Frissen, A.E., Boeriu C.G., **2018**. Water-soluble chitosan derivatives and pH-responsive hydrogels by selective C-6 oxidation mediated by TEMPO-laccase redox system. Carbohydr Polym. 186, 299–309

**Krolicka, M.**, Hinz, S.W.A., Koetsier, M.J., Joosten, R., Eggink, G., van den Broek, L.A.M., Boeriu C.G., **2018**. Chitinase Chi1 from *Myceliophthora thermophila* C1, a thermostable enzyme for chitin and chitosan depolymerization. J. Agric. Food. Chem. 66, 1658–1669

**Krolicka, M.**, Hinz, S.W.A., Koetsier, M.J., Eggink, G., van den Broek, L.A.M, Boeriu, C.G., **2018**.  $\beta$ -N-Acetylglucosaminidase *MthNAG* from *Myceliophthora thermophila* C1, a thermostable enzyme for production of N-acetylglucosamine from chitin. Appl. Microbiol. Biotechnol. 102,7441–7454

**Krolicka, M.**, Boeriu, C.G., Hinz, S.W.A., Koetsier, M.J., van den Broek, L.A.M., Boer, E.P.J., Eggink, G., **2018**. Biocatalytic cascade for the production of N-acetyl-D-glucosamine from chitin with Chitinase Chi1 and N-acetylglucosaminidase *MthNAG* from *Myceliophthora thermophila* C1, submitted

**Krolicka, M.**, Koetsier, M.J., van den Broek, L.A.M., Boeriu, C.G., **2018**. Enzymatic production of N-acetylglucosamine: achievements and perspectives. Appl. Microbiol. Biotechnol., submitted

Mozejko, J., **Krolicka, M.**, Ciesielski, S., **2011**. Analysis of DNA transcription termination sequences of gene coding for phaC1 polymerase in *Pseudomonas* species. Environ. Biotechnol. 7, 12–16

## Overview of completed training activities

### Discipline specific activities

4th International Environmental Best Practice Conference, Olsztyn (PL), **2013**<sup>1</sup>

Summer Course Glycosciences, Wageningen, **2014**

COST Training School, Food waste processing in the frame of the biorefinery concept, Lisbon, **2014**

1st International EPNOE Junior Scientists Meeting, Future Perspectives in Polysaccharide Research, Wageningen, **2014**<sup>2</sup>

12th International Conference of the European Chitin Society/13th International Conference on Chitin and Chitosan, Münster (DE), **2015**

Technical workshop, Basic and advanced techniques of chitin and chitosan analysis, Münster (DE), **2015**

Training school "Polysaccharides in health and well-being", Wageningen, **2015**

Pre-conference course "Physics and Chemistry in Polysaccharide Science: *From Molecules to Materials*", Warsaw (PL), **2015**

4th EPNOE International Polysaccharide Conference "Polysaccharides and polysaccharide-based advanced materials: from science to industry", Warsaw (PL), **2015**<sup>2</sup>

3rd Wageningen PhD symposium, Wageningen, **2016**

8th International Congress on Biocatalysis (Biocat2016), Hamburg (DE), **2016**<sup>2</sup>

2nd International EPNOE Junior Scientists Meeting, Sophia-Antipolis (FR), **2016**<sup>2</sup>

### General courses

VLAG PhD week, Baarlo (NL), **2013**

Project and time management, Wageningen, **2014**

Data Management Planning, Wageningen, **2016**

Techniques for Writing and Presenting a Scientific Paper, Wageningen, **2016**

BioBusiness Summer School, Amsterdam, **2016**

### Optionals

Preparation of research proposal, **2013**

International PhD study trip (Portugal), **2014**<sup>2</sup>

Weekly group meetings, **2013—2017**

PhD Day, **2013—2017**

<sup>1</sup> – Poster, <sup>2</sup> – oral presentation

The research presented in this thesis was conducted at the Bioprocess Engineering group in collaboration with Wageningen Food & Biobased Research, both part of Wageningen University & Research and DuPont Biosciences. This research received funding from the Netherlands Organisation for Scientific Research (NWO) in the framework of the TASC Technology Area BIOMASS.

Cover designed by Iliana Boshoven-Gkini || [www.agilecolor.com](http://www.agilecolor.com)

Printed by Digiforce || [www.proefschriftmaken.nl](http://www.proefschriftmaken.nl)