Development of a fully enzymatic conversion process from marine chitin to chitosan oligomers

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DISSERTATION

to obtain the degree of Doctor at the Maastricht University, on the authority of the Rector Magnificus, Prof.dr. Rianne M. Letschert in accordance with the decision of the Board of Deans, to be defended in public on Wednesday, the 3rd of November at 16:00 hours

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Glossary

°C	Degree Celsius
x g	Multiples of g-force
A	Alanin
A	Acetylated glucosamine
6xHis tag	Hexa-histidine purification tag
AA	Amino acid
AU	Absorption units
bp	Base pair
BCA	Bicinchoninic acid assay
BSA	Bovine serum albumin
BsaI	Type IIs restriction enzyme used for golden gate cloning
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
C	Cytosin
CAPS	N-cyclohexyl-3-aminopropanesulfonic acid
CDA	Chitin-deacetylase
Contig	Consensus region of overlapping DNA
COS	Chitosan oligosaccharide
CV	Column volume
d	Days
D	Deacetylated glucosamine
DA	Degree of acetylation
de novo	From the beginning
DNA	Desoxyribonucleic acid
DPn	Degree of polymerization
DoE	Design-of-experiments
dsbA	Bacterial thiol disulfide oxidoreductase
EC	Enzyme commission number
E. coli	*Escherichia coli*
E. coli BL21 (DE3)	*Escherichia coli* expression strain
E. coli DH5α	*Escherichia coli* cloning strain
FF	Fast flow
FPLC	Fast protein liquid chromatography
Fw	Forward (primer)
G	Guanine
GGDC	Genome-to-genome distance calculator
GH	Gylcosyl hydrolase

GlcN	Glucosamine
GlcNAc	N-acetyl glucosamine
In silico	Performed on a computer
IMAC	Immobilized metal affinity chromatography
In vitro	Studies outside of biological contexts
In vivo	Studies inside biological contexts
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	Kilo basepairs
kDa	Kilo dalton
K_m	Michaelis-Menten constant
kV	Kilo volt
LB	Lysogeny broth
LC-MS	Liquid chromatography – mass spectrometry
Log-phase	Exponential growth phase
M	Molarity
MES	2-(*N*-morpholino) ethanesulfonic acid
MW	Molecular weight
m/z	Mass-to-charge ratio
NBT	Nitro blue tetrazolium chloride
NGS	Next generation sequencing
NodB	Chitin deacetylase domain
OD_{600nm}	Optical density at 600 nm
OFAT	One-factor-at-a-time approach
PA	Pattern of acetylation
PBS	Phosphate buffered saline buffering solution
PCR	Polymerase chain reaction
PelB	Leader sequence of *Erwinia carotovora* for protein secretion
Pfam	Protein family database
pH	*Pondus hydrogenii*
Rev	Reverse (primer)
rpm	Revolutions per minute
RSM	Response surface methodology
RT	Room temperature

SignalP	Software to predict the presence of signal peptides
SDS-PAA	Sodium dodecyl sulfate polyacrylamide activity gel
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sp. nov.	*Species nova*
T	Tyrosine
T7	Promoter sequence from phage T7
TB	Terrific broth
TLC	Thin layer chromatography
TRIS	Tris(hydroxymethyl) aminomethane
TSA	Tryptic soy agar
U	Enzymatic catalytic activity unit
UV	Ultraviolet light
V_{max}	Maximum reaction velocity
v/v	Volume per volume
Wt	Wild-type strain
w/v	Weight per volume

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Summary

Polysaccharides such as cellulose and chitin belong to the most abundant biopolymers in the world and take over essential roles in the structural conformations in plant- and animal kingdoms. Both polymers are also widely integrated in chemical industries and secondary products are already fully integrated in our daily lives. Recent structural and functional descriptions of chitin-based degradation products such as chitosan and chitosan oligomers revealed an impressive variety of intrinsic properties that are however highly sensitive to changes of physicochemical characteristics. As the predominant harsh chemical degradation processes of chitin lack the required reaction specificity and selectivity, an efficient production process for defined chitosan products still remains a major challenge. Enzymes are natural functional macromolecules and key factors in biocatalytic processes typically employed for a more specific and selective conversion of molecules. As an alternative to chemical conversions, enzymatic processes can furthermore foster a reduction of environmental impact and elimination of toxic production residues.

The objective of this thesis is to integrate chitinolytic enzymes for the controlled degradation of chitin to defined chitosan oligomers, which can be used for fiber functionalization in medical applications. In particular, novel chitinases and chitin-deacetylases originating from a novel marine bacterial strain will be implemented in *in vitro* enzyme cocktails for the controlled depolymerization and deacetylation of marine chitin. Furthermore, the hydrolytic potential of enzyme cocktails will be assessed to alter physicochemical properties of chitosan oligomers, thus changing intrinsic physiological effects.

An overview on the currently established methods for the preparation of defined chitosan oligomers is given in **Chapter 2**. Chemical methods and their optimization approaches for depolymerization and deacetylation of chitin are summarized and limitations are highlighted. The role and function of chitinolytic enzymes by means of chitinases and chitin-deacetylases and state-of-the-art of enzymatic degradation

processes are stressed. Statistical methodologies and biotechnological approaches to improve the relevance of fully enzymatic approaches are discussed.

In order to establish a fully enzymatic production process for chitosan oligomers, effective chitinolytic enyzmes are required to carry out depolymerization and deacetylation reactions. A polyphasic characterization and phylogenetic classification of a novel chitinolytic bacterium (Chi5) are described in **Chapter 3**. Experimental evidence is given confirming the capability of the bacterium to hydrolyze marine chitin using mixtures of chitinolytic enzymes.

Five different genes each for chitinases and chitin-deacetylases were identified after a whole-genome sequencing of Chi5 and recombinant techniques were used to express the genes by *Escherichia coli* BL21. The enzymes are further explored and characterized in **Chapter 4** and **Chapter 5** to assess reaction optima, enzyme kinetics, substrate specificities and product characteristics. It is revealed that the enzymes are active on soluble as well as insoluble substrates and are capable to degrade chitin to oligomers of a low degree of polymerization and carry out a partial deacetylation.

A holistic fully enzymatic approach for the generation of chitosan oligomers is explored in **Chapter 6**. The design-of-experiments approach is used to model optimum enzyme combinations for depolymerization and sequential deacetylation reactions in order to increase conversion rates and to alter product properties in a controllable manner.

Overall conclusions, future prospects and further recommendations of this thesis are summarized in **Chapter 7**. The scientific and social impact of the research in this thesis is presented in **Chapter 8**.

Chapter 1

Introduction

1.1 Chitin, chitosan and chitosan oligomers

Chitin is a natural polysaccharide predominantly found in the cell walls of fungi and the exoskeletons of crustaceans and insects (Figure 1.1). It is the second most abundant polysaccharide in the world after cellulose, and the global annual production is ~10^{11} tons [1]. The seafood industry generates ~10^{7} tons of chitin annually as a waste stream, much of which is composted or converted to low-value products such as fertilizers, pet foods and fishmeal [2]. The chemical structure of chitin (*N*-acetyl-1,4-β-D-glucopyranosamine) is similar to that of cellulose due to the β(1-4) linkage of the monomers – these are glucose in cellulose and predominantly *N*-acetylglucosamine (GlcNAc) in chitin (Figure 1.2). Although chitin is generally defined as a polymer of GlcNAc, natural chitin is actually a co-polymer of randomly distributed glucosamine (GlcN) and GlcNAc units, with the latter much more abundant. Three allomorphs of chitin occur naturally, differing in terms of microfibril orientation: α-chitin, β-chitin and γ-chitin [3-5]. Chitin is tough, inelastic and insoluble in aqueous media, reflecting the abundance of hydrogen bonds that form between acetamido groups in adjacent polymer chains. Chitin therefore has few industrial applications, although it can be used as a matrix material for the purification of enzymes with carbohydrate-binding domains [6-8] and as membrane material for the retention of proteins [9, 10]. Additionally, smaller chitin oligomers are used in the agricultural industry as plant growth stimulators [11-13].

Chitin is the primary feedstock for the production of the commercially more relevant polymer chitosan (Figure 1.2) as well as chitosan oligomers (COS). Like chitin, chitosan is a co-polymer of GlcN and GlcNAc units, but GlcN is the predominant constituent [3]. Chitosan is therefore considered a deacetylated derivative of chitin, often defined by the degree of acetylation (DA). Chitin becomes chitosan when the DA reaches 50%, but for practical reasons the name chitosan continues to be used for molecules with a lower DA, and chitin is typically reserved to describe polymers where the DA is ~100%. In addition to the DA, the properties of chitin and chitosan also depend on the degree of polymerization (DP), which indicates the chain length and correlates with the molecular weight (MW) in kDa [14].

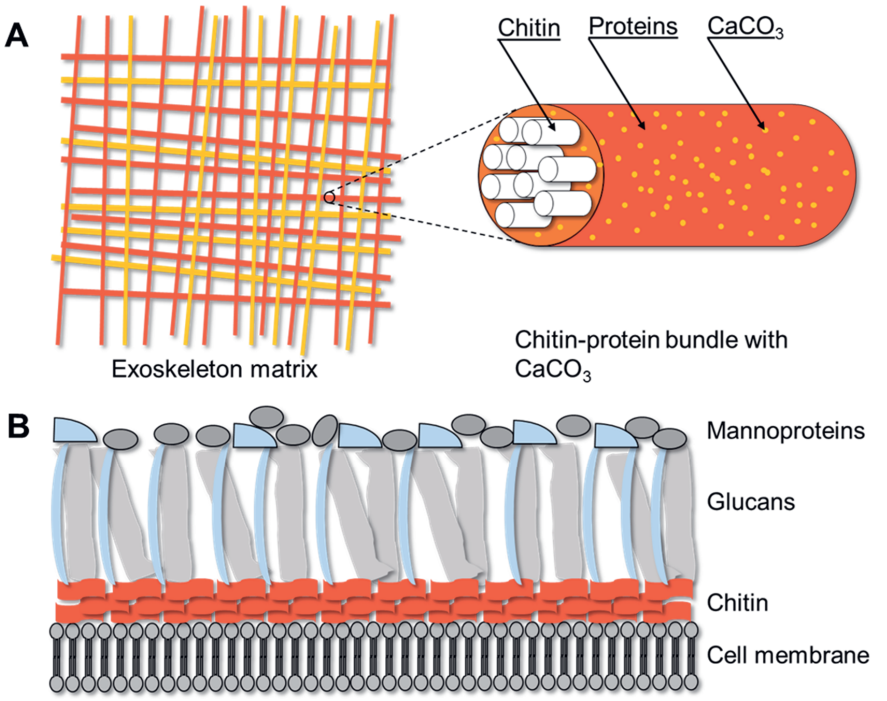

Figure 1.1. Natural occurrences of chitin in biological matrices. A: A fibrous composite of chitin, structural proteins and $CaCO_3$ assembles the exoskeleton matrix of crustacean animals. B: Chitin is embedded in fungal cell walls as a structural component to enhance rigidity. Figures adapted from [15, 16]

The DP is a useful term when the chain length is precisely defined, for example DP6 is a hexamer. For larger oligomers and polymers, the chain length tends to vary within a certain range, and the DP may be given as an approximate or average value, or the average MW in kDa may be used instead. Unlike chitin, chitosan is soluble in acidic aqueous solutions. Chitosan can therefore interact with other molecules in solution and shows wide-ranging biological effects, including antibacterial, antifungal, anti-inflammatory and anti-cancer activities, as well as fat-binding, film-forming, antioxidant and chelating capacities, leading to applications in several industry sectors [17, 18]. In the food industry, chitosan is used as a preservative, a packaging additive, a dietary supplement, and as a nutrient encapsulation system [19]. In the cosmetic industry, chitosan is used as an antioxidant and antibacterial agent in skin protection products, toothpaste and mouthwash, and as a film-forming agent in shampoos and lotions [20].

In the agricultural industry, chitosan is used to protect plants from bacteria, fungi and viruses, as a plant growth regulator, and as a fertilizer additive [21-25]. In the wastewater treatment industry, the fat-binding and chelating properties of chitosan are exploited for the removal of fats, dyes and heavy metals [26-30].

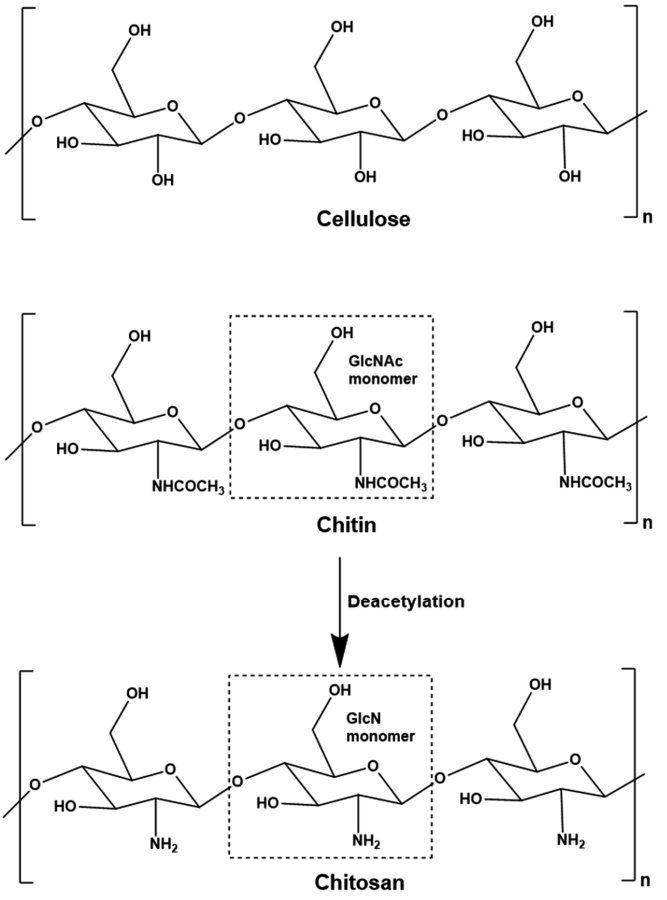

Figure 1.2. Chemical structures of cellulose, chitin and its fully deacetylated derivative chitosan. Whereas cellulose is a polymer of glucose, N-acetylglucosamine (GlcNAc) and glucosamine (GlcN) are the monomeric constituents of the chitinous polysaccharides.

Pure chitosan is biocompatible, biodegradable and non-toxic, so its antimicrobial and anti-inflammatory properties are also ideal for medical applications [31-33]. Chitosan is therefore used as an antibacterial agent in wound dressings, as a non-viral

vector for gene therapy, and chitosan hydrogels are used as drug delivery systems and for the treatment of cancer [34-36]. Finally, chitosan-based scaffolds are used in tissue regeneration [37] (Figure 1.3). The medical and technical applications of chitosan are however restricted because of its low solubility at physiological pH, but COS are more soluble and are therefore more suitable for medical and cosmetic applications [38]. COS also possess bioactive properties lacking in the longer chitosan polymer because these small molecules can penetrate cell membranes, allowing them to influence gene expression and biological processes such as apoptosis [39, 40]. The medical applications of COS therefore combine the antimicrobial, anti-inflammatory and antioxidant properties of chitosan with enhanced solubility and the ability to specifically bind nucleic acids and certain drugs [41-48]. Recent studies have revealed a strong structure–function relationship for chitosan and COS [14, 49, 50]. The DP and DA determine general properties such as solubility and mode of chemical interactions, but another important property is the pattern of acetylation (PA), which can be defined as the sequence of GlcN and GlcNAc units along the molecular backbone [51]. Chitosan with a higher molecular weight (28–1671 kDa) has stronger antimicrobial effects than COS with a lower molecular weight (1–22 kDa) [14]. However, the antimicrobial activity of chitosan and COS reflect three different modes of action dependent on the DP and DA: (1) interaction with molecules on the cell surface, (2) interaction with the cell membrane, and (3) interactions with intracellular targets [52]. COS within the DP range 2–15 show higher antifungal activity than chitosan (MW 2000 kDa) [53]. The anti-tumor effects of COS are 100% higher when using short and fully deacetylated chains (DP1–DP8, DA=0%) rather than chitosan (MW ~1900 kDa, DA=1.5%) [39].

These findings indicate that (1) the reliable production of chitosan/COS with defined properties is necessary to ensure consistent biological and physiological activity and (2) COS are more soluble and in some cases more physiologically active than chitosan. Given the infinite variety of molecules that could be produced by systematically controlling the DA, DP and PA, the production of COS that do not occur in nature could allow the discovery of new mechanisms of action and potentially new biological and physiological functions.

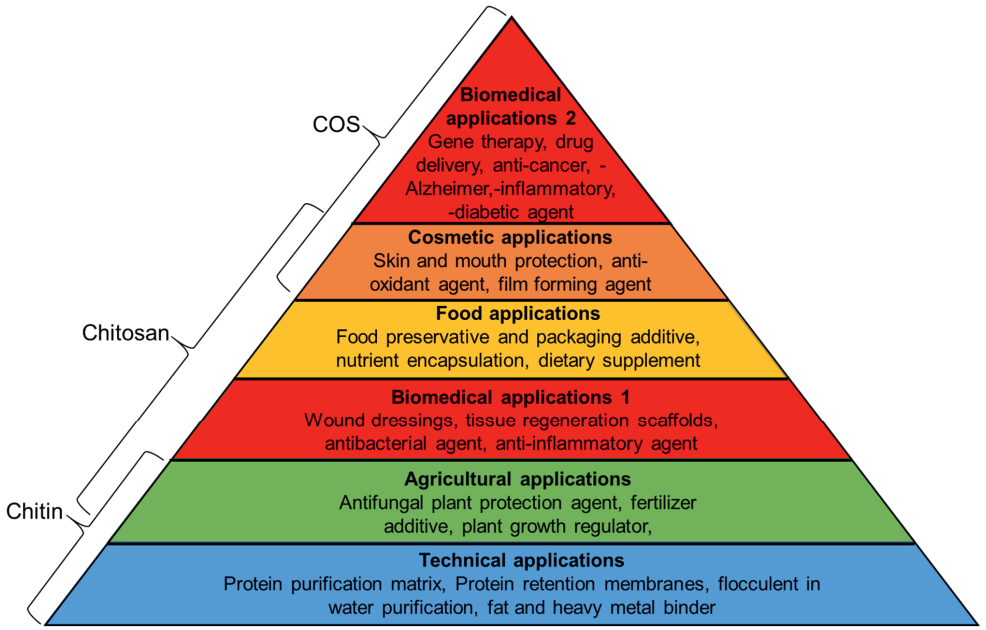

Figure 1.3. Applications of chitin, chitosan and chitosan oligomers (COS) ordered according to their application fields, market volume and product quality. Biomedical applications are split into two segments: applications for bulk chitosan and applications for more defined COS [17, 31, 48].

1.2 Aim and scope of this thesis

Until now, the production of COS is mainly achieved by chemical depolymerization and deacetylation reactions that give limited control on product characteristics and require substantial efforts in downstream processing to isolate single products. Hazardous chemical residues in products furthermore restrict the implementation of COS in medical and cosmetic applications. More specific and selective chemo-enzymatic hybrid processes have been explored by exchanging either depolymerization or deacetylation reactions with chitinases and chitin-deacetylases. However, so far no fully enzymatic conversion process from insoluble chitin to COS has been established yet. The limited accessibility of crystalline substrates, product inhibitions, high enzyme production costs, and deficiency of appropriate enzymes are few typical bottlenecks for enzymatic reactions.

The work of this thesis will focus on the establishment of a fully enzymatic conversion process of marine chitin to COS exploiting the novel marine chitinolytic bacterium Chi5 (planned final name: *Photobacterium orarium)*. Therefore, novel chitinases and chitin-deacetylases isolated from the bacterium will be implemented in *in vitro* enzyme cocktails. The physiologically active oligomers are considered as coating agents for fiber functionalization and potential future applications in the medical sector are discussed. The multiple objectives and milestones of this thesis are summarized schematically in Figure 1.4.

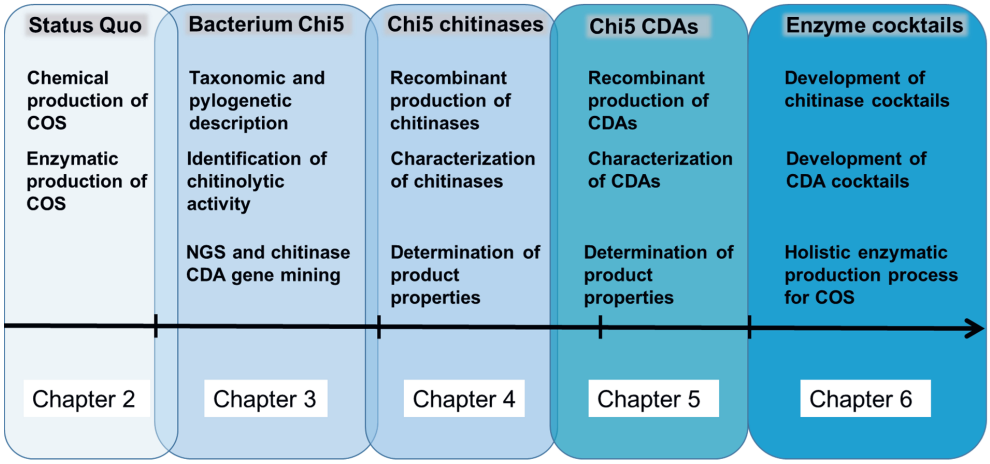

Figure 1.4. Schematic workflow of this thesis. (NGS: next generation sequencing, COS: chitosan oligomer, CDA: chitin deacetylase).

1.3 Outline of this thesis

The following questions are addressed in this thesis:

- **How do chitinolytic enzymes compare to the chemical production of COS? What is the potential of enzymatic processes? Will enzymatic reactions replace chemical reactions?**

  The state of the art of chemical and enzymatic conversion processes of chitin to COS are presented in **Chapter 2**. Optimization approaches for chemical depolymerization and deacetylation processes are critically discussed. Current literature also gives an overview on chitinolytic enzymes used for

the production of COS. Optimization approaches are explored that give the opportunity to develop fully enzymatic processes.

- **Is Chi5 an entirely novel bacterial strain and a source for chitinolytic enzymes?**

  The marine bacterium Chi5 will be characterized polyphasically concerning its growth characteristics, cellular compositions, morphology, genetic relatedness and metabolism in **Chapter 3**. Growth studies on chitin substrates and hydrolysis experiments with Chi5 culture supernatant will be used to assess the chitinolytic potential.

- **Which enzymes are responsible for chitinolytic activity? Which are the genes encoding the enzymes? Can the novel enzymes be expressed recombinantly? Is enzymatic activity maintained when expressed recombinantly by *Escherichia coli* BL21?**

  Enzymatic depolymerization and deacetylation steps of chitin are typically mediated by chitinases and chitin-deacetylases. In order to assess the presence of such enzymes, a next generation sequencing of the Chi5 genome will be carried out and gene mining will be used to identify homologous genes encoding the respective enzymes in **Chapter 4** and **Chapter 5**. The enzymes derived from Chi5 are entirely novel thus no predictions can be made whether they can be expressed efficiently by the host *E. coli* BL21. Therefore, the expression of diverse enzyme variants will be investigated. Likewise it will be assessed if the enzymes maintain hydrolytic activity when produced heterologously. The best enzyme variants with respect to specific activity will be selected for further analysis.

- **What are the reaction optima of the novel enzymes and on what substrates are the enzymes active? What products can be generated?**

  Selected chitinases and chitin-deacetylase variants will be produced at larger scale, purified and subsequently characterized regarding their pH-, temperature-, and salinity-optima, co-factors, substrate specificities and kinetics in **Chapter 4** and **Chapter 5**. Product properties regarding DP and DA will be determined. In order to mimic realistic bioprocess conditions,

chitin powder will be used as a substrate for all experiments instead of common artificial or chemically pre-treated materials.

- **How can process optimization help to tailor enzymatic processes? Can design-of-experiments be used to model optimum enzyme mixtures? Can product properties of chitosan oligomers be changed?**

  A holistic enzymatic COS production approach is explored in **Chapter 6**. Mixtures of recombinant chitinases and chitin-deacetylases will be assessed using the design-of-experiments approach to tailor optimized enzyme cocktails. Statistical modeling will be used to determine enzyme formulations for increased production rates and yield and putative changes in COS product distributions. Sequential enzymatic depolymerization and deacetylation reactions of chitin using optimized mixtures will be validated.

- **What further approaches can be applied to modify products from enzyme reactions? Which additional valorization strategies for chitin can be considered using the chitinolytic bacterium Chi5? What is the impact of the research?**

  The main conclusions of the thesis, additional optimization strategies with regards to enzyme immobilization and enzyme evolution are discussed in **Chapter 7**. Future technical applications for bacterium Chi5 growing on chitin are explored. Potential medical application for enzymatically produced COS are highlighted. The impact of the research for the scientific community and society is assessed in the concluding **Chapter 8**.

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Chapter 2

Conversion of chitin to defined chitosan oligomers: Current state and considerations

In this chapter, current advances in the generation of defined COS are reviewed. COS can exhibit many intrinsic functions resulting in versatile application spectra. These functions are however dependent on the DP, DA and PA thus requiring controllable production processes. Current chemical and biological fractionalization approaches of crustacean shell biomass are summarized. Various chemical depolymerization and deacetylation routes are described and optimization approaches for enhanced control of physicochemical properties are discussed. Differences of chemical and enzymatic approaches and the state-of-the-art for the enzymatic depolymerization and deacetylation reactions are highlighted. Strategies for enzymatic process optimization and increased control are reviewed.

This chapter is based on the following publication:

Schmitz, C.; Gonzales-Auza, L.; Koberidze, D.; Rasche, S.; Fischer, R.; Bortesi, L. Conversion of chitin to defined chitosan oligomers: current state and future prospects. *Marine Drugs* **2019**, 17(8), doi: 10.3390/md17080452.

2.1 Chitin extraction from marine bio-waste

Chitin is mainly found in fungal cell walls and in the exoskeletons of crustaceans and insects, but also in some algae and micro-algae. Although crustacean shells are the most abundant and easily accessible source of chitin [1], the other sources yield α-chitin and β-chitin of either similar or more consistent quality than crustaceans [2-9]. Furthermore, algal sources of chitin are gaining attention in the cosmetic and food industries as animal-free/vegan products [10-12]. Most chitin/chitosan is nevertheless derived from crustacean shell biomass, which can be considered a natural composite material comprising 30–40% structural proteins, 30–50% calcium carbonate and various forms of calcium phosphate, and 20–30% chitin [13] along with smaller quantities of pigments and lipids [14].

The processing and valorization of this material begin with the extraction of chitin, and this involves washing and grinding, the two major steps of demineralization and deproteination (Figure 2.1) [15], and the removal of pigments and lipids using $KMnO_2$. Demineralization or decalcification typically involves treatment with HCl or other acids such as HNO_3, H_2SO_4, CH_3COOH or HCOOH to produce soluble calcium salt [14-17]. The deproteination of shrimp waste for industrial applications is usually achieved by mixing with NaOH at temperatures up to 160 °C, but other alkalis such as KOH, Na_2CO_3, $NaHCO_3$, K_2CO_3, $Ca(OH)_2$, Na_2S, $CaHSO_3$ and Na_3PO_4 have also been used successfully [14, 18-20]. While effective, these treatments cause the random cleavage of the chitin backbone and random deacetylation, resulting in chitosans and COS that are undefined in terms of DP and DA [14, 18, 20, 21].

Biological methods can reduce the environmental burden of the alkali/acid steps and also avoid unwanted changes to the chitin structure [22]. In such processes, naturally produced lactic acid is used for demineralization and proteases are used for deproteination. Additionally, whole bacterial extraction procedures have been developed involving the co-fermentation of proteolytic and lactic acid bacteria on shrimp waste, resulting in milder treatment conditions and a better-defined chitin product [23-25].

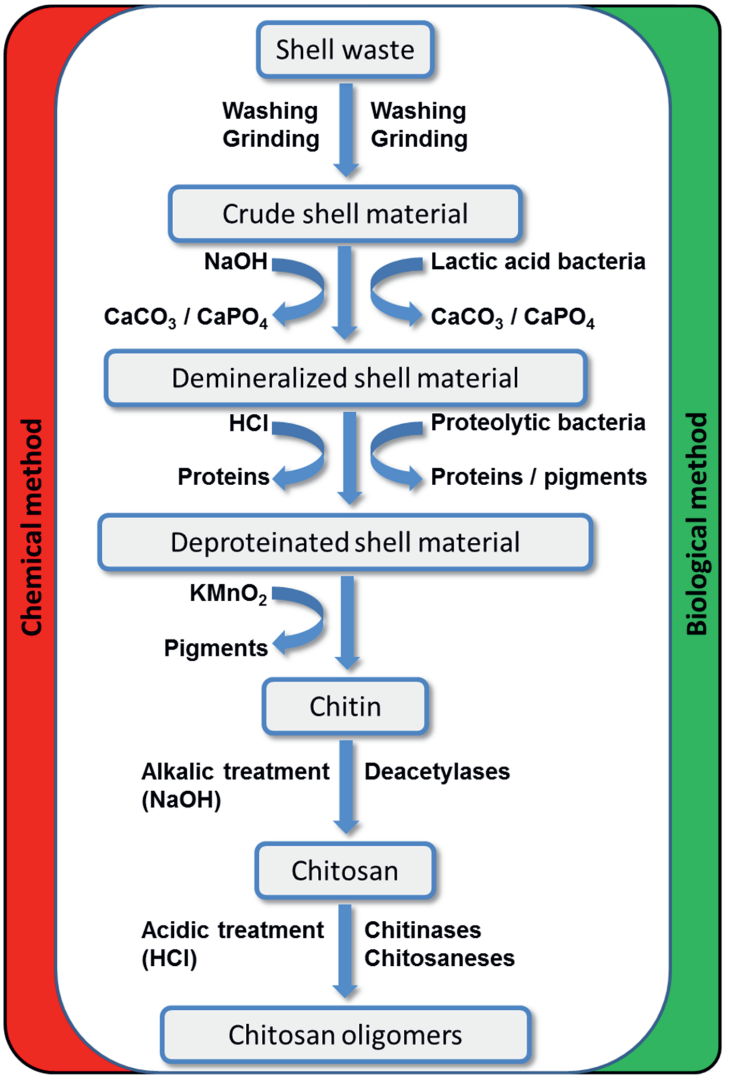

Figure 2.1. Chemical and biological methods for the fractionated extraction of shell components and the sequential conversion of chitin to chitosan and chitosan oligomers (COS).

2.2 Chemical conversion of chitin to chitosan oligomers

2.2.1 Chemical deacetylation of chitin

The chemical production of COS is usually initiated by the deacetylation of insoluble chitin raw material. This increases the solubility of the substrate for the subsequent

depolymerization step, which yields water-soluble COS molecules. Because the biological functions of COS depend on the DA, further limited deacetylation is carried out to generate oligomers with a DA in the range 0–50%. The chemical removal of acetyl groups from chitin can be achieved either by acid or alkali hydrolysis. However, acidic deacetylation also causes the cleavage of the chitin backbone thus reducing the DP. Therefore, hot alkaline deacetylation (>80 °C) is the method of choice for the production of chitosan and COS because depolymerization is less prevalent [26], although prolonged high-temperature alkali treatment also causes depolymerization [27]. The most common reagent is NaOH, and the DA is determined by the NaOH concentration (25–50%), reaction temperature (80–120 °C) and reaction time (1–24 h). An extensive investigation of these process parameters as well as the effect of additives ($NaBH_4$ and thiophenol) revealed that the high-molecular-weight (50–1000 kDa) chitosan can be produced with a well-controlled DA by using fixed reaction conditions (50% NaOH, 120 °C in the presence of $NaBH_4$) and varying the reaction time between 3 and 12 h [17, 28]. Similarly, chitin processing conditions have been screened systematically (25% or 50% NaOH, for 2, 5 or 10 h, 100 °C) revealing that the higher NaOH concentration significantly increased the reaction rate and prolonged incubation favored the recovery of low-molecular-weight chitosan [15]. In another investigation, chitin was incubated at 80 °C or 90 °C in 45% NaOH for 20, 60 and 120 min with similar results [29]. Chitosans with DA values of 21–29% and 15.6% have been produced by varying the NaOH concentration, temperature and time in a screening experiment, revealing that all three parameters must be high (120 °C, 24 h, 40% (w/v) NaOH) to reduce the DA, but this also resulted in a significant reduction in the DP [27].

Statistical experimental designs using response surface methodology (RSM) have also been used to find optimum conditions for efficient deacetylation while avoiding severe depolymerization. For example, one study revealed interdependencies between the incubation time, temperature and NaOH concentration and showed a synergistic effect in which the DA (4.3–32.7%) and DP (100–1100 kDa) both decreased as the temperature, reaction time and NaOH concentration increased [30]. RSM has also been used to optimize the deacetylation/depolymerization relationship, revealing that a temperature of 130 °C and a reaction time of 90 min allowed the

production of COS with an average MW of 150 kDa and a DA of 10% [31]. Based on these empirical optimization studies, alkaline deacetylation conditions can now be selected to produce chitosan within a desired DA range of 0–50%. However, the harsh conditions inevitably have a simultaneous effect on the DP, which makes it difficult to produce specific COS using chemical methods. It would therefore be desirable to decouple the deacetylation and depolymerization reactions. Chemical deacetylation methods are also nonspecific so it is not currently possible to control the pattern of acetylation (PA).

2.2.2 Chemical depolymerization of chitin and chitosan

The conversion of chitin/chitosan to shorter oligomers requires the hydrolysis of the glyosidic bond between the GlcNAc and/or GlcN units. Various approaches for chemical depolymerization have been tested using HNO_2, HF and H_2O_2 [32, 33], but acid hydrolysis with HCl remains the most common and effective method [34-37]. However, chemical hydrolysis of chitin and chitosan achieves relatively low yields of COS (up to 28 %) and favors the production of monomeric GlcNAc/GlcN [38-40]. Additionally, COS prepared by acid hydrolysis are usually excluded from human medical applications because the toxic hydrolysis reagents cannot be removed completely [41]. A defined and consistent DP can only be achieved by the assessment and control of physicochemical factors that influence the depolymerization process. HCl has selective activity according to the specific nature of the glycosidic bond in the chitin/chitosan backbone, with threefold higher activity on GlcNAc–GlcNAc and GlcNAc–GlcN bonds compared to GlcN–GlcN bonds [42]. However, given the random distribution of GlcNAc and GlcN in natural chitinous polymers, this selectivity cannot be exploited to achieve a specific DP.

Therefore, the DP has been optimized by manipulating various physicochemical process parameters, as discussed below. A mixture of chitin and COS ranging from dimers (DP2) to dodecamers (DP12), was prepared by the acid hydrolysis of fully deacetylated chitosan (DA=0%) using 12 M HCl followed by differential precipitation to first remove larger oligomers using NaOH and sequentially adding ethanol to precipitate the desired smaller oligomers. Acetic anhydride was then added in different amounts to partially re-acetylate the COS, achieving specific DA values of 25%, 40%,

60%, 80% and 90% [32]. A similar selective fractionation approach using methanol (70%, 80% and 90%) was used to enrich for COS with a DP > 6 following the acid hydrolysis of chitosan [43]. The inclusion of small quantities of H_2O_2 in the acid hydrolysis mix yielded COS mixtures of DP ≤ 9, and increasing the H_2O_2 content (1–5%), reaction time (0–6 h) and temperature (60–100°C) led to higher reaction rates, product yields and a broader COS distribution in terms of DP [33]. Acidic hydrolysis has also been carried out using acetic acid spiked with H_2O_2 to partially degrade the chitosan, followed by ultrafiltration to isolate COS with different DP ranges [44]. The correlation between the H_2O_2 concentration and incubation time has been investigated with or without microwave radiation, revealing conditions that allow the production of COS with a better-defined MW, but it was not possible to produce COS with a MW below 2000 Da using this microwave enhanced degradation procedure [36]. Hydrolysis of chitosan in the presence of diluted HCl (1.8 M) and three different zeolite adsorbents (HZSM-5, molecular sieves beads 0.3 nm and 1.0 nm) was also carried out and yielded COS up to DP 9. Furthermore, with this method it could be shown that higher yields can be achieved as the hydrolysis equilibrium is shifted more towards the products [45]. Finally, chitin tetramers, pentamers and hexamers have been produced by the acid hydrolysis of chitin with concentrated HCl at 40°C followed by a sequential differential acetone precipitation method [46]. The chemical hydrolysis of chitin can rapidly yield mixtures of COS. However, inorganic catalysts such as HCl and H_2O_2 do not achieve sufficient intrinsic substrate specificity and reaction selectivity to effectively produce COS with a defined DP. Laborious additional purification steps such as preparative gel permeation chromatography and cation exchange chromatography are therefore required to separate COS into size-specific fractions [34]. Chemical hydrolysis also affects the DA and there is no way to control the PA. These issues can be addressed by replacing chemical hydrolysis with biological methods for the preparation of COS, specifically the use of chitinolytic enzymes such as chitinases, chitosanases and chitin deacetylases.

2.3 Biological conversion of chitin to chitosan oligomers

2.3.1 Enzymatic deacetylation of chitin

Chitin deacetylases (CDAs, EC 3.5.1.41) catalyze the hydrolysis of GlcNAc in chitin and COS, to produce chitosan, partially acetylated COS, or fully deacetylated glucosamine oligomers [47]. CDAs have been identified in marine and soil bacteria, several fungi, a few insects and at least one virus. All CDAs belong to the carbohydrate esterase family 4 (CE4) and share a conserved NodB homology domain (polysaccharide deacetylase domain) which is required for their catalytic activity [48]. The first active CDA to be purified and characterized was from the fungus *Mucor rouxii* in 1970, and it has a MW of 75 kDa and an optimum pH of 5.5 [49]. Since then, CDAs have also been isolated from the fungi *Absidia coerulea, Aspergillus nidulans,* and *Colletotrichum lindemuthianum* [50, 51]. Their physicochemical properties (MW, optimum pH and temperature, effect of metal ions, and substrate specificity) have been studied extensively [51].

In order to identify enzymes that produce well-defined COS and understand the reaction process, substrate specificity and catalytic mechanism, further CDAs from organisms such as archaea, marine bacteria and insects have been isolated, purified and characterized in addition to those from fungi (Table 2.1) [51, 52]. Depending on the substrate and the reaction conditions, CDAs can produce different fully or partially deacetylated COS that are well defined in terms of DA and PA. Two fungal CDAs have been studied in particular detail, revealing distinct catalytic mechanisms known as the multiple-attack (Figure 2.2A) and multiple-chain (Figure 2.2B) mechanisms, respectively. The CDA from *M. rouxii* (Zygomycetes) binds to the polysaccharide chain and several sequential deacetylations take place [53]. This exo-type enzyme deacetylates chitin oligomers with a DP > 2, with sequential deacetylation at the non-reducing end of the oligomer, yielding D_n as the acetylation pattern (Figure 2.2A) [54]. In contrast, the CDA from *C. lindemuthianum* (Ascomycetes) uses the multiple-chain mechanism in which the enzyme forms an active enzyme–polymer complex and hydrolyzes a single acetyl group before dissociating and forming a new active complex, which binds to another chain [55]. The enzyme fully deacetylates DP3 and DP4 chitin oligomers (Figure 2.2B), but the reducing end $(GlcNAc)_2$ cannot be deacetylated [51].

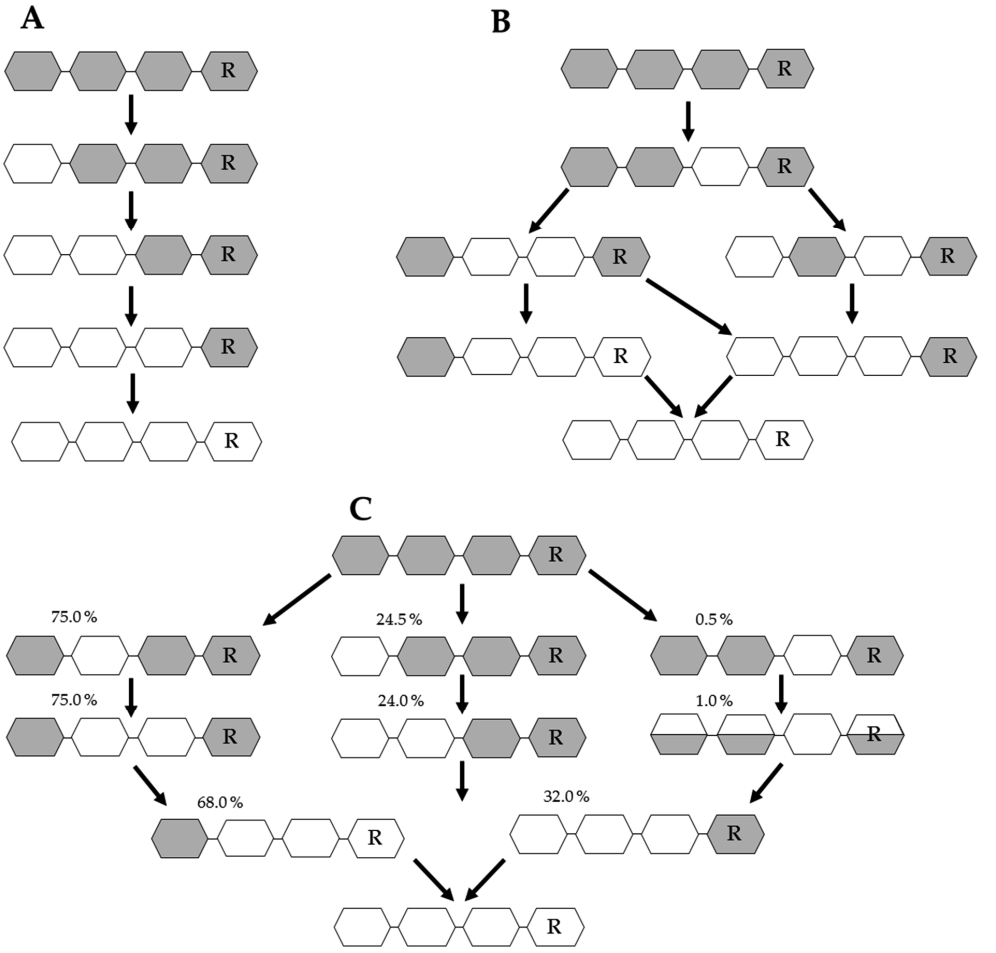

Figure 2.2. Overview of different pathways of the enzymatic deacetylation of chitin oligomers. A: Deacetylation of a chitin tetramer by *M. rouxii* CDA in a 'multiple attack mode'. B: A chitin tetramer that is deacetylated by a *C. lindemuthianum* CDA. This is done in a 'multiple chain mode'. C: Subside capping deacetylation model used to explain the generation of partially deacetylated COS using a recombinant CDA from *Puccinia graminis f. sp. Tritici.* R: sugar with reducing end; grey box: GlcNAc; white box: GlcN; %: relative conversion yield; halved box: three different variants can be generated from the precursor oligomer as only one of the acetylated sugars gets deacetylated. Figures adpted from ref [54, 56, 57].

An additional deacetylation mechanism, the "subside capping deacetylation model", has also been identified, in which several catalytic events take place on a single

substrate molecule and lead to sequential deacetylation (Figure 2.2C). This mechanism is used by bacterial CDAs such as *Rhizobium* spp. NodB and *Vibrio* spp. CDA, which deacetylate a specific position leading to deacetylated products [56].

Most bacterial CDAs preferably act on low-molecular-weight COS and are essentially inactive on polymeric chitin and chitosans. Such enzymes have been found in *Vibrio* spp. and more recently in *Shewanella*, a genus of marine bacteria living in extreme aquatic habitats [58, 59]. The best-known CDAs that produce COS with a defined DA are *Rhizobium* spp. NodB and VcCOD from *Vibrio choloreae* [48, 60, 61]. Both enzymes can produce COS with the same deacetylation pattern (ADA_{n-2}) (Table 2.1). The enzymes have been combined in a single reaction to generate double-deacetylated COS with a novel and defined PA ($DDA_{(n-2)}$) [61, 62]. This was the first study describing the preparation of novel COS with a previously unreported PA. More recently a CDA from the soil bacterium *Arthrobacter* sp. (ArCE4A) was found to accept COS substrates with a DP ≥ 2. This enzyme uses a multiple-chain mechanism to produce different mono/di-deacetylated products with a defined PA ($D_{(n-1)}A$) [63].

Defined COS can also be produced using fungal CDAs. The CDA from *Puccinia graminis* (PgtCDA) has been expressed in *Escherichia coli* and its regioselectivity was tested on different chitinous substrates [57]. The enzyme showed activity towards soluble glycol chitin and partially acetylated chitosan polymers, yielding a product with a novel PA (AAD_n, where n > 1) (Table 2.1). The expression of several recombinant fungal CDAs has been achieved in various eukaryotic hosts and in *E. coli* by codon optimization, fusion to solubility-promoting proteins and targeting to different subcellular compartments [57, 64-71] (Table 2.1).

Table 2.1. Isolated or recombinant fungal (above the dotted line) and bacterial (below the dotted line) chitin deacetylases (CDAs) that convert oligomeric chitin substrates to partially deacetylated chitosan oligomers (COS) with various patterns of acetylation. Abbreviations: n.r. = not reported; DP = degree of polymerization; A = acetylated monomer; D = deacetylated monomer; index n-m = terminal distance and locus where CDA converts A to D.

Enzyme	Source Organism	Expression host	Substrate	COS Product	Literature
ClCDA	*Colletotrichum lindemuthianum*	Natural isolate	DP2-DP6	D_n	[48]
MrCDA	*Mucor rouxii*	Natural isolate	≥DP3	D_n	[49]
ScCDA1	*Saccharomyces cerevisiae*	*S. cerevisiae*, *Pichia pastoris*	DP2-DP6	n.r.	[59]
ScCDA2	*S. cerevisiae*	*S. cerevisiae*, *Pichia pastoris*, *E.coli*	DP2-DP7	n.r.	[60,61]
FvCDA	*Flammulina velutipes*	*Pichia pastoris*	DP2-DP6	n.r.	[62]
RcCDA	*Rhizopus circinans*	*Pichia pastoris*	DP6	n.r.	[63]
PaCDA	*Podospora anserinas*	*Hansenula polymorpha*	≥DP2	D_n	[64]
AnCDA	*Aspergillus nidulans*	*E.coli*	DP2 -DP5	D_n	[65]
PgtCDA	*Puccinia graminis*	*E.coli*	DP4-DP6	AAD_{n-2}	[57]
PesCDA	*Pestolotiopsis sp.*	*E.coli*	DP4-DP6	$AAD_{n-3}A$	[66]
PcCDA	*Pochonia chlamydosporia*	*E.coli*	DP4, DP5	$ADDA_{n-3}$	[51]
SwCOD	*Shewanella woodyi*	*E.coli*	DP2-DP4	AD; $[ADA_{n-2}]$	[53]
SbCOD	*Shewanella baltica*	*E.coli*	DP2-DP4	AD; $[ADA_{n-2}]$	[54]
ArCE4A	*Arthrobacter sp.*	*E.coli*	DP2-DP6	D_{n-1} A	[55]
NodB	*Rhizobium spp.*	*E.coli*	DP1-DP6	ADA_{n-2}	[58]
VcCOD	*Vibrio chloreae*	*E.coli*	DP2 -DP6	ADA_{n-2}	[65]

The recent expression of a CDA from the nematophagous fungus *Pochonia chlamydosporia* (PcCDA) revealed its activity on COS with a DP > 3, generating mono/di-deacetylated products with a different PA compared to closely-related fungal CDAs [52]. PcCDA selectively converts a DP5 substrate into single mono-deacetylated products in the penultimate position from the non-reducing end (ADAAA), and then transforms them into a di-deacetylated product (ADDAA). This novel PA produced by this enzyme provides further insight into the substrate specificity of this enzyme family. Finally, 14 partially-acetylated chitosan tetramers have been produced by combining different bacterial, fungal and viral chitin deacetylases [72]. This study showed for the first time that a range of CDAs from different origins can acetylate COS with the same regioselectivity displayed during the conventional deacetylation reaction. Forward and

reverse reactions were performed for each enzyme, with the forward reaction causing the tetramer to be partially deacetylated and the reverse reaction resulting in glucosamine re-acetylation [72]. The PA of oligomers produced by CDAs have recently been explained based on the subsite capping model, developed by comparing the structures of five CDAs in the CE4 family [57] (Figure 2.2). The model provides insight into the position of secondary protein-structures such as α-helices, β-sheets and loops. Furthermore, it was shown that the position of loop- structures decorating the active site are key elements in the substrate specificity of different CDAs. Understanding the structure–function relationship of enzymes in the presence of their substrate can help to explain the regioselectivity of deacetylation and the resulting PA [56, 57, 73].

In summary, several diverse CDAs have been characterized in terms of their physicochemical properties and deacetylated products. The substrate specificity and selective reaction mechanisms of CDAs can, in contrast to chemical methods, consistently generate COS with a defined DA and PA. Further studies are required to understand the structure–function relationships of partially acetylated COS, which will enable targeted applications in the food industry, agriculture and medicine.

2.3.2 Enzymatic depolymerization of chitin and chitosan

The biological conversion of chitin polysaccharides into shorter oligomers requires hydrolytic enzymes that contain conserved chitin-binding domains and chitin-specific active sites. Many chitinolytic enzymes are produced by plants, fungi and bacteria (Table 2.2). All of them are glycosyl hydrolases, but they differ in terms of reaction mechanism, thermostability and product characteristics [74]. Chitinolytic hydrolases can be categorized according to their mode of action. Endo-chitinases (EC 3.2.1.14) bind randomly to a chitin polysaccharide strand and hydrolyze internal glycosidic bonds producing various fragment sizes ranging from dimers to polymers. These enzymes are required for the production of COS because they partially reduce the DP of chitin.

In contrast, exo-chitinases (EC 3.2.1.29) bind to the reducing or non-reducing end of chitin and release monomeric (DP=1) and to lesser extent dimeric (DP=2) GlcNAc units. These enzymes are necessary for the complete degradation of chitin, and are therefore of only secondary interest for the production of larger COS (DP > 2). Chitosanases (EC 3.2.1.132) are more selective chitinolytic enzymes because they

hydrolyze GlcN-GlcN bonds and their activity is therefore dependent on the DA and PA [75]. Finally, chitobiases (EC 3.2.1.29) cleave GlcNAc dimers to release GlcNAc monomers [76]. Other enzymes such as cellulase and lysozyme are also known to exhibit some hydrolytic activity towards chitin and chitosan but are not specific for these substrates [44, 77]. COS with a size range of 5–30 kDa have been produced by fractionated enzymatic hydrolysis using a mixture of a cellulase, pepsin and lysozyme [44].

Table 2.2. Isolated or recombinant fungal (above the dotted line) and bacterial (below the dotted line) chitinolytic glycosyl hydrolases used for the conversion of chitinous substrates to chitosan oligomers (COS) with different degrees of polymerization (DP). DA = degree of acetylation. GlcNAc = N-acetylglucosamine.

Enzyme	Source Organism	Expression host	Substrate	COS Product	Literature
Chitinosanase	*Alternaria alternata*	Natural isolate	Chitosan DA 40-70 %	Cleavage after GlcN-GlcNAc	[74]
Chitosanase	*Purpureocillium lilacinum* CFRNT12	Natural isolate	Colloidal and crystalline chitosan	DP 2-6	[80]
Chitinase Chi1	*Myceliophthora thermophila* C1	*Myceliophthora thermophila* C1	Chitosan Mw (100, 600, and 3000 kDa); DA (77, 78, 88, 90 %)	DP 2-12	[78]
Chitosanase	*B. subtilis*	*B. subtilis* PT5	α and β type chitosan	DP 2 -4	[81]
Chitinase-D	*Serratia proteamaculans*	*Escherichia coli*	Chitosan DA 35 % and 61 %	DP 2-12	[79]
Chitinase-D	*Serratia marcescens* GPS5	*E. coli*	Colloidal chitin, chitosan DA 10 %	DP 1-8	[87]
GH46 family chitosanase	*Bacillus subtilis (*BsCsn46A*)*	*E. coli*	Chitosan DA 15, 30 and 60 %	DP 2-15	[85, 86]
GH46 family chitosanase	*Gynuella sunshinyii*	*E. coli*	Chitosan DA 95 % COS DP 2-6	DP 2-7	[83]
GH8 family chitosanase	*Bacillus strain*	*E. coli*	Chitosan DA > 90 %	DP 5.5 (mean)	[84]

Chitinases and chitosanases from various sources have been expressed as recombinant proteins in heterologous host systems such as *E. coli* and *Bacillus subtilis* [78-84]. Chitinase D from *Serratia proteamaculans* was used to convert chitosan polymers (DA=35% and DA=61%) to undefined COS mixtures varying in DA and with a DP ≥ 6 after incubation for 90 min. Purified specific COS were also obtained after size exclusion chromatography for bioactivity testing [85]. RSM has been used to statistically optimize the production of a *Purpureocillium lilacinum* CFRNT12

chitosanase by solid-state fermentation and the enzyme was used to produce mixtures of different COS from colloidal chitosan [86]. Interestingly, a new class of chitosanases from the fungus *Alternaria alternata* has been identified that selectively cleaves after GlcN-GlcNAc pairs and thus shows no enzymatic activity against fully acetylated or fully deacetylated substrates, making the enzyme ideal for the production of novel structurally defined COS [78]. The secretion of a heterologous chitosanase by *B. subtilis* was optimized to generate mixtures of chitosan dimers, trimers and tetramers from chitosan [87]. Eight different chitinolytic bacteria and 20 chitinolytic fungi were isolated from soil samples, and by incubating the secretomes with chitosan polymers it was possible to produce diverse mixtures of COS (DP1–DP6) as well as larger oligomers, providing another potential source of novel COS for medical and industrial applications [88]. Recently, a GH46 family endo-chitosanase isolated from the rhizobacterium *Gynuella sunshinyii* was expressed in *E. coli* and used to produce short COS with a consistent DP when incubated with chitosan [89]. Similarly, a GH8 family chitosanase expressed in *E. coli* was suitable for the large-scale production of COS because 1 g of enzyme could hydrolyze up to 100 kg of chitosan [90]. A *B. subtilis* GH46 family chitosanase with fast reaction times has been expressed in *E. coli* and used to produce COS with a size range of DP1–DP6 starting with chitosan samples differing in DA [91, 92]. The product distribution could be shifted to favor lower or higher DP by changing the type of chitosan substrate, the incubation temperature (30 °C, 50 °C) and the incubation time (2 min to 3 h) [91, 92]. A unique chitinase D from *Serratia marcescens* (SmChiD) was found to display chitobiase activity on DP2 substrates and transglycosylation activity on substrates with a DP ≥ 3, resulting in products of DP1 and DP2 and the formation of oligomers of DP3–DP6 [93]. This enzyme is ideal for the production of longer-chain bioactive COS. Many chitinolytic and chitosanlytic enzymes have been characterized in natural extracts or produced as recombinant proteins for testing, revealing different reaction mechanisms and product properties. This provides a vast spectrum of different enzymes to generate products with a range of DP values, but the diversity and complexity of the enzymes makes it difficult to find the optimal candidate. In this regard, the enzyme yield achieved by natural or heterologous expression is a key factor which influences overall process productivity. Process development for the production of novel COS products must

therefore balance the need for a specific reaction mechanism with the ability to produce sufficient quantities of each functional enzyme.

2.4 Fully enzymatic production of chitosan oligomers

Many microbes can utilize chitin from marine or terrestrial organisms as a source of carbon and nitrogen [94-96]. Chitin is usually degraded extracellularly to monomers and dimers using mixtures of endo-chitinases, exo-chitinases and chitosanases. The simpler carbohydrates are then assimilated and metabolized. Despite these effective enzymatic mechanisms, a whole-bacterial process that converts chitin to COS has not yet been achieved. This reflects the fact that exo-chitinases completely degrade chitin to yield monomers. Deleting or silencing these enzymes could potentially allow the production of COS but this would also prevent the bacteria utilizing chitin as a nutrient, therefore inhibiting their growth. The provision of alternative carbon sources such as glucose would allow bacterial growth but would suppress the expression of chitinolytic enzymes because bacteria tend to switch off unnecessary metabolic circuits if a preferred substrate is available. Therefore, whole-bacterial degradation processes are currently used solely for the commercial production of monomeric GlcNAc [97].

To overcome these limitations, *in vitro* degradation processes can be established using either naturally extracted or recombinant chitinolytic enzymes for the development of specific enzyme cocktails. A fully enzymatic process for the *in vitro* conversion of chitin into defined COS (i.e. enzymatic deacetylation and depolymerization steps using chitinases and/or chitosanases and CDAs) has not been reported yet. However, hybrid processes combining chemistry and biology have been established at the laboratory scale to generate defined COS. These semi-biological processes usually require chitin standard oligomers with a highly-defined DP and use CDAs alone or in combination to generate defined COS [61]. Alternatively, chitosan produced by chemical deacetylation can be depolymerized using chitinases and chitosanases to yield defined COS [85]. The environmentally friendly production of COS requires the establishment of fully enzymatic processes for the conversion of chitin to defined COS by combining chitinases and CDAs in a single process, thus avoiding the use of hazardous chemicals. However, due to the abundance of natural chitinolytic enzymes and their diverse substrate specificities and product

characteristics, the underlying biocatalytic mechanism and process-relevant enzyme properties must be characterized first. Furthermore, for multi-step enzymatic reactions carried out using enzyme cocktails, the reaction conditions must be optimized for all enzymes in the process simultaneously.

2.5 Design of experiments approach for multi-enzyme process optimization

Standard process optimization involves the variation of one parameter while the others are held constant, which is known as the one-factor-at-a-time (OFAT) approach. This does not account for interactions between factors, so statistical experimental designs, also known as the design-of-experiments (DoE) approaches, are more widely used for bioprocess optimization and were successfully applied to significantly improve biomass, protein and metabolite yields [98-103]. Statistical analysis can be used to simultaneously examine the effects of multiple input parameters on a given output, allowing conversion processes to be optimized in terms of product accumulation and any other relevant characteristics by considering how the input parameters interact [104, 105]. As the OFAT does not take these factor interactions into account due to the sequential optimization of individual factors, putative beneficial effects are not assessed and often the true optimum is missed. Various specified designs are available for process optimization studies, including RSM for the quantitation of factor impacts on responses, mixture designs for the optimization of mixtures, and factorial designs to test screening factors (Figure 2.3) [106].

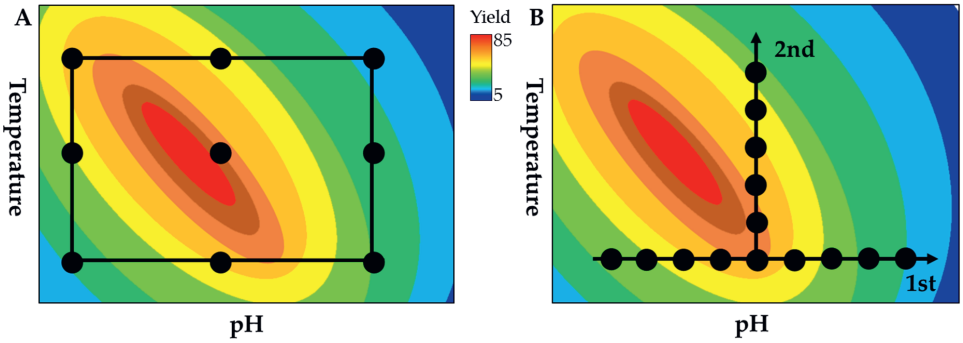

Figure 2.3. Comparison of a factorial design experiment (A) and the one-factor-at-a-time (OFAT) approach (B) for the maximization of the product yield by optimizing two representative process factors: pH and temperature. A: The factorial experimental design allows the investigation of all possible combinations of the two factors on different levels considering potential interdependencies between the factors within the design-space. Optimum conditions for both factors that give maximum yield can be determined. B: In the OFAT approach, both factors are investigated sequentially (1^{st}, 2^{nd}) neglecting factor interactions, leading to the loss of conclusive information and thereby missing the true maximum.

As mentioned above, statistical experimental designs have been successfully applied for the chemical degradation of chitin to chitosan and COS in order to assess factor interactions between the process variables reagent type and content, reaction time and process temperature [30, 31]. For complex enzyme reactions, the product-related effects of various physical and biological factors can be investigated and quantified, including pH, temperature, reaction time, salt concentration, co-factors, enzyme combinations, and enzyme mixture ratios. Because a DoE analysis reveals factor interactions, predictive models can be built that offer further information about process parameters to increase product yields and process efficiency [107, 108]. Sequential rounds of DoE optimization can be carried out to optimize the overall process, reflecting the complexity and number of investigated factors. For example, initial factorial screening involves the investigation of multiple factors on different levels, revealing the significance of basic factor interactions. In the sequential approach, factors showing no (or insignificant) interdependencies can be excluded from the experiment and RSM is used to assess factor interdependencies in more detail [109]. The final predictive model can determine optimal factor combinations as

well as predicted and extrapolated factor combinations to achieve a specific, desired output.

Recently, a DoE approach was used to create optimized enzyme cocktails for the complete enzymatic hydrolysis of marine chitin to monomeric GlcNAc by testing 41 enzyme mixtures each comprising five different chitinases. A full cubic model and D-optimal design was used for optimization and similar saccharification yields (70-75 %) and enzyme dosages to that of current lingocellulose processings could be achieved with the optimized cocktail [110]. A similar DoE approach has been described for the hydrolysis of lignocellulosic biomass. A quadratic design including five different enzyme mixture components was used for a two stage optimization process. Higher hydrolysis yields (% of glycan conversion) of the optimized cocktails (82.3±4.4 %) could be observed compared to the two commercial cellulose cocktails Cellic® CTec2 (70.0±4.7 %) and Cellic® CTec3 (76.3±0.8 %)[107]. Synergistic minimal enzyme cocktails have also been developed for the saccharification of sugarcane biomass using a mixture model (simplex-centroid model) in which precise mixture ratios of three enzymes were compared at their reaction optima. As in the optimization study above, the optimized enzyme cocktail was compared to two commercial cellulose cocktails (Celluclast 1.5 L and Cellic CTect2) and comparable reducing sugar yields could be achieved [111]. For the conversion of chitin to COS, the DoE factors (enzymes, mix ratios, temperature, pH, salt, and cofactors) could be tested not only to increase overall product yields but also to achieve specific values for DP and DA.

In conclusion, it can be pointed out that the large amount of different chitinolytic enzymes and variable physicochemical parameters allow for a far more open process development in terms of altered product properties compared to the chemical degradation process that is restricted by the limited amount of different process parameters. The discovery and isolation of chitinolytic bacteria from different environments is furthermore of great interest as they represent a great source for novel chitinases and CDAs that could facilitate the development of enzymatic COS production processes. The DoE approach could furthermore be implemented for effective process optimization in order to maximize yields and to potentially alter product properties. Thereby the development of enzymatic processes that are

competitive to chemical processes with regards to COS conversion yields could become technically more feasible.

2.6 References

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Chapter 3

Chi5: A novel marine chitinolytic bacterium

In this chapter, the novel marine bacterium Chi5 is characterized polyphasically and its chitinolytic potential for the degradation of marine chitin is explored. Following a selective isolation of the strain, the characterization comprises the assessment of morphological properties, essential metabolic pathways, cellular composition as well as growth characteristics. Sequencing of the 16S rDNA and the genome is used to generate a phylogenetic tree of all known *Photobacterium* species and to carry out a genome-to-genome hybridization for the conclusive determination of the genetic relatedness. Multiple analytical methods are used to assess the chitinolytic potential of the novel strain and to identify putative target enzymes.

Keywords: chitinolytic potential, Chi5, phylogenetic tree, polyphasic characterization

This chapter is based on the following publication:

Schmitz, C.; Agdour, S.; Jablonka, N.; Fritsch, L.; Fischer, R.; Schillberg, S.; Bortesi, L.; Rasche, S., *Photobacterium orarium* sp. nov., a bacterium isolated from coastal seawater. *Int J System Evol Micr*. Prepared for submission.

3.1 Introduction

Marine environments are a promising source for novel chitinolytic bacteria as they represent the ecosystem with the worldwide highest abundance of chitin [1]. The relatively low overall density of organic nutrients in sea-water results in high selective pressure on microorganisms and therefore well-adapted bacteria can be obtained from water samples that can potentially utilize any accessible nutritional resource [2-4]. As described in **Chapter 2,** microorganisms thus are a potent source for the discovery of novel chitinolytic enzymes as many bacteria are capable to carry out a complete degradation of insoluble marine chitin for subsequent metabolization.

In this chapter, the morphological, metabolic, phylogenetic and genomic characteristics of Chi5, a novel bacterial isolate from North Sea coastal sediments are described. Based on polyphasic evidence, Chi5 represents a new *Photobacterium* species. *Photobacterium* is a genus of Gram-negative, motile, rod-like marine bacteria with a facultative fermentative metabolism [5]. The first *Photobacterium* species was discovered in 1889 [6], and at the time of writing the genus comprises 27 recognized species (including two subspecies), namely *P. ghanghwense* [7], *P. halotolerans* [8], *P. aphoticum* [9], *P. aquae* [10], *P. galatheae* [11], *P. gaetbulicola* [12], *P. lutimaris* [13], *P. rosenbergii* [14], *P. marinum* [15], *P. jeanii* [16], *P. swingsii* [17], *P. leiognathi* [18], *P. panulari* [19], *P. angustum* [20], *P. aquimaris* [21], *P. phosphorum* [22, 23], *P. illiopiscarium* [24], *P. kishitanii* [25], *P. aplypisae* [26], *P. alginatilyticum* [27], *P. aestuarii* [28], *P. lipolyticum* [29], *P. profundum* [30], *P. frigidiphilum* [31], *P. indicum* [32], *P. damselae* subsp. *damselae* [33-35], and *P. damselae* subsp *piscicida* [33, 36]. All species in this genus require NaCl for growth and are positive for oxidase and catalase activity [5]. The overall chitinolytic potential of the novel strain was assessed in *in vitro* hydrolysis studies for the initial identification of putative target enzymes.

3.2 Results and discussion

3.2.1 Polyphasic characterization

The Chi5 cells were found to be Gram-negative with a rod-like morphology (approximate size 1.5–3.5 x 0.8–0.9 µm) and motility was conferred by a polar flagellum. Colonies on TSA were circular, creamy and opaque. They remained viable within the temperature range 15–37 °C, within the pH range 6.0–10.0, and in the presence of up to 5% (w/v) NaCl (optimal growth at 28 °C, pH 7.0 and 1–2% (w/v) NaCl). Colonies grew more rapidly under aerobic rather than anaerobic conditions (data not shown).

Strain Chi5 was able to utilize as sole carbon sources D-glucose, D-fructose, D-mannitol, *N*-acetyl-D-glucosamine, D-maltose, D-trehalose, adipic acid, malic acid and citrate, and was able to ferment D-glucose, mannitol and D-mannose but not inositol, L-arabinose, sorbitol, rhamnose, sucrose, melibiose or amygdalin. The cells were unable to produce indole, H_2S or acetoin, but could reduce nitrate and hydrolyze gelatin.

The enzyme assays revealed that Chi5 possesses esterase (C4), esterase lipase (C8), lipaseleucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, acid and alkaline phosphatase, naphthol-AS-BI-phosphotohydrolase, α-glucosidase and *N*-acetyl-β-glucosaminidase activities, but there was no evidence for α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, α-mannosidase, α-fucosidase, arginine dihydrolase, tryptophan deaminase, lysine decarboxylase, ornithine decarboxylase or urease activities. The Chi5 cells were also positive for catalase and oxidase activities, and were susceptible to all tested antibiotics.

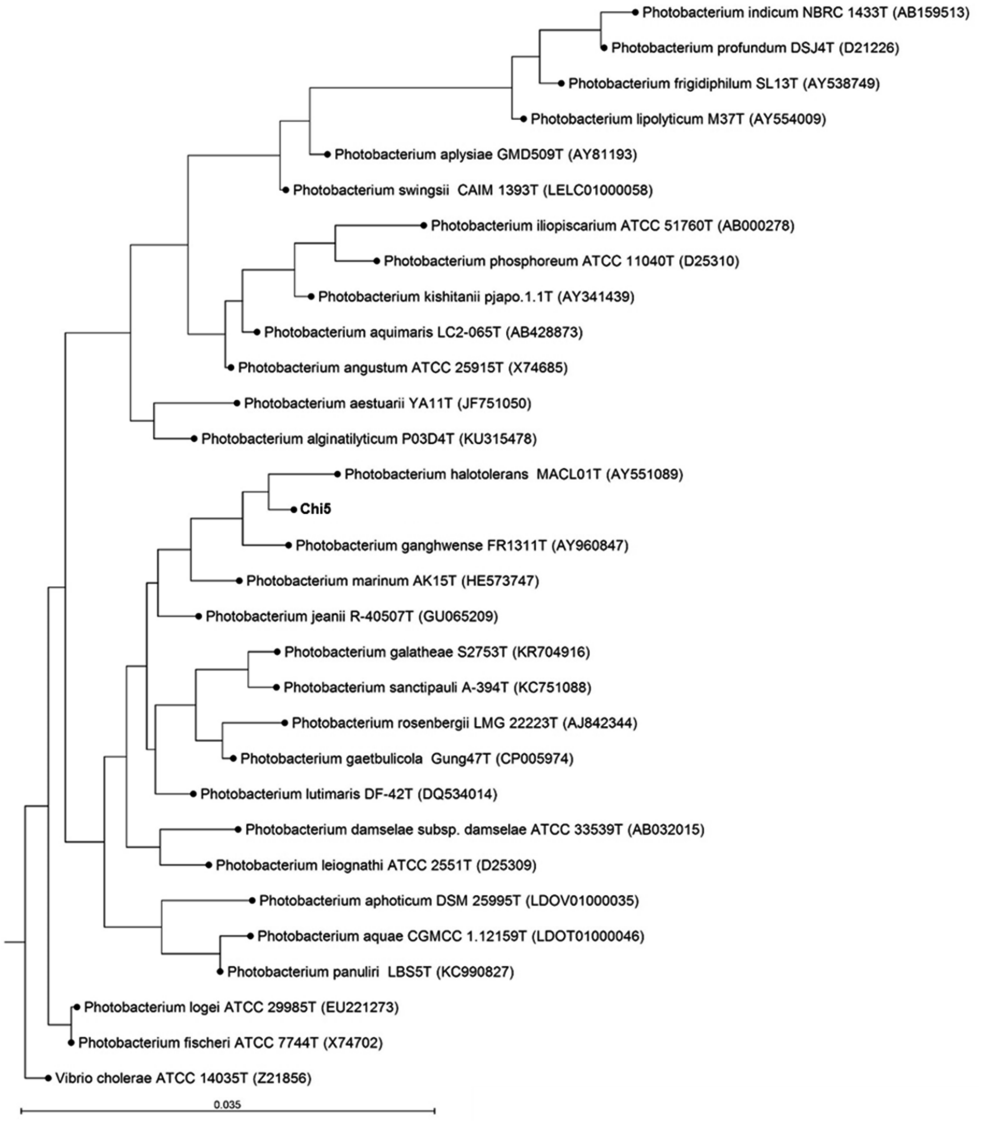

Figure 3.1. Neighbor-joining tree based on nearly complete 16S rDNA sequences indicating the relationship between Chi5 and other members of the genus *Photobacterium*. Bootstrap values (≥ 30%) based on 1000 re-samplings are shown. *Vibrio cholerae* 14035^{T} was used as an outgroup. Bar = 0.035 nucleotide substitutions per position.

Approximately 95% of the Chi5 16S rDNA sequence was established by direct sequencing of the PCR product, and comparative analysis indicated that the strain belongs to the genus *Photobacterium* and clusters with *P. galatheae* (98.3% identity

to *P. galatheae* S2753^T), *P. halotolerans* (98.3% identity to *P. halotolerans* MACL01^T) and *P. ganghwense* (97.5% identity to *P. ganghwense* DSM 22954^T) (Figure 3.2). The level of sequence identity with other species included in the phylogenetic analysis was lower than 94.8%. The DNA G+C content of strain Chi5 was 49.1 mol%.

Whole-genome sequencing data were used to further asses the relationship between Chi5 and similar species/strains of the genus *Photobacterium*. The genomes of *P. galatheae* S2753^T (KR704916), *P. halotolerans* MACL01^T (AY551089) and *P. ganghwense* DSM 22954^T (LDOU01000025) were available on GenBank. The genomes were compared using Genome-to-Genome Distance Calculator v2.1 (GGDC) available from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (http://ggdc.dsmz.de/) [37]. The mean DNA-DNA hybridization values (GGDC) were 22.70% [range 20.4–25.2%] for *P. galatheae* S2753^T (KR704916), 52.30% [49.6–54.9%] for *P. halotolerans* MACL01^T (AY551089) and 20.80% [18.6–23.3%] for *P. ganghwense* DSM 22954^T (LDOU01000025). Usually, the lower threshold for species identity is 70%. The GGDC results thus indicated that Chi5 is a distinct species of *Photobacterium* rather than a strain of an existing species [37].

Fatty acid profiling revealed the presence of fatty acids in the range C_{12}–C_{18}. The most abundant fatty acids were $C_{16:1}$ ω7c and/or $C_{16:1}$ ω6c (38.7%), which are described as 'summed feature 3' in Table 3.2, $C_{16:0}$ (22.6%), $C_{18:1}$ ω7c (22.3%), $C_{12:0}$ (6.9%), $C_{12:0}$ 3-OH (5.3%), 'summed feature 2', which includes $C_{14:0}$ 3-OH, iso-$C_{16:1}$, an unidentified fatty acid with an equivalent chain length of 10.928 and/or $C_{12:0}$ aldehyde (4.6 %), $C_{16:1}$ ω9c (1.9%) and $C_{14:0}$ (1.1%). The fatty acid composition of Chi5 is compared to that of other *Photobacterium* species in Table 3.1.

Table 3.1. Fatty acid composition of Chi5 compared with its closest relatives in the genus Photobacterium. 1, Chi5; 2, *P. galatheae* S2753T (Machado et al., 2015), 3, *P. halotolerans* MACL01T (Rivas et al., 2006); 4, *P. ganghwense* FR1311T (Park et al., 2006); 5, *P. gaetbulicola* Gung47T (Kim et al., 2010). Values are percentages of total fatty acids, -: not detected or not described.

Fatty acids	1	2	3	4	5
Straight-chain					
$C_{12:0}$	6.9	3.9	5.9	3.4	1.7
$C_{14:0}$	1.1	1.32	1.0	3.6	4.2
$C_{15:0}$	–	1.3	–	-	2.9
$C_{16:0}$	22.6	22.1	21.2	21.1	14.0
$C_{17:0}$	0.8	2.0	–	–	1.0
Branched					
iso $C_{13:0}$	0.3	–	–	–	–
iso $C_{15:0}$	0.2	–	–	–	6.9
iso $C_{16:0}$	0.5	–	1.0	–	0.4
iso $C_{17:0}$	0.5	–	–	–	3.5
iso $C_{17:1}$ω9c	–	–	–	–	1.4
Unsaturated					
$C_{16:1}$ω7c alcohol	–	–	–	–	–
$C_{16:1}$ω9c	1.9	2.2	2.5	2.5	–
$C_{17:1}$ω8c	–	2.2	–	–	0.6
$C_{18:1}$ω7c	22.3	21.4	19.9	29.6	10.3
Hydroxy					
$C_{12:0}$ 3-OH	5.3	6.1	6.6	2.1	2.8
iso-$C_{15:0}$ 3-OH	–	–	–	–	–
Summed features					
2 (1)	4.6	4.2	3	2.7	3.7
3 (2)	38.7	27.9	32.6	27.8	33.6

(1) Summed feature 2 contained $C_{14:0}$ 3-OH, iso-$C_{16:1}$, an unidentified fatty acid with equivalent chain length of 10.928 and/or $C_{12:0}$ aldehyde.
(2) Summed feature 3 contained $C_{16:1}$ ω7c and/or $C_{16:1}$ ω6c.

The main biochemical and physiological properties that distinguish strain Chi5 from known *Photobacterium* species are summarized in Table 3.2. Compared with *P. galatheae*, its closest phylogenetic neighbor, Chi5 utilizes different carbon sources, produces acids from various fermentable substrates, and synthesizes iso $C_{13:0}$ fatty acids. These data clearly indicate that Chi5 is a novel species in the genus *Photobacterium*.

Table 3.2. Differential physiological and biochemical characteristics of Chi5 and its closest phylogenetic neighbors. Strains: 1, Chi5; 2, *P. galatheae* S2753T (data from Machado et al., 2015); 3, *P. halotolerans* MACL01T (data from Rivas et al., 2006; Machado et al., 2015); 4, *P. ganghwense* FR1311T (data from Park et al., 2006; Machado et al., 2015); 5, *P. gaetbulicola* Gung47T (data from Kim et al., 2010). +: Positive reaction or production; –: negative reaction or no production; ND: data not available.

Characteristic	1	2	3	4	5
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
Growth at:					
4° C	+	-	+	-	-
35 °C	+	+	+	+	+
Production of H_2S	-	-	-	-	+
Reduction of nitrate	+	+	+	+	+
Hydrolysis of gelantine					
Indole production	-	-	-	+	ND
Arginine dihydrolase	-	-	-	+	-
β-Galactosidase	-	-	+	+	+
Assimilation of:					
D-mannose	-	-	+	+	+
N-acetyl-D-glucosamine	+	+	+	+	+
L-arabinose	-	+	+	-	-
Fermentation of:					
D-mannose	+	-	+	-	+
L-arabinose	-	-	+	-	-
Rhamnose	-	-		+	-
Melibiose	-	-	-	-	+
Sucrose	-	-	+	-	+
DNA G+C content (mol%)	49.1	49.5	49.8	44.0	50.6

3.2.2 Taxonomic description of the novel bacterial strain

Cells are Gram-negative and rod-like with motility conferred by a polar flagellum. Colonies on TSA are circular, cream-colored and opaque, and test positive for oxidase and catalase activity. Optimal growth occurs at 28 °C and pH 7.0 in the presence of 1–2% (w/v) NaCl. The cells are susceptible to the antibiotics ampicillin, carbenicillin, chloramphenicol, kanamycin, rifampicin, streptomycin, tetracycline and zeocin. They can utilize D-glucose, D-fructose, D-mannitol, *N*-acetyl-D-glucosamine, D-maltose, D-lactose, adipic acid, malic acid and citrate as sole carbon sources, and can ferment D-glucose, mannitol and D-mannose, but not inositol, L-arabinose, sorbitol, rhamnose, sucrose, melibiose or amygdalin. They can reduce nitrate and hydrolyze gelatin. The cells are positive for the following enzyme activities: esterase (C4), esterase lipase (C8), lipaseleucine acrylamidase, valine acrylamidase, cystine acrylamidase, trypsin, acid and alkaline phosphatase, naphthol-AS-BI-phosphotohydrolase, α-glucosidase and *N*-acetyl-β-glucosaminidase. The cells show no evidence of α-chymotripsin, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, α-mannosidase, α-fucosidase, arginine dihydrolase, tryptophan deaminase, lysine decarboxylase or ornithine decarboxylase activities. The cells cannot produce indole, H_2S or acetoin. The DNA G+C content is 49.1 mol%. The major fatty acids are $C_{16:1}$ ω7c and/or $C_{16:1}$ ω6c ('summed feature 3', 28.67%), $C_{16:0}$ (22.59%), $C_{18:1}$ ω7c (22.34%), $C_{12:0}$ (6.86%) and $C_{12:0}$ 3-OH (5.32%), with smaller amounts of $C_{14:0}$ 3-OH and/or $C_{16:1}$ iso, and an unidentified fatty acid with an equivalent chain length of 10.928 and/or 12:0 aldehyde ('summed feature 2', 4.60%), $C_{16:1}$ ω9c (1.94%) and $C_{14:0}$ (1.11%). Strain Chi5 was isolated from coastal sediment samples collected near Ostend, Belgium.

3.2.3 Assessment of chitinolytic activity

Chitinolytic activity of the culture supernatant of Chi5 was assessed using a reducing end assay, zymography as well as thin layer chromatography. All analytical methods confirmed that Chi5 is capable to degrade marine chitin. The reducing end assay demonstrated that, relative to the *E. coli* BL21 (DE3) negative control, substantial amounts of reducing sugars are released after incubation of the Chi5 supernatant with chitin powder for 16 h at 30 °C (Figure 3.3A).

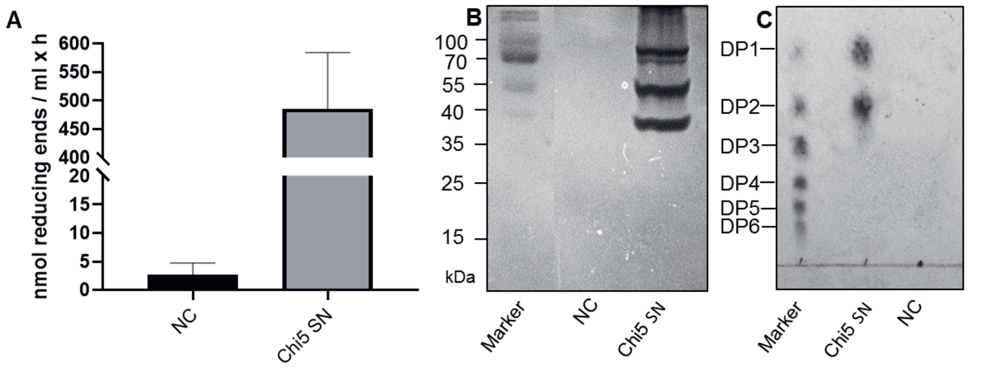

Figure 3.3. Analysis of Chi5 supernatant for the hydrolysis of chitin. A: Quantification of chitinolytic activity of culture supernatant (SN) from Chi5 after incubation with chitin powder as a substrate for 16 h at 30 °C. *E. coli* BL21 wild-type culture supernatant was used as a negative control (NC). Reducing sugars were quantified using a reducing end assay and a calibration curve of N-acetyl glucosamine was prepared freshly. The reducing end assays were carried out in three replicates (n=3). B: Zymogram of freshly prepared supernatant of a Chi5 culture grown on chitin as the sole carbon source. For the identification of active chitinolytic enzymes, 10 % (v/v) glycol chitin was incorporated in the gel and fluorescent dye was applied after incubation of the gel at 30 °C for 2h. *E. coli* BL21 supernatant was used as a negative control (NC). C: Fresh Chi5 cultures supernatant was incubated with 5 % (w/v) chitin powder at 30 °C for 16 h. After heat inactivation (95 °C, 10 min) 0.3 µl of the hydrolysate was separated by TLC. *E. coli* BL21 supernatant was used as a negative control (NC) and commercial chitin standard molecules (monomer-hexamer) were used as a size marker. kDa= kilo Dalton; DP= degree of polymerization.

The release of free sugars is thus associated with the presence of chitinolytic enzymes that hydrolyze the glycosidic bonds within chitin. Zymography confirmed at least four chitinolytic enzymes that are involved in the degradation process ranging from ~37 kDa to over 100 kDa (Figure 3.4B). Product analysis by TLC further revealed that monomeric and dimeric compounds are generated (Figure 3.4C) indicating the presence of exo-chitinase activity within the supernatant.

3.3 Conclusions

Chi5 proved to be an entirely novel bacterial strain and a source for enyzmes with exo-chitinase activity that can be used for conversion of insoluble chitin to monomeric

and dimeric sugars. In this respect, the characteristic of Chi5 to utilize chitin as a sole carbon and nitrogen source could point to novel and unique chitinolytic enzymes with high conversion rates for crystalline chitin. As it is the intention to generate chitosan oligomers with a DP higher than 2, the strain cannot be utilized directly for the production of such compounds. As a consequence, the individual chitinases produced by Chi5 need to be expressed recombinantly and characterized separately in order to establish a limited *in vitro* degradation process from chitin to COS. For further investigations and characterizations of the degradation process, the next generation sequencing data of the Chi5 genome was further analyzed to identify the genes encoding the single chitinolytic enzymes as well as putative chitin-deacetylases

Future exploitations for Chi5 for the development of a versatile biological production platform for GlcNAc, recombinant proteins as well as polyhydroxalkanoats (PHA) as a building block for bioplastics are discussed in the outlook of this thesis.

3.3 Experimental section

Selective strain isolation

Strain Chi5 was isolated from seawater samples collected on the North Sea coast near Ostend, Belgium. After storage at 4 °C, three rounds of culture enrichment were carried out to screen for chitinase-producing microorganisms. In the first round, 5 ml of sea water were transferred into three flasks each containing 25 ml minimal medium with chitin as the sole carbon source (50 mM phosphate buffer (pH 7.3), 0.5% (w/v) chitin, and 1% (v/v) salt solution comprising 0.5% (w/v) $Ca(NO_3).4H_2O$, 2% (w/v) $MgSO_4.7H_2O$, 10% (w/v) $(NH_4)_2SO_4$, 0.1% (w/v) $Fe(III)NH_4$ citrate and 10% (v/v) trace element solution SL6). Flasks were incubated for 7 d at 28 °C at 160 rpm. In the subsequent rounds, 2-ml aliquots of culture from the previous enrichment step were transferred to flasks containing 25 ml fresh medium and incubated as above. After the third round, a 2-ml aliquot was plated on minimal agar containing 0.001% (w/v) Fluorescent Brightener 28 (Sigma-Aldrich) and 0.5% (w/v) chitin as the sole carbon source, and incubated at 28 °C. By viewing the plates under UV light, colonies displaying a dark halo indicating chitin degradation were observed. These were picked and transferred to liquid M9 minimal medium containing 1% (w/v) colloidal chitin as the sole carbon source. Strain Chi5 was selected for further characterization on the basis of its strong chitin-degrading activity.

Morphological characterization

The cells were plated on tryptone soy agar (TSA) supplemented with 0.5% (w/v) NaCl, and the colony visual characteristics were recorded, as well as investigating cell morphology and motility by light microscopy using an Olympus BX40, and by scanning electron microscopy using a Philips XL-30 ESEM. To determine the flagellum type, cells were seeded onto glass slides pre-coated with poly-L-lysine and grown for 18 h at 28 °C, then dehydrated with ethanol and acetone via critical point drying as previously described [38]. Gram staining was carried out using a commercial gram staining kit for microscopy (Merck Millipore).

Biochemical and metabolic characterization

Chi5 cells were suspended in commercial (a) CHB/E medium, (b) AUX medium (bioMérieux), and (c) sterile 0.85% (w/v) NaCl. These three suspension were used to test for (a) carbon source utilization with API 50 CH strips, (b) enzyme activity with API ZYM strips, and (c) various other physiological and biochemical characteristics with API 20E and API 20NE strips (all API stripes from bioMérieux). Anaerobic growth was evaluated on TSA by incubation in a gas-tight container containing an Anaerocult A chemical oxygen scrubber (Merck). Sensitivity to antibiotics was evaluated using the agar diffusion assay, in which bacteria growing on TSA plates are exposed to sterile paper discs impregnated with 10 µg ampicillin, 100 µg carbenicillin, 100 µg chloramphenicol, 30 µg kanamycin, 50 µg streptomycin, 30 µg tetracycline, 100 µg rifampicin or 100 µg zeocin [39].

Fatty acid profile, DNA G+C content and 16S rDNA sequencing

The fatty acid profile, DNA G+C content and 16S rDNA sequence were determined by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). The composition of fatty acids was determined by gas chromatography according to the Microbial Identification System (MIDI). Genomic DNA extraction, 16S rDNA sequence amplification by PCR and product purification were carried out as previously described [40]. The resulting sequence data were analyzed using Molecular Evolutionary Genetic Analysis v7 (MEGA7) [41], the EzTaxon server [42-44] and BlastN [45]. For further comparison, 16S rDNA sequences were obtained from the EMBL or RDP databases [46]. A similarity matrix was calculated by pairwise comparison of sequences within the alignment and a phylogenetic tree was constructed using the neighbor-joining method with the Jukes-Cantor nucleotide distance measure [47]. Bootstrap analysis was based on 1000 re-samplings. The G+C content of the DNA was determined by HPLC using an Agilent 1260 Infinity II HPLC system [48]. Chromatograms were evaluated using OpenLAB 2 software (Agilent) and the G+C content was calculated based on the ratio of deoxyguanidine to thymidine [49].

Whole genome sequencing

In order to sequence the Chi5 genome, 400-bp libraries were prepared using the Ion Xpress Plus gDNA Fragment Library Preparation Kit (Thermo Fisher Scientific). The libraries were sequenced using the Ion Torrent technology (Thermo Fisher Scientific) and sequence assembly was performed using the Lasergene genomic suite software (DNASTAR). The sequence was condensed onto 60 scaffolds with a 10-fold coverage. The reads were assembled into contigs using CLC main workbench v7.7 (Qiagen).

Sample incubation and activity assay

For the preparation of chitinolytic active culture supernatant, first a 100 mL starter culture of Chi5 in TS medium was incubated overnight at 28 °C and 180 rpm. For the main culture, 200 mL liquid M9 minimal medium containing 1% (w/v) chitin (chitin powder extracted from shrimp shells [~400.000 g/mol; Carl Roth]) were inoculated 1:100 using the starter culture and then incubated for 72 h at 30 °C and 180 rpm. Cells and culture supernatant were separated by centrifugation (7000 *x* g, 20 min), the supernatant was sterile filtrated and stored at -20 °C until further use.

A reducing end assay was carried out to assess the chitinolytic activity of Chi5 culture supernatant [50]. Therefore, 100 µl of Chi5 culture supernatant were incubated with 5 % (w/v) chitin and 900 µl MAT buffer (33 mM MES, 33 mM sodium acetate, 33 mM TRIS; pH 8.0) for 16 h in an overhead shaker at 30 °C (standard conditions). Samples were centrifuged at 13000 x g for 2 min and 40 µl of the supernatant was mixed with 40 µl 0.5 M NaOH followed by the addition of 40 µL of a solution containing 1.5 mg/ml 3-Methyl-2-benzothiazolinonhydrazon and 0.75 mg/ml Dithiothreitol. Samples were incubated at 80 °C for 15 min and then thoroughly mixed with 80 µl of 0.5 % (w/v) $FeNH_4(SO_4)_2$) x $12H_20$, 0.5 % (w/v) sulfamic acid and 0.25 M HCl. Samples were cooled down to room temperature and absorption was measured at 620nm. A calibration curve of N-acetyl-glucosamine was prepared freshly for each measuring cycle. One unit [U] was defined as the amount of enzyme required to release 1 µmol of reducing sugars per hour.

Preparation of glycol chitin for zymography

In order to prepare soluble glycol chitin for the use in zymography, the method described by Trudel and Asselin was adopted [51]. Briefly, 1 g of glycol chitosan (Sigma-Aldrich) was dissolved in 20 ml 10 % (v/v) acetic acid and stirred at ambient temperature for 24h. Methanol (90 ml) was added gradually and the suspension was vacuum filtrated through a 0.45 µm bottle top filter, followed by the addition of 1.5 ml acetic anhydride and incubation for 30 min at room temperature. The resulting gel was blendend with equal amounts of methanol and then centrifuged at 27.000 *x* g for 15 min at 4 °C. After repetition of the previous step, glycol chitin was re-suspended in 100 ml demineralized water, spiked with sodium azide as a preservative and stored at 4 °C.

Zymography

Optical analysis of active chitinolytic enzymes within the supernatant samples was carried out by zymography using SDS-PAA gels. The gels were prepared using 12 % (v/v) acrylamide and 10 % (v/v) of glycol chitin as a substrate. Supernatant samples (16 µl) were mixed with 5 x native SDS buffer (4 µl) and 15 µl of this mixture were loaded on the gel. Electrophoretic separation was carried out at constant 100 V for 2 h. After separation, gels were incubated and developed according to the procedure summarized in Table 3.3.

Table 3.3. Incubation steps for determination of chitinolytic activity of enzymes by zymography. *contains 0.5 M TRIS and 0.01 % (w/v) Calcoflour white M2R (Sigma Aldrich).

Incubation solution	Duration	Temperature
Wash buffer (PBS + 0.05 % Triton X100)	2 x 20 min	RT
TRIS (pH 7)	2 h	30 °C
H_2O	10 min	RT
Developing solution*	10 min	RT
H_2O	2 x 20 min	RT
Evaluation of gel under UV light (302 nm) using the GelDoc XR system		

Product analysis by thin-layer-chromatography

Products were identified after enzymatic hydrolysis using 10x10 cm TLC Silica gel 60 F254 plates (Merck). For separation, a mixture of butanol: methanol: 25 % ammonia: H_2O (5:4:2:1) was utilized as mobile phase. A total volume of 0.5 µl was applied in 0.25 µl spots for hydrolysis samples and chitin standard sugars (Megazyme, Chicago, USA) (5 mg/ml). After separation, the plate was air-dried and the developing solution (200 ml Acetone, 30 ml phosphoric acid (85 %), 4 ml aniline, 4 g diphenylamine) was applied followed by spot visualization at 300 °C using a heat gun [52].

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Chapter 4

Recombinant production and characterization of five novel chitinolytic enzymes for the *in vitro* degradation of chitin

In this chapter, homologous genes of the chitinolytic enzymes will be identified after next generation sequencing of the Chi5 genome using protein family databases. Multiple target gene constructs will be cloned into *E. coli* BL21 (DE3) using the golden gate cloning approach and expressed recombinantly. Optimum enzyme variants with respect to expression levels and activity will be selected for scaled-up expression and purification by immobilized metal affinity chromatography from the culture supernatant using a 6xHis tag. Optimum pH, temperature and salinity for all enzymes are determined on insoluble chitin as a substrate. Co-factors are explored as putative reaction activators or inhibitors. Enzyme kinetics are monitored in order to determine maximum reaction rates and optimum enzyme-to-substrate ratios. Oligomeric hydrolysis products are identified for all novel enzymes by thin layer chromatography.

Keywords: chitin oligomers, enzyme characterization, gene mining, golden gate cloning, recombinant production

This chapter is based on the following publication:

Schmitz,C.; Jablonka, N.; Schillberg, S.; Fischer, R.; Rasche, S.; Bortesi, L., Recombinant production and characterization of five novel chitinolytic enzymes isolated from a marine *Photobacterium* strain for the *in vitro* degradation of chitin. Prepared for submission.

4.1 Introduction

In **Chapter 3**, a novel marine chitinolytic bacterial strain was isolated from coastal seawater samples and the initial genetic analysis of the 16S rDNA revealed that it belongs to the genus *Photobacterium*. The strain is capable to utilize marine chitin from shrimp shells as carbon and nitrogen source after carrying out an extracellular hydrolysis of chitin to GlcNAC and $GlcNAC_2$ by secreting a cocktail of multiple chitinolytic enzymes. This chapter summarizes the gene mining of putative chitinases, their cloning and recombinant expression in *E. coli* BL21 and sequential characterization of the respective enzymes. Furthermore, soluble hydrolysis products are identified by thin layer chromatography by testing various chitin substrates and chitin standard molecules.

As shown in **Chapter 2**, the partially deacetylated chitin-derivative chitosan currently receives great attention as it possesses a variety of intrinsic physiological effects by virtue of anti-bacterial,-inflammatory and –fungal properties and therefore has found a many different applications in agrochemical, cosmetic, food, waste-water treatment and medical fields [1-7]. Chitosan however possesses limited solubility in aqueous and pH neutral media and therefore applications especially in cosmetic and medical fields are still restricted. Shorter COS are therefore getting increasing attention as they exhibit similar or even enhanced physiological effects while being highly water soluble and therefore can open up even more application fields especially in human health contexts [8-13].

Conventionally, chitosan and COS are produced by a chemical deacetylation and depolymerization of chitin extracted from mainly shrimp shells or alternatively from insects and fungi [14]. However, several issues accompany the chemical degradation of chitin such as: (1) a limited controllability of the reaction process resulting in less defined products in terms of degree of polymerizations (DP) and degree of acetylation (DA); (2) an increase of environmental pollution due to the excessive use of chemical reagents (e.g., sodium hydroxide and hydrochloric acid); (3) product contaminations from residual reactants that may cause health issues especially for the applications in cosmetic, food industry, and medical products [15, 16].

Alternative COS production approaches using chitinolytic enzymes therefore received increasing attention in recent researches [17-21]. Chitinases belong to the group of glycosyl hydrolases and cleave glycosidic bonds in chitin and can be grouped in three classes: endo-chitinases (EC 3.2.1.14) that randomly hydrolyze chitin into smaller fragments and oligomers; exo-chitinases degrade chitin to release diacylchitobiose and GlcNAc (EC 3.2.1.30) and N-acetyl-β-glucosaminidases (EC 3.2.1.52) hydrolyse oligomeric chitin to give free GlcNAc [22-26]. A large variety of different chitinolytic enzymes were isolated so far from different organisms of various habitats and some of these enzymes are also already integrated in hybrid-processes to generate COS from chitosan (see Table 2.2 for an overview) [27-31].

In order to employ chitinases for the production of COS, a limited degradation of chitin by endo-chitinases must be ensured while avoiding a hydrolysis by exo-chitinases, chitobiases and N-acetyl-β-glucosaminidases to GlcNAc. As Chi5 secretes a potent mixture of chitinolytic enzymes in order to depolymerize chitin to assimilable GlcNAC and $GlcNAC_2$, the active culture supernatant cannot be utilized readily for the production of COS with a DP higher than 2 thus indicating the existence of exo-chitinase activity. In this chapter, all genes coding for chitinases were therefore identified from the Chi5 genome data and expressed recombinantly in order to yield single enzymes for the identification of endo- and exo-chitinases and for the determination of activity optima.

4.2 Results

4.2.1 Cloning and expression of putative chitinases

Gene mining was carried out after next generation sequencing and 60 contigs were screened for chitinase homologues using the Pfam database (version 2.9). Five genes (C1-C5) were identified that showed several characteristic chitinase domains (Table 4.1). The chitinase A and glycosyl hydrolase 18, 19 and 20 domains have previously been reported to be specific for chitinases and chitobiases that differ from each other in structures and modes of action [32]. Compared to the currently reported chitinases that range between 20-90 kDa, the predicted molecular weight of the novel chitinases in general is ranked high [33]. C1, 3 and 4 are significantly larger with 83.9 – 86.8 kDa

compared to C2 and 5 with 56.5 and 65.4 kDa, respectively. However, no additional or unknown domains were identified within the genes. Natural signal peptides were discovered for C1, C2, C3 and C4, suggesting that C5, containing also a putative chitobiase domain, remains intracellular in order to further hydrolyze $GlcNAG_2$ to GlcNAG.

Table 4.1. Chitinase specific domains for the five putative chitinases (C1-C5) that were identified in the genome of Chi5. AA=amino acid. kDa= kilo dalton.

Enzyme	Size AA	Molecular weight [kDa]	Domain 1	Domain 2	Domain 3	Signal peptide
C1	815	85.5	Chitinase A, N-terminal domain	Glycosyl hydrolase family 18	-	✓
C2	515	56.5	Carbohydrate binding domain	Glycosyl hydrolase family 19	-	✓
C3	826	86.8	Chitinase A, N-terminal domain	Glycosyl hydrolase family 18	-	✓
C4	777	83.9	Glycosyl hydrolase family 18	Chitinase C	Cellulose-binding domain	✓
C5	594	65.4	Glycosyl hydrolase family 20, domain 2	Glycosyl hydrolase family 20, catalytic domain	Chitobiase domain	-

A total of eight constructs comprising a cytosolic form, a fusion to the dsbA protein, variants with the natural and PelB signal peptide and the 6xHis tag as well as Tag54/6xHis combi-tag were cloned for each putative chitinase gene (Figure 4.1) in *E.coli* DH5α.

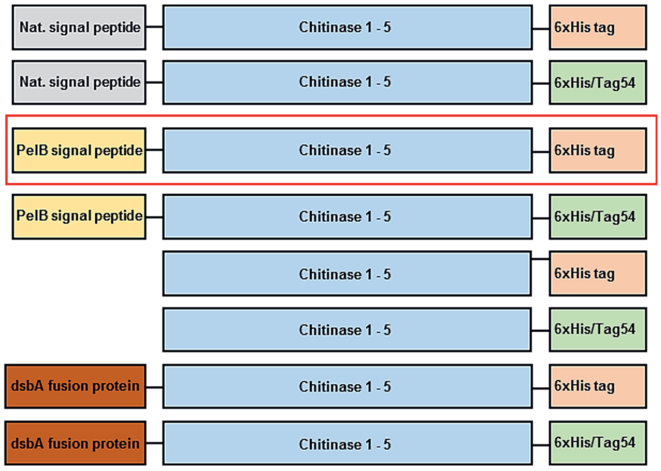

Figure 4.1. Construct variants for each putative chitinase gene cloned in *E. coli* DH5α using the golden gate cloning technology. Variants comprising the PelB signal peptide and 6xHis tag (in red frame) were selected for upscaled expression and purification.

Integrity and correctness of the constructs was confirmed by colony PCR and Sanger-sequencing. All constructs were transformed into *E. coli* BL21 and expressed recombinantly. Expression levels of the target enzymes were determined densitometrically after immunoblot detection for cytosolic and secreted expression. Enzymatic activity was determined for all samples using a reducing end assay. Data from expression levels and activity were combined to obtain the specific enzyme activity (nmol reducing ends/mg enzyme) and it was revealed that variants comprising the PelB signal peptide exhibit the overall highest activity (Figure 4.2). Furthermore, it was confirmed that these enzymes are efficiently released into the culture supernatant rendering, cell lysis unnecessary for future production cycles. Based on these data, the enzyme versions with the PelB signal peptide were selected for further characterization studies.

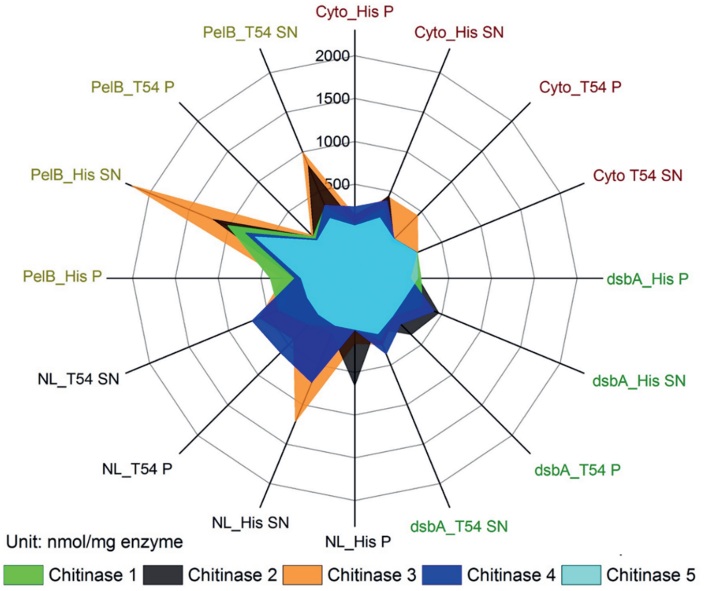

Figure 4.2. Spider plot summarizing the specific activity of all chitinase variants generated, expressed as nmol reducing ends/mg enzyme. Cell lysates and culture supernatant were assessed separately for each variant. Enzyme activity was quantified using the reducing end assay and enzyme quantities were determined densitometrically after immunoblot analysis. PelB: PelB signal peptide; cyto: cytosolic expression; dsbA: dsbA fusion protein; NL: natural signal peptide; His: 6xHis tag; T54: Tag54/6xHis combi tag; P: cell pellet lysate; SN: culture supernatant.

4.2.2 Purification of recombinant chitinases

A two-step purification of the selected recombinant enzymes with PelB signal peptide form the culture supernatant was carried out with an initial ammonium sulfate precipitation step and a sequential IMAC purification of the solubilized protein precipitate. Elution fractions were pooled and an SDS-PAGE and immunoblot was prepared loading the same volume for each sample (Figure 4.3). Expression levels of the five chitinases varied largely, and C1 was detected in much greater abundance relative to the others. On the Coomassie stained SDS-PAGE gel, specific bands for C2 could not be detected after purification due to low expression levels, however immunoblot analysis revealed the presence and integrity of the enzyme.

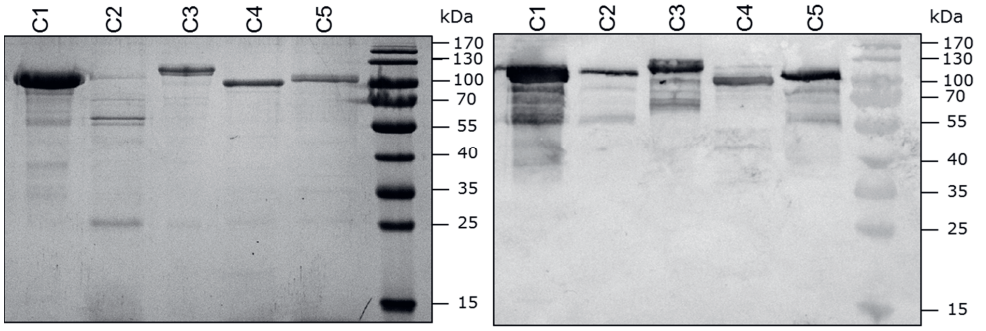

Figure 4.3. Coomassie R-250 stained SDS-PAGE gel (left panel) and corresponding immunoblot (6xHis tag detection; right panel) of the elution fractions of chitinases 1-5 (C1-C5) after IMAC purification. For the Coomassie stained gel, 8 µl pf pure sample were loaded. For immunoblot analysis, samples for C1 was pre-diluted 1:5 with PBS (pH 7.4) and 4 µl were loaded for each sample. kDa= kilo dalton; M= protein marker. The results shown are representative of at least three replicates.

The observed molecular weight of all enzymes in SDS-PAGE gel and immunoblot was not in accordance with the expected theoretical molecular weight taking into account that a 6xHis tag was added C-terminally to the enzymes and in general, bands for all enzymes were detected at a higher molecular weight. For all samples, unspecific bands could detected at ~25 kDa and ~50 kDa after IMAC purification, most likely representing host cell protein-contaminations. The amount of protein in the culture media, ammonium sulfate concentrates and the final dialyzed elution fractions was determined using the BCA assay and the same fractions were incubated with chitin

powder for 16 h at 30 °C followed by the quantification of reducing sugars to measure specific chitinase activity (Table 4.2). Thereby it was confirmed that all target enzymes were successfully enriched during purification according to the specific activity.

Table 4.2. Summary of purification efficiency of recombinant chitinases C1-C5 against chitin powder as a substrate. Total activity was assessed by the reducing end assay and total protein content of the individual fractions was quantified by the BCA assay. Both data were used to calculate the specific activity in U/mg. Data represents means of three replicate experiments (n=3).

Enzyme/ Purification fraction	Volume [mL]	Total activity [U]	Total protein [mg]	Specific activity [U/mg]	Purification [-fold]	Yield [%]
C1 Culture supernatant	800	127	1292	0.10	1.0	100
C1 Dialysed elution	20	49	14	3.52	35.6	39.0
C2 Culture supernatant	800	24	670	0.04	1.0	100
C2 Dialysed elution	20	12	2.3	5.36	146.3	49.9
C3 Culture supernatant	800	143	1104	0.13	1.0	100
C3 Dialysed elution	20	53	6.5	8.13	62.9	36.9
C4 Culture supernatant	800	57	791	0.07	1.0	100
C4 Dialysed elution	20	19	3.1	6.07	84.1	33.1
C5 Culture supernatant	800	25	965	0.03	1.0	100
C5 Dialysed elution	20	12	1.9	6.67	258.9	50.0

4.2.3 Characterization of chitinolytic enzymes

All enzymes were first characterized regarding their temperature and pH-optima as well as the optimum NaCl content as the enzymes were isolated from a sea-water microorganism. As the future intention is to integrate these novel enzymes in a degradation processes of natural chitin, all parameters were determined using chitin powder as a substrate instead of pre-treated analogs such as colloidal chitin, glycol chitin or artificial fluorogenic substrates. Investigating the effect of temperature showed that C1, 3 and 4 are highly active within a temperature range of 10-50 °C, with their optimum temperature at 30 °C. C2 and C5 are less tolerant and showed highest activity at 30 °C and 40 °C, respectively, and lost more than 50 % of activity at 50 °C and 60 °C (Figure 4.3 A). Next, enzyme temperature stability was investigated (Figure 4.3 B). All enzymes showed reduced activity after 15 min incubation at 60 °C. Enzyme C1 retained a 50 % residual activity after 240 min incubation at 60 °C, while the activity of the other chitinases dropped below 50% after 15 min and remained on that level.

With regard to the pH, C1, 3 and 4 showed similar behavior and had the optimum at pH 8, while C2 and C5 showed optimum reaction conditions at pH 9 and pH 10, respectively (Figure 4.4 A).

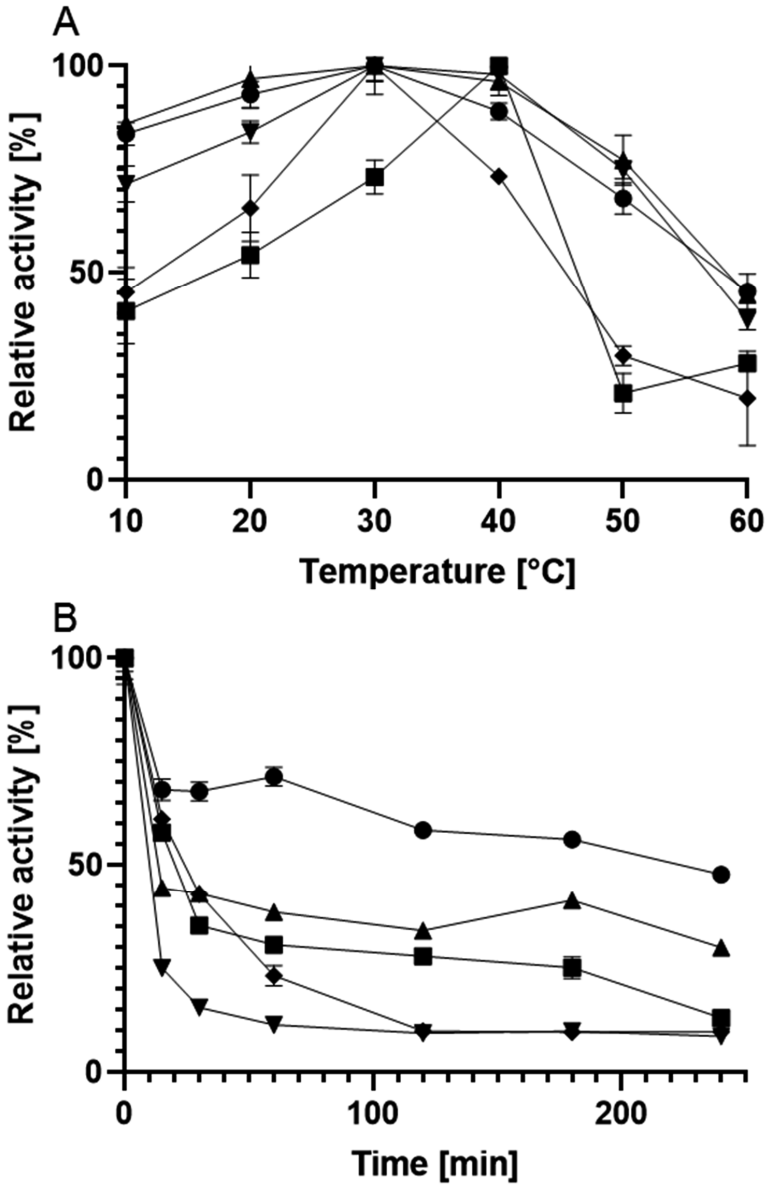

Figure 4.3. Temperature optima and stability of recombinant chitinases (C1-C5) produced in *E. coli* BL21. Activity was determined using a reducing end assay with chitin powder as substrate. Highest measured absolute activity was taken as 100 %. A: Activity within the temperature range (10-60 °C) at pH 8. B: Temperature stability at 60 °C. Residual enzyme activity was determined at standard conditions after pre-incubation of enzymes for 0-240 min. ●: C1, ■: C2, ▲: C3, ▼: C4, ♦: C5. Data represents means of three replicate experiments (n=3).

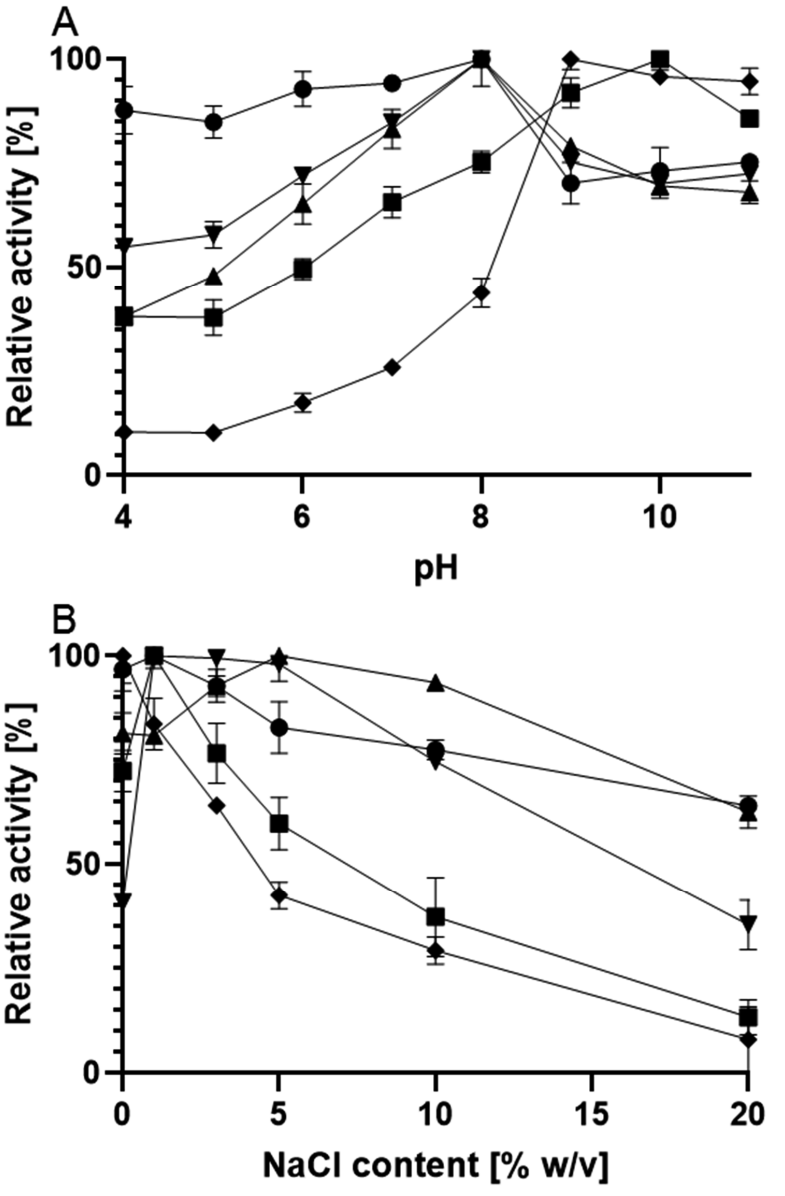

Figure 4.4. Optimum pH and salinity of recombinant chitinases (C1-C5) produced in *E. coli* BL21. Activity was determined using a reducing end assay with chitin powder as substrate. Highest measured absolute activity was taken as 100 %. A: Effect of different pH on enzymatic activity (pH 4-11) at 30 °C. B: Activity within different NaCl contents (0-20 % w/v) at pH 8, 30 °C. ●: C1, ■: C2, ▲: C3, ▼: C4, ♦: C5. Data represents means of three replicate experiments (n=3).

Given the marine origin of the Chi5 strain, the effect of salt was also evaluated, testing the addition of NaCl to the standard buffer. NaCl amounts up to 5 % (w/v) have a beneficial effect on enzyme activity for C1-C4 reflecting the average 3.5 % salt content of the North Sea environment from which the bacterium was isolated (Figure 4.4 B). Increasing NaCl amounts up to 20 % (w/v) reduced enzyme activity substantially to 10 % for C2, 4 and 5, with C1 and C3 showing highest tolerance (65 % activity). As expected, C5 that is not secreted by Chi5 showed the lowest tolerance to increasing salt contents reflecting the overall lower intracellular salt concentrations relative to salt contents in the North Sea. Enzyme optima for temperature, pH and salinity are summarized in Table 4.3.

Table 4.3. Optimum temperature, pH and salinity conditions for recombinant chitinases (C1-C5) determined on chitin powder.

Enzyme	Temperature [° C]	pH	Salinity [% NaCl]
C1	30	8	1
C2	40	10	1
C3	30	8	5
C4	30	8	1
C5	30	9	0

The addition of different co-factors and chemicals was assessed to determine whether they stimulate or inhibit enzymatic activity and the effect was evaluated relative to the control in standard MAT buffer. The selection of tested compounds at the respective concentrations was based on findings for different chitinases so far [34]. Incubation of 2 µM of enzymes with different metallic ions and chemical compounds revealed activity inhibitors (Table 4.4). All enzymes were inhibited the strongest by Zn^{2+} and Cu^{2+} and SDS (0.5 % w/v) resulting in a relative loss of activity of up to 99 %. C2 and 5 were furthermore substantially inhibited by all tested ions and no enhancers of enzymatic activity were identified. Inhibitory effects of Ni^{2+} and imidazole on enzymatic activity also underline the requirement for dialysis of the elution fractions after purification in order to remove residual Ni^{2+} and imidazole from the elution buffer.

Table 4.4. Effect of selected metallic ions (1 mM) and chemical compounds at on chitinase activity (C1-C5) relative to the standard MAT buffer control (100 %). Data represents means of three replicate experiments (n=3).

Metal ion/Chemical compound 1mM	[%] Residual activity C1	[%] Residual activity C2	[%] Residual activity C3	[%] Residual activity C4	[%] Residual activity C5
Control	100.0	100.0	100.0	100.0	100.0
Mg^{2+}	99.8±3.1	38.7±1.7	68.8±2.8	3.3±0.2	25.8±0.9
Zn^{2+}	30.5±0.3	2.3±0.4	21.7±0.1	8.1±0.7	0.6±0.5
Ca^{2+}	92.7±2.1	35.6±1.7	94.7±2.0	3.8±0.4	21.3±0.1
EDTA	68.9±0.7	20.4±1.6	80.8±4.8	73.9±4.1	28.0±0.9
Cu^{2+}	11.1±3.7	12.3±0.9	16.9±0.2	14.8±3.4	3.9±2.9
Ni^{2+}	76.6±5.1	23.0±0.7	64.8±1.2	38.8±1.7	5.6±0.3
K^{+}	100.1±2.8	44.8±2.3	72.6±1.7	3.7±0.2	28.6±0.4
Tween 20 (0.5 % v/v)	84.3±4.1	4.2±4.4	38.3±5.4	63.6±4.0	36.2±1.3
SDS (0.5 % w/v)	1.5±0.2	21.6±1.0	2.4±1.9	33.2±3.4	19.9±0.3
Triton X100 (0.5 % v/v)	87.8±8.5	59.1±2.4	47.4±4.1	60.2±3.0	35.6±2.2
Imidazole (100 mM)	84.3±10.3	52.8±1.4	41.1±1.8	47.2±2.3	31.9±2.7

Table 4.5. Relative (in % activity of chitin powder) activity testing of purified recombinant chitinases (C1-C5) with chitin (powder), chitosan (medium molecular weight), colloidal chitin and cellulose. Enzymes were incubated for 16 h at optimum conditions with 50 mg/ml of the respective substrates and the reducing end assay was used for quantification of free sugars. Data represents means of three replicate experiments (n=3).

Enzyme	Chitin [%]	Chitosan [%]	Colloidal chitin [%]	Cellulose [%]
C1	100	117.1±9.1	159.9±4.8	14.3±5.7
C2	100	71.4±6.7	191.4±4.7	10.0±1.1
C3	100	113.4±9.0	133.4±6.5	9.4±0.9
C4	100	141.3±5.5	117.7±6.3	10.4±0.9
C5	100	117.8±1.7	164.1±2.8	11.2±1.1

Different chitinous substrates were tested to assess the substrate affinity of the novel chitinases relative to untreated chitin powder. Testing of different substrates revealed that chitin powder and colloidal chitin in general are degraded by all enzymes (Table 4.5). As expected, the conversion of colloidal chitin is more efficient compared to chitin powder as it provides an overall smaller particle size and thereby larger surface area. Chitosan powder (DA: 15 %) was also degraded efficiently and high chitosanase activity was observed especially for C1, C3, C4 and C5. Merely basal activity was detected when cellulose was utilized as a substrate for all enzymes.

4.2.4 Enzyme kinetics on chitin powder

Enzyme kinetics were determined using chitin powder as a substrate and the optimum enzyme to substrate ratio could be determined as well as V_{max} and K_m values. The concentrations of chitin powder varied from 0 mg/ml up to 150 mg/ml while amount of enzyme was kept constant (2µM). The initial velocities (V_0) versus substrate concentration ([S]) were plotted as Lineweaver–Burk and Michaelis-Menten curves using GraphPad software (Figure 4.5).

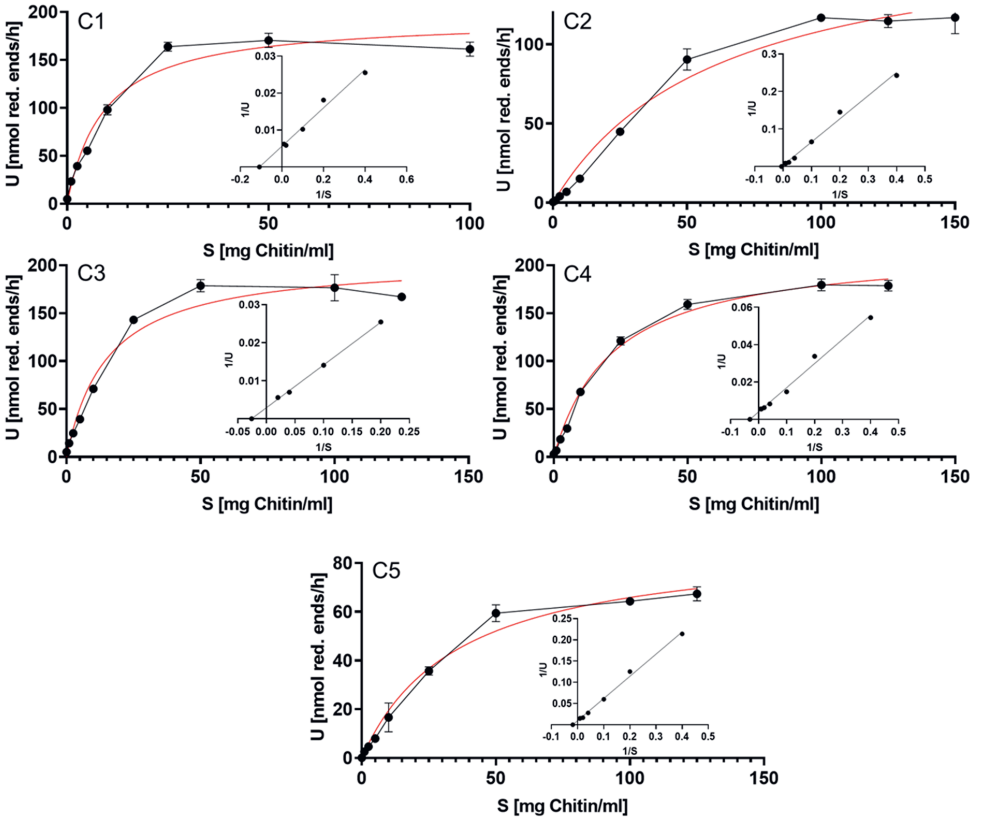

Figure 4.5. Michaelis-Menten fitted line plot of recombinant chitinases (C1-C5) (GraphPad software). The inserted Lineweaver-Burke plot relates reaction velocities to released GlcNAc associated with the concentration of chitin powder (0-150 mg/ml). V_{max} and K_m were calculated from the non-linear fit model. Data represents means of three replicate experiments (n=3).

Maximum reaction velocities (V_{max}) and Michaelis-Menten constants (K_m) were determined using a non-linear fit Michaelis-Menten model (Table 4.6). Thereby, optimum enzyme-substrate ratios were determined that would allow a maximization of substrate conversion rates. Furthermore, the K_m indicated the reciprocal enzyme-substrate affinity for C1-C5. Higher K_m values reflect an overall lower affinity and thus reduced conversion efficiencies. The data indicated that C1 and C3 have the highest affinity to insoluble chitin while maintaining an overall high V_{max}. In comparison, higher K_m values for C2 and C5 reflected an overall lower affinity to insoluble chitin.

Table 4.6. Enzyme and substrate specific kinetic parameters V_{max} (maximum reaction velocity) and K_m (enzyme-substrate affinity) for recombinant chitinases determined on insoluble chitin powder using a non-linear fit model.

Enzyme	V_{max} [U/ml]	K_m [mg/ml]
C1	197.7	9.1
C2	175.4	61.8
C3	206.2	15.2
C4	218.6	22.2
C5	89.5	36.0

4.2.5 Analysis of hydrolysis products

The five recombinant chitinases were tested with different substrates at optimum reaction conditions, and hydrolysis products were identified using TLC (Figure 4.6). Depending on the substrates, the different enzymes yielded different product ranges. Colloidal chitin and chitin powder were mainly degraded to dimeric, trimeric and monomeric oligomers. Chitosan was degraded to a crude mixture of multiple oligomers ranging from dimers to pentamers and even larger undefined yet water-soluble fragments that migrated slightly from the starting point. Further analysis of the cleavage patterns was carried out using chitin standard molecules of DP2-DP6 and incubating them at 4 mg/ml concentration with the individual enzymes (Figure 4.7).

Dimeric chitin molecules were not converted by any of the enzymes. Surprisingly, as the Pfam search revealed that C5 contains a chitobiase domain supposed to cleave dimers to yield GlcNAc, no exochitinase activity was observed. Solely C1, 3 and 4 cleaved chitin trimers to dimers and monomers, with C2 and C5 showing no activity on trimers. All chitinases degraded chitin tetramers to dimers as a single product. Chitin pentamers were converted to dimers and monomers by C1 and C3 and to trimers and dimers by C2, 4 and 5. Chitin hexamers were degraded to dimers and trimers. Although all chitinases are different in their structure and present different glycosyl hydrolase domains hydrolysis products in general were similar.

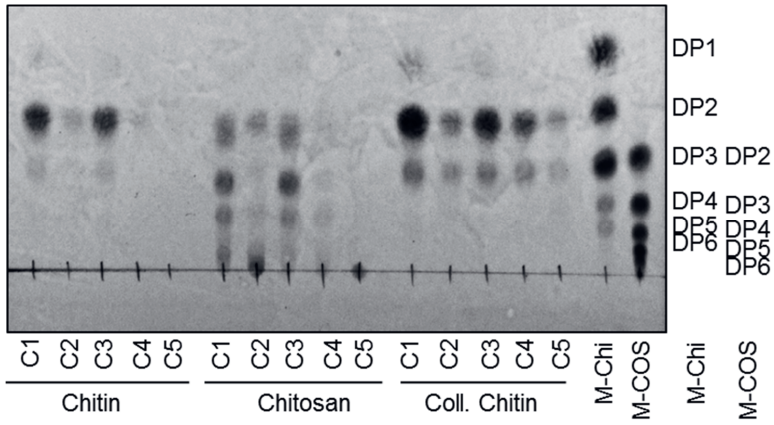

Figure 4.6. Thin layer chromatogram of enzymatic hydrolysis products from recombinant chitinases 1-5 (C1-C5) incubated for 16 h at 30 °C with chitin powder, chitosan or colloidal chitin. Chitin and chitosan standard molecules ranging from degree of polymerization (DP) 1 up to DP 5 were used to identify hydrolysis products. The results shown are representative of three replicates.

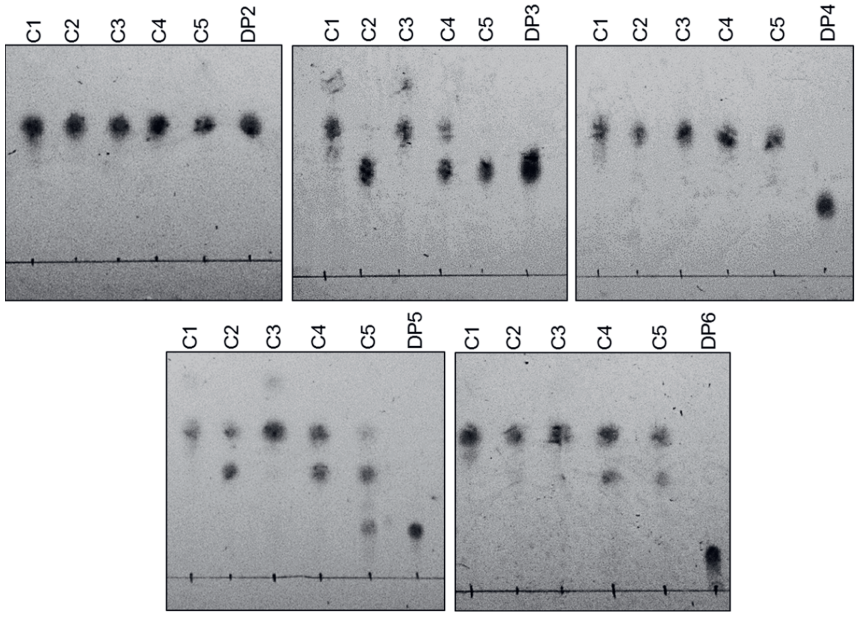

Figure 4.7. Thin layer chromatograms of enzymatic hydrolysis products from recombinant chitinases (C1-5) incubated for 16 h with chitin oligomers of degree of polymerization 2-6 (DP2-DP6) at a concentration of 4 mg/ml. Spots on the far right are untreated standard molecules. The results shown are representative of three replicates.

4.3 Discussion

The recombinant overexpression of chitinolytic enzymes attracted great attention in the last few decades, as chitinases are either utilized directly as antifungal agents or alternatively to elucidate novel mechanisms to enzymatically degrade chitin into functional oligomers [27-29, 31, 34, 35]. Chitinolytic bacteria utilize chitin for their metabolism and therefore represent a great source for endo-, and exo-chitinases that are essential to convert chitin into GlcNAc [36]. Five different putative chitinolytic enzymes were discovered within the genome of Chi5 that are utilized for a complete degradation of insoluble chitin to GlcNAC and $GlcNAC_2$. Pfam searches revealed homologies to glycosyl hydrolase family 18, 19 and 20 characteristic for endo, and exo-chitinase activity [37, 38]. Natural signal peptides were identified for C1-C4 indicating that the respective enzymes are secreted by Chi5 in order to carry out an extracellular degradation. The sequence for C5 did not contain any specific signal peptide signifying that the enzyme remains intercellular for the further processing of assimilated dimeric chitin. This is further supported by the presence of a chitobiase domain.

In order to carry out an individual and adequate characterization of the identified chitinases, singular enzymes must first be isolated and purified to remove potential interferences by residual medium constituents as well as similar chitinolytic enzymes. The isolation and purification of single chitinases from the complex Chi5 supernatant was investigated earlier using ion-exchange chromatographic approaches. However, results revealed that a complete isolation of single enzymes could not be achieved (data not shown). Therefore, a recombinant approach was followed to produce all chitinases separately using *E. coli* BL21.

The golden gate cloning approach was used to introduce different variants of the genes-of-interest into a bacterial expression plasmid. The natural signal peptide and the PelB signal peptide were tested both for a secretion by *E. coli* BL21 thereby exploiting the oxidative milieu in the periplasmatic space that promotes correct protein folding [39]. Similarly, an N-terminal fusion of the target enzymes to the dsbA protein was investigated to enhance the formation of disulfide bonds for the generation of active enzymes. Two different purification tags (6xHis tag and Tag54/6xHis tag) were tested to investigate whether their introduction will interfere with protein expression and

activity. For all five genes, constructs with a N-terminal PelB signal peptide for secretion and a C-terminal 6xHis tag for purification encoded enzymes with the highest specific activity and were selected for further research. An initial experiment of enzyme purification from the culture supernatant injected directly on the Ni^{2+} charged Chelating Sepharose FF column, revealed that the spent medium contained an unknown metal-complexing constituent causing ion leakage that resulted in a significant loss of binding capacity (data not shown). Thus, it became necessary to carry out an ammonia sulfate precipitation step before IMAC purification to remove interfering substances by buffer exchange to PBS (pH 8.0).

Typically, chitin powder is not utilized to determine chitinase parameters, as it is highly crystalline, less defined concerning particle size and DA, has a relatively low specific surface area and the initial molecular mass is difficult to calculate. However, in comparison to more artificial and pre-treated substrates such as glycol chitin, colloidal chitin or synthetic fluorogenic substrates that are often used to determine enzyme properties, powdery chitin reflects realistic process parameters for later applications of the enzymes in degradation processes.

Enzyme characterization studies identified optimum conditions with regards to temperature, pH and NaCl content, functional substrates as well as effects of putative co-factors for all five enzymes. Furthermore, the determination of enzyme kinetics indicate optimum enzyme to substrate ratios. Optimum temperatures for C1-C5 (30-40 °C; Figure 4.4 A) are overall lower than those reported in the literature for bacterial chitinases, as these enzymes are predominantly reported as thermophilic or thermo-tolerant enzymes with optima in the 40-60 °C range [30, 40-42]. Typically in industrial processes, high temperatures are favored as solubility of hydrophobic compounds and the overall biodegradation process can be enhanced. Thus thermophilic enzymes are implemented in such processes as the can withstand higher temperatures while maintaining activity over a longer period of time. Such thermophilic enzymes are typically isolated from thermophilic bacteria and fungi or modified synthetically by protein engineering. The novel chitinases of Chi5 showed to be highly active at more moderate temperatures and can maintain up to 50 % activity at 60 °C. The overall lower optima can be linked to the natural sea-water habitat of Chi5 with an average temperature of 5-25 °C. Overall for an industrial chitin degradation process these

properties could become beneficial as the enzymes are highly active on chitin powder that would not require elevated temperature thus reducing the total energy costs and process efforts [43]. Furthermore, thermal stability of COS is decreasing proportional to its molecular weight which implies that milder reaction temperature will improve product stability [44]

Optimum pH conditions were determined to be between pH 8 and 10 for the five novel enzymes (Figure 4.5 A) reflecting adaptation to the slightly basic conditions in sea water (pH 7.5 – 8.4) the Chi5 strain was isolated from. Literature data reported various pH optima for different chitinolytic enzymes ranging from pH 4 – 8, depending on the environment the enzymes were originally isolated from [30, 38, 40, 41, 45-47]. The addition of salt (1 – 5 % (w/v) NaCl) resulted in increased enzyme activity (Figure 4.5 B) reflecting adaptation of the chitinases to marine conditions. C1, 3 and 4 maintained a relative activity of up 75 % at 20 % (w/v) salt content. Although several chitinases were also isolated from marine sources before and salinity is an essential parameter as the enzymes may have evolved to possess higher activity at a certain salt content, only limited data is published in literature on the determination of optimum salinity for chitinases. However, the findings for C1-C5 are in accordance with the data reported on preservation of activity at salinity up to 20 % (w/v) [48]. As the mild temperature optima of the chitinases (30°- 40 °C) could promote bacterial contaminations in non-sterile reaction conditions, additions of salt to the reaction mix could minimize the risk of contaminations.

Several co-factors were tested to elucidate whether enzyme activity can be improved and to identify potential reaction inhibitors. Co-factor analysis revealed that all tested metal ions (Mg^{2+}, Zn^{2+}, Ca^{2+}, Cu^{2+}, Ni^{2+}, K^{+}), chemicals (EDTA, Imidazole) and detergents (SDS, Tween 20, Triton X100) can be considered activity repressors and no activators were identified. C1 exhibits relatively high tolerance to Mg^{2+}, Ca^{2+}, K^{+} ions, Tween 20, Triton X100 and Imidazole with a minimal residual activity of 84.3 % relative to the control. On the other side C2, C4 and C5 were strongly inhibited by almost all compounds with most residual activities reduced below 40 % of initial activity (Table 4.4). These results may furthermore prove that the studied chitinases are not metalloenyzmes, as also reported for various bacterial chitinases [49].

Enzyme kinetics were determined using chitin powder as a substrate and absolute K_m values are substantially higher compared to data reported in the literature for different chitinases using colloidal chitin as a substrate (K_m Chi5 chitinases: 9.1 – 61.8 mg/ml; K_m literature: 1.4 – 3.0 mg/mL) [34, 50, 51]. Substrate properties can have major impact on enzyme kinetics and colloidal chitin and untreated chitin powder differ largely in terms of their overall material properties: 1) The particle size and crystallinity of colloidal chitin is substantially lower than chitin powder 2) the surface area of colloidal chitin is higher resulting in increased enzyme accessibility. These different material parameters greatly affect the overall enzymatic activity of the chitinases and thereby conversion rates are increased substantially for colloidal chitin. However, the utilized substrate for enzyme characterizations should always be selected based on the intended future application to assess enzyme parameters under accurate process conditions. The novel chitinases will be implemented in a fully enzymatic conversion process of chitin to COS and harsh chemical substrate pre-treatment methods should be minimized or even eliminated completely. Therefore, the use of chitin powder helped to assess optimum enzyme - substrate ratios in order to maximize reaction rates.

Digestion experiments revealed that mixtures of soluble oligomers are produced ranging mainly from monomeric GlcNAG to $GlcNAG_3$ with chitin as a substrate, and larger oligomers (≥pentamers) were observed when chitosan was degraded. Further analysis on the cleavage mechanisms revealed that C1 and C3 did exhibit exo-chitinase activity independent on oligomeric or polymeric chitin substrates (Figure 4.8, Figure 4.9) as monomeric GlcNAC was detected by TLC. C2, 4 and 5 yielded similar product patterns when incubated with insoluble substrates. As it was further revealed after digestion of standard molecules, C2, 4 and 5 did not yield GlcNAc, thereby exhibiting endo-chitinase activity. The presented data underline that the novel chitinases are all suited for the degradation of powdery chitin without any further harsh chemical pre-treatment.

Enzymatic degradation reactions will be further investigated in multiple aspects: 1) stimulate the production of single oligomers with a specific DP and reduce the amount of undesired oligomers and GlcNAc; 2) produce oligomers with DP>3; 3) maximize the overall conversion rates and product yields. Therefore, synergistic effects of multi-

enzyme reactions will be assessed and process optimization will be carried out using the design-of-experiments approach.

4.4 Conclusions

The genes for five novel potential chitinases were identified from genome data of the novel bacterial strain Chi5 and successfully expressed and purified from *E. coli* BL21. The novel enzymes are relatively large compared to current reported bacterial chitinases and also show lower temperature and pH optima with chitin powder as substrate, as well as a substantial tolerance to salinity. The lower temperature optimum at 30 °C will be beneficial especially when considering reactions at larger scales as it involves less effort and expenses when establishing and maintaining the process. The high salt tolerance could furthermore be exploited to establish non-sterile degradation reactions with higher salt content potentially inhibiting bacterial contaminations. It was also discovered that all chitinases convert chitin and colloidal chitin to mainly dimeric, trimeric and monomeric compounds and chitosan to a cruder mixture of different COS. Future analyses will focus on the implementation of the different chitinases in *in vitro* cocktails to maximize the conversion rates and to investigate whether different degradation products with regards to the DP can be obtained by multi-enzyme reactions.

4.5 Experimental section

Materials

All chemicals used in the study were of highest purity and obtained from Carl-Roth (Germany). Chelating Sepharose FF was purchased from GE Healthcare (Sweden). All utilized buffers were prepared freshly in demineralized water.

Bacterial strains and plasmids

The novel marine chitinolytic Chi5 strain was isolated from seawater samples from Oostende, Belgium. The *E. coli* strain DH5α dam-/dcm- (New England Biolabs, Ipswich, USA) was grown in lysogeny-broth (LB) medium. The *E. coli* BL21 (DE3) (New England Biolabs) strain was grown in terrific-broth (TB) medium. If needed, both media were supplemented with appropriate concentrations of ampicillin (100 µg/ml) or kanamycin (50 µg/ml).

Next generation sequencing and gene mining

Genomic DNA was extracted from a liquid culture of Chi5 using the "NucleoBond AxG500" kit (Machery Nagel) according to the manufacturers instructions. The DNA sample was further processed for the *de novo* whole genome sequencing using the "Ion Xpress Plus gDNA Fragment Library Preparation" kit (Machery Nagel) and ion torrent sequencing was carried out by the Fraunhofer Institute for Molecular Biology and Applied Ecology IME, Aachen (Ion Torrent Personal Genome Machine PGM, Thermo Fisher Scientific). The DNA-STAR assembly method lead to 60 contigs that were assembled onto scaffolds (10-fold coverage). The protein family database (Pfam; https://pfam.xfam.org) was used to identify homologous polysaccharide binding domains and chitinases active sites in the scaffolds reflecting the presence of putative chitinases. The SignalP 4.1 server was used to obtain potential signal peptides responsible for the secretion of the enzymes (http://www.cbs.dtu.dk/services/SignalP-4.1).

Cloning of chitinase genes and sequencing

The golden gate cloning technology was employed to clone the target genes into the pET39b(+) expression vector resulting in multiple constructs for each gene-of-

interest. Golden gate cloning enables a simultaneous directed ligation of multiple genetic fragments or bricks into a vector backbone using type IIs restriction enzymes that cut outside their recognition site [52, 53]. A preceding *in silico* design of specific cleavage sites and overhangs allows a high-throughput assembly of constructs from multiple gene-bricks in a one-pot restriction and ligation step. The general principle of golden gate cloning is displayed in Figure 4.8.

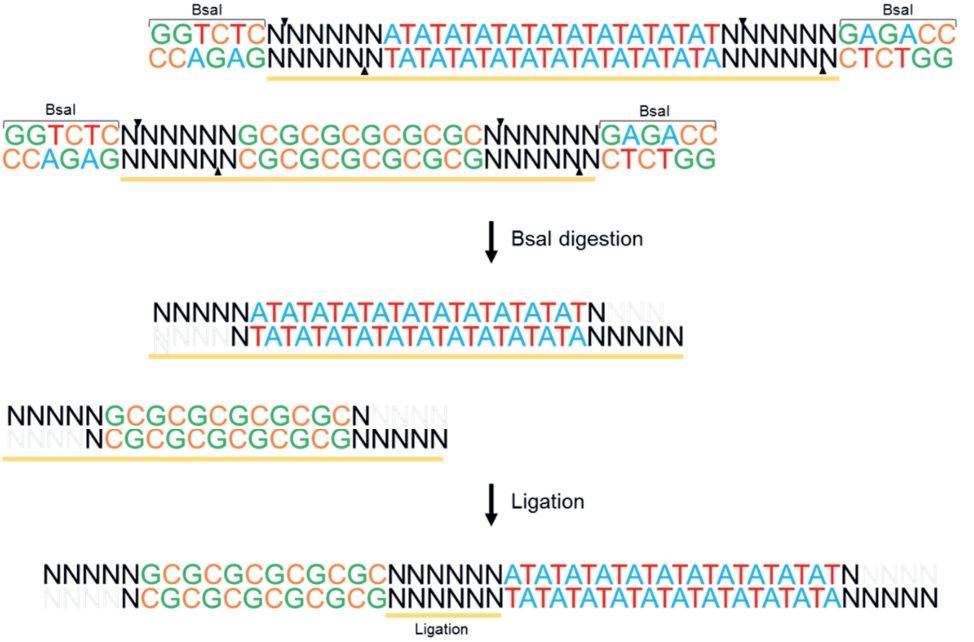

Figure 4.8. Principle of the golden gate cloning technology. Type IIs restriction enzymes such as BsaI cut outside of their recognition site (GAGACC) resulting in single stranded overhangs and the loss of the recognition site itself. The overhangs (N) are specifically designed *in silico* to allow a directed ligation of the gene fragments and the vector backbone. The order of the individual genes is thus predetermined and cloning efficiency is increased. Digestion and ligation can be carried out in one reaction vessel.

Multiple genetic elements that allow a successful integration and functional expression of the novel enzymes by *E. coli* BL21(DE3) were assessed. Natural signal peptides identified after the Pfam search and the commonly used PelB signal peptide from *Erwinia carotovora* were tested N-terminally for secretion of the target enzymes into the culture medium [39]. A fusion to a bacterial periplasmatic oxidoreductase

(dsbA) acting as a folding enhancer to oxidize disulfide bonds as well as a variant for cytosolic expression were tested [54]. A 6xHis tag or alternatively a tag54/6xHis combi-tag were both introduced C-terminally as purification tags [55, 56]. Three distinct entry vectors were designed from the pET39b(+) to assemble the constructs in the correct orientation (Table 4.7): pGR_SigP for secretion of the target enzymes; pGR_dsbA for fusion to the dsbA protein and pGR_cyto for cytosolic expression. A complete list of all constructs is given in table A1.2 in the **Appendix A** section. Vector maps of the individual entry vectors and exemplary vectors for gene-bricks as well as final constructs are depicted in **Appendix B** in figures A2.1-A2.6.

Table 4.7. Overhang design of genetic bricks and vectors utilized in golden gate cloning. The 5´and 3´ single stranded overhangs are generated after digestion with the BsaI restriction enzyme and allow a directed assembly of the constructs.

Genetic element	5´overhang	3´overhang
pGR_SigP	TGAG	TGTA
pGR_dsbA	TGAG	CCGG
pGR_cyto	TGAG	CCGG
Natural signal peptide	ACAT	CCGG
PelB signal peptide	ACAT	CCGG
Gene-of-interest	GGCC	GCTT
6xHis-tag	CGAA	ACTC
Tag54/6xHis-tag	CGAA	ACTC

All genetic bricks and vector backbones to assemble the final constructs were modified and synthesized (Thermo Fisher Scientific, Waltham, USA) with flanking BsaI recognition sites (5´…GGTCTC(N)$_1$▼…3´ and 3´…CCAGAG(N)$_5$▲…5´) with the proper overhangs in the correct orientation to ensure the directed assembly of the constructs (Table 4.1). Cloning was carried out using the golden gate assembly mix according to the manufacturer´s instructions (New England Biolabs). Integration and correctness of inserts was verified by colony PCR and Sanger-sequencing using T7 primers T7$_F$(5´AAATTAATACGACTCACTATAGGG3´) and T7$_R$ (5´ATGCTAG TTATTGCTCAGCGG3´) flanking the cloned constructs.

Expression of recombinant chitinases in *E. coli* BL21 (DE3)

For the expression analysis of the chitinase constructs, freshly heat-shock transformed *E. coli* BL21 (DE3) cells were used to inoculate an overnight starter culture (180 rpm, 37 °C) in 20 mL TB medium supplemented with 50 µg/ml kanamycin. The starter culture was used to inoculate the main culture 1:100 in 25 ml TB-medium using 100 mL Ultra Yield™ flasks (Thomson Instrument Company, Oceanside, USA). Cultivation was carried out at 37 °C in orbital shakers at a shaking velocity of 200 rpm. After 4 h of cultivation (OD_{600nm}=4), cells were induced in the middle of the log-phase by addition of 1 mM isopropyl-β-D-thio-galactopyranoside (IPTG) to the culture broth and temperature was decreased to 28 °C. After reaching a total cultivation time of 18 h, cells and supernatant were separated by centrifugation (8000 *x* g, 30 min). For cell lysis, an aliquot of the pellet was resuspended in BugBuster® master mix (Merck Millipore, USA) and incubated at RT for 2 h in an overhead shaker (60 rpm). Cell debris were separated by centrifugation (8000 *x* g, 2 min) and the resulting lysis supernatant as well as culture supernatant were subjected to densitometric analysis of immunoblot images and enzyme activity assay. Selected optimal enzyme candidates were produced at larger scale using 800 ml TB-medium and 2.5 L Ultra Yield™ flasks. Cultivation of starter cultures, induction and harvesting procedures were analogous to those above mentioned for small-scale expression.

Purification of chitinases from culture supernatant

The protocol for the purification of 6xHis tagged chitinases was adapted from “Affinity Chromatography Vol.2: Tagged Proteins” (GE Healthcare) and consisted of two steps:

Step 1. Ammonium sulfate precipitation

Solid ammonium sulfate was slowly added to the culture supernatant to a final concentration of 70 % and was stirred for 2 h at room temperature. The precipitate was collected by centrifugation (8000 *x* g, 30 min) and dissolved in 0.1x volumes of PBS (pH 8.0) relative to the starting volume. Concentrated samples were centrifuged (8000 *x* g, 10 min) and filtrated (0.45 µM) in order to remove any insoluble particles.

Step 2. Immobilized metal affinity chromatography (IMAC)

Samples from step 1 were applied to a Chelating Sepharose FF (GE Healthcare, Sweden) resin [column volume (CV) 5 ml] charged with 0.2 M $NiSO_4$. The column was connected to an ÄKTA pure 25 (GE Healthcare), equilibration was carried out using 3 CV PBS (pH 8.0) and samples were injected directly on the column using a sample pump (2 ml min^{-1}). The column was washed with equilibration buffer until the UV_{280nm} signal reached <30 mAU and weakly bound unspecific protein was eluted first from the column using 50 mM imidazole in PBS (pH 8.0) followed by a second elution step with 250 mM imidazole in PBS (pH 8.0). All steps were carried out at a constant flow rate of 2 ml min^{-1}. Flow-through-, wash- and elution-fractions were collected using a fraction collector and elution fractions containing chitinases were pooled and dialyzed against a mixture of 33 mM MES, 33 mM CAPS, 33 mM TRIS (MAT-buffer; pH 8).

SDS-PAGE, immunoblot analysis and enzyme assay

Protein analysis was carried out by SDS-PAGE according to the method described by Laemmli *et al.* using 12 % separating gels and protein bands were visualized by staining with Coomassie Brilliant Blue R-250 [57]. For immunoblot analysis, samples from SDS-PAGE gel were transferred to a nitrocellulose membrane by tank-blotting; detection was carried out with a primary mouse monoclonal 6xHis-tag antibody [0.2 µg/ml] (Thermo Fisher Scientific) and sequential band visualization with the secondary anti-mouse antibody alkaline phosphatase conjugate [0.2 µg/ml] (Thermo Fisher Scientific) using NBT/BCIP as a substrate. Samples were quantified densitometrically using the AIDA 5 software (Raytest Isotopenmessgeräte GmbH) relative to the 6xHis tagged protein K12v105 (Fraunhofer IME). Quantification of purified proteins was carried out via the bicinchoninic acid assay (BCA) using bovine serum albumin as standard for calibration [58].

For the determination of enzymatic activity of recombinant chitinases, a reducing end assay was carried out [59]. Fractions containing chitinases were incubated with 5 % (w/v) chitin powder extracted from shrimp shells (~400.000 g/mol; Carl Roth) in MAT buffer for 2 h in an overhead shaker at ambient temperature (standard conditions). Samples were centrifuged at 13000 x g for 2 min and 40 µl of the supernatant was mixed with 40 µl 0.5 M NaOH and 40 µL of a reagent containing 1.5 mg/ml 3-Methyl-

2-benzothiazolinonhydrazon and 0.75 mg/ml Dithiothreitol. Samples were incubated at 80 °C for 15 min and then thoroughly mixed with 80 µl of 0.5 % (w/v) $FeNH_4(SO_4)_2$)x $12H_20$, 0.5 % (w/v) sulfamic acid and 0.25 M HCl. After cooling down to room temperature, 100 µl of the samples were measured at 620nm. A calibration curve of N-acetyl-glucosamine was constructed freshly for each measuring cycle. One unit [U] was defined as the amount of enzyme required to release 1 µmol of reducing sugars per hour.

Preparation of homogenous chitin powder

Chitin from shrimp shells (~400.000 g/mol; Carl Roth) was mechanically pre-treated using a GyroGrinder (Fritsch GmbH, Germany) at 6000 rpm and thereby converted to a homogeneous powder of 80 µM average particle diameter. The powder was used without any further treatment as a substrate for all chitinolytic degradation experiments.

Preparation of colloidal chitin

The preparation of colloidal chitin was similar to the method described by Murthy and Bleakley with some minor differences [60]. Chitin powder (Carl Roth) was dissolved in concentrated hydrochloric acid (HCl) (5 g in 100 mL) and stirred at 4 °C for 24 h, after which the mixture was centrifuged at 4000 *x* g for 15 min at 4 °C. The precipitate was washed to neutral pH with distilled water and stored at 4 °C.

Characterization of recombinant chitinases

Enzyme samples were incubated at different temperature (10-60 °C, 10 °C increment) and pH (4-11, increment 1) using different buffer systems (Acetate, Tris-HCl, MES, total molar concentration 100 mM), as well as NaCl content (0, 1, 2.5, 5, 10 and 20 % w/v) to determine optimum reaction conditions. All experiments were carried out at 1.0 ml scale using 2µM of the respective enzymes and a 5 % (w/v) suspension of chitin powder extracted from shrimp shells (~ 400.000 g/mol; Carl, Roth) in 100 mM MAT buffer. For the determination of the substrate specificity, chitin powder (5 % w/v), glycol chitin (10 % v/v), colloidal chitin (5 % w/v), chitosan [DA: 15-25 %, medium molecular weight, Sigma-Aldrich] (5 % w/v) and microcrystalline cellulose powder (Sigma Aldrich) (5 % w/v) were investigated, respectively. The effect of several potential co-factors on activity was elucidated at a concentration of 1 mM. Additionally,

the effect of detergents [SDS 0.5 % (w/v), Tween-20 and Triton X-100 both at 0.5 % (v/v)] and chemicals (Imidazole 100mM, EDTA 1 mM) on enzymatic activity was assessed. Samples were incubated in a thermomixer for 16 h at 900 rpm. After incubation, samples were analyzed using the enzyme assay. Thermostability was assessed after incubation of recombinant chitinases at 60 °C for different durations followed by the quantification of residual enzyme activity under standard assay conditions. Enzyme kinetics of recombinant chitinases were assessed by determination of K_m and V_{max} by Lineweaver-Burke representation of the Michaelis-Menten model preceding an incubation of constant amounts of enzyme (2 µM) with 0 – 150 mg/ml chitin and sequential quantification of reducing sugars.

Product analysis by thin-layer-chromatography

Hydrolysis products were analyzed using 10x10 cm TLC Silica gel 60 F254 plates (Merck, Darmstadt, Germany) and a mixture of butanol: methanol: 25 % ammonia: H_2O (5:4:2:1) as mobile phase. A total sample volume of 0.5 µl was applied in 0.25 µl spots. Chitin standard sugars (Megazyme, Chicago, USA) (5 mg/ml) were applied to the plate (0.5 µL in 0.25 µL spots) as size standard for the determination of the DP. After separation, the plate was air-dried and the developing solution (200 ml Acetone, 30 ml phosphoric acid (85 %), 4 ml aniline, 4 g diphenylamine) was sprayed on the plate followed by spot visualization at 300 °C using a heat gun [61].

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Chapter 5

Recombinant production and characterization of five novel chitin-deacetylases for the *in vitro* deacetylation of chitin

In this chapter, the genes for polysaccharide deacetylases are isolated using the genomic data generated after next generation sequencing of Chi5. All target genes are cloned using the analogous golden gate cloning approach into *E. coli* BL21 and expressed recombinantly as described for the chitinase genes in **Chapter 4**. The PelB signal peptide is introduced for secretion of the target enzymes to the culture supernatant. Immobilized metal affinity chromatography is applied for purification from the culture supernatant using a 6xHis tag. Optimum pH, temperature and salinity for all enzymes are determined on chitin as a substrate. Co-factors are explored as putative reaction activators or inhibitors. Enzyme kinetics are monitored in order to determine maximum reaction rates and optimum enzyme-to-substrate ratios. Partially deacetylated COS generated by the recombinant enzymes are identified using thin layer chromatography and the degree of deacetylation is determined by LC-MS.

Keywords: chitosan oligomers, enzyme characterization, enzymatic deacetylation, golden gate cloning, recombinant protein production

This chapter is based on the following publication:

Schmitz, C.; Schillberg, S.; Fischer, R.; Rasche, S.; Bortesi, L., Recombinant production and characterization of five novel chitin-deacetylases isolated from a marine *Photobacterium* strain for the *in vitro* deacetylation of chitin. Prepared for submission.

5.1 Introduction

As presented in **Chapter 2**, chitosan and COS are functional molecules derived from chitin that can exhibit antibacterial antifungal and anti-inflammatory effects and have therefore found many diverse applications especially in food cosmetic and medical industry [1-5]. The intrinsic physiological properties are however influenced by the physicochemical characteristics that are the degree of polymerization (DP) and especially the degree and pattern of acetylation (DA, PA) [6-10]. Dependent on the DA and PA, different or additional effects of chitosan and COS can arise or disappear, making it essential to generate products with consistent and reliable properties and quality. Typically, chitosan and COS are generated by chemical deacetylation of chitin and chitin-oligomers by employing NaOH as an alkaline reagent at elevated temperatures (80-120 °C) [11-14]. This deacetylation process is an inexpensive and efficient approach to rapidly convert chitin to chitosan. However, some key drawbacks have to be taken into consideration in particular regarding the generation of chitosan and COS of consistent and defined quality: (1) alkaline deacetylation uses NaOH at elevated temperature that may result in random hydrolysis of glycosidic bonds thus leading to a less specific and uncontrolled generation of COS (2) generation of products with one specific DA is hardly achievable (3) controlling the PA is not feasible and (4) product contaminations by residual reactants are problematic for human-related applications [15-17]. Consequently, green chemistry approaches and in particular enzymatic reactions are of high interest as they can address all of the above mentioned issues and increase product quality substantially. In addition, fully biological conversion processes can minimize process energy consumption and reduce the overall environmental pollution as hazardous acidic and alkaline solutions are omitted [18].

Enzymatic deacetylation processes have proven to be a valuable alternative for the production of chitosan and COS from chitin due to the increased specificity and selectivity of enzyme reactions. Therefore, controlling the physicochemical properties of chitosan and COS becomes more feasible compared to chemical processes. Chitin deacetylases (CDAs, EC 3.5.1.41) belong to the group of carbohydrate esterase family 4 and usually share a conserved NodB homology domain that is responsible for the

catalytic activity [19, 20]. Several CDAs have been studied extensively and the assessment of their catalytic mechanisms revealed different deacetylation pathways that would allow to generate COS with specific DA and PA [21-24]. As described in detail in **Chapter 2**, the “multiple attack mode” allows for a sequential deacetylation starting from the non-reducing end. The “multiple-chain mode” results in a more random deacetylation as the enzyme dissociates from the chitin polymer after one deacetylation reaction and then binds to another position for the next reaction. Similarly, the “subside capping model “ was established to explain the generation of COS with different PA [25]. So far, a plethora of different CDAs were successfully isolated and characterized from various bacteria, fungi and insect species and subsequently implemented in the *in vitro* generation of defined chitosan and partially acetylated COS [6, 7, 23, 26].

In theory, COS can be generated from chitin by two distinct enzymatic strategies: 1) Enzymatic deacetylation of chitin by CDAs and subsequent depolymerization to oligomers by chitinases and 2) Enzymatic depolymerization of chitin to oligomers by chitinases followed by a deacetylation to COS. The CDAs reported by literature so far are however rarely active on powdered chitin due to the lack of chitin-binding domains and thus still require the pretreatment of crystalline chitin e.g. by hydrolysis with HCl. As such chemical pretreatment processes in general result in similar drawbacks as those mentioned above, it is desired to either minimize or even eliminate these processes and establish a green chemistry approach. Consequently, an enzymatic depolymerization-deacetylation approach is preferred for the generation of COS.

In **Chapter 3**, the chitinolytic bacteria Chi5 was isolated from coastal seawater samples and that can utilize marine chitin from shrimp shells as carbon and nitrogen source by carrying out an extracellular hydrolysis of chitin to GlcNAC and $GlcNAC_2$ using a cocktail of multiple chitinolytic enzymes. The aim of this chapter is to identify putative CDAs containing homologous NodB domains within the genome data. Target genes will be cloned and expressed recombinantly by *E. coli* BL21. Enzymes will be characterized using chitin powder as well as chitin oligomers and hydrolysis products will be characterized to determine the DA.

5.2 Results

5.2.1 Cloning and expression of putative chitin-deacetylases

Next generation sequencing was used to generate 60 contigs comprising the sequence of the genomic DNA. All contigs were screened for CDA homologies using the Pfam database. Five open reading frames were discovered containing characteristic NodB and a divergent polysaccharide deacetylase 2 domains (Table 5.1). Enzyme D3 contains an associated domain of unknown function in addition to the NodB polysaccharide deacetylase 1 domain. Natural signal peptides were discovered for D1 and D5 leading to the conclusion that the secreted enzymes are deacetylating chitin extracellularly in contrast to D2, D3 and D4.

Table 5.1. Homologous characteristic domains for chitin deacetylases (D1-D5) that were identified within the genome of the Chi5 strain. AA=amino acid. kDa = kilo Dalton.

Enzyme	Size AA	Molecular weight [kDa]	Domain 1	Domain 2	Signal peptide
D1	419	48.1	NodB polysaccharide deacetylase 1	-	✓
D2	238	30.3	NodB polysaccharide deacetylase 1	-	-
D3	312	38.0	NodB polysaccharide deacetylase 1	Domain of unknown function (DUF3473)	-
D4	353	42.6	NodB polysaccharide deacetylase 1	-	-
D5	221	26.9	Divergent polysaccharide deacetylase 2	-	✓

Integrity and correctness of all constructs was confirmed by colony PCR and Sanger-sequencing. All target CDA constructs obtained by golden gate cloning were expressed recombinantly by *E. coli* BL21 (DE3) analogous to the chitinase constructs generated in **Chapter 4** (Figure 5.1).

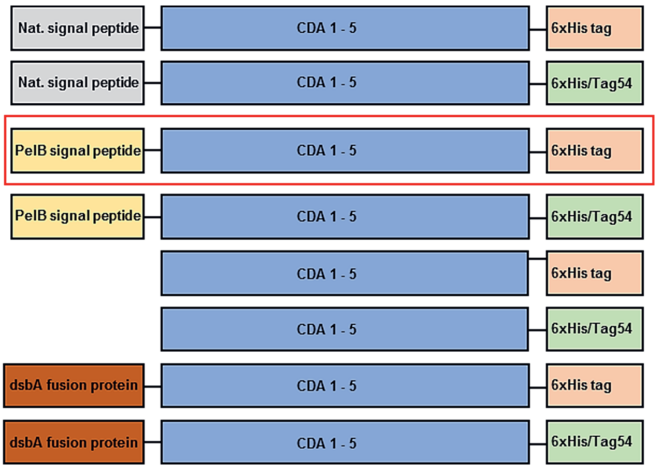

Figure 5.1. Construct variants for each putative CDA gene cloned in *E. coli* DH5α using the golden gate cloning technology. Variants comprising the PelB signal peptide and 6xHis tag (in red frame) were selected for upscaled expression and purification.

Enzyme quantities were determined for cell lysate and supernatant after immunoblot analysis, and enzyme activity was assessed using the acetic acid assay measurement. The data from three replicates were used to calculate the specific enzyme activity in µg acetic acid/ mg enzyme (Figure 5.2). Comparison of the individual enzyme variants and both expression compartments revelead that, similarly to the chitinase expression study, variants comprising a PelB signal peptide and 6xHis tag yielded the overall highest specific activity. As a result, the respective constructs were selected for further production and characterization of CDAs.

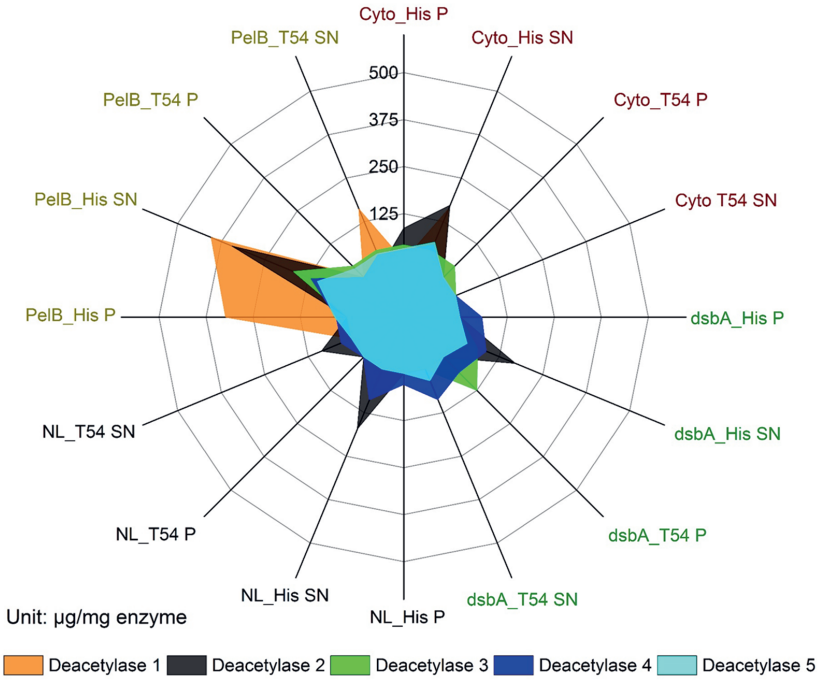

Figure 5.2. Spider plot summarizing the specific activity of all chitin-deacetylase variants generated by golden gate cloning. Cell lysates and culture supernatant were assessed separately for each variant to identify successful enzyme secretion. Enzyme activity was quantified using the acetic acid assay and enzyme were quantified after immunoblot analysis. PelB: PelB signal peptide; cyto: cytosolic expression; dsbA: dsbA fusion protein; NL: natural signal peptide; His: 6xHis tag; T54: Tag54/6xHis combi tag; P: cell pellet lysate; SN: culture supernatant.

5.2.2 Purification of recombinant CDAs

All five recombinant enzymes were enriched by a two-step purification with an initial ammonia sulfate precipitation at 70 % saturation and a sequential IMAC purification of resolubilized protein precipitate. Elution fractions containing target enzymes were pooled and subjected to analysis by SDS-PAGE and immunoblot (Figure 5.3). Substantial differences in expression levels were identified as D1, D3 and D4 were detected in greater abundance compared to D2 and D5. Distinct bands for D2 and D5 could only be detected by immunoblot analysis due to low expression levels.

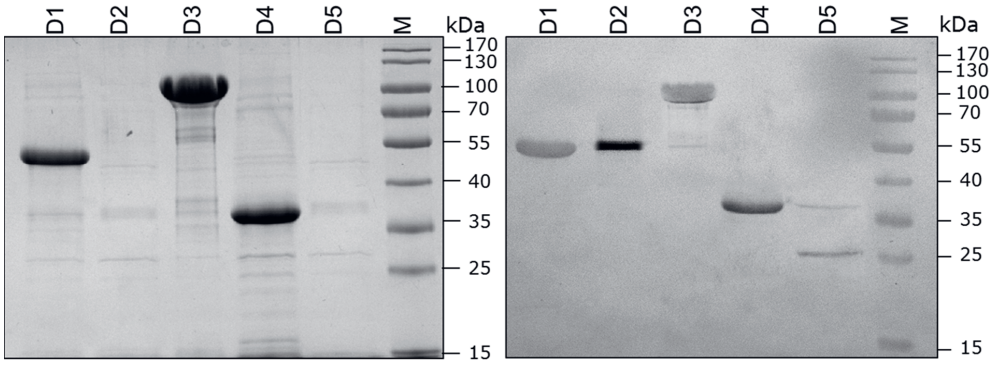

Figure 5.3. SDS-PAGE gel (left panel) and corresponding immunoblot (6xHis tag detection, right panel) of the elution fractions of chitin deacetylases 1-5 (D1-D5) after IMAC purification. For the Coomassie stained gel, 8 µl pf pure sample were loaded. For immunoblot analysis, samples for D1 and D3 and D4 were pre-diluted 1:5 with PBS (pH 7.4) and 4 µl of each sample were loaded. The protein band for D1 (48.1 kDa) was detected at the expected height and the target band for and D4 (42.6 kDa). Bands for D2 (30.3 kDa), D3 (38.0 kDa) and D5 (26.9 kDa) were detected at higher heights compared to the theoretical absolute molecular weight deduced from the amino acid sequence. For all enzymes, bands were detected at 100-120 kDa which may result from protein aggregates. kDa= kilo Dalton; M=protein marker. The results shown are representative of at least three replicates.

Target band for D1 (48.1 kDa) was identified at the expected height and D4 (42.6 kDa) was detected at a lower height. Compared to the theoretical absolute molecular weight deduced from the amino acid sequence, D2 (30.3 kDa), D3 (38.0 kDa) and D5 (26.9 kDa) were detected at higher heights. Total protein content of the fermentation media, ammonium sulfate concentrates and the final dialyzed elution fractions was determined using the BCA assay and the respective fractions were incubated with chitin powder for 16 h at 30 °C followed by the quantification of released acetic acid to measure the specific chitin-deacetylase activity (U/mg) and to confirm enrichment of the target enzymes (Table 5.2).

Table 5.2. Summary of purification efficiency of recombinant chitin-deacetylases D1-D5 against chitin powder as a substrate. Total activity was assessed by the commercial acetic acid assay and total protein content of the individual fractions was quantified by the BCA assay. Both data were used to calculate the specific activity in U/mg. Data represents means of three replicate experiments (n=3).

Enzyme/ Purification fraction	Volume [mL]	Total activity [U]	Total protein [mg]	Specific activity [U/mg]	Purification [-fold]	Yield [%]
D1 Culture supernatant	800	121	724	0.17	1.0	100
D1 Dialysed elution	20	31	4.3	7.33	43.6	26.1
D2 Culture supernatant	800	92	1140	0.08	1.0	100
D2 Dialysed elution	20	8	1.8	4.82	59.3	9.5
D3 Culture supernatant	800	67	1081	0.06	1.0	100
D3 Dialysed elution	20	18	8.9	2.02	32.3	26.7
D4 Culture supernatant	800	127	1008	0.13	1.0	100
D4 Dialysed elution	20	29	7.8	3.75	29.6	22.9
D5 Culture supernatant	800	119	1135	0.11	1.0	100
D5 Dialysed elution	20	15	2.4	6.38	60.8	13.0

5.2.3 Characterization of chitin-deacetylases

All five chitin deacetylases were studied concerning their temperature and pH-optima as well as the optimum NaCl content. Chitin powder as well as chitin pentamers were selected as substrates for the characterization in order to assess optimum reaction conditions for sequential depolymerization-deacetylation as well as deacetylation-depolymerization reactions. CDA 2 and 3, however, did not exhibit enzymatic activity on chitin-pentamers. Therefore, the data relative to these two enzymes solely refer to chitin powder as a substrate.

D1, D2, D4 and D5 showed high relative activity (75 -100 %) within the temperature range of 20-50 °C. Reduced activity was assessed at 60-70 °C. D3 exhibited a more narrow range of high activity from 20-30 °C, with residual activity (≤ 25 %) at 10 °C and 40-70 °C (Figure 5.4 A). Temperature optima were determined to be at 30 °C for D3 and D5, 30 °C for D2, 40 °C for D4 and 50 °C for D1. Temperature stability tests at 60 °C revealed that D4 lost more than 65 % activity 15 min post incubation and residual activity remained on the same level. Similar behavior was observed for D2, although residual activity settled at 50 – 60 %. Inactivation curves for D3 and D5 showed a gradual decrease of residual activity 15 min after incubation reaching residual activities at 240 min incubation of 23 % (D3) and 50 % (D5). D1 showed no substantial change in activity within the first 60 min of incubation. Thereafter, a decrease to 38 % final residual activity was observed (Figure 5.4 B).

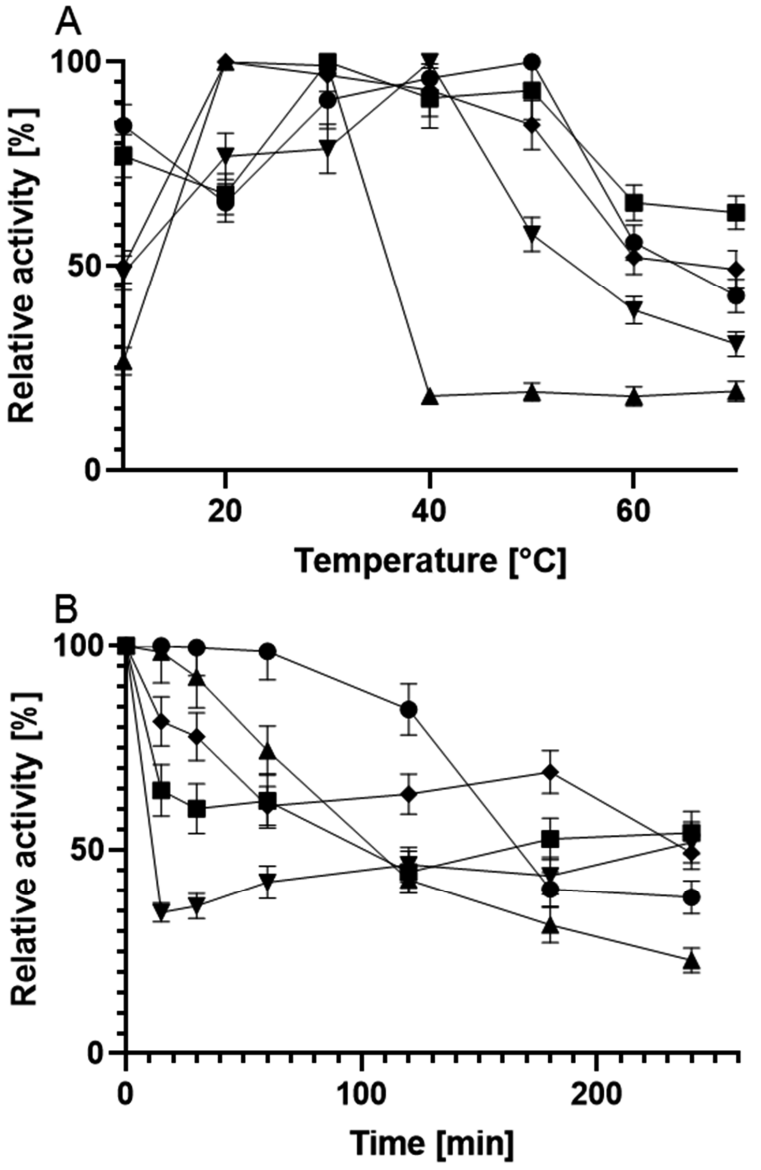

Figure 5.4. Optimum temperature activity and stability of recombinant deacetylases (D1-D5) produced by *E. coli* BL21. Activity was assessed using a commercial acetic acid assay with chitin powder and chitin trimers as substrates. Highest measured absolute activity was taken as 100 % A: Activity within the temperature range (10-70 °C) at pH 8. B: Temperature stability at 60 °C. Residual activity was determined at standard conditions after pre-incubation of enzymes for 0-240 min. ●: D1, ■: D2, ▲: D3, ▼: D4, ♦: D5. Data represents means of three replicate experiments (n=3).

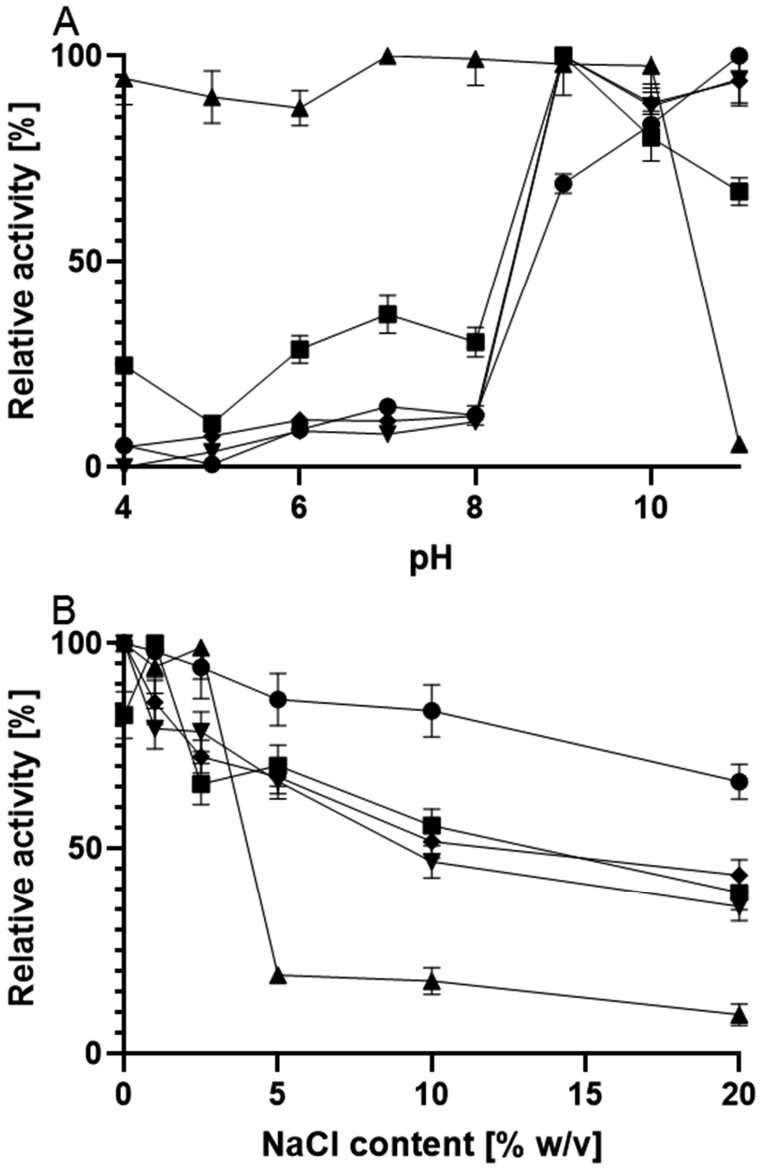

Figure 5.5. Optimum pH and salinity for activity of recombinant deacetylases (D1-D5) produced in *E. coli* BL21. Activity was assessed using a commercial acetic acid assay with chitin powder and chitin trimers as substrates. Highest measured absolute activity was taken as 100 % A: pH dependent activity (pH 4-11) at 30 °C. B: Enzymatic activity in the presence of 0- 10% (w/v) NaCl at pH 9, 30 °C. ●: D1, ■: D2, ▲: D3, ▼: D4, ♦: D5. Data represents means of three replicate experiments (n=3).

D1, D2, D4 and D5 all showed low activity (<30 %) within the pH range 4-8. D2, D4 and D5 reached the peak activity at pH 9 and the optima for D1 was determined to be at pH 11. D3 showed a different behavior as it maintained a residual activity close to 100 % within the pH range 4-10. At pH 11, however, the enzyme was inactivated (Figure 5.5 A). Salinity tests revealed that D1, D2, D4 and D5 are halotolerant up to 10 % (w/v) NaCl (1.72 M) with at least 50 % residual activity. D3 showed less halotolerance: up to 2.5 % (w/v) activity remained close to 100 %, while at 5 % NaCl (w/v) activity was reduced to less than 10 % (Figure 5.5 B). Optimum temperature, pH and salinity conditions for D1-D5 are summarized in Table 5.3.

Table 5.3. Optimum temperature, pH and salinity conditions for recombinant deacetylases (D1-D5) determined on chitin powder

Enzyme	Temperature [° C]	pH	Salinity [% NaCl]
D1	50	11	0
D2	30	9	1
D3	20	7	0
D4	40	9	0
D5	20	9	0

Different co-factors and chemicals were supplemented to the reaction mix at 1 mM to determine whether they stimulate or inhibit enzymatic activity and the effect was evaluated relative to the control in standard MAT buffer. Enzymes were incubated with a selection of metallic ions and chemical compounds and activators and inhibitors of activity were identified (Table 5.4). Surfactants such as Tween 20 and Triton X100 were identified as generally beneficial for enzyme activity. Furthermore, Mg^{2+} was strongly promoting activity for D5 and moderately for D1, and K^{+} resulted in increased activity for D1, D3 and D4 compared to the control. The tested concentration of Zn^{2+} completely inactivated all enzymes, while Cu^{2+}, Ni^{2+} and SDS had strong inhibitory effects on the enzymes.

Table 5.4. Effect of selected metallic ions (1 mM) and chemical compounds on enzymatic activity relative to the standard MAT buffer control (100 %). Data represents means of three replicate experiments with standard deviation (n=3).

Metal ion/Chemical compound 1mM	[%] Residual activity D1	[%] Residual activity D2	[%] Residual activity D3	[%] Residual activity D4	[%] Residual activity D5
Control	100.0	100.0	100.0	100.0	100.0
Mg^{2+}	115.8±10.0	99.1±9.6	37.8±3.9	85.9±2.4	198.4±8.3
Zn^{2+}	0	0	0	0	0
Ca^{2+}	78.3±5.1	67.5±9.3	29.9±3.4	50.0±2.9	82.1±4.5
EDTA	46.1±5.8	86.2±7.2	21.8±4.7	54.5±3.1	115.1±8.1
Cu^{2+}	26.5±4.3	31.7±6.0	12.3±5.6	5.8±0.8	76.7±9.6
Ni^{2+}	70.9±2.1	23.8±12.2	20.9±9.9	38.3±2.1	29.0±8.0
K^{+}	133.0±2.4	95.7±9.6	128.9±8.8	116.8±3.1	98.2±5.8
Tween 20 (0.5 % v/v)	127.5±7.1	114.1±6.8	141.5±8.3	104.9±4.1	142.4±8.0
SDS (0.5 % w/v)	37.2±8.6	32.4±5.4	15.5±2.3	29.3±2.7	52.9±11.0
Triton X100 (0.5 % v/v)	128.1±3.7	96.2±7.2	137.3±8.5	91.3±1.3	78.7±11.7
Imidazole (100 mM)	103.7±4.7	100.7±9.4	37.1±1.7	117.2±2.7	127.1±18.4

5.2.4 Enzyme kinetics on chitin powder

The kinetic parameters V_{max} and K_m were determined empirically using chitin powder as a substrate in increasing concentration from 0 mg/ml up to 100 mg/ml while keeping the amounts of enzyme constant at 2 µM. Michaelis-Menten curves of the reaction velocities (U) versus the substrate concentrations (mg/ml) and double reciprocal Lineweaver-Burk representations were plotted using GraphPad software. Non-linear regression models were applied to determine the kinetic parameters (Figure 5.6).

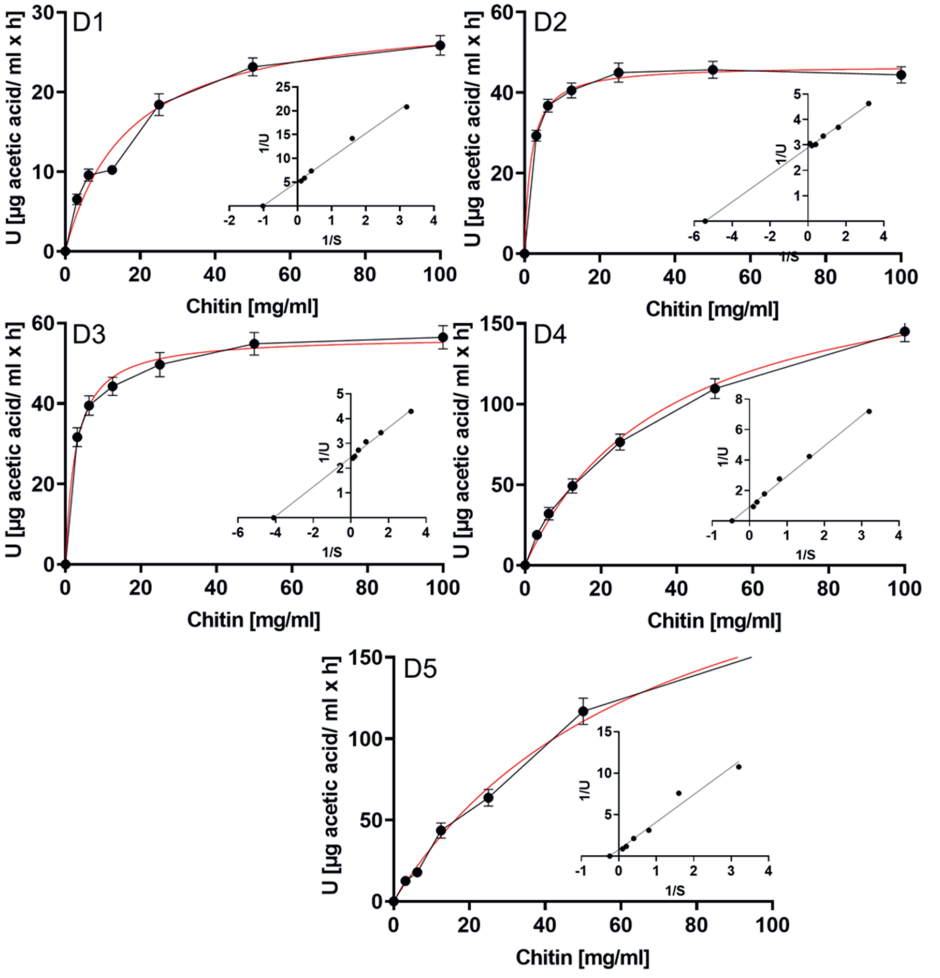

Figure 5.6. Michaelis-Menten fitted plots of recombinant chitin CDAs (D1-D2) on insoluble chitin powder (GraphPad software). The inserted double reciprocal Lineweaver-Burke plot relates D1-D2 inverted reaction velocities to released acetic acid associated with the inverted concentration of chitin (0-100 mg/ml). V_{max} and K_m were calculated from the non-linear fit model. Data represents means of three replicate experiments (n=3).

Table 5.5. Enzyme and substrate specific kinetic parameters V_{max} (maximum reaction velocity) and K_m (enzyme-substrate affinity) for CDAs determined on insoluble chitin using a non-linear fit model.

Enzyme	V_{max} [U/ml]	K_m [mg/ml]
D1	30.3	16.8
D2	46.8	1.8
D3	56.7	2.7
D4	196.5	37.5
D5	265.7	70.0

Maximum reaction velocities (V_{max}) and Michaelis-Menten constants (K_m) on insoluble chitin were determined using a non-linear fit Michaelis-Menten model (Table 5.5). Optimum enzyme-substrate ratios were determined that would allow a maximization of substrate conversion rates. Furthermore, the K_m indicated the general enzyme-substrate affinity for D1-D5 towards chitin. The data indicated that D2 and D3 have the highest affinity to insoluble chitin. In comparison, higher K_m values were determined for D1, D4 and D5 indicating that the respective enzyme-substrate affinity is low and the substrate is not converted efficiently.

5.2.5 Enzyme kinetics on chitin trimers

As mentioned above, D1, D4 and D5 exhibited limited enzymatic activity on insoluble chitin reflected by relatively high K_m values. Enzyme kinetics were also determined on soluble chitin-trimers as a substrate in order to determine V_{max} and K_m values (Figure 5.7). D2 and D3 did not exhibit enzymatic activity on oligomeric chitin, measured as the release of acetic acid, and were thus excluded from the Michaelis-Menten models. As expected, K_m values for D1, 4 and 5 were significantly lower on chitin trimers as a substrate compared to chitin powder, indicating that these enzymes exhibit a higher affinity towards soluble chitin (Table 5.6).

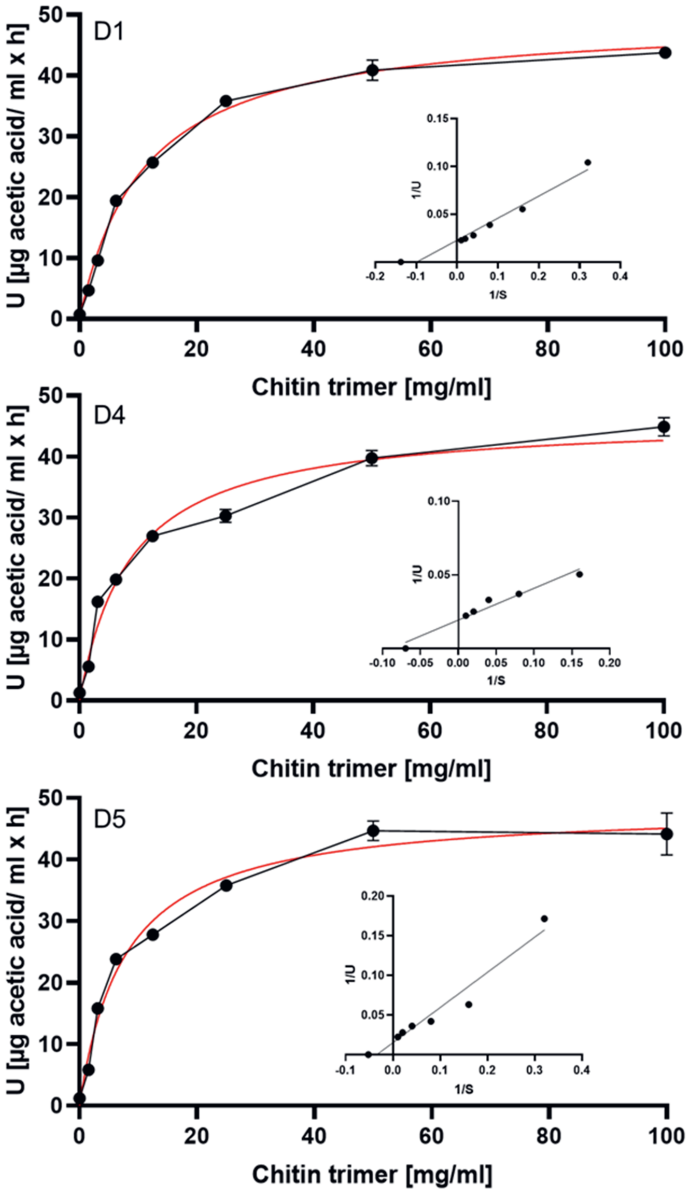

Figure 5.7. Michaelis-Menten fitted plots of recombinant CDAs (D1, D4 and D5) on chitin trimers and associated double reciprocal Lineweaver-Burke plots relating reaction velocities determined by released acetic acid and substrate concentration (0-100 mg/ml). V_{max} and K_m were determined from the non-linear fit model in the GraphPad software. Enzymes D2 and 3 did not exhibit activity on chitin trimers and were excluded from the calculation. Data represents the mean of three technical replicates (n=3).

Table 5.6. Enzyme and substrate specific kinetic parameters V_{max} (maximum reaction velocity) and K_m (enzyme-substrate affinity) for D1, 4 and 5 determined on chitin trimers using a non-linear fit model. D2 and 3 did not exhibit enzymatic activity on chitin trimers and were excluded from the models.

Enzyme	V_{max} [U/ml]	K_m [mg/ml]
D1	49.6	10.9
D4	46.4	8.7
D5	48.5	7.7

In conclusion, all recombinant CDAs show deacetylation activity on insoluble chitin powder however, K_m values indicate that affinity towards soluble chitin substrates is higher for D1, 4 and 5. Characterization of deacetylated soluble substrates will therefore further reveal what products can be generated efficiently by the novel enzymes.

5.2.7 Characterization of deacetylated chitosan oligomers

Chitin powder that was deacetylated enzymatically could not be investigated further regarding specific product properties as the products remain in a water-insoluble state. Therefore, chitin standard molecules (10 mg/ml each) from monomeric GlcNAc (DP1) up to hexameric chitin (DP6) were incubated for 16h at optimum reaction conditions and products were subjected to analysis by TLC (Figure 5.8).

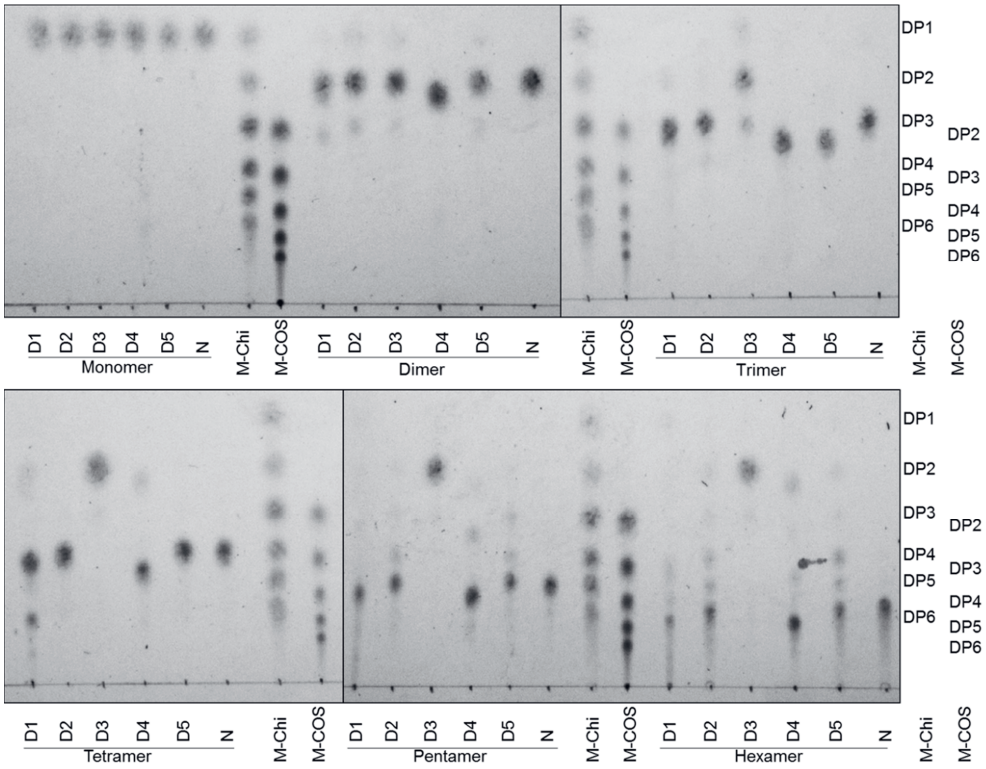

Figure 5.8. Thin layer chromatograms of enzymatic reaction products from recombinant chitin deacetylases (D1-D5) incubated for 16 h with GlcNAc and chitin oligomers (dimers-hexamers) at a concentration of 7.5 mg/ml. Spots on the far right are untreated standard molecules as negative controls (N).. M-Chi: Chitin oligomer standard mixture (DP1-DP6); M-COS: Fully deacetylated chitosan oligomers standard mixture (DP2-DP6).

Chromatograms revealed that GlcNAc is unaffected by all five enzymes and not converted to glucosamine. D4 completely converts dimeric chitin to a partially deacetylated derivative. Partially deacetylated derivatives with a different migration behaviour could also be overserved for D1, D2, D3 and D5. Chitin trimers are efficiently converted by D1, D3 D4 and D5. The migration behavior, however, is different for products generated by D1, indicating that the product is more polar and thereby contains more acetyl groups relative to the products of D4 and D5. D3 presumably exhibits chitinolytic activity on trimers as they are split to dimers and GlcNAc. D1 and D4 yield different deacetylated products when incubated with tetrameric chitin. Again, D3 reduces the DP of the substrate to DP2 and no characteristic deacetylation activity

could be observed for all chosen substrates (DP1-DP6). Tetramers are also unaffected by D2 and D5. Similar results for all enzymes were obtained for pentameric chitin. Changes in migration behavior were however observed for D2 and D5 when incubated with hexameric chitin. Again, deacetylation of hexameric chitin was also detected for D1 and D4.

Representative chitin standard samples (0.5 µM) were analysed by LC-MS for further characterization and assessment of the products' DA (Figure 5.9 – 5.11 a-c). Results showed that D1, D4 and D5 successfully deacetylate the tested substrates and acetyl-groups (m/z 42) are released from the substrate resulting in an increase of retention time (Table 5.7). Characteristic double peaks for each COS compound were detected which is typical for chitin oligomers due to the α/β anomeric splitting [27]. A reference chromatogram of chitin standard oligomers (DP2-DP6) is included in the Appendix B section (Figure A2.7). D1 also di-deacetylates chitin trimers and pentamers, a characteristic that was not observed for the other enzymes. No apparent deacetylation activity was observed for D2 on oligomeric substrates. It was further confirmed that D3 exhibits chitinolytic activity on chitin tetramers resulting in fully acetylated chitin dimers and trimers. However, no deacetylation activity on the chosen substrates could be determined by LC-MS.

Table 5.7. Expected molecular weight by MS analysis of fully deacetylated, mono-deacetylated and di-deacetylated GlcNAc and chitin oligomers displayed as mass-to-charge ratios (m/z) for single protonated compounds.

GlcNAc/ GlcNAc oligomer	Molecular weight + H [m/z]	Mono-deacetylated product + H [m/z]	Di-deacetylated product + H [m/z]
Monomer	222	180	-
Dimer	425	383	341
Trimer	628	586	544
Tetramer	830	789	746
Pentamer	1035	993	951
Hexamer	1238	1196	1154

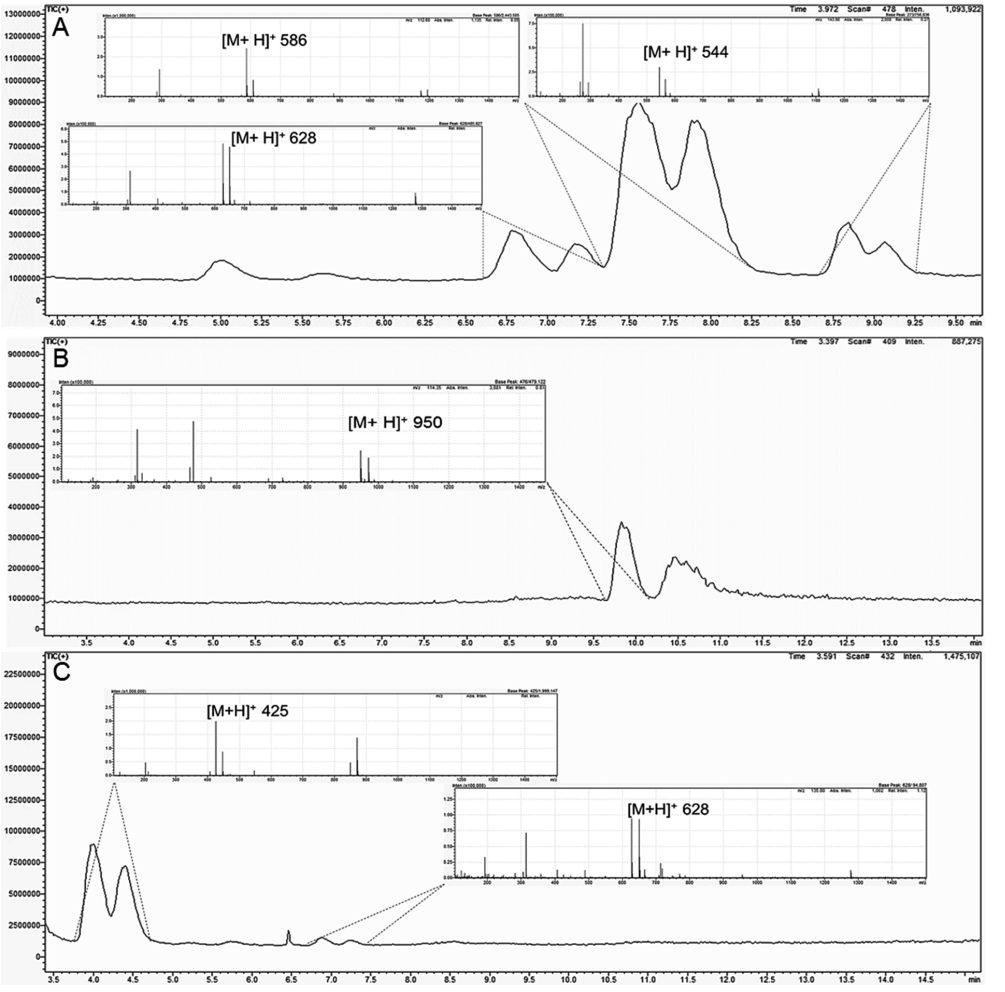

Figure 5.9. Characterization of chitin and chitosan products by LC-MS analysis of enzymatically deacetylated chitin oligomers. Recombinant deacetylase 1 (D1) was used to deacetylate chitin trimers (A) and pentamers (B); deacetylase 3 (D3) was used to convert chitin tetramers (C). The analysis revealed that D1 mono- and di-deacetylates chitin trimers and di-deacetylates chitin pentamers. In accordance with TLC, D3 revealed chitinolytic activity, as fully acetylated chitin dimers and trimers were yielded from chitin pentamers as a substrate.

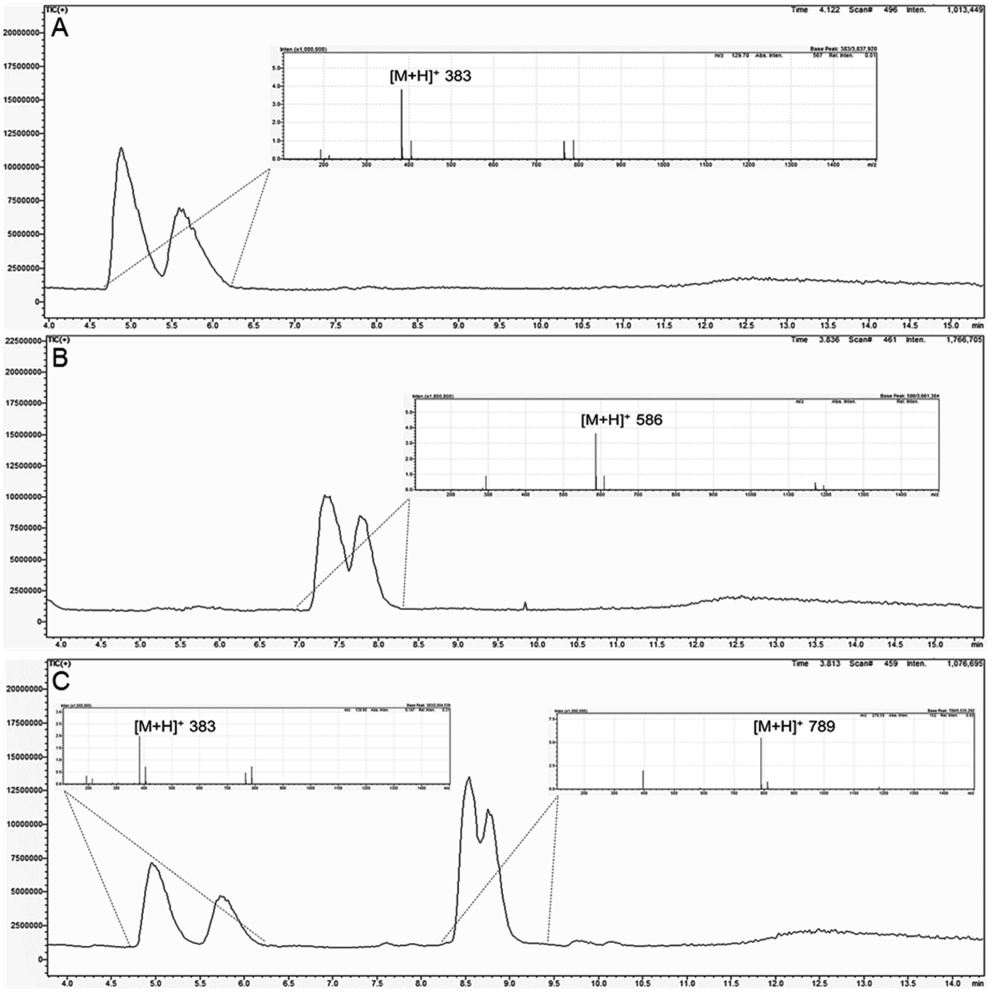

Figure 5.10. Characterization of chitin and chitosan products by LC-MS analysis of enzymatically deacetylated chitin oligomers. Recombinant deacetylase 4 (D4) was used to deacetylate chitin dimers (A), trimers (B) and tetramers (C). The analysis revealed that D4 mono-deacetylates chitin dimers and trimers. With chitin tetramers as a substrate, additional hydrolytic properties were revealed, resulting in mono-deacetylated chitin dimers as well as mono-deacetylated chitin tetramers.

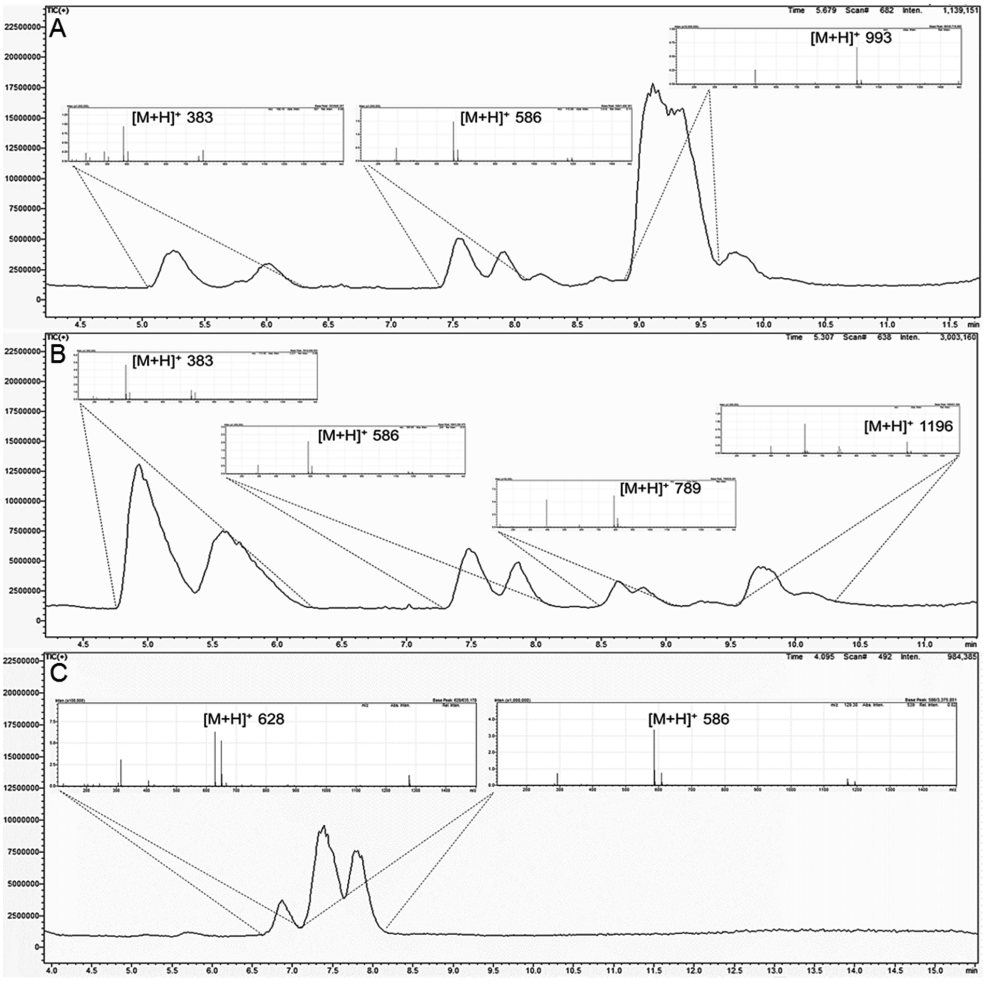

Figure 5.11. Characterization of chitin and chitosan products by LC-MS analysis of enzymatically deacetylated chitin oligomers. Recombinant deacetylase 4 (D4) was used to deacetylate chitin pentamers (A) and hexamers (B); Deacetylase 5 was used for deacetylation of chitin trimers (C) and hexamers (results anaologous to (b). Similar to chitin tetramers as a substrate, chitinolytic behavior and simultaneous deacetylation was observed for chitin pentamers and hexamers as mono-deacetylated chitin dimers, trimers and tetramers alongside the mono-deacetylated pentamers and hexamers were yielded. Comparable results were also obtained for D5 when incubated with chitin hexamers. Chitin trimers incubated with D5 yielded mono-deacetylated trimers alongside non-converted substrate.

Using D4 a mono-deacetylation of chitin dimers and trimers was achieved. For larger chitin oligomers, chitinolytic activity was observed in addition to deacetylation activity resulting in multiple mono-deacetylated side-products such dimers, trimers and tetramers (Figure 5.10c; Figure 5.11a and b). Comparable results were also obtained for D5 when incubated with chitin hexamers (data not shown). Incubation of chitin trimers with D5 also resulted in mono-deacetylated products. Full conversion of the substrate was however not achieved.

5.3 Discussion

Chitosan and COS can be generated from chitin by either a chemical or enzymatic deacetylation step. Typical bulk chemicals by means of NaOH for deacetylation enable a rapid and accessible approach for the generation of chitosan and COS from chitin. Such chemically deacetylated chitosan and COS products are however reluctantly implemented in cosmetic, food or medical products, as they still contain the risk of hazardous residual solvents [17, 18, 28]. Green chemistry enzymatic approaches could thus pave the way to eliminate this risk and furthermore complement and potentially replace chemical processes [29]. However, thus far, fully enzymatic deacetylation processes of insoluble chitin to chitosan only have limited effectiveness due to the low substrate accessibility. The absence of chitin binding domains within the CDAs was identified as the major limitation, as it typically enables enzymes to interact with the insoluble substrate and reduce crystallinity [24].

In **Chapter 4**, five novel chitinases were discovered within the genome of Chi5, expressed heterologously and characterized on chitin powder as a substrate. In addition to these chitinolytic enzymes, five genes for chitin-deacetylases were discovered within the same genome. For all enzymes, characteristic NodB polysaccharide deacetylase domains and a divergent polysaccharide deacetylase 2 domain were discovered. In addition, for D3, a second domain of unknown function (DUF3473) was identified that is however associated with a polysaccharide deacetylase domain according to Pfam (Table 5.2).

All genes were successfully cloned and expressed by *E. coli* BL21 using a C-terminal PelB signal peptide for secretion and a C-terminal 6xHis tag for purification. A

two-step purification procedure was adapted from **Chapter 4** to prevent ion leakage from the Ni^{2+} charged Chelating Sepharose FF resin. All enzymes were successfully enriched when comparing the specific activity (Table 5.3). Production levels for the five enzymes varied strongly, as D1, D3 and D4 showed higher expression compared to D2 and D5, which were only detectable on the immunoblot (Figure 5.2). Literature reports that both fungal and bacterial chitin-deacetylases isolated so far usually range from 25 up to 80 kDa [7, 23, 26, 30-32]. The predicted molecular mass based on the amino acid sequence of the five novel putative chitin-deacetylases named D1-5 lies within the range 26.9 – 48.1 kDa. Enzymes were characterized regarding optimum pH, temperature and salinity using chitin powder as a substrate, as it reflects the actual non-pretreated substrate that will be implemented in future fully enzymatic degradation processes. Enzymatic activity on water-soluble chitin oligomers is however also of interest as it might also promote the controlled deacetylation and transformation of chitin oligomers to partially deacetylated COS that could exhibit further physiological properties.

Different CDAs reported so far from either fungal or bacterial sources have shown very broad spectra of optima regarding pH and temperature ranging from pH 4.5 up to pH 11.5 and 30- 55 °C [6, 23, 24, 31]. Optima of the five novel enzymes were determined to be within pH 9-11 and 30-50 °C, showing comparable results to those reported. Optimum salinity was not taken into consideration for any of the CDAs described in the literature so far. However, salinity is a crucial factor that could have substantial influence especially for enzymes isolated from aquatic environments. Additions of NaCl did not stimulate enzymatic activity, however at up to 10 % (w/v) NaCl the residual activity of D1, D2, D4 and D5 was above 50 % relative to the control. This underlines that non-secreted (D2-D4) and secreted (D1 and D5) CDAs work best in physiological environments as provided by the cytosol. Enzymes D1, D2, D4 and D5 maintained activity above 50 % relative to the control at 10 % (w/v) NaCl indicating a high halotolerance. For the secreted D1 and D5 a higher halotolerance was expected. The high halotolerance of D2 and D4 could indicate that the respective enzymes are leaking into the Chi5 culture supernatant without any known signal peptide. The lower halotolerance of D3 could indicate that the enzyme remains in the Chi5 cytosol [33].

Enzyme kinetics of CDAs are typically determined on soluble substrates such as chitin oligomers or glycol chitin, and chitin powder is usually not selected as a substrate as either no or very low enzymatic activity is detected due to the high crystallinity and thus low accessibility of the substrate [34-36]. Interestingly, all novel bacterial CDAs exhibited quantifiable activity on chitin powder and D1, 4 and 5 were active on chitin oligomers. As it is intended to integrate the enzymes in deacetylation processes for both types of substrates, kinetics were determined on both chitin powder as well as chitin trimers. Analysis revealed that D1, 4 and 5 are more active on chitin trimers reflected by a lower K_m value compared to chitin powder. Several co-factors were assessed showing substantial effects on enzyme activity. Surprisingly, Zn^{2+} was identified as a major inhibitor of enzymatic activity for all novel enzymes. In previously reported studies on CDAs, Zn^{2+} and other divalent metal ions such as Ca^{2+}, Co^{2+}, Mg^{2+} were identified as an important cofactor for enhancing activity at 1 mM concentration [30]. Stimulation of activity was however observed for D5 when using Mg^{2+} and for D1 and D3 when K^+ was added. Moreover, an increase of enzyme activity was also observed for all enzymes by addition of the non-ionic surfactants Tween 20 and Triton X100. A possible explanation for this is an accelerated enzyme-substrate dissociation rate due to the reduction of surface tension resulting in overall higher turnover [37]. Interestingly, the surfactant SDS did inhibit activity substantially at the tested concentration (0.5 % w/v) which may be due to anionic nature of SDS resulting in interactions with the charged protein surface and reduced enzyme-substrate interactions [38].

Product analysis by TLC and LC-MS confirmed that the CDAs were active on various chitin oligomers (DP2-DP6). Different product properties were identified for the individual enzymes. However, due to the lack of further structural product properties, no conclusions can be made regarding the deacetylation model (multiple attack, multiple chain and subside capping) of the respective CDAs. Although D1 shares the same NodB polysaccharide deacetylase 1 domain with D2, D3 and D4 it generates di-deacetylated products. Conversely, D2 does not exhibit deacetylation activity on oligomers with DP1-DP5; however, using DP6 as a substrate, depolymerization or degradation effects were observed. As activity was also assessed for chitin powder, it can be deduced that D2 is an enzyme that is only active on larger oligomers and

polymers. D3 contains the NodB domain as well as an associated domain of unknown function. Product analysis revealed no deacetylation activity but chitinolytic activity on the substrates resulting in fully acetylated chitin oligomers. Deacetylation activity was however observed for chitin powder. One explanation could be that the active site of the NodB domain of D3 does not recognize shorter oligomers and therefore the domain of unknown function that supposedly conveys the chitinolytic activity is the only domain active on oligomers. It is likely that for larger chitin polymers, both domains exhibit catalytic activity resulting in simultaneous degradation and deacetylation reactions. D4 exclusively carries out a mono-deacetylation on DP2 and further mono-deacetylation was also observed for DP3. Analysis of larger deacetylated oligomers (DP4-DP6) by D4 as well as D5 revealed mono-deacetylated and depolymerized products as it was observed by TLC and LC-MS. For instance, DP6 was mono-deacetylated by D4 as well as degraded to mono-deacetylated DP2, DP3 and DP4. As no further homologous catalytic domain with chitinolytic activity was found within the genes for D4 and D5, this might be an indication for the presence of a non-homologous glycoslyl hydrolase domain that were discovered by the previous Pfam domain search. Thermal stability of low molecular weight COS has shown to decrease proportional to the molecular weight and DA [39, 40]. The partially deacetylated COS products may not be degraded to shorter COS by an undiscovered enzymatic mechanisms but due to reduced thermal stability at 30 °C.

The chitinases characterized in **Chapter 4** are capable to release small (DP1-DP4), water-soluble chitin oligomers from insoluble chitin substrate. The CDAs characterized in this chapter exhibit activity both on chitin oligomers as well as solid chitin powder. As enzymatic deacetylation activity on chitin powder is relatively limited concerning the increase of the DA, the novel CDAs can be implemented more effectively in a sequential depolymerization-deacetylation approach.

5.4 Conclusions

In addition to the previously discovered chitinases within the genome of Chi5 in **Chapter 4**, five novel genes for CDAs were identified, successfully expressed and purified from *E. coli* BL21 (DE3). Activity optima were determined on chitin powder and

chitin trimers, which revealed similar ranges to currently reported CDAs. Chitin oligomers were successfully converted to mono- as well as di- deacetylated COS. As a consequence, the novel enzymes will be tested on oligomeric chitin substrates generated by an enzymatic hydrolysis carried out by the chitinases characterized in the previous chapter. Thus, subsequent work will focus on the implementation of chitinases and CDAs in a successive *in vitro* conversion process in order to generate COS from chitin powder. Therefore, the CDAs of this chapter will be implemented in combined reactions to assess whether lower DAs can be achieved and overall conversion rates be maximized.

5.5 Experimental section

Materials

All chemicals used in the study were purchased from Carl-Roth (Germany) and were of highest purity, Chelating Sepharose FF from GE Healthcare (Sweden). All buffers were prepared in demineralized water.

Bacterial strains and plasmids

Coastal seawater samples from Oostende, Belgium, were used for the selective isolation of Chi5. The *E. coli* strains DH5α dam-/dcm- and BL21 (DE3) (New England Biolabs, Ipswich, USA) were used for cloning (LB medium) and expression (TB medium), respectively. Both media were spiked with ampicillin (100 µg/ml) or kanamycin (50 µg/ml).

Next generation sequencing and gene mining

Genomic DNA was extracted from Chi5 and the ion torrent sequencing approach (Ion Torrent Personal Genome Machine PGM, Thermo Fisher Scientific) was applied followed by the DNA-STAR assembly method. The resulting 60 contigs were assembled to scaffolds (10-fold coverage) and characteristic NodB domains were identified using the protein family database (Pfam version 2.9; https://pfam.xfam.org). The SignalP 4.1 server was used to predict and isolate signal peptides responsible for the secretion of the enzymes (SignalP-5.0).

Cloning of chitin deacetylase genes and sequencing

Golden gate cloning was used to generate multiple CDA constructs analogous to those described in **Chapter 4** for the expression of recombinant chitinases [41, 42]. Constructs comprised a secreted version with either the N-terminal natural signal peptide or the PelB signal peptide, a version fused to an oxidoreductase to form disulfide bonds (dsbA) as well as a cytosolic version [43-45]. C-terminally, the 6xHis tag and tag54/6xHis combi-tag used for purification and specific detection by antibodies were both tested in the setup. Single stranded overhangs flanked by the BsaI recognition sites (5´…GGTCTC$(N)_1$▼…3´ and 3´…CCAGAG$(N)_5$▲…5´) were designed for all genetic elements to allow a directed assembly of the constructs in the

three entry vectors pGR_SigP, pGR_dsbA and pGR_cyto (Table 5.8). Cloning was carried out using the golden gate assembly mix according to the manufacturer´s instructions (New England Biolabs). Verification of successfully integration of the inserts was carried out by colony PCR and DNA sequencing using T7 primers $T7_F$ (5´AAATTAATACGACTCACTATAGGG3´) and $T7_R$ (5´ATGCTAGTTATTGCTCAGCG G3´) adjoining the cloned constructs. A complete list of all constructs is given in table A1.1 in **Appendix 1**.

Table 5.8. Overhang design of genetic elements utilized in golden gate cloning. The 5´and 3´ single stranded overhangs are generated after digestion with the BsaI restriction enzyme and allow a directed assembly of the constructs.

Genetic element	5´overhang	3´overhang
pGR_SigP	TGAG	TGTA
pGR_dsbA	TGAG	CCGG
pGR_cyto	TGAG	CCGG
Natural signal peptide	ACAT	CCGG
PelB signal peptide	ACAT	CCGG
Gene-of-interest	GGCC	GCTT
6xHis-tag	CGAA	ACTC
Tag54/6xHis-tag	CGAA	ACTC

Expression of recombinant CDAs in *E. coli* BL21 (DE3)

For the expression analysis of the different deacetylase constructs, freshly heat-shock transformed *E. coli* BL21 (DE3) cells were cultivated in 25 ml TB-medium using 100 mL Ultra Yield™ flasks (Thomson Instrument Company, Oceanside, USA). Cultivation was carried out at 37 °C in orbital shakers at a shaking velocity of 200 rpm. After 4 h of cultivation (OD600=4), cells were induced in the middle of the log-phase by addition of 1 mM isopropyl-β-D-thio-galactopyranoside (IPTG) to the culture broth and temperature was decreased to 28 °C. After reaching a total cultivation time of 18 h, cells and supernatant were separated by centrifugation (8000 *x* g, 30 min). For cell lysis, an aliquot of the pellet was resuspended in BugBuster® master mix (Merck Millipore, USA) and incubated at RT for 2 h in an overhead shaker (60 rpm). Cell debris were separated by centrifugation (8000 *x* g, 2 min) and lysis supernatant as well as

culture supernatant were subjected for analysis by immunoblot and the enzyme activity assay.

Selected optimal enzyme candidates were produced at larger scale using 800 ml TB-medium and 2.5 L Ultra Yield™ flasks (Thomson Instrument Company, Oceanside, USA). Cultivation, induction and harvesting procedures were analogous to the above mentioned small-scale expression.

Purification of CDAs

The protocol for the purification of 6xHis tagged CDAs was adapted from **Chapter 3** and consisted of two steps:

Step 1. Ammonium sulfate precipitation

Solid ammonium sulfate was slowly added to 750 ml culture supernatant to a final concentration of 70 % and was stirred for 2 h at room temperature. The precipitate was collected by centrifugation (8000 *x* g, 30 min) and dissolved in 100 ml PBS (pH 7.4).

Step 2. Immobilized metal affinity chromatography (IMAC)

Samples from step 1 were applied to a Chelating Sepharose FF (GE Healthcare, Sweden) resin (column volume (CV) 5 ml) charged with 0.2 M $NiSO_4$. The column was connected to an ÄKTA pure 25 (GE Healthcare, Uppsala, Sweden) and equilibration was carried out using 3 CV PBS (pH 8.0). The column was washed with equilibration buffer until the UV_{280nm} signal fell below 30 mAU and weakly bound unspecific protein was then eluted from the column using 50 mM imidazole in PBS (pH 8.0) followed by a second elution step at 250 mM imidazole in PBS (pH 8.0). All steps were carried out at a constant flow rate of 2 ml min^{-1}. Fractions of 2 ml containing chitin-deacetylases were pooled and dialyzed against a mixture of 33 mM TRIS, 33 mM CAPS, 33 mM MES (MAT-buffer) (pH 8).

SDS-PAGE, immunoblot analysis and enzyme assay

Protein analysis was carried out by SDS-PAGE according to the method described by Laemmli *et al.* using 12 % separation gels and protein bands were visualized by staining with Coomassie Brilliant Blue R-250 [46]. For immunoblot analysis, samples

from SDS-PAGE gel were transferred first to a nitrocellulose membrane and detection was carried out by 6xHis-tag antibodies [0.2 µg/ml] (Thermo Fisher Scientific) and sequential band visualization by the secondary antibody [0.2 µg/ml] using alkaline phosphatase as a reporter enzyme (Thermo Fisher Scientific) converting NBT/BCIP. Protein quantification was carried out via the bicinchoninic acid assay (BCA) using bovine serum albumin as standard for calibration. Samples were quantified densitometrically using the AIDA 5 software (Raytest Isotopenmessgeräte GmbH) relative to the 6xHis tagged protein K12v105 (Fraunhofer IME). Quantification of purified proteins was carried out via the bicinchoninic acid assay (BCA) using bovine serum albumin as standard for calibration [47].

For the determination of enzyme activity, a commercial acetic acid assay kit (K-ACETRM; Megazyme, Bray, Ireland) was used in the multitier plate format, measuring the amount of released acetate by the action of chitin deacetylases on various chitinous substrates. For quantification, a calibration curve was generated freshly for each measuring cycle using acetic acid as a standard. One unit [U] was defined as the amount of enzyme required to release 1 µg of acetic acid per hour.

Preparation of homogenous chitin powder

Chitin from shrimp shells (~400.000 g/mol; Carl Roth) was mechanically pre-treated using a GyroGrinder (Fritsch GmbH, Germany) at 6000 rpm and thereby converted to a homogeneous powder of 80 µM average particle diameter. The powder was used without any further treatment as a substrate for deacetylation experiments.

Characterization of recombinant chitin deacetylases

Enzyme samples were incubated at different temperature (10-70 °C, 10 °C increment) and pH (4-11, increment 1) using different buffer systems (CAPS, Tris-HCl, MES, 100 mM) as well as NaCl content (0, 1, 2.5, 5 and 10 % w/v) to determine optimum reaction conditions. All experiments were carried out at 1.0 ml scale using 2µM of the respective enzymes and a 5 % (w/v) suspension of chitin powder extracted from shrimp shells (~ 400.000 g/mol; Carl, Roth, Karlsruhe, Germany) in 100 mM MAT buffer. Reaction optima for CDA 1, 4 and 5 were also assessed using 10 mg/ml chitin trimers in 100 mM MAT buffer. The effect of several potential co-factors on activity

was elucidated at a concentration of 1 mM. Additionally, the effect of detergents (SDS 0.5 % (w/v), Tween-20 and Triton X-100 both at 0.5 % (v/v)) and chemicals (Imidazole 100mM, EDTA 1 mM) on activity was assessed. Samples were incubated in a thermomixer for 16 h at 900 rpm. After incubation, samples were analyzed using the enzyme assay. Thermostability was assessed after incubation of recombinant chitin deacetylases at 60 °C for different durations followed by the quantification of residual enzyme activity under standard assay conditions. Enzyme kinetics of recombinant deacetylases were assessed by determination of K_m and V_{max} by Lineweaver-Burke representation of the Michaelis-Menten model preceding an incubation of constant amounts of enzyme (2µM) with 0 – 100 mg/ml chitin powder as well as chitin trimers and sequential quantification of released acetic acid.

Product analysis by thin-layer-chromatography and LC-MS

Hydrolysis products were analyzed using 10x10 cm TLC Silica gel 60 F254 plates (Merck, Darmstadt, Germany) and a mixture of butanol: methanol: 25 % ammonia: H_2O (5:4:2:1) as mobile phase. A total sample volume of 1 µl was applied in 0.2 µl spots and 0.5 µl of chitin standard sugars (Megazyme, Chicago, USA) (1 mg/ml) were applied to the plate. After separation, the plate was air-dried and the developing solution (200 ml Acetone, 30 ml phosphoric acid (85 %), 4 ml aniline, 4 g diphenylamine) was sprayed on the plate followed by incubation in an oven at 180 °C for 2 min or until spots appeared [16].

The protocol for analysis by LC-MS was developed based on the method described by Hamer *et al.* [7]. Analysis of oligomeric chitin and chitosan samples was carried out using a Shimadzu LC-30 AD system coupled to a SIL-30AC autosampler, a CTO-20AC column oven and a LCMS-2020 mass spectrometer (Shimdazu, Kyõto, Japan). Samples of 1 µl were separated by hydrophilic interaction chromatography using an Acquity UPLC BEH Amide column (1.7 µm, 2.1 x 150 mm) coupled to an Acquity UPLC BEH Amide 1.7 µm VanGuard pre-column (2.1 x 5 mm) (both Waters Corporation, Milford, USA). Flowrate was set to 0.5 ml min^{-1} and the column oven temperature to 30 °C. Samples were eluted from the column with a gradient of A (Acetonitrile + 0.1 % (v/v) formic acid) and B (water). Sample separation was done over 16 min using the following gradient: 0-2.5 min isocratic 80 % A; 2.5 – 12.5 min, linear from 80 % to 35 % (v/v) A, followed by column re-equilibration 12.5 – 13.5 min, linear from 35 % - 80 % A

(v/v); 13.5 – 16.0 min, isocratic 80 % A. MS-detection was carried out in positive mode with the interface voltage at 4.5 kV, the nebulizer gas flow rate at 1.5 L/min, drying gas flow at 15.0 L/min and the dry temperature at 249 °C. Mass spectra were recorded over a scan range from m/z 100-1500.

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Chapter 6

Towards a fully enzymatic chitin degradation process for the generation of partially deacetylated chitosan oligomers

In this chapter, a fully enzymatic conversion process from mechanically homogenized chitin to partially deacetylated COS is explored using the design-of-experiments approach. The chitinases and chitin-deacetylases characterized in **Chapter 4** and **5** are implemented in two distinct mixtures designs to assess significant factors and factor interactions, their effect on product accumulation and influence on product distribution. Both designs are further used to predict optimum factor combinations for maximized conversion rates and multiple combinations are validated in sequential depolymerization and deacetylation reactions. Total substrate conversion yields of chitin and the proportional amount of single COS compounds in the product mixtures are determined.

Keywords: conversion rate, design-of-experiments, factor optimization, mixture *d*esign, statistical modelling

This chapter is based on the following publication:

Schmitz, C.; Lijnen P.; Schillberg, S.; Fischer, R.; Rasche, S.; Bortesi, L., Towards a fully enzymatic conversion process for the generation of partially deacetylated chitosan oligomers from chitin. Prepared for submission.

6.1 Introduction

Chitosan and COS have become a remarkable object of research as a broad variety of intrinsic properties are featured in various fields of application [1-5]. Conversion reactions from chitin to COS typically comprise sequential depolymerization and deacetylation steps. At present, conventional production processes for COS are based on high temperature chemical conversion by means of hydrochloric acid (HCl) for depolymerization, and sodium hydroxide (NaOH) for deacetylation [6-14]. Such processes not only generate large amounts of chemical waste, waste water and potential product contaminations, but controlling DP, DA and PA is hardly achievable in practice due to the lack of substrate specificity and selectivity of the catalysts. In addition to the unsystematic reaction behavior, adverse reactions also occur frequently during chemical deacetylation resulting in further undesired depolymerization effects [9]. Approaches to optimize the chemical production of defined COS were pursued by investigating the effects of multiple process parameters such as temperature, reagents, and incubation time [13, 15-17]. Slight improvements of controllability on physicochemical properties and reduction of adverse reactions were achieved, but reductions of chemical waste was usually not taken into consideration during optimization.

The goal of the work presented in this chapter is to develop a sequential fully enzymatic depolymerization and deacetylation process for the controlled degradation of chitin powder to COS using the chitinases and CDA produced recombinantly and characterized in **Chapters 4** and **5**. Because the product spectrum of individual enzyme reactions concerning DP and DA did not show major differences, synergistic effects of various chitinase and CDA combinations will be investigated. Mixing enzymes with different hydrolytic domains can yield different products compared to single enzyme reactions, as cascade enzyme-substrate interactions and feed-back effects can occur [18-22]. In particular, products of one enzymatic reaction can serve as substrates for different enzymes, thereby yielding final products with different properties (Figure 6.1). Further potential benefits from multi-enzyme processes are synergistic effects that could result in higher overall products yields due to the shift of the reaction equilibrium along with a reduction of product inhibitions.

Multi-enzyme processes have been explored extensively in the field of biocatalysis. For instance, optimized *in vitro* multi-component mixtures for the enhanced degradation of lignocellulosic biomass were successfully established [23-27].

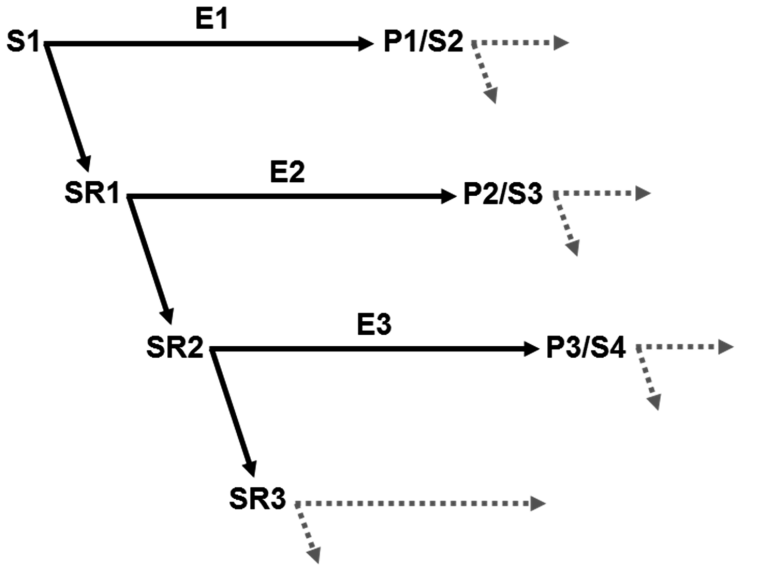

Figure 6.1. Schematic overview of an exemplary cascading multi-enzyme process using chitin as insoluble substrate. Solid substrate residues (SR) and products (P) may serve as additional substrates (S) for sequential reactions by different enzymes (E). Thereby, different final products could be obtained. Enzymatic feed-back reactions further increasing complexity are not considered in this scheme.

Although enzymatic reactions are superior to chemical reactions in terms of controllability and selectivity, major drawbacks are relatively low conversion rates due to product inhibitions as well as limited substrate availability. So far, no fully cascade multi-enzyme conversion process from insoluble chitin to defined COS has been reported, probably due to the aforementioned drawbacks and limitations compared to chemical processes. However, several hybrid processes were established, comprising either a chemical deacetylation of chitin to chitosan and sequential enzymatic depolymerization to COS or a chemical depolymerization of chitin to chitin oligomers and sequential enzymatic deacetylation to COS [28-30]. Still, it is desired to eliminate chemical pre-treatment and degradation steps entirely and to establish fully enzymatic processes for the efficient generation of defined COS. Due to the diverse nature of

chitinolytic enzymes with different catalytic mechanisms and substrate specificities, intensification of the process can be achieved by modeling multi-enzyme reactions. In addition, modeling of such processes could also reveal whether product properties of COS could be altered concerning DP and DA.

Process analysis and optimization are essential methodologies to assess complex multi-factor procedures such as mixed enzyme reactions. A common method to investigate and optimize processes is to sequentially vary one putative influential factor and keep all other factors constant. This one-factor-at-a-time (OFAT) approach, however, conceals factor interactions and cannot expose synergistic interdependencies of two or more factors on a given output, thereby missing the true optimum [31]. In contrast, the design-of-experiments (DoE) approach is a mathematical tool to carry out a reduced number of experiments in a systematic way to obtain meaningful information on the factor influences and factor interactions. The underlying statistical analysis allows to 1) assess all significant factors and factor interactions simultaneously 2) identify optimum factor combinations 3) extrapolate and interpolate factor combinations to achieve a desired output [32-35].

There are several specialized designs for mixtures (mixture designs), screening factors (factorial designs) and the quantitation of factor impacts on responses (response surface methods) available for process optimization studies. The advantages of DoE in comparison to the OFAT approach can easily be illustrated with an example for a factorial design. Assuming that the effect of two factors A0 and B0 on the desired output are investigated, information could be gained by varying (index “1”) the factors one at a time. For the OFAT approach this results total in two factor level scenarios: A1 B0 and A0 B1. If the OFAT design showed that A1 B0 and A0 B1 gave better results than A0 B0 the conclusion would be that A1 B1 should improve the results even further. However, if there is an interaction or interdependency between both factors this conclusion may be false. Moreover, an interaction between A and B means that the effect of A on the results depends on the level of B and vice versa. In a factorial design, an additional combination where both factors are varied simultaneously is added (A1 B1). By this approach it is possible to obtain data just as concise as those from the OFAT approach and additionally decipher potential interactions of the investigated factors. In figure 6.2, a factorial design (a) and the

OFAT approach (b) are displayed side by side [36] demonstrating that the factorial design investigates a larger experimental design space than the OFAT approach.

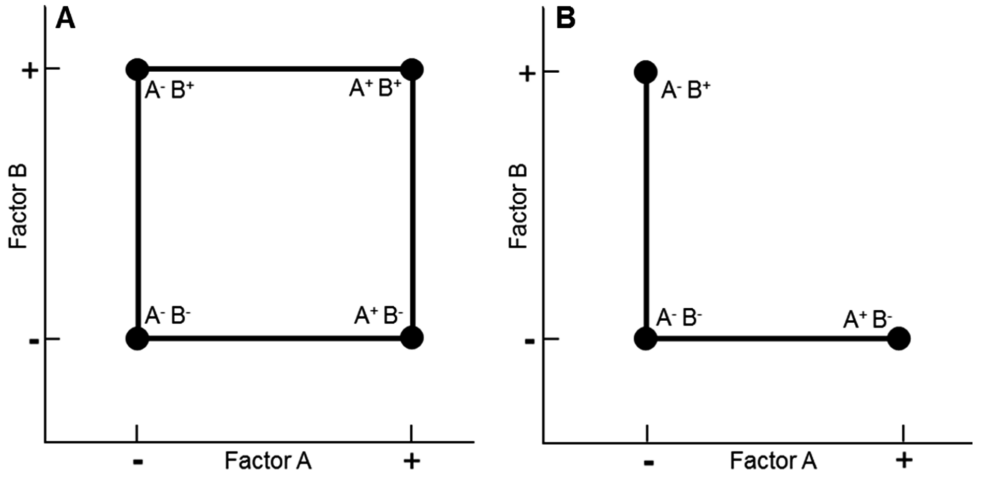

Figure 6.2. Comparison of a factorial design experiment (A) and the OFAT approach (B). A: The factorial experiments allows the investigation of all possible combinations of two factors (A, B) on two levels (-: low level; +: high level) thus considering potential interactions (A+ B+) between the factors. B: In the OFAT approach, apart from the original factor levels (A-, B-) two factor combinations are investigated neglecting factor interactions that might lead to the loss of meaningful information.

In this chapter, separate special cubic mixture designs are generated for chitinases as well as CDAs. Each enzyme is considered a separate factor in a mixture and the effect on total conversion rate and product properties is studied. The I-optimal design type is selected for both mixture designs as the average variance of predictions is minimized resulting in more precise predictions of optimal mixtures [37].

Optimized mixtures for chitinases and CDAs are validated experimentally and implemented in sequential depolymerization and deacetylation reactions for the conversion of chitin powder to partially deacetylated COS.

6.2 Results

6.2.1 Chitinase mixture design

All five chitinases implemented in the DoE originated from the novel marine Chi5 strain expressing these enzymes to carry out a conversion of crystalline chitin to monomeric and dimeric polysaccharides. All chitinases were produced recombinantly using *E. coli* BL21 (DE3) and characterized regarding optimum reaction conditions and product properties in **Chapter 4**. As so far only singular enzyme reactions were carried out, the DoE approach was used to 1) determine ideal enzyme combinations to maximize product rates 2) identify possible enzyme combinations to alter product distribution in terms of DP. To assess the effects of the individual factors (chitinase 1-5) as well as two- and three-factor interactions, an I-optimal mixture design was set up and a special cubic model was implemented for evaluation. Enzymes were dialyzed and pre-diluted using MAT buffer (pH8) in order to add equal volumes recommended by the mixture design plan. Chitin and enzymes were used from the same batches to ensure consistent experimental conditions. The amount of released reducing sugars from the enzymatic degradation was quantified by the reducing end assay and used as a primary response.

Analysis of variance (ANOVA) indicated that all main factors had highly significant impact on the degradation of the substrate. Furthermore, highly significant two factor and three factor interactions between the main factors were identified (Table 6.1). Significance of the model was confirmed by the not-significant lack-of-fit test and the predicted R^2 value was in reasonable agreement with the adjusted R^2 value (Table 6.2).

Table 6.1. Significant factors and factor interactions for the chitinase mixture design. A reduced cubic model was used to analyze the response data (released reducing sugars in nmol/ml x h). Significant main factors are A (chitinase 1), B (chitinase 2), C (chitinase 3), D (chitinase 4) and E (chitinase 5). Significant factor interdependencies were preselected by an automated backward selection with a p-value threshold of 0.05. Factors that were required to maintain model hierarchy were not excluded.

Factor	F- value	P-value
Model	168.97	<0.0001
Linear mixture	585.86	<0.0001
AB	12.12	0.0016
AD	87.53	<0.0001
AE	162.03	<0.0001
BC	21.41	<0.0001
CD	88.40	<0.0001
CE	32.21	<0.0001
ABE	17.98	0.0002
BCD	16.15	0.0004
BCE	20.65	<0.0001

*significant main factors: A, B, C, D and E.

Table 6.2. Model parameters to confirm significance for chitinase mixture design.

Parameter	Value
R^2	0.9906
Adjusted R^2	0.9847
Predicted R^2	0.9381
Lack of Fit	0.7747

The reduced cubic model revealed further that factors A and C take part in multiple highly significant (p-value <0.0001) two factor (AB, AD, AE, BC, CD, CE) and three-factor interactions (ABE, BCD, BCE). Interestingly, no direct interactions between factor A and C were significant in the model. The overall highest conversion rate was achieved by run 19 (2 µM Factor A; 502 nmol/mL x h). Hydrolysis products were subjected to analysis by TLC (Figure 6.3) and LC-MS (Figure 6.4) in order to identify

products and to reveal potential alterations in product properties with regards to the DP.

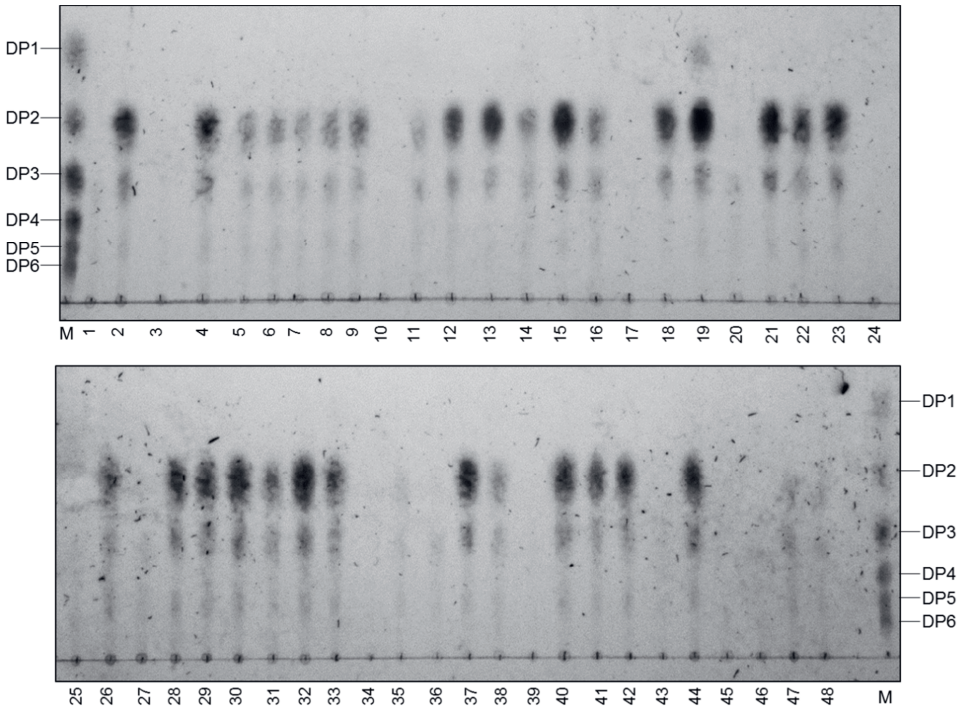

Figure 6.3. Thin layer chromatogram of hydrolysis products from mixture DoE of recombinant chitinases 1-5 with chitin as a substrate. A total sample volume of 0.1 µl was loaded by sequential application of 0.1 µl. A mixture of chitin oligomers was used a size marker to identify products. Differences were identified regarding the total product yields reflected also by the quantitative data from the reducing end assay. M= chitin size marker; DP=degree of polymerization.

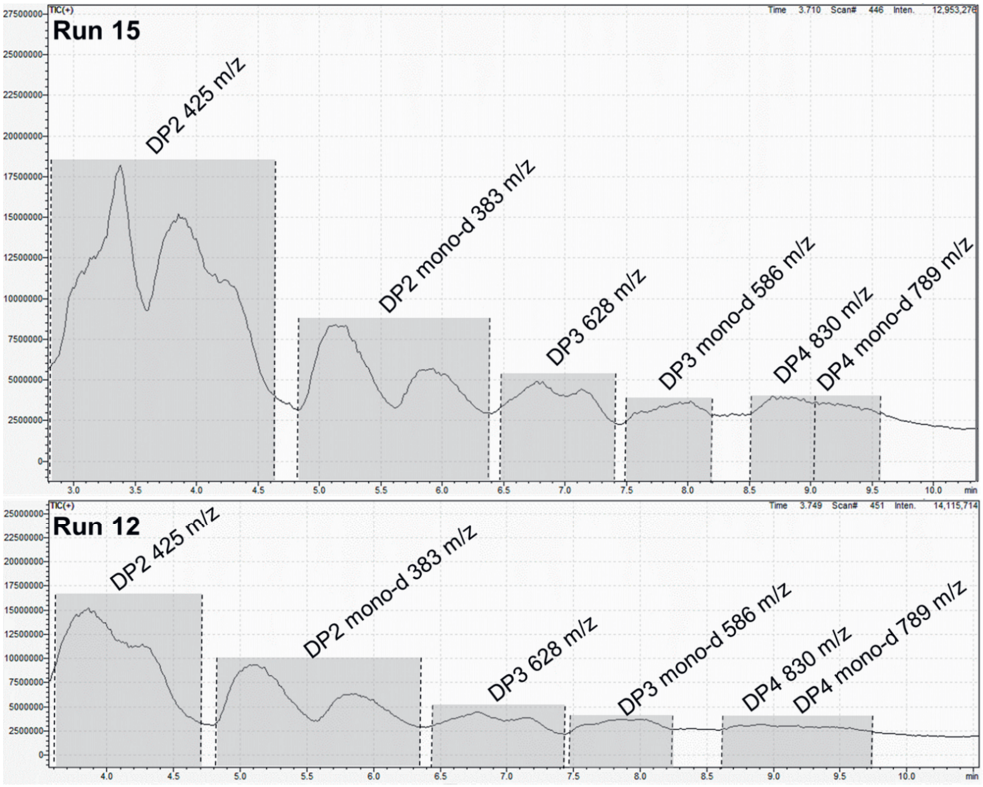

Figure 6.4. Exemplary LC-MS spectra of hydrolysis sample from chitinase mixture design (Run 15 and 12). Oligomeric chitin products were identified by their respective molecular weights. Peak integration was used to determine average mixture compositions. DP= degree of polymerization. Mono-d: mono-deacetylated product.

The relative distribution of DP2, DP2 mono-d, DP3, DP3 mono-d, DP4 and DP4 mono-d was anticipated as a further responses to evaluate the model in order to identify chitinase mixtures to predict the DP and to improve the production of chitin oligomers with a DP>2. Therefore the individual compound peaks of the LC-MS spectra were integrated for the design runs to use the relative abundances as responses. However, 16 runs could not be evaluated quantitatively as the overall product yield was too low to carry out a peak integration (runs 3, 10, 17, 20, 24, 25, 27, 34, 35, 36, 39, 43 and 45-48). The response for these runs was therefore set to zero. However, ANOVA evaluation resulted in non-statistically significant models.

The mean was calculated for 10 representative runs and confirmed that the overall proportional distribution of chitin oligomers is similar between the individual runs (Table

6.3). Therefore, it was concluded that the individual chitinase mixtures did not change the overall product composition and only affected the overall conversion rate.

Table 6.3. Average proportional product composition of calculated from integrated peak areas of 10 representative runs from the chitinase mixture design.

Compound	Average proportion
DP2	37.2±2.4
DP2 mono-deacetylated	20.0±6.4
DP3	6.4±2.5
DP3 mono-deacetylated	5.1±2.0
DP4	1.0±0.1
DP4 mono-deacetylated	5.0±0.4

The optimization function in Design Expert was used to model enzyme mixtures to maximize the overall conversion rate of chitin to oligomeric chitin mixtures. Three mixture solutions for maximized chitin (S1-S3 Max) oligomer rates were tested and quantitative data was compared to the values predicted by the model. In addition, three mixture solutions for minimized chitin oligomer rates (S1-S3 Min) were tested to validate the model´s predictability.

All solutions for maximized conversion rates included a mixture of factor A (chitinase 1) and factor B (chitinase 2) with factor A being the predominant component (Table 6.5). Solution 3 also introduced factor C (chitinase 3) as a minor component. All solutions were validated experimentally at a 1 mL scale in three technical replicates. The achieved rates (Rate ach.) were in accordance with the predicted values (Rate pred.) and a substantial increase in conversion rates were achieved compared to the best run in the design (run 19; 502 nmol/ml x h) (Table 6.4). The optimized mixture were able to improve the rates by 80 % (S1 Max), 73 % (S2 Max), 53 % (S3 Max) relative to run 19. The results for minimized solutions further confirmed that the model could be used to carry out reliable predictions of conversion rates.

Table 6.4. Optimized chitinase mixtures suggested by the design model. Multiple solutions were recommended and three (1-3 Max) predicting the highest rate of reducing sugars (Rate pred.) were selected for validation. In addition, three solution (1-3 Min) for minimal rates were included to validate the predictive power of the model. Analysis was carried out in three technical replicates (n=3). A: chitinase 1, B: chitinase 2, C: chitinase 3, D: chitinase 4, E: chitinase 5.

Solution	Factor A [µM]	Factor B [µM]	Factor C [µM]	Factor D [µM]	Factor E [µM]	Rate pred. [nmol/ ml x h]	Rate ach. [nmol/ ml x h]
1 Max	1.7	0.3	0	0	0	869	904±27
2 Max	1.6	0.2	0.1	0	0	843	869±26
3 Max	1.8	0.2	0	0	0	753	769±15
1 Min	0	0.4	0	0.6	1.1	3.9	20±1
2 Min	0	0	0	1.8	0.2	4.1	18±1
3 Min	0	0.6	0.7	0	0.7	8.6	19±1

A response plot of the mixture model is displayed in Figure 6.5. Tested optimized mixtures and corresponding responses for all solutions (1-3 Max black dots; 1-3 Min grey dots) were included in the plot indicating that the Max solutions were meeting the highest achievable conversion rates within the design constrains. Thus the response plot displays that the model could be used to successfully predict optimum enzyme combinations for maximized rates. Tested solutions for minimized rates were included to further validate the models predicative power and the response plot could be used to successfully predict enzyme combination to achieve minimum rates.

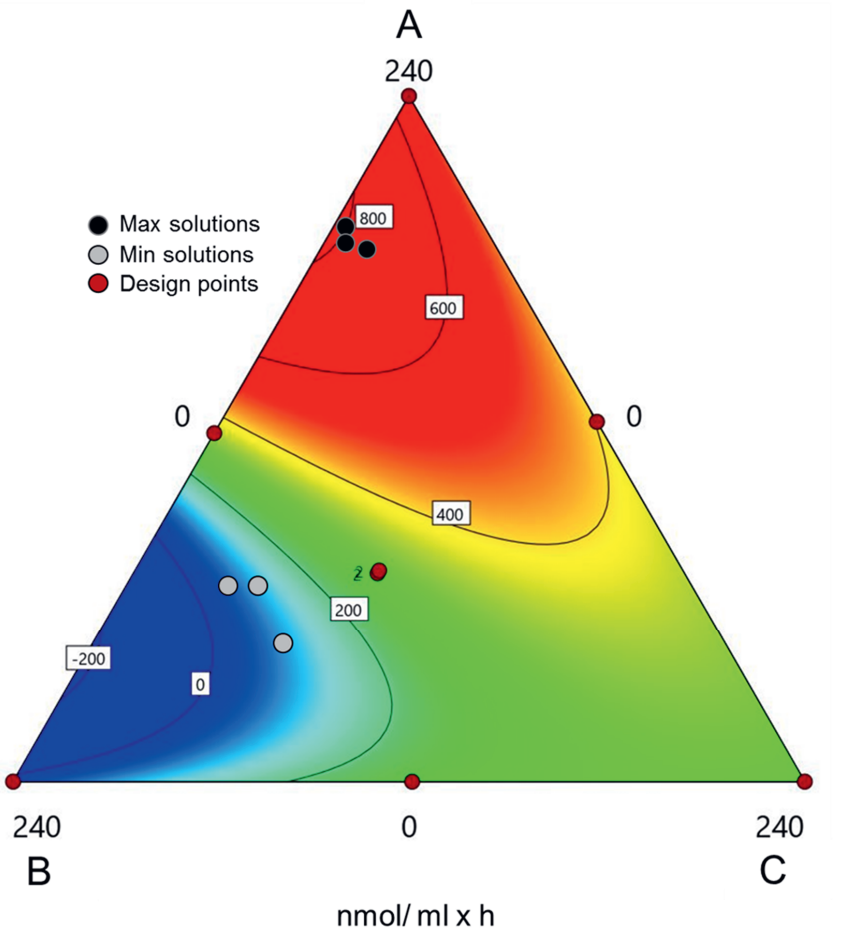

Figure 6.5. Response plot (nmol/ml x h) for tested optimized solutions from the chitinase mixture design. Three solutions were tested to maximize the conversion rate of chitin to chitin oligomers (Black dots) and three solutions for minimized conversion rate (grey dots). The minimized solutions were used to further validate the predictability of the design model. The total molar enzyme concentration was set to 2 µM. Response plots highlight which factor combinations are necessary to achieve the desired output (red color = high response; blue color =low response). A: chitinase 1; B: chitinase 2; C: chitinase 3.

Product analysis by TLC and LC-MS further revealed the composition of the product mixture (Figure 6.6 and Figure 6.7). Product compositions of S1 Max – S3 Max were identical and did not change from the composition of the design runs. Chitin oligomers with DP2 were the predominant product and compounds with DP1, DP3 and DP4 were identified at lower quantities by TLC. LC-MS data further revealed the presence of

partially deacetylated chitin oligomers that could not be detected by TLC due to the limited seperative resolution and lack of partially deacetylate standard molecules.

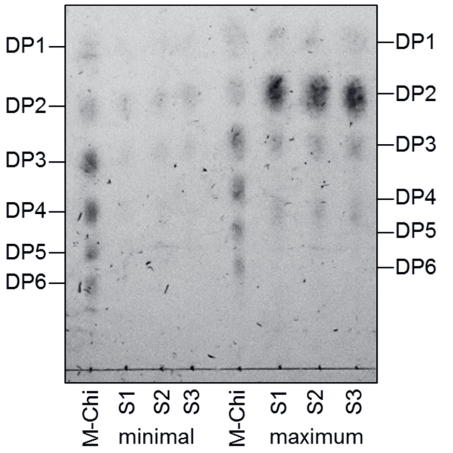

Figure 6.6. Thin layer chromatogram of oligomeric chitin products for tested solutions for minimized (S1-S3) and maximized (S1-S3) chitin conversion rates. A total sample volume of 0.3 µl were loaded sequentially in 0.1 µl spots. Samples for S1-S3 Max were diluted 1:3 with dH_20 before application. Oligomeric chitin standards were used as a size marker. DP= degree of polymerization; M-Chi: size marker chitin oligomers; M-COS: size marker chitosan oligomers.)

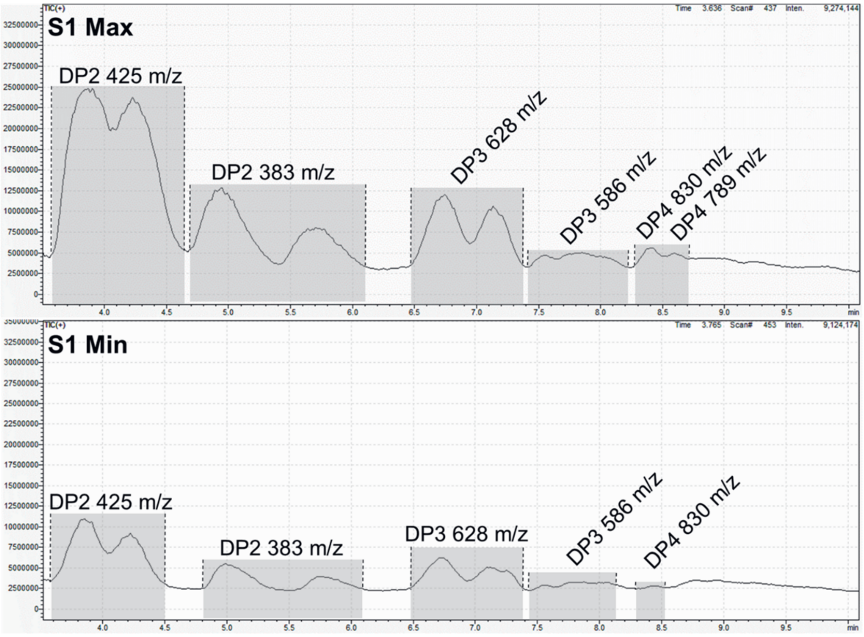

Figure 6.7. Exemplary LC-MS spectra from two optimized solutions for maximized (S1 Max) and minimized (S1 Min) chitin conversion rates. Oligomeric chitin products were identified by their respective molecular weights. Peak integration was used to determine average mixture compositions. DP= degree of polymerization.

The overall substrate conversion yield was determined for all maximized solutions after determining the residue substrate after the reaction, relative to the starting material. With solutions S1 Max, the highest substrate conversion yield of 28.9±0.7 % (28.9 mg/mL) and an improvement to 58 % compared to run 19 (18.3±0.5 %) was achieved.

In conclusion, the chitinase mixture model was used successfully to increase the overall conversion rates substantially relative to single enzyme reactions. However, the model could not be used to predict the DP of products and thereby altering product properties.

6.2.2 Deacetylase mixture design

In line with the chitinases, the deacetylases implemented in the DoE also originated from the same marine *Photobacterium* strain. Enzymes were produced recombinantly using *E. coli* BL21 (DE3) and characterized in terms of optimum reaction conditions and product properties in **Chapter 5**. Combined enzymatic deacetylation reactions

were not investigated until now, therefore the DoE approach was used to assess significant main factors (deacetylase 1-5) and factor interactions and to elucidate potential changes in the product characteristics as well as to maximize the DA.

A special cubic mixture design with 48 runs was set up and a special cubic model was implemented for data evaluation. Deacetylases were dialyzed and pre-diluted to identical molar concentrations using MAT buffer (pH 9) to ensure equal volume additions recommended by the mixture design plan (Table 6.2). A chitin oligomer mixture freshly generated by the optimized chitinase mixture S1 Max was used as a substrate at a concentration of 4 mg/ml. The amount of released acetic acid by enzymatic deacetylation was quantified by the acetic acid assay and used as a response for the model evaluation.

Upon analysis of the results by ANOVA, three main factors (A, B and D) had significant impact on the deacetylation of the substrate. Furthermore, multiple significant (p-value <0.05) and highly significant (p-value <0.0001) two-factor and three-factor interactions between the main factors were identified (Table 6.5). Single factors C and D did not have a significant effect on the response, however interactions with these factors showed high significance. Overall significance of the model was confirmed by the not-significant lack-of-fit test and the predicted R^2 value was in reasonable agreement with the adjusted R^2 value (Table 6.6).

Table 6.5. Factor and factor interdependencies used to identify deacetylase combinations for maximum conversion rates. A reduced quadratic model was implemented to analyze the response data (released acetic acid in µg/ml x h). Significant factor interactions were preselected by an automated backward selection with a p-value threshold of 0.05. Factors that were required to maintain model hierarchy were not excluded.

Factor	F- value	P-value
Model	57.53	<0.0001
Linear mixture	178.31	<0.0001
BC	36.48	<0.0001
BE	41.59	<0.0001
CD	42.28	<0.0001
DE	35.32	<0.0001
BCD	23.92	<0.0001
BCE	6.26	0.0187
BDE	27.22	0.0002
CDE	11.08	0.0025

*significant main factors: A, B and D.

Table 6.6. Model parameters to endorse significance for deacetylase mixture design.

Parameter	Value
R^2	0.9770
Adjusted R^2	0.9600
Predicted R^2	0.8490
Lack of Fit	0.5737

The overall highest rate of released acetic acid (75 µg/ml x h) was achieved by runs 11, 12 and 15 (see table A1.4 in the appendix). Deacetylated samples were subjected to analysis by TLC and LC-MS in order to identify the product spectrum with regards to the DA (Figure 6.8 and Figure 6.9). TLCs revealed that the chitin oligomers of DP2 and DP3 were successfully deacetylated resulting in an increase of retention due to stronger interaction with the polar stationary phase. Monomeric GlcNAc was unaffected by all enzyme mixtures. For DP2 it was confirmed that the substrate was mono-deacetylated by all enzyme mixtures as the fully deacetylated standard molecule (M-COS, DP2) is retained even stronger by the stationary phase. Therefore it could be concluded that independent of the enzyme mixture, an enzymatic deacetylation of DP2

will result in mono-deacetylation. As no further product properties could be identified using TLC, samples were further investigated by LC-MS.

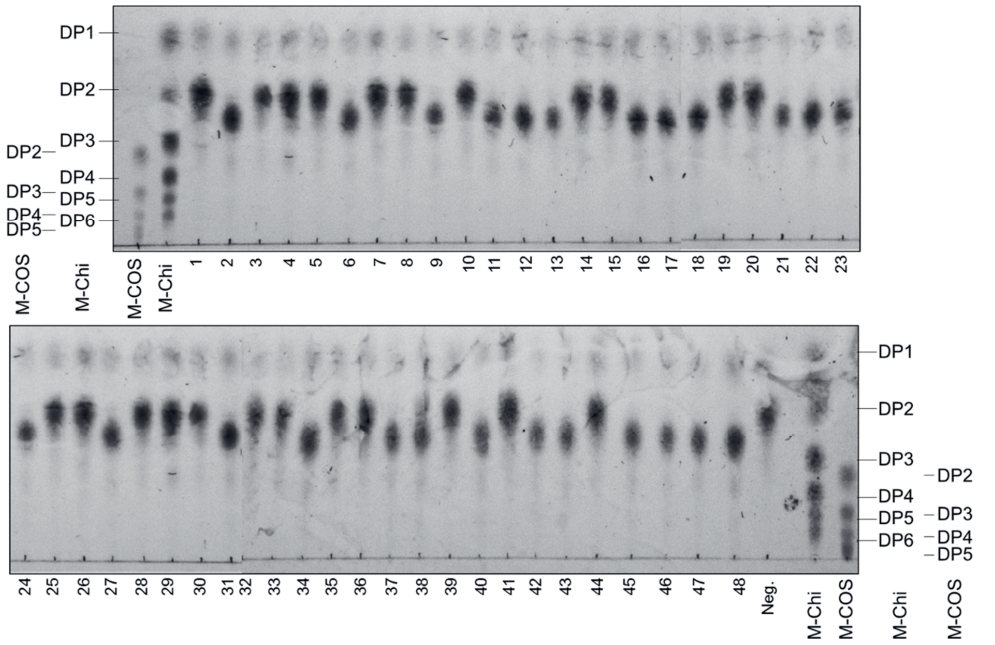

Figure 6.8. Thin layer chromatogram of deacetylated products from CDA mixture design of recombinant CDAs 1-5. A total sample volume of 0.3 µl was loaded by sequential application of 0.1 µl. Relative to the substrate (Neg.), changes in migration for deacetylated products can be identified as deacetylated oligomers are retained stronger by the stationary phase. DP= degree of polymerization; Neg.: negative control (=substrate); M-Chi: chitin size marker; M-COS: fully deacetylated chitosan oligomer size marker.

Two representative LC-MS spectra from the design (run 11= high conversion, run 13= low conversion) are depicted in figure 6.9 including untreated chitin oligomer substrate as a negative control. Analysis indicated that all chitin oligomers (DP2-DP4) inside the substrate and product mixture are mono-deacetylated and no products with higher DA were generated.

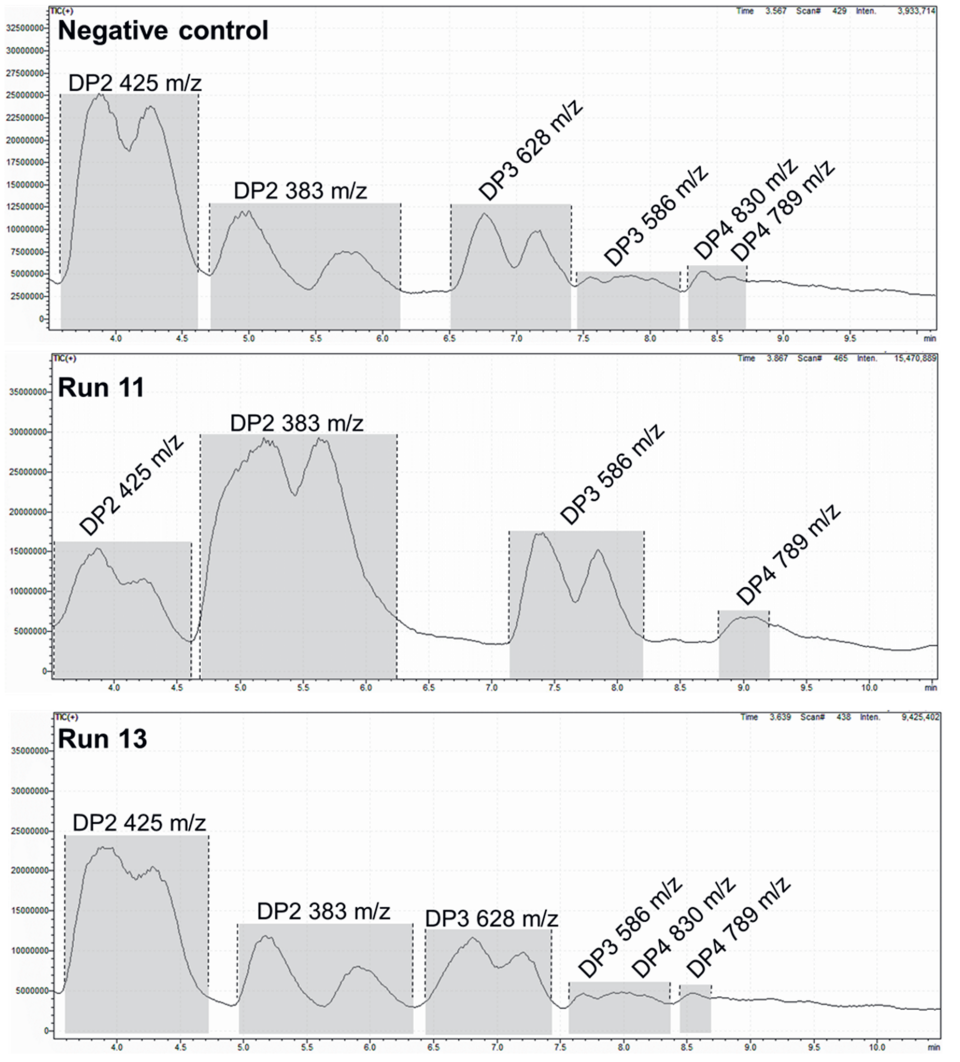

Figure 6.9. Exemplary LC-MS spectra of substrate (negative control) used in the CDA design and enzymatically deacetylated samples from the design (Run 11 and 13). Oligomeric chitin and mono-deacetylated chitosan products were identified by their respective m/z. DP= degree of polymerization. Mono-d: mono-deacetylated product.

It was concluded that the mixture design could not be used to predict and alter the DA beyond a mono-deacetylation, thus the design was evaluated in order to maximize to overall deacetylation efficiency to increase the yields of mono-deacetylated chitosan oligomers. The optimization function of the DesignExpert software was thus used to

develop CDA mixtures that would allow a maximization of the response. Three solutions for maximized conversion rate (S1-S3 Max) were tested alongside three solutions for minimized rates (S1-S3 Min) in order to validate the predictive power of the model (Table 6.7). For minimized solutions, the mixtures were more complex and only solution S1 Max contained factor D at low concentrations (0.6 µM). This suggests that factor D has the strongest positive effect on deacetylation. Furthermore, it was concluded that the significant factor interactions between A, B, C and E do have a negative influence on the overall deacetylation efficiency. The actual rates achieved (Rate ach.) were in agreement with the predicted values (Rate pred.) (Table 6.7). A rate-increase was achieved relative to the best runs of the mixture design (run 11, 12 and 15; 75 µg/ml x h). The optimized mixture were able to improve the rates by 13 % (S1 Max), 19 % (S2 Max), 20 % (S3 Max) relative to runs 11, 12 and 15. Solutions for minimized conversion rates further confirmed that the model could be implemented to predict deacetylation conversion rates. The optimized mixtures for maximized rates all contain factor D (deacetylase 4) as a major constituent and factor C and E (deacetylase 3 and 5) as minor components.

Table 6.7. Optimized CDA mixtures suggested by the CDA design model. Multiple solutions were recommended and three (1-3 Max) predicting the highest rates of released acetic acid (Rate pred.) were selected for validation. In addition, three solution (1-3 Min) for minimal rates were included to validate the predictive power of the model. Analysis of released acetic acid was carried out in three technical replicates (n=3). A: deacetylase 1, B: deacetylase 2, C: deacetylase 3, D: deacetylase 4, E: deacetylase 5.

Solution	Factor A [µM]	Factor B [µM]	Factor C [µM]	Factor D [µM]	Factor E [µM]	Rate pred. [µg/ ml x h]	Rate ach. [µg/ ml x h]
1 Max	0	0.3	0	1.45	0.55	88.7	84.8±0.8
2 Max	0	0	0.32	1.29	0.39	88.2	89.3±0.2
3 Max	0	0	0.33	1.42	0.25	85.9	89.9±0.3
1 Min	0.36	0.64	0.07	0.6	0	5.1	5.2±0.4
2 Min	0.33	1.14	0.53	0	0	6.2	5.7±0.3
3 Min	0	0.58	0.55	0	0.87	7.1	6.2±0.5

A response plot was created using the DesignExpert software indicating the factor influences on the response. The tested solutions (S1-S3 Max black dots, S1-S3 Min

grey dots) are marked in the plot revealing the within the mixture model, optimum enzyme combinations were discovered to maximize the conversion rate (Figure 6.10).

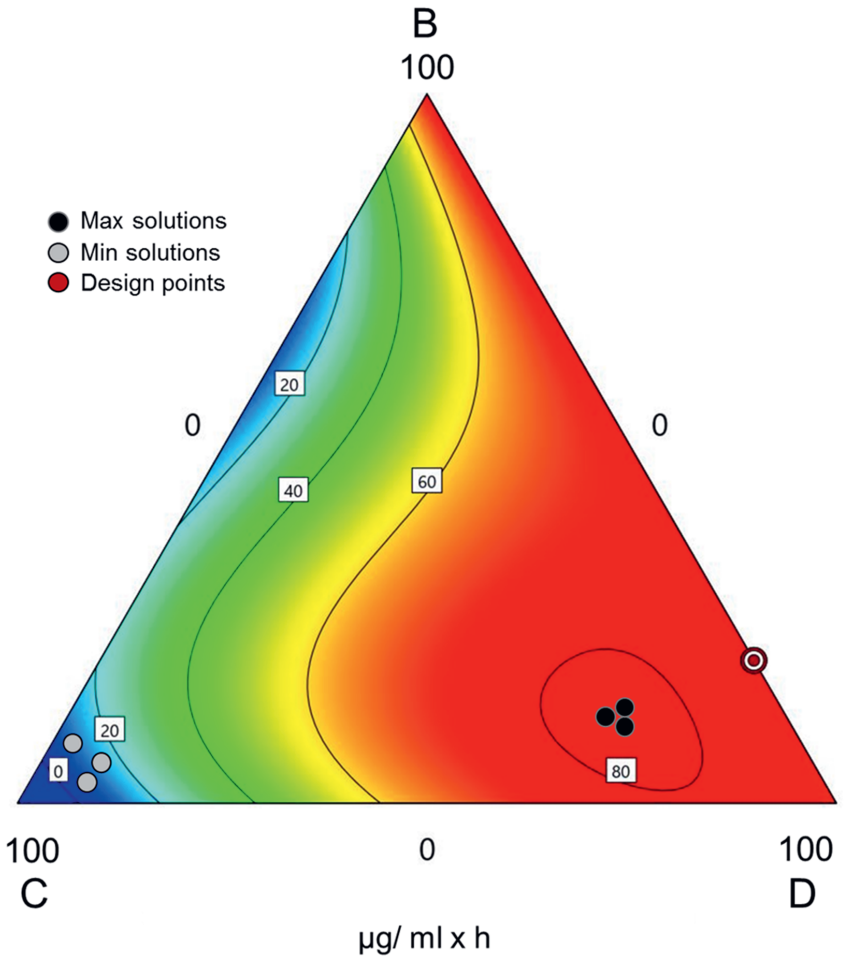

Figure 6.10. Response plot (µg acetic acid/ml x h) for tested optimized solutions from the CDA mixture design. Three solutions were tested to maximize the deacetylation effect (black dots) and three solutions for minimized conversion rates (grey dots). The minimized solutions were used to further validate the predictability of the design model. The total molar enzyme concentration was set to 2 µM. Response plots highlight what factor combinations are necessary to achieve the desired output. B: deacetylase 2; C: deacetylase 3; D: deacetylase 4.

Product analysis by TLC revealed that solutions for minimized rates did not convert the fully acetylated substrates to mono-deacetylated products, as expected (Figure 6.11). All solutions for maximized deacetylation efficiency resulted in overall similar

product properties as DP2 was converted to mono-deacetylated DP2 and DP3 to mono-deacetylated DP3 respectively.

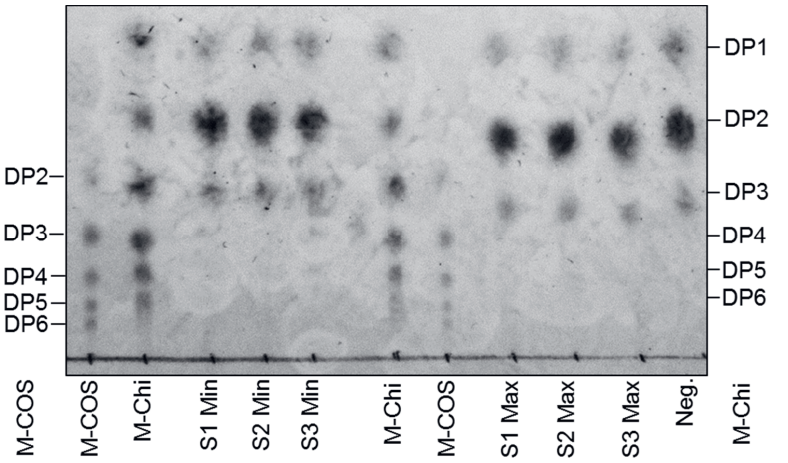

Figure 6.11. Thin layer chromatograms of products for tested solutions for minimized (S1-S3 Min) and maximized (S1-S3 Max) deacetylation effect. A total sample volume of 0.3 µl were loaded sequentially in 0.1 µl spots. Oligomeric chitin (M-Chi; DP1-DP6) and fully deacetylated chitosan standards (M-COS, DP2-DP6) were used as a size marker.

Products were further characterized by LC-MS analysis (Figure 6.12). Results showed that for S1 Max, an almost complete conversion to mono-deacetylated DP2, DP3 and DP4 was achieved and besides DP2, no fully acetylated substrate for DP3 and DP4 were detected. Peak integration of DP2 and DP2 mono-deacetylated revealed that 5.8 % of DP2 was not deacetylated. Compared to run 11 (15.6 % leftover), the overall yield of mono-deacetylated DP2 was improved substantially. It can be concluded that the optimized mixtures were capable to increase the overall conversion efficiency relative to best runs from the mixture design.

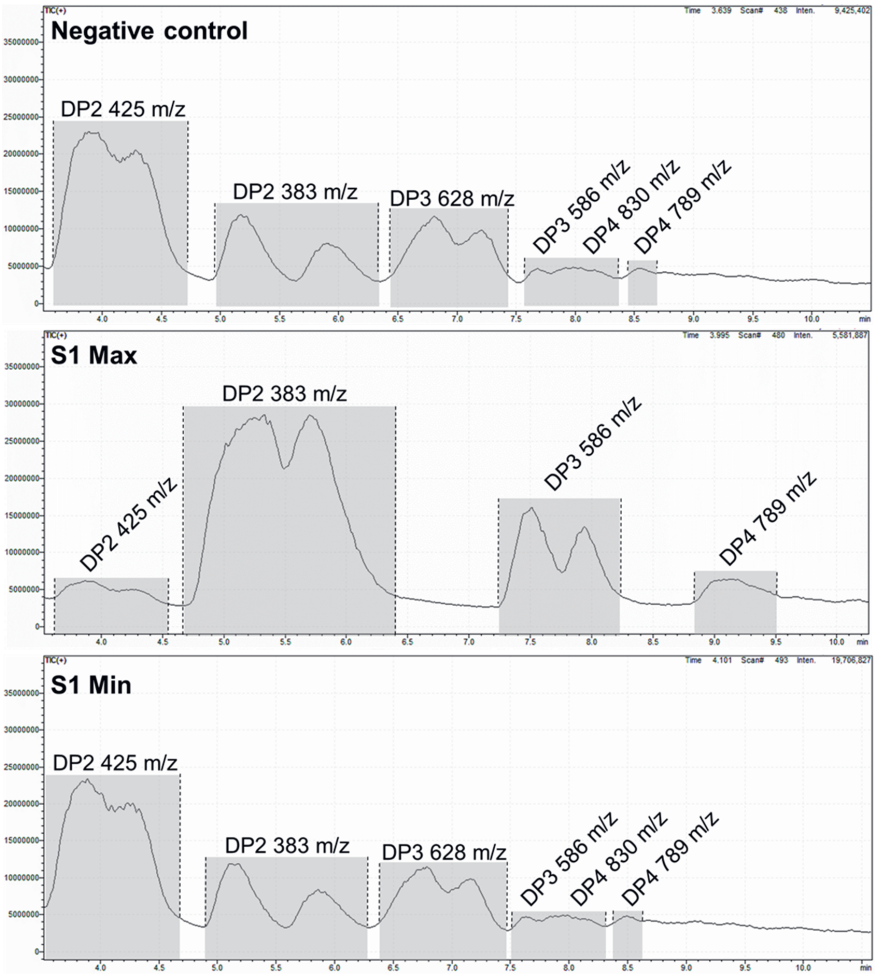

Figure 6.12. Exemplary LC-MS spectra of tested optimized solutions for CDA mixture design. Three CDA solutions were tested for a maximized deacetylation effect (S1-S3 Max). In addition three solutions for minimized deacetylation yields were tested to validate the predictive power of the design model (S1-S3 Min). MS-spectra were used to identify the individual oligomeric products based on their m/z.

6.3 Discussion

The development of multi-enzyme “one-pot” reactions is a challenging procedure compared to single enzyme reactions, as multiple relevant parameters must be assessed simultaneously. Complexity of such processes increases almost proportionally with the amount of enzymes included, as coupled and parallel reactions

as well as enzyme interactions can no longer be comprehended. Thus mathematical modelling of the process is required to identify enzyme interdependencies and to carry out process optimization [25, 38].

The design-of-experiments approach enables the assessment of complex biological systems using solely statistical modelling. Thereby, significant factors and factor interactions, optimum factor combinations and rate projections can be carried out for real systems [32]. In order to obtain a robust process modeling, it is essential to carry out a factor pre-selection and to optimize the system focusing on the most crucial factors, thereby improving overall significance of the models. For this research, the different chitinolytic enzymes were analyzed to develop two individual mixture models to assess enzyme-substrate interactions and to tailor optimized enzyme cocktails for improved product rates. Furthermore, it was investigated whether the different enzyme combinations had a significant effect on altering product distributions in terms of DP and DA. Evaluation of both models revealed that different combinations of chitinases or CDAs had significant effect on the product rates. Significant factors and factor interactions were identified that indicated synergistic enzyme effects.

The chitinase mixture model revealed that all main factors (chitinase 1-5) as well as multiple two-factor and three-factor interactions had a significant impact on the overall product rate. As the model calculates any statistically significant interaction, further analysis has yet to reveal whether the response is reduced or increased by the respective factors and interactions. The evaluation of the model by using the relative abundance of chitin oligomers with DP2, DP3 and DP4 as a response demonstrated that the product spectrum could not be changed by using different enzyme mixtures. This underlines the hypothesis that all implemented chitinases hydrolyse chitin by an overall related reaction mechanism. Therefore, further analysis of the model focused on the maximization of the production rate.

In order to assess the effect on the product rate more accurately, the optimization function in the DesignExpert software was used to specify enzyme cocktails for maximized conversion rates. Three solutions with the highest predicted rates were validated. Furthermore, the testing of minimized solutions were carried out to further validate the predictability of the model and achieved data was in agreement with the

predicted rate. All tested mixtures revealed that chitinase 1 (factor A) had the strongest positive impact on the production rate and chitinase 2 and 3 (factor B and C) enhanced the overall conversion. The predicted rates exceeded the highest yield achieved in the design (run 19) by 73 % and experimental validation of all solutions were in great accordance with the predictions. One possible explanation for the substantial increase of activity by adding minor amounts of chitinase 2 to chitinase 1 is a synergistic effect coming from the different enzyme domains. Both enzymes contain different chitin-binding domains that are responsible for increased accessibility of the active site to the substrate [39-41]. As chitinase 2 has an overall low chitinolytic activity, the chitin-binding domain could potentially enhance the overall accessibility for chitinase 1 resulting in an increased overall conversion rate.

So far, many chitinolytic enzymes from different bacterial or fungal sources were tested for the degradation of chitin to oligomers yielding a broad spectrum of different oligomers [42, 43]. Typically, chitin is first pre-treated using harsh chemical or mechanical methods in order to break the crystal structure and to increase the overall substrate availability. Chitin oligomers of DP 1-5 with an overall yield of 23.6 % were obtained after steam explosion pre-treatment and a sequential enzymatic hydrolysis for 6 days [44]. Size distributions of enzymatically produced chitin oligomers vary largely between DP1 up to DP9 depending on the utilized enzyme and substrate. Using chitin as a substrate, crude chitinase mixtures and pure enzymes from different sources typically yield GlcNAc and dimeric chitin from trace amounts up to 95 % after an incubation for 2-10 days. Chitosan as a substrate can yield an even broader spectrum of products due to the increased solubility and higher oligomers (DP3-DP9) can be generated using chitosanases [42]. In comparison to the data reported in the literature, the optimized enzyme cocktails were able to generate similar overall product yields ranging from DP1-DP4. Thus, even higher product rates and yields can be expected from the optimized enzyme cocktails using e.g. chemically pre-treated chitin such as colloidal chitin as it provides a higher surface area and lower crystallinity compared to chitin powder.

In summary the implementation of the chitinase mixture model successfully revealed significant enzyme interdependencies and the model could predict optimized enzyme combinations that allowed a substantial increase in conversion rates of chitin

powder compared to single enzyme reactions. The reliable predictive power even above the design constrains (502 nmol / ml x h) of the model further demonstrated that DoE can be used to develop highly specific enzyme cocktails that allow a substantial increase of conversion efficiency. Both designs were furthermore carried out and evaluated within two working days leading to the optimized mixtures. Subsequently, statistically driven optimization studies is a time-and resource efficient process compared to empirically non-strategic optimization studies such as the OFAT approach. In further consequence, the presented data indirectly demonstrated the restrictions of the OFAT approach where influential factors are varied sequentially and significant factor interactions as well as the true optimum can hardly be determined strategically.

The purpose of the deacetylase mixture design was to control and maximize the enzymatic deacetylation effects on oligomeric chitin. Therefore, five different CDAs were implemented in the design to reveal putative enzyme-synergies. The amount of released acetic acid was used as a response as it is a mean to characterize the overall degree of deacetylation of the substrate. Product analysis by TLC and LC-MS revealed further that for all substrates a mono-deacetylation could be achieved. Thus, changes of the product´s retention time and thereby the achieved DA could be correlated directly to the released acetic acid. This response was used to evaluate the model in order to identify enzyme combinations to maximize the deacetylation efficiency.

Main factors A, B and D (deacetylase 1, 2 and 4) and multiple two-factor and three-factor interactions were identified to have a significant impact on the response. Interestingly, factors C and E (deacetylase 3 and 5) took part in significant interactions. In order to evaluate which factors and interactions result in an increase of the deacetylation efficiency, the optimization function was used to model optimum deacetylase mixtures. Three solutions for maximized yield and three solutions for minimized yields were tested to validate the models predictability. The desirability and response plots for the tested solutions indicated that factor D did has the highest positive impact on substrate deacetylation as it was suggested as the dominant component in all maximized solutions. Solutions for minimized yields further confirmed this circumstance. The high accordance of achieved yields to predicted yields for all

tested solutions implied that the model is suitable to predict optimum deacetylase combinations.

The overall yield improvement using optimized mixtures was 20 % compared to the best run from the design. This implies that the best runs from the design were already close to the true optimum and maximized solutions were able to tweak beneficial deacetylase synergies. Product analysis by LC-MS revealed further that besides DP2 (5.8 % fully acetylated DP2 left) all chitin oligomers were converted entirely to mono-deacetylated products. Longer incubation times than the ones implemented for the design (16 h) could be tested with the optimized mixtures in order to completely deacetylate the residue DP2.

6.4 Conclusions

This chapter described the first fully enzymatic depolymerization and deacetylation process of chitin powder to generate partially deacetylated COS with a maximum DP of 4. Both mixture models for chitinases and CDAs were capable to identify significant main factors and factor interactions between the enzymes. Optimized enzyme mixture suggested by the design were validated and it was revealed that highly specific enzyme mixtures are required to achieve a substantial increase of the conversion rates. The design´s predictability was exceptionally reliable as extrapolated responses with a rate increase of up to 58 % were achieved. Such specific mixtures could not be determined strategically using the OFAT approach as it does not take any factor interactions into consideration. Furthermore, using the DoE approach all optimum mixtures were determined systematically using a minimum amount of individual experiments making it a time-and resource-efficient approach. Optimized mixtures were furthermore capable to produce mono-deacetylated COS of DP2-DP4 from chitin powder with overall yields of 28.9 % which is competitive to current chemical degradation reactions. COS with a DP higher than 4 could not be detected thus far and as a consequence future research could focus on approaches to generate larger COS. Strategies to achieve this goal are discussed in detail in the outlook chapter

6.5 Experimental section

Materials

All chemicals used in the study were purchased from Carl-Roth (Germany) and were of highest purity; Chelating Sepharose FF was from GE Healthcare (Sweden). All buffers were prepared in demineralized water.

Recombinant production of enzymes

Previously cloned constructs for chitinases (**Chapter 4**) and CDAs (**Chapter 5**) were all transformed in *E. coli* BL21 (DE3) cells and expressed recombinant. Starter cultures in 100 mL TB medium were cultivated overnight (180 rpm, 37 °C) and 10 mL of these culture were used to inoculate the main cultures (OD_{600nm}: 0.1). Ultra Yield™ flasks (2.5 L; Thomson Instrument Company, Oceanside, USA) were used for propagation of the main culture in 1000 ml TB-medium at 37 °C in orbital shakers and a shaking velocity of 200 rpm. Induction was carried out in the middle of the log-phase after 4 h of cultivation (OD600=4) by addition of 1 mM isopropyl-β-D-thio-galactopyranoside (IPTG) to the culture broth and temperature was then reduced to 28 °C. Cells and supernatant were separated by centrifugation (8000 *x* g, 30 min) after a total cultivation time of 18 h was reached.

Purification of enzymes from culture supernatant

The protocol for the purification of 6xHis tagged chitinases and CDAs was adapted from **Chapter 4 and 5** and two sequential steps were carried out:

Step 1. Ammonium sulfate precipitation

Solid ammonium sulfate was slowly added to 950 ml culture supernatant to a final concentration of 70 % and was stirred for 2 h at room temperature. The precipitate was collected by centrifugation (8000 *x* g, 30 min), dissolved in 100 ml PBS (pH 8.0) and filtrated (0.45 µm).

Step 2. Immobilized metal affinity chromatography (IMAC)

Dissolved samples from step 1 were applied to a Chelating Sepharose FF (GE Healthcare, Sweden) resin (column volume (CV) 15 ml) charged with 0.2 M NiSO4. The column was connected to an ÄKTA pure 25 (GE Healthcare, Uppsala, Sweden)

and equilibrated using 3 CV PBS (pH 8.0). Samples were injected directly using a sample pump and followed by a washing-step with equilibration buffer until the UV_{280nm} signal fell below 30 mAU. Weakly bound unspecific protein was eluted from the column using 50 mM imidazole in PBS (pH 8.0) followed by a second elution step at 250 mM imidazole in PBS (pH 8.0). All steps were carried out at a constant flow rate of 5 ml min-1. Elution fractions of 2 ml containing the respective enzyme were pooled and dialyzed against MAT buffer (33 mM TRIS, 33 mM CAPS, 33 mM MES [pH 8]). Proteins were quantified by the bicinchoninic acid assay (BCA) using bovine serum albumin as standard for calibration [45].

Substrate preparation

Chitin from shrimp shells (~400.000 g/mol; Carl Roth) was mechanically pre-treated using a GyroGrinder (Fritsch GmbH, Germany) at 6000 rpm and thereby converted to a homogeneous powder of 80 µM average particle diameter. The powder was used without any further treatment as a substrate for all chitinolytic degradation experiments.

Quantification of chitinase and chitin-deacetylase activity

Enzymatic activity of recombinant chitinases was determined by carrying out a reducing end assay [46]. Hydrolysis samples were centrifuged at 8000 *x* g for 2 min and 40 µl of the supernatant was mixed with 40 µl 0.5 M NaOH and 40 µL of a aqueous MBTH reagent solution (1.5 mg/ml 3-Methyl-2-benzothiazolinonhydrazon and 0.75 mg/ml Dithiothreitol). Samples were incubated at 80 °C for 15 min and then mixed with 80 µl developing solutions (0.5 % (w/v) $FeNH_4(SO_4)2)x\ 12H_20$, 0.5 % (w/v) sulfamic acid and 0.25 M HCl). Samples were cooled down to room temperature and absorption was determined at 620nm. A calibration curve of N-acetyl-glucosamine was prepared freshly for each measuring cycle. One activity unit [U] was defined as the amount of enzyme required to release 1 nmol of reducing sugars per hour.

A commercial acetic acid assay kit (K-ACETRM; Megazyme, Bray, Ireland) was used in multitier plate format measuring the amount of released acetate during CDA reactions. A calibration curve was generated freshly for each measuring cycle using acetic acid as a standard. One activity unit [U] was defined as the amount of enzyme required to release 1 µg of acetic acid per hour.

Product analysis by thin-layer-chromatography and LC-MS

For the identification of hydrolysis products, soluble samples were subjected to TLC analysis using 10x10 cm TLC Silica gel 60 F254 plates (Merck, Darmstadt, Germany) and a mixture of butanol: methanol: 25 % ammonia: H_2O (5:4:2:1) as mobile phase. A total volume of 0.3 µl for samples and chitin standard sugars (Megazyme, Chicago, USA) (1 mg/ml) were applied to the plate. After separation, the plate was air-dried and developed using a solution containing 200 ml Acetone, 30 ml phosphoric acid (85 %), 4 ml aniline and 4 g diphenylamine. Spots were made visible using a heat gun at 300 °C [47].

The protocol for analysis by LC-MS was developed based on the method described by Hamer *et al.* [28]. Analysis of soluble oligomeric samples was carried out using a Shimadzu LC-30 AD system coupled to a SIL-30AC autosampler, a CTO-20AC column oven and a LCMS-2020 mass spectrometer (Shimdazu, Kyõto, Japan). A sample volume of 1 µl was separated by hydrophilic interaction chromatography using an Acquity UPLC BEH Amide column (1.7 µm, 2.1 x 150 mm) coupled to an Acquity UPLC BEH Amide 1.7 µm VanGuard pre-column (2.1 x 5 mm) (both Waters Corporation, Milford, USA). Flowrate was set to constant 0.5 ml min-1 and the column oven temperature to 30 °C. Samples were eluted from the column with a gradient of A (Acetonitrile + 0.1 % (v/v) formic acid) and B (water). Sample separation was done over 16 min using the following gradient: 0-2.5 min isocratic 80 % A; 2.5 – 12.5 min, linear from 80 % to 35 % (v/v) A, followed by column re-equilibration 12.5 – 13.5 min, linear from 35 % - 80 % A (v/v); 13.5 – 16.0 min, isocratic 80 % A. MS-detection was carried out in positive mode with the interface voltage at 4.5 kV, the nebulizer gas flow rate at 1.5 L/min, drying gas flow at 15.0 L/min and the dry temperature at 249 °C. Mass spectra were recorded over a scan range from m/z 100-1500.

Peaks from target compounds were identified and integrated automatically using the Chromatopac algorithm of the post run analysis software (Shimadzu). Peak smoothing was inactivated and the baseline following degree was set to 1 while disabling the baseline correction method. Noise was calculated using the ASTM method.

Design-of-experiment models

Chitinase mixture model

A mixture design with cubic model order was generated including all five chitinases as variable factors using the Design Expert 11 software (Stat-Ease inc. Minneapolis, USA) in order to optimize enzyme combinations for improved conversion efficiency of chitin powder to COS. In addition, the design was used to assess whether product properties can be altered in order to yield single chitin oligomers. A total molar concentration range of 0-2.0 µM (representing a volume of 0-240 µl) was selected for all chitinases (Table 6.8). The total reaction volume was fixed to 1000 µL for all experiments and within this volume the total molar enzyme concentration was set. The special cubic model was created in order to assess three-factor interactions resulting in a total of 48 runs. All runs were prepared in 2 ml centrifuge tubes using 100 mg of ground chitin powder at a working volume of 1000 µl using MAT buffer at pH 8. Samples were incubated at 30 °C for 16 h in a thermomixer at 1000 rpm. After incubation, a reducing end assay was carried out to determine the total amount of reducing sugars and TLC was used to assess changes in product properties. These responses were used to evaluate the design by analysis of variance (ANOVA) within the Design Expert 11 software revealing optimum enzyme combinations.

Table 6.8. Factors and range limits used in the chitinase mixture design. The upper limit (240 µl) represents the maximum enzyme concentration of 2 µM.

Factor	Name	Lower limit [µl]	Upper limit [µl]
A	Chitinase 1	0	240
B	Chitinase 2	0	240
C	Chitinase 3	0	240
D	Chitinase 4	0	240
E	Chitinase 5	0	240

Deacetylase mixture model

A mixture design with cubic model order for 48 runs was created in Design Expert 11 for combined CDA reactions to alter the DA of the products. Five recombinant deacetylases were implemented in the design that made up a total molar concentration of 2 µM (0-100 µl) (Table 6.9). All runs were carried out in 2 ml centrifuge tubes using

4 mg/ml of chitin oligomers prepared from chitin powder using an optimized mixture of chitinases. Samples were incubated in a thermomixer at 1000 rpm at 30 °C for 16 h in 100 mM MAT buffer (pH 9). After incubation, the acetic acid assay was used to quantify the amount of released acetic acid and this response was further processed by ANOVA to evaluate the design in terms of optimized enzyme combinations for maximized rates.

Table 6.9. Factors and limits of factor ranges applied in the deacetylase mixture design. The Upper limit (100 µl) represents an enzyme concentration of 2 µM.

Factor	Name	Lower limit [µl]	Upper limit [µl]
A	Deacetylase 1	0	100
B	Deacetylase 2	0	100
C	Deacetylase 3	0	100
D	Deacetylase 4	0	100
E	Deacetylase 5	0	100

Optimized depolymerization and deacetylation reactions

Optimized chitinase and CDA mixtures were used to carry out sequential depolymerization and deacetylation reactions. Therefore, chitin powder (100 mg chitin) was incubated for 16 h to carry out the chitinolytic enzyme reaction first using one optimized chitinase mixture at a total molar concentration of 2 µM at pH 8 and 30 °C. Subsequently, the chitinolytic reaction was stopped by heat inactivation of enzymes (95 °C 10 min). The second reaction was started by addition of 2 µM of the optimized deacetylase mixture and again incubated for 16 h at 30 °C. Enzymes were again deactivated as described above and products were analyzed after freeze-drying using TLC and LC-MS.

Quantification of products

Individual oligomeric chitin and chitosan products were quantified using TLC by establishing calibration curves of monomeric, dimeric, trimeric and tetrameric chitin standard oligosaccharides within a concentration range of 0.3125, 0.625, 1.25, 2.5 and 5 mg/ml. A total standard volume of 1 µl was applied and analysis was carried out as described in 2.7. A densitometrical sample quantification was carried out using the

AIDA image analyzer software (Raytest Isotopenmessgeräte GmbH, Straubenhardt, Germany).

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Chapter 7

Conclusions and outlook

The aim of this thesis was to establish a fully enzymatic *in vitro* conversion process of chitin to partially deacetylated COS using recombinant chitinases and CDAs isolated from the novel chitinolytic bacterial strain Chi5. Multiple objectives were formulated for this thesis:

- The verification of Chi5 as a novel bacterial chitinolytic strain using a polyphasic characterization;
- The identification of homologous chitinolytic enzymes within the Chi5 genome;
- The recombinant production of novel chitinases and CDAs in *E. coli* BL21 (DE3) and their subsequent characterization;
- The determination of physicochemical properties of oligomeric products generated by enzymatic hydrolysis;
- The exploration of a holistic enzymatic degradation process of chitin optimized by the design-of-experiments approach.

The most crucial results as well as future prospects regarding enzymatic production of COS are highlighted in this chapter.

7.1 Conclusions

This work stems from the interest in chitosan and COS given their broad application spectrum of in multiple sectors, and the access to a new bacterium as source of novel enzymes for a more environmental friendly and controlled chitin conversion. The highly sensitive structure-function relationship of chitosan and COS reflects the need for a controlled and targeted production process to ensure consistent product activity and quality. The numerous chemically catalyzed depolymerization and deacetylation approaches reported until now demonstrate that a systematic production of defined chitosan and COS has not yet been achieved. Chitinases and CDAs were identified as potent substitutes of harsh chemicals, as they provide increased substrate specificity and selectivity enabling a better controllable degradation process. In addition, the environmental footprint of currently used harsh chemical routes can be improved by the implementation of enzymatic reactions. However, the limited accessibility of crystalline substrates such as raw chitin and thus the relatively low conversion rates compared to chemical processes require the implementation of potent enzymes as

well as sophisticated optimization methodologies in order to establish an enzymatic conversion process as efficient as the chemical COS production processes. Conversion yields of common chemical approaches typically yield up to 30 % of COS from chitosan or chitin as a raw material. In this regard, COS yields for enzymatic process should at least be able to achieve similar yields in order to consider them efficient. Using the optimized enzymatic process product yields in similar ranges were achieved and furthermore process conditions were set at substantially milder temperature conditions compared to high-temperature chemical hydrolysis reactions (**Chapter 2**).

In **Chapter 3**, the marine bacterial strain Chi5 was characterized with the aim to identify novel sources for chitinolytic enzymes. Morphological, metabolic, biochemical and phylogenetic classifications revealed that it is a distinct strain in the *Photobacterium* genus. The novel strain is capable to grow on marine chitin as a sole source for carbon and nitrogen. Analysis of supernatant samples from Chi5 grown on chitin revealed the presence of multiple chitinolytic enzymes that carry out an extracellular degradation of crystalline chitin to absorbable monomeric and dimeric GlcNAc.

Next generation sequencing of the Chi5 genome was carried out and the assembled data was used for gene mining of homologous and characteristic enzyme domains for chitinases (glycosyl hydrolases) and chitin-deacetylases (NodB). In **Chapter 4**, five different target genes for chitinases were cloned successfully into *E. coli* BL21 as different constructs using the golden gate cloning technology. Constructs comprising a PelB signal peptide for secretion and 6xHis tag for purification by IMAC were identified as the most suitable candidates for expression. Purified chitinases were characterized using chitin powder regarding their pH,-temperature and salt optima and their respective substrate specificity and kinetics. Molecular masses and chain lengths of oligomeric products were determined. It was determined that the novel enzymes differ in size from typical reported chitinases and exhibit in general higher pH (8-10) and lower temperature (30-40 °C) optima. Furthermore, salt contents of 1-5 % (w/v) NaCl were beneficial for enzyme activity. Tetrameric trimeric, dimeric and monomeric oligomers were mostly obtained after enzymatic digestion of chitin and colloidal chitin in different quantity distributions. The results suggested that the novel enzymes can

be used for a biological degradation process at moderate temperatures of insoluble chitin to chitin-oligomers, although with a rather limited range of DP.

Five genes for chitin-deacetylases were discovered within the genome of Chi5 and analogous constructs to those from the chitinases were cloned into *E. coli* BL21 and expressed recombinantly. In **Chapter 5**, the purified enzymes were characterized accordingly on insoluble chitin as well as chitin trimers and reaction optima of the five novel enzymes were determined to be within pH 9-11, and between 30-50 °C which were in line with most of the currently reported CDAs. Product analysis of converted chitin oligomers revealed that three of the novel enzymes are capable to mono as well as di-deacetylate the substrates. Two enzymes did not exhibit deacetylation activity on chitin oligomers but on chitin powder. Enzymes were furthermore capable to partially deacetylate chitin. The results suggested that the novel enzymes can be applied for the deacetylation of soluble chitin oligomers and for the partial deacetylation of chitin polymers.

The chitinases and chitin-deacetylases were used to explore a fully enzymatic conversion process to directly generate partially deacetylated COS from chitin powder. Thus, specific enzyme cocktails were developed using the design-of-experiments approach to elucidate optimum enzyme combinations for maximized production rates and putative changes on product characteristics with regards to DP and DA. In **Chapter 6**, two mixture designs for chitinases and CDAs were used to generate enzyme mixtures to maximize conversion rates. It was discovered that total conversion rates could be increased by 80 % (chitinase mixture) and by 20 % (CDA mixture) compared to single and non-optimized enzyme reactions. Different enzyme mixtures did however not alter the overall product composition concerning DP and DA of COS. Furthermore, contrary to the findings in **Chapter 5**, no di-deacetylation and tri-deacetylation of the products was observed. A total substrate conversion yield of 28.9 % (28.9 mg/mL) from chitin powder to mono-deacetylated chitosan oligomers with DP2, DP3 and DP4 as well as GlcNAc was achieved resulting in an overall yield improvement of 58 % compared to the previously best single enzyme reaction. Compared to current chemical COS production processes yields of the novel enzymatic process are in a smilar range and thus the process can be considered efficient. Furthermore, the DoE approach demonstrated to be a more useful and rapid

tool than the OFAT approach for modeling complex multi-enzyme reactions and to determine optimum enzyme combinations to maximize conversion rates.

This thesis describes the first fully enzymatic conversion process of non-chemically pre-treated chitin to partially deacetylated COS using chitinases and CDA all originating from a novel chitinolytic bacterium. Future research focusing on the intensification of the enzymatic process from alternative perspectives as well as the further exploitation of the unique growth characteristics of bacterium Chi5 for the development of new products are highlighted in the following chapters.

7.2 Outlook and future prospects

Enzymatic conversion processes play a substantial role in the transformation of biomass to functional compounds. In this thesis the potential of a novel fully enzymatic *in vitro* process for the conversion of chitin powder to partially deacetylated COS was demonstrated. This outlook discusses further optimization approaches with regards to continuous COS production processes as well as enzyme engineering approaches to increase conversion yields and to eventually alter reaction mechanisms to expand the product spectra even further. Additional valorization approaches for chitin using the unique growth characteristics of the Chi5 strain are explored (Figure 7.1).

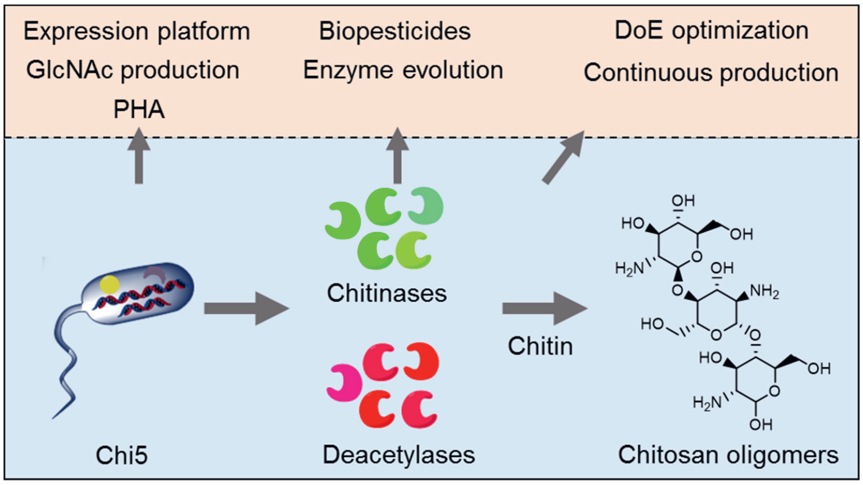

Figure 7.1. Graphical overview of the basic development process for the enzymatic production of COS from chitin including alternative optimization and valorisation considerations. The chitinolytic potential of bacterium Chi5 could be exploited for the development of a versatile production platform for recombinant proteins, GlcNAc or polyhydroxyalkanoats (PHA) which are used as a raw material for the development of bioplastics. The novel chitinolytic enzymes could further be employed for the development of biological fungicides. Enzyme evolution could be used to modify essential enzyme properties to manipulate COS properties and increase overall conversion rates. Further optimization studies for the production of COS could employ the process intensification by DoE studying the effects of physical process parameters. A continuous COS production system could furthermore be established to alter product properties with regards to the DP and DA while increasing conversion rates simultaneously.

7.2.1 Additional strategies for the enzymatic production of defined COS

This thesis explored the establishment of a novel i*n vitro* conversion process from chitin powder to COS exploiting the enzymes produced by the new bacterium Chi5. The DoE approach was successfully integrated to maximize the conversion efficiency based on statistical modelling by mixing the chitinolytic enzymes. Three alternative optimization approaches to maximize productivity and to eventually alter product characteristics could be explored in the future.

Design of experiments optimization

Other DoE approaches could be implemented to optimize physicochemical reaction conditions of enzyme cocktails thus conversion rates and yields could eventually increase beyond the current levels. Again, effects of parameter variation on product compositions could be investigated. Temperature, pH and NaCl-optima for singular

enzyme reactions were determined in this thesis and for both mixture DoEs, these factors not considered for the optimization. Therefore, factorial designs could be implemented to assess incubation temperature, incubation duration, pH as well as NaCl as variable factors. This allows to determine optima for mixed enzyme reactions that could eventually deviate for single reactions. The singular effect of potential co-factors was assessed for chitinases as well CDAs. For future optimization approaches the combinatory effect of two or more co-factors on enzyme activity could be assessed using DoE and factorial designs.

Continuous COS production process

Common advantages of non-continuous batch enzyme reactions implemented also in this thesis are the relatively quick and easy setup of the reaction itself and the fact that more sophisticated equipment such as peristaltic pumps, flow meters, reservoirs and enzyme immobilization/retention systems are not required to maintain the process. However, during batch reactions, enzymes, substrates and products are not separated from each other resulting in major constraints that could have a negative influence on the overall product yields, product quality as well as the economy of the process: 1. Enzyme activity could be reduced during the process due to increasing product concentrations and inhibitory effects. 2. Products need to be isolated from potential residual substrate contaminations and enzymes. 3. Enzymes cannot be recycled efficiently for additional processes. A continuous enzyme process can address all of these drawbacks by immobilizing the enzymes on a matrix without disrupting their activity, and delivering the substrate continuously while the products are continuously removed. The space–time yields can therefore be increased and the costs simultaneously reduced due to the enzyme recycling. [1, 2].

Multiple approaches for the establishment of a continuous COS production system have been reported. A column reactor with chitosanases immobilized on chitin was tested for effective production of chitosan pentamers and hexamers from chitosan. It was however discovered that substrate affinity and chitosanase activity is reduced substantially due to the immobilization [3]. Also an ultrafiltration membrane reactor was coupled to a column reactor with immobilized enzymes that fostered not only the production of COS of even larger DP than hexamers but also provided in general more

control on molecular weight distributions. The reason for this is the quick removal of oligomeric degradation products from the reaction by ultrafiltration avoiding further hydrolysis reactions to shorter COS of e.g. DP2-DP4 [4]. The chitinases and CDAs investigated in this thesis could be used to develop a similar production process by coupling the chitin-degradation and deacetylation reaction in one continuous system to produce partially deacetylated COS (Figure 7.2). For such as setup, chitinases could be immobilized directly on the solid substrate allowing chitin oligomers to be released into the aqueous phase and transported to the second reactor for deacetylation using free deacetylases. Ultrafiltration membranes (UF) are implemented to seperate COS from the reaction mixtures and to control the permeation rate in the attempt to isolate partially deacetylated COS with DP>4.

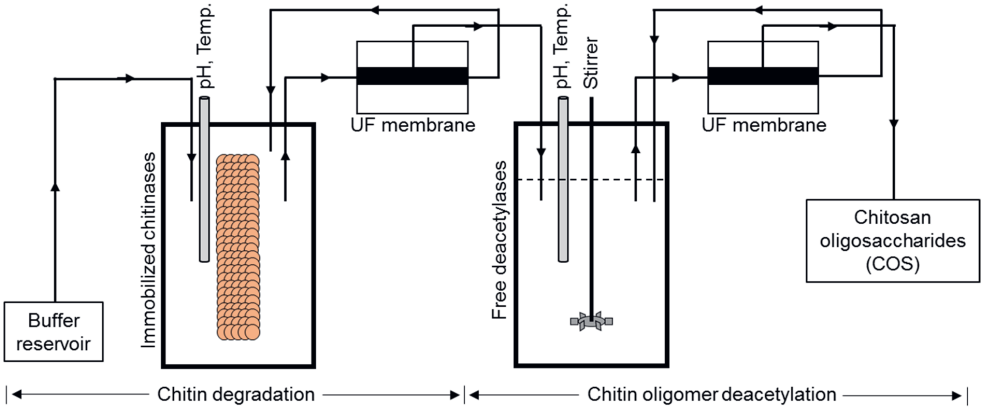

Figure 7.2. Schematic diagram of a potential reactor system for the continuous enzymatic production of COS from chitin. Temp.: temperature; UF: ultra filtration.

An alternative approach to influence and eventually change product properties of COS with regards to DP and DA is enzyme engineering that could be used to alter the chitinase and deacetylase reaction mechanisms [5]. Enzyme evolution can be used to modify various properties including enzyme activity, temperature/pH stability, substrate specificity, and reaction mechanisms. This can be achieved by random mutagenesis followed by library screening using a method that selects for enzymes with improved properties. Alternatively, if the enzyme structure is known, amino acids occupying key positions in and around the catalytic site or substrate-binding site can be systematically

exchanged by site-directed mutagenesis followed by direct comparisons to determine activity and specificity [6-8].

Directed enzyme evolution

Multiple directed evolution approaches by means of error-prone PCRs of chitinases and chitosanes have been reported and a 2.7-fold and respectively 16.9-fold increase of activity compared to the wildtype variant was achieved [9, 10]. So far, nothing is reported on the directed evolution of chitinases to alter the reaction mechanisms in order to manipulate the substrate specificity or mode-of–action. However directed evolution was successfully implemented to alter the substrate specificity of a P450 monooxygenase and resulted in the generation of a novel P450 propane monooxygenase [11]. Site saturation mutagenesis was also used successfully to alter the enantioselectivity of a lipase from *Pseudomonas aeruginosa* [5]. These examples demonstrate that enzyme engineering has great potential for the overall optimization of hydrolytic processes and in particular chitinolytic enzymes. Likewise directed evolution approaches can eventually be used as a tool to develop entirely novel chitinolytic enzymes with unique properties that cannot be found in nature.

7.2.2 Further chitin valorization approaches

Besides the fully enzymatic COS production process developed in this thesis, additional valorisation approaches of chitin using the novel Chi5 strain can be explored. The remarkable ability of Chi5 to convert crystalline chitin and/or shrimp shells to bacterial biomass can be exploited to establish Chi5 as an attractive platform organism for the synthesis of products of higher value.

In nature, Chi5 enzymatically degrades chitin substrates to GlcNAc and dimeric chitin using a complex mixture of endo- and exo-chitinases. Degradation products are typically assimilated by Chi5 for further metabolic reactions. The pure chitinolytic culture supernatant could thus be utilized to develop a production process for the generation of GlcNAc. The amino sugar GlcNAc takes over essential roles in the human body in glycoproteins, proteoglycans and other building blocks in the connective tissue. As a results, GlcNAc has found multiple applications in tissue regeneration, in joint damage prevention, anti-inflammatory reactions as well as an

agent to improve skin quality. [12, 13]. Current harsh chemical GlcNAc production methods from chitin are not widely commercialized due to increased environmental concerns from high amounts of chemical waste as well as product contaminations by residual reactants. Multiple enzymatic GlcNAc production were investigated so far as substitutes, using mainly isolated and purified chitinolytic enzymes. Although high yields (77 %) of GlcNAc could be achieved, the overall productivity and commercial attractiveness is still considered low [14]. Bacterium Chi5 thus harbours the potential to establish a more efficient GlcNAc production process as it is not required to isolate single enzymes and crude substrates e.g. shrimp shell waste could be utilized directly in the process.

Chi5 belongs to the *Vibrionaceae* bacterial family and multiple member of this family showed to produce natural polyesters (polyhydroxyalkanoates [PHA]) as storage compounds predominantly during nutrient limitations and excess of carbon sources [15-18]. Biobased PHA gains increasing attention as a substitute for petrol-based polymers for the production of plastics. Currently however, production costs for PHA are still not competitive to conventional plastics as costs for raw materials are too high [19]. Thus a cost-effective alternative would be the conversion of less expensive biological waste materials to PHA. The unique growth characteristics of Chi5 on shrimp waste material could further help to develop an economically attractive PHA production process as it would eventually eliminate additional chitin-extraction or pre-treatment of the shrimp raw material. The halophilic nature of Chi5 could furthermore be exploited to develop a non-sterile conversion process in the presence of high salt contents minimizing the chances of contaminations by other bacteria. As a result, the PHA production process by Chi5 could be integrated directly in shrimp processing facilities with less efforts. In summary, the genetic relatedness of Chi5 and its unique potential to grow on chitin opens the chance to develop a novel and economically attractive production process for PHA.

Lastly the potential of Chi5 to be converted into a platform for the expression of recombinant proteins could be explored while maintaining the growth on inexpensive shrimp shells. Such platform would be of economic interest especially for large-scale fermentations where costs for cultivation media become a crucial factor in total production costs [20, 21]. Therefore, the transformability of Chi5 needs to be assessed

first in order to determine what genetic elements (promotors, origins of replication, regulators) and transformation techniques (heath-shock, electroporation, particle bombardment) are required to introduce and replicate foreign DNA in Chi5. In addition, suitable conditions and requirements to induce the expression of recombinant proteins are vital to be investigated (type of inductor, codon-usage, protein folding and secretion). In case transformation and expression protocols are successfully established, the entire process chain needs to be characterized concerning expression levels, productivity, product stability and toxicity, product isolation as well as purification and data must be compared to already well-established bacterial expression system such as *E. coli* BL21 and *Bacillus subtilis*. Further advantages over common bacterial hosts using Chi5 as an expression platform need to be assessed such as growth rates and biomass production as well as protein yields. Thereby potential benefits of Chi5 as a novel expression system can be pointed out, possibly expanding the current list of bacterial expression systems further and allowing to obtain a proprietary expression host. [22, 23].

7.2.3 Potential medical applications for enzymatically generated COS

The diverse properties and intrinsic functionalities of different chitosan and COS molecules led to very broad application spectra including the agrochemical, food, cosmetic, waste-water-treatment, and medical segments. The restrictive use of COS generated by chemical degradation processes and benefits of enzymatically produced COS were discussed in this thesis and a novel entirely biocatalytic process was demonstrated for the generation of partially acetylated COS from marine chitin. While antibacterial, antifungal and anti-inflammatory COS hold interest for all target markets, enzymatically generated COS are considered non-hazardous and thus attract great interest in particular for biomedical and pharmaceutical applications. Likewise, the biomedicine and pharmaceutic chitosan sector is the second most lucrative application segment after water treatments and a continuous growth of global production and revenue was projected from 2013 -2020 (Figure 7.3). The overall market attractiveness is graded high, implying that the market is open for future progress and the development of novel applications and products [24].

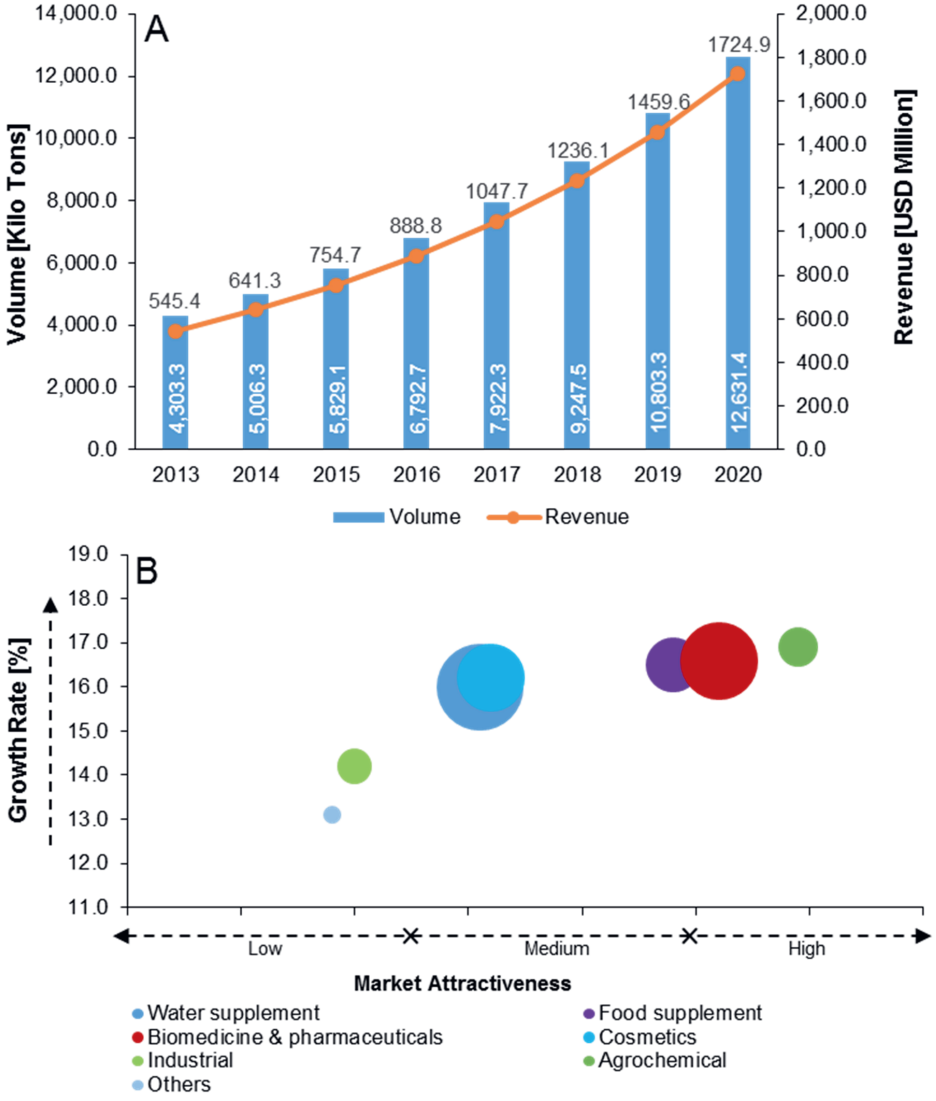

Figure 7.3. Global chitosan market analysis. A: global production volume (Tons) and revenue (USD Million) of chitosan for biomedicine and pharmaceutical applications 2013-2020. B: Chitosan market attractiveness analysis of application segments. Source: Transparency market research [1].

Due to the elevated biocompatibility of chitosan and COS, multiple applications of COS can be realized for biomaterials and regenerative medicine for systemic and percutaneous treatments. Modern implants are fully or partly plastic-based and typically built from PET, PTFE (Gore-Tex) and PE (Med-Por) fibers [25-29]. Until now,

artificial implants harbor the potential of post-surgical pathogenic infections, inflammations and reduced wound healing [30, 31]. Integration of active COS in the respective implant carrier material either during the manufacturing process or alternatively during post-processing as a coating could not only reduce the risk of infection posed by the implant itself but also contain any follow-up infections on the surgical site by slowly diffusing into surrounding body regions providing a protected milieu. This implementation of COS as a direct and preventive protective could be translated to diverse body implants such as stents, artificial heart valves, dental roots and joint replacements [32-34]. Potential applications for percutaneous medicinal products are the implementation of COS in wound dressings and surgical sutures in order to minimize the risk of bacterial infections and inflammations during the healing process [35-37].

7.2.4 Biorefinery of sustainable biological waste streams

Until now, fossil fuels remain the central resource for the production of chemicals by petroleum oil refinery. However, diminishing fossil reserves and rising emissions of carbon-based greenhouse gases due to an open carbon cycle further intensifies the shift towards a circular economy using renewable resources for the production of platform and bulk chemicals. Current biomass refinery processes, however, hold several disadvantages compared to conventional oil refinery that are also the main reasons that impede integration into industries: 1) Lower overall energy density 2) higher complexity of biomass composition 3) decentralized biomass production and processing requires a high level of logistical expenditures [38].

The utilization of marine crustacean waste as a renewable resource is one possible opportunity to address these issues and to develop an economically more feasible biorefinery route. Annually, up to 10 million tonnes of shrimp and crab shells emerge as a by-product in food industry and most of this biowaste is currently underutilized due to the predominant disposal into landfills and the ocean or incineration [4]. In addition, seafood industries are typically localized semi-central at coastal regions close to the respective fishing zones and the less complex composition of three major constituents (proteins, calcium carbonate and chitin) make shell waste preferable for biorefinery processes compared to woody biomass [39]. Fractionated isolation of the

individual shell compounds makes them either readily available for multiple industrial applications (proteins, calcium carbonate) or yields a new resource for further valorization processes (chitin) (Figure 7.4).

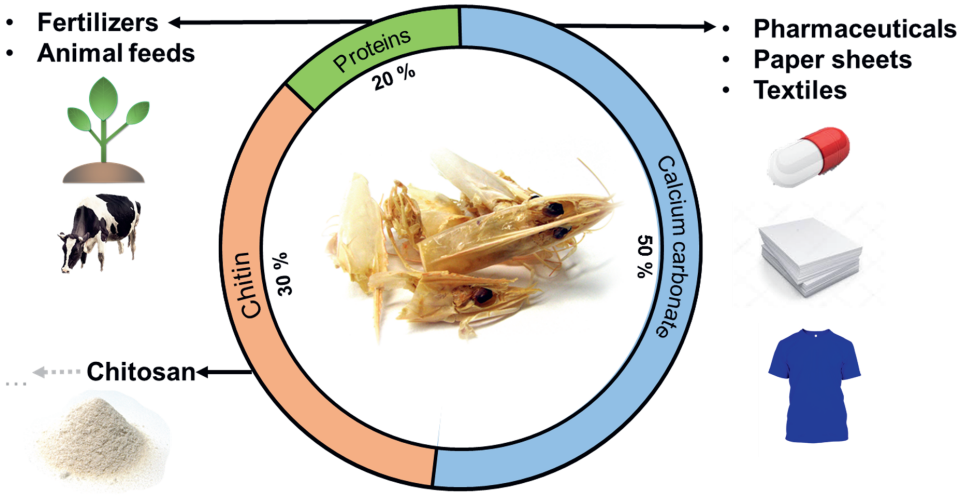

Figure 7.4. Average composition of crustacean shell material and respective application segments after fractionated biorefinery. Chitosan is sequentially implemented in multiple industrial and medical segments as described previously.

Industrial fractionation of shrimp shells typically involves demineralization, deproteination, and decolorization steps applying harsh chemical treatments at elevated temperatures (up to 100 °C). Due to the unfavorable environmental impact of this process, production volumes of individual shell components are restricted substantially by environmental regulations and, as a result, prices for pure chitin are relatively high. Substitutions by fully biological fractionation approaches were discussed in **Chapter 2** and although these novel processes are not yet competitive on an industrial scale, the increasing future demand for pure chitin will inevitably lead to a stronger implementation of green chemistry approaches [40-42].

In conclusion, chitosan and COS are remarkably versatile compounds and are firmly consolidated in numerous markets today. Novel biotechnological production processes for chitosan and COS may reveal novel functions thus stimulating the development of innovative applications and open new markets. Along with a

competitive biological fractionation and valorization approach for crustacean biowaste, a stronger biobased and circular economy can be fostered.

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Chapter 8

Impact of the research

The novel enzymatic process developed in this thesis for the production of COS from chitin proved to be a viable alternative to current chemical processes when comparing production rates and total conversion yields. Until now, COS are mainly produced from chitin by harsh chemical treatments resulting in less defined products and contaminations by residual chemical reactants. Thus, the implementation of chemically produced COS in medical products is strictly regulated and opportunities for the development of novel products is thereby limited. The novel enzymatic process will eliminate the use of harsh chemicals for decrystallization-, depolymerisation- and deacetylation- reactions and increases biocompatibility and safety of products simultaneously. The novel production process could stimulate the development of novel medical products and applications implementing functional COS molecules with increased biocompatibility. Healthcare sectors and subsequently social fields could further profit from a stronger implementation of COS in medicinal products as novel disease treatments and preventive medication approaches could be developed and explored. As a further consequence, the enzymatic process will support the value chain by creating a new and more direct link between raw material producers (e.g. shrimp industry) and biomedical industries. The implementation of the novel process may be part of the increase in profits for these industries. Future projects could bridge both fields directly by establishing a close cooperation with biomedical R&D institutions for the implementation of COS into novel products. The scope of this project is communicated openly and frequently using multiple channels such as conferences, exhibitions, as well as the press. Thereby, novel contacts from research institutions as well as companies operating at both ends of the value chain were established successfully resulting in novel projects and fruitful cooperations. Currently, the cooperation with partners from chemical industry is being established to explore future applications for enzymatically produced chitosan and COS.

Besides the novel COS production pathway the novel bacterial strain Chi5 as well as the chitinolytic enzymes could help to develop alternative technologies and products that can be used to transit to a more circular and biobased society and minimize the accumulation of chemical waste streams. In a running project, the unique growth characteristics of Chi5 are explored and channelled into a more product-oriented manner e.g. recombinant protein and bioplastic (PHA) production thus further

strengthening the paradigm “from waste-to-product”. Momentarily, feasibility studies are carried out in cooperation with a shrimp processing company and a university of applied science to assess the potential of Chi5 as a novel sustainable and economically attractive PHA production platform. In this context, partners from chemical industries were contacted and currently potential applications for PHA produced by Chi5 are explored. The three expression vectors developed in this project were successfully implemented in additional projects as a cloning and expression platform for recombinant proteins. Chitinases have shown to exhibit antifungal properties by degrading fungal cell walls and currently applications as a biological crop-protection agent are explored together with partners from agrochemical industries. Patent submissions for this application are currently in preparation. Chi5 is currently implemented in an European project aiming for the development of new marine ingredients and products using underutilized marine bioresources such as jellyfish, starfish and invasive crab species as a raw material. In this context the increasing competition for limited natural resources due to the estimated demographic growth is addressed thus the transition to a more biobased society is further stimulated.

Peer-reviewed publications describing the entire process development could further be used to communicate the most essential findings in this thesis to the scientific community potentially stimulating more research in the enzymatic production of COS from marine chitin instead of e.g. harshly chemically pre-treated colloidal chitin or chitosan powder.

In summary, the findings summarized in this thesis will cover a wide spectrum of future applications for enzymatically produced COS as well as the origin bacterium Chi5. The current scientific impact can be determined by the high interest from industry as well as running projects exploring the development of a range of different applications. Future social impact of the research could be evaluated after the implementation of potentially novel biobased products derived from chitin.

Appendix A

Multiple constructs for the expression of chitinases and CDAs were generated in **Chapters 4** and **5** using the golden gate cloning technology to test whether functional enzymes can be expressed by *E. coli* BL21.

Table A1.1. Composition of genetic constructs for all five CDAs generated using the golden gate cloning technology.

Name	Signal peptide	Gene-of interest	Purification tag	Vector backbone
DK1	-	Deacetylase 1	6xHis	pGR_cyto
DK2	-	Deacetylase 1	Tag54/6xHis	pGR_cyto
DK3	-	Deacetylase 1	6xHis	pGR_dsbA
DK4	-	Deacetylase 1	Tag54/6xHis	pGR_dsbA
DK5	PelB	Deacetylase 1	6xHis	pGR_SigP
DK6	PelB	Deacetylase 1	Tag54/6xHis	pGR_SigP
DK7	Natural	Deacetylase 1	6xHis	pGR_SigP
DK8	Natural	Deacetylase 1	Tag54/6xHis	pGR_SigP
DK9	-	Deacetylase 2	6xHis	pGR_cyto
DK10	-	Deacetylase 2	Tag54/6xHis	pGR_cyto
DK11	-	Deacetylase 2	6xHis	pGR_dsbA
DK12	-	Deacetylase 2	Tag54/6xHis	pGR_dsbA
DK13	PelB	Deacetylase 2	6xHis	pGR_SigP
DK14	PelB	Deacetylase 2	Tag54/6xHis	pGR_SigP
DK15	-	Deacetylase 3	6xHis	pGR_cyto
DK16	-	Deacetylase 3	Tag54/6xHis	pGR_cyto
DK17	-	Deacetylase 3	6xHis	pGR_dsbA
DK18	-	Deacetylase 3	Tag54/6xHis	pGR_dsbA
DK19	PelB	Deacetylase 3	6xHis	pGR_SigP
DK20	PelB	Deacetylase 3	Tag54/6xHis	pGR_SigP
DK21	-	Deacetylase 4	6xHis	pGR_cyto
DK22	-	Deacetylase 4	Tag54/6xHis	pGR_cyto
DK23	-	Deacetylase 4	6xHis	pGR_dsbA
DK24	-	Deacetylase 4	Tag54/6xHis	pGR_dsbA
DK25	PelB	Deacetylase 4	6xHis	pGR_SigP
DK26	PelB	Deacetylase 4	Tag54/6xHis	pGR_SigP
DK27	Natural	Deacetylase 4	6xHis	pGR_SigP
DK28	Natural	Deacetylase 4	Tag54/6xHis	pGR_SigP
DK29	-	Deacetylase 5	6xHis	pGR_cyto
DK30	-	Deacetylase 5	Tag54/6xHis	pGR_cyto
DK31	-	Deacetylase 5	6xHis	pGR_dsbA
DK32	-	Deacetylase 5	Tag54/6xHis	pGR_dsbA
DK33	PelB	Deacetylase 5	6xHis	pGR_SigP
DK34	PelB	Deacetylase 5	Tag54/6xHis	pGR_SigP

Table A1.2. Composition of genetic constructs for all five chitinases generated using the golden gate cloning technology.

Name	Signal peptide	Gene-of interest	Purification tag	Vector backbone
CS1	-	Chitinase 2	6xHis	pGR_cyto
CS2	-	Chitinase 2	Tag54/6xHis	pGR_cyto
CS3	-	Chitinase 2	6xHis	pGR_dsbA
CS4	-	Chitinase 2	Tag54/6xHis	pGR_dsbA
CS5	PelB	Chitinase 2	6xHis	pGR_SigP
CS6	PelB	Chitinase 2	Tag54/6xHis	pGR_SigP
CS7	Natural	Chitinase 2	6xHis	pGR_SigP
CS8	Natural	Chitinase 2	Tag54/6xHis	pGR_SigP
CS9	-	Chitinase 1	6xHis	pGR_cyto
CS10	-	Chitinase 1	Tag54/6xHis	pGR_cyto
CS11	-	Chitinase 1	6xHis	pGR_dsbA
CS12	-	Chitinase 1	Tag54/6xHis	pGR_dsbA
CS13	PelB	Chitinase 1	6xHis	pGR_SigP
CS14	PelB	Chitinase 1	Tag54/6xHis	pGR_SigP
CS15	Natural	Chitinase 1	6xHis	pGR_SigP
CS16	Natural	Chitinase 1	Tag54/6xHis	pGR_SigP
CS17	-	Chitinase 3	6xHis	pGR_cyto
CS18	-	Chitinase 3	Tag54/6xHis	pGR_cyto
CS19	-	Chitinase 3	6xHis	pGR_dsbA
CS20	-	Chitinase 3	Tag54/6xHis	pGR_dsbA
CS21	PelB	Chitinase 3	6xHis	pGR_SigP
CS22	PelB	Chitinase 3	Tag54/6xHis	pGR_SigP
CS23	Natural	Chitinase 3	6xHis	pGR_SigP
CS24	Natural	Chitinase 3	Tag54/6xHis	pGR_SigP
CS25	-	Chitinase 4	6xHis	pGR_cyto
CS26	-	Chitinase 4	Tag54/6xHis	pGR_cyto
CS27	-	Chitinase 4	6xHis	pGR_dsbA
CS28	-	Chitinase 4	Tag54/6xHis	pGR_dsbA
CS29	PelB	Chitinase 4	6xHis	pGR_SigP
CS30	PelB	Chitinase 4	Tag54/6xHis	pGR_SigP
CS31	Natural	Chitinase 4	6xHis	pGR_SigP
CS32	Natural	Chitinase 4	Tag54/6xHis	pGR_SigP
CS33	-	Chitinase 5	6xHis	pGR_cyto
CS34	-	Chitinase 5	Tag54/6xHis	pGR_cyto
CS35	-	Chitinase 5	6xHis	pGR_dsbA
CS36	-	Chitinase 5	Tag54/6xHis	pGR_dsbA
CS37	PelB	Chitinase 5	6xHis	pGR_SigP
CS38	PelB	Chitinase 5	Tag54/6xHis	pGR_SigP

Two mixtures designs with 40 individual runs each for chitinases and CDAs were generated in **Chapter 6** using the Design Expert 11 software.

Table A1.3. I-optimal mixture design plan for the assessment of optimum chitinase combinations for maximized yield of released reducing sugars. Maximum volumes of 240 µL represent a molar enzyme concentration of 2 µM. Factor A-E: chitinase 1-5.

Run	Factor A [µL]	Factor B [µL]	Factor C [µL]	Factor D [µL]	Factor E [µL]	nmol/ mL x h
1	0	0	0	0	123	55
2	73	93	74	0	0	272
3	0	81	0	81	78	27
4	78	81	0	81	0	274
5	0	0	78	82	80	152
6	78	0	82	0	80	101
7	0	0	78	82	80	166
8	57	60	62	0	61	98
9	80	0	0	80	80	133
10	0	0	0	118	122	37
11	0	53	62	53	72	96
12	37	0	155	24	24	296
13	126	0	114	0	0	394
14	82	76	0	0	82	53
15	70	66	66	38	0	399
16	63	58	63	0	56	144
17	0	0	0	240	0	45
18	0	0	240	0	0	287
19	240	0	0	0	0	502
20	39	68	0	65	68	58
21	81	0	78	81	0	420
22	63	0	67	48	62	218
23	122	118	0	0	0	341
24	0	81	0	80	79	31
25	0	24	30	30	156	32
26	80	0	0	80	80	164
27	30	154	1	30	25	69
28	74	92	74	0	0	271
29	0	79	74	87	0	265
30	125	0	0	115	0	401
31	0	119	121	0	0	252
32	153	25	0	28	34	380
33	45	0	70	63	62	226
34	0	0	0	0	240	30
35	118	0	0	0	122	65
36	0	78	82	0	80	53
37	72	24	76	68	0	402
38	78	0	82	0	80	130
39	0	121	0	0	119	40
40	0	78	84	78	0	326
41	40	0	35	158	7	314
42	0	0	124	116	0	306
43	0	78	82	0	80	39
44	78	81	0	81	0	311
45	0	240	0	0	0	34
46	0	119	0	121	0	47
47	0	59	52	60	69	102
48	67	68	0	35	70	106

Table A1.4. I-optimal mixture design plan for the assessment of optimum chitin-deacetylase combinations for maximized yield of released acetic acid. Maximum volumes of 100 µL represent a molar enzyme concentration of 2 µM. Factor A-E: deacetylase 1-5.

Run	Factor A [µL]	Factor B [µL]	Factor C [µL]	Factor D [µL]	Factor E [µL]	µg / mL x h
1	0	0	0	200	0	72
2	63	72	65	0	0	29
3	200	0	0	0	0	64
4	65	0	68	0	67	38
5	65	0	71	64	0	70
6	0	101	0	0	99	4
7	46	57	48	0	49	21
8	68	0	0	67	65	73
9	0	0	0	0	200	5
10	47	58	0	47	48	74
11	25	33	0	131	11	75
12	58	46	47	49	0	75
13	48	58	49	0	45	22
14	0	0	200	0	0	4
15	0	66	0	69	65	75
16	0	0	0	99	101	71
17	101	0	99	0	0	46
18	0	66	67	0	67	7
19	47	58	0	47	48	62
20	63	72	65	0	0	33
21	66	66	0	0	68	26
22	63	70	0	67	0	70
23	0	31	134	0	35	3
24	58	0	54	58	30	69
25	0	0	100	100	0	71
26	135	0	30	0	35	69
27	65	0	0	66	69	65
28	98	0	0	0	102	49
29	0	102	0	98	0	70
30	58	46	47	49	0	64
31	0	66	0	69	65	64
32	0	0	69	65	66	69
33	66	66	0	0	68	36
34	63	70	0	67	0	68
35	111	89	0	0	0	60
36	3	197	0	0	0	64
37	0	49	52	42	57	71
38	38	127	6	11	18	69
39	0	0	100	0	100	4
40	0	0	34	33	133	68
41	0	99	101	0	0	4
42	0	52	41	51	56	67
43	99	0	0	101	0	66
44	66	0	65	0	69	34
45	0	69	65	66	0	68
46	65	0	71	64	0	69
47	0	62	70	68	0	70
48	26	0	58	61	55	66

Appendix B

Vector maps of the three different entry plasmids utilized as a vector backbone for golden gate cloning of the target genes for chitinases and CDAs (A2.1-A2.3), plasmids carrying gene-bricks for subsequent cloning (A2.4-A2.5) and one exemplary final construct (A2.6). BsaI recognition sites in the entry vectors were introduced to the plasmids via standard cloning of the respective entry sites.

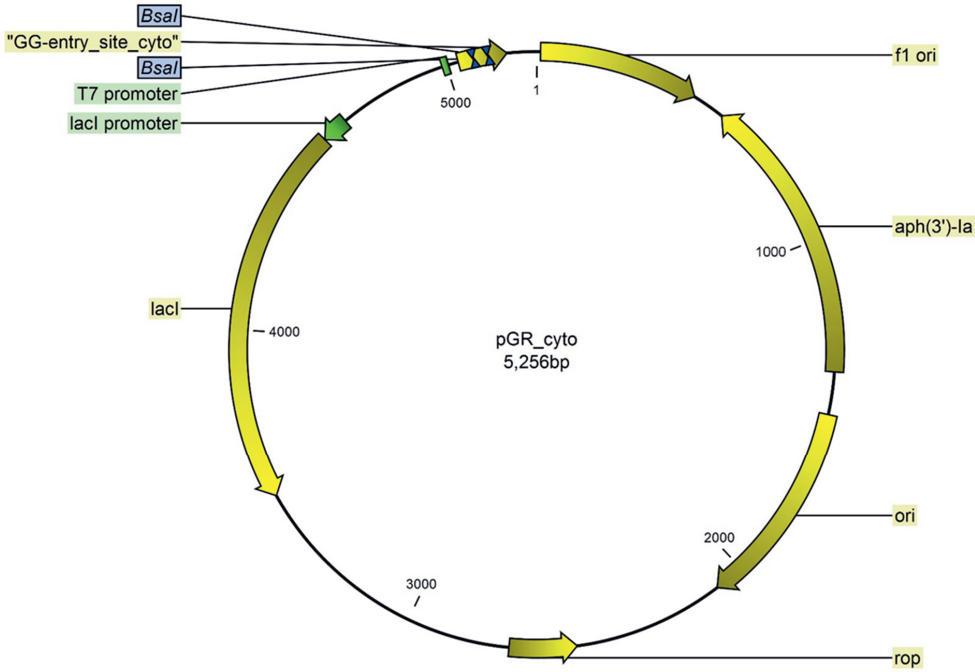

Figure A2.1. Annotated vector map of the pGR_cyto plasmid used for cytosolic expression of target genes. Two BsaI recognition sites were introduced to the plasmid by standard cloning of the GG-entry_site_cyto. T7 promoter: promoter for bacteriophage T7 RNA polymerase. LacI: The lac repressor binds to the lac operon to inhibit expression in *E. coli*. Addition of lactose or IPTG can relieve this repression and initiate expression; Rop: The repressor of primer, regulates the replication of the plasmid inside the bacterium; ori: origin of replication; aph(3´)-Ia: confers resistance to kanamycin; f1 ori: bacteriophage origin of replication.

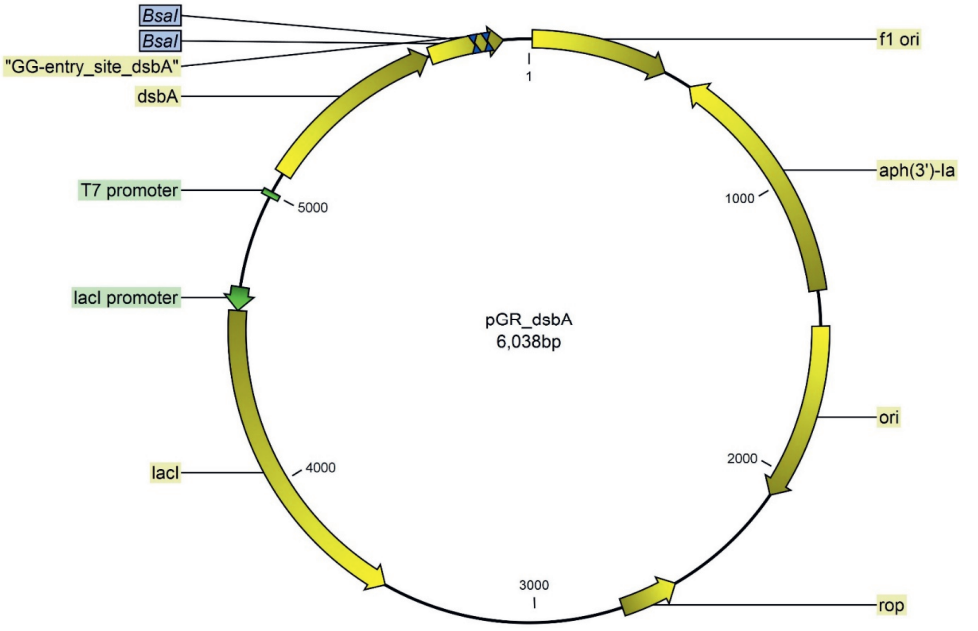

Figure A2.2. Annotated vector map of the pGR_dsbA plasmid used for the expression of target genes fused to the dsbA protein that acts as a folding enhancer. Two BsaI recognition sites were introduced to the plasmid by standard cloning of the GG-entry_site_dsbA. dsbA: bacterial periplasmatic oxidoreductase; T7 promoter: promoter for bacteriophage T7 RNA polymerase. LacI: The lac repressor binds to the lac operon to inhibit expression in *E. coli*. Addition of lactose or IPTG can relieve this repression and initiate expression. Rop: The repressor of primer, regulates the replication of the plasmid inside the bacterium; ori: origin of replication; aph(3´)-Ia: confers resistance to kanamycin; f1 ori: bacteriophage origin of replication.

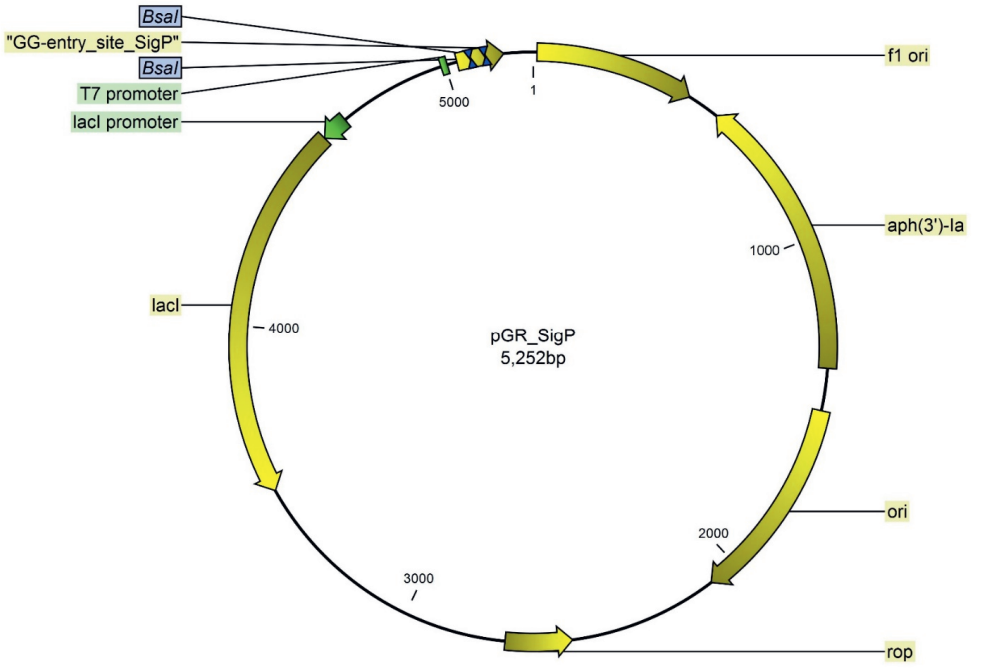

Figure A2.3. Annotated vector map of the pGR_SigP plasmid used for expression of target genes and sequential secretion of enzymes. Two BsaI recognition sites were introduced to the plasmid by standard cloning of the GG-entry_site_SigP. Assembly of the construct require the addition of N-terminal signal peptides. T7 promoter: promoter for bacteriophage T7 RNA polymerase. LacI: The lac repressor binds to the lac operon to inhibit expression in *E. coli*. Addition of lactose or IPTG can relieve this repression and initiate expression; Rop: The repressor of primer, regulates the replication of the plasmid inside the bacterium; ori: origin of replication; aph(3´)-Ia: confers resistance to kanamycin; f1 ori: bacteriophage origin of replication.

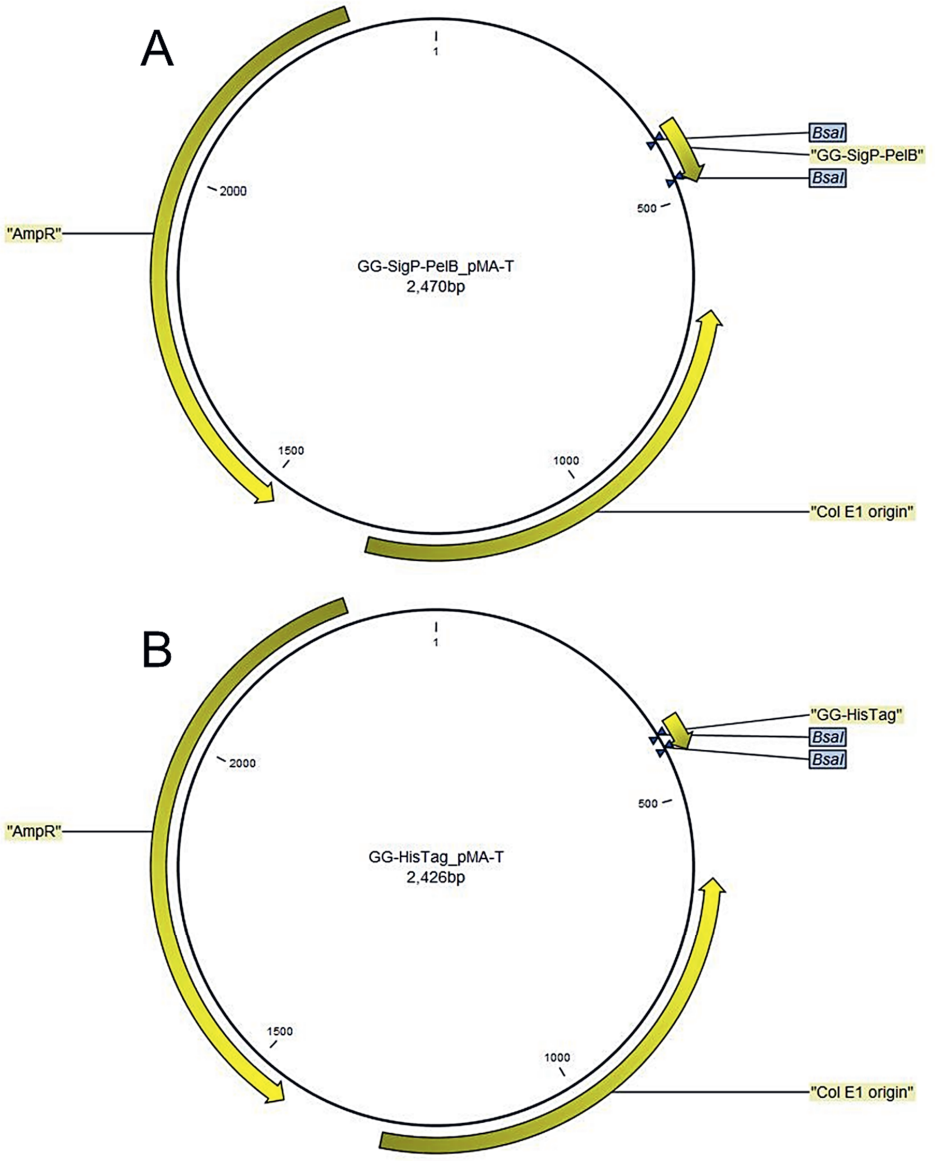

Figure A2.4. Annotated vector maps of plasmids providing gene bricks for golden gate cloning. A: Plasmid containing gene sequence for PelB signal peptide (GG-SigP-PelB) flanked by BsaI recognition site. Selection is carried out via ampicillin resistance gene (AmpR). Plasmid is analogous to those carrying the natural signal peptide. B: Plasmid carrying the 6xHis purification tag flanked by BsaI recognition sites. Selection is conveyed by the ampicillin resistance gene (AmpR). Plasmid structure carrying the 6xHis/tag54 combi tag is analogous. Col E1 ori: origin of replication for *E. coli.*

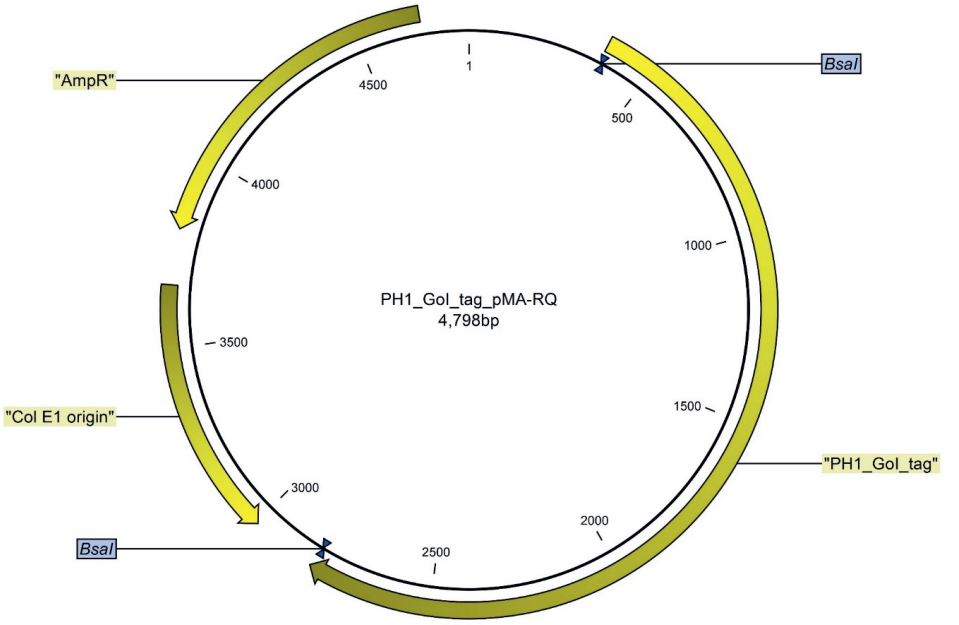

Figure A2.5. Exemplary annotated vector map of plasmid carrying the target genes (chitinase, CDA) used for golden gate cloning. The gene-of-interest (PH1_GoI_tag) is flanked by BsaI recognition sites. Antibiotic resistance is conveyed by the ampicillin resistance gene (AmpR). Col E1 origin: origin of replication for plasmid amplification in *E. coli*.

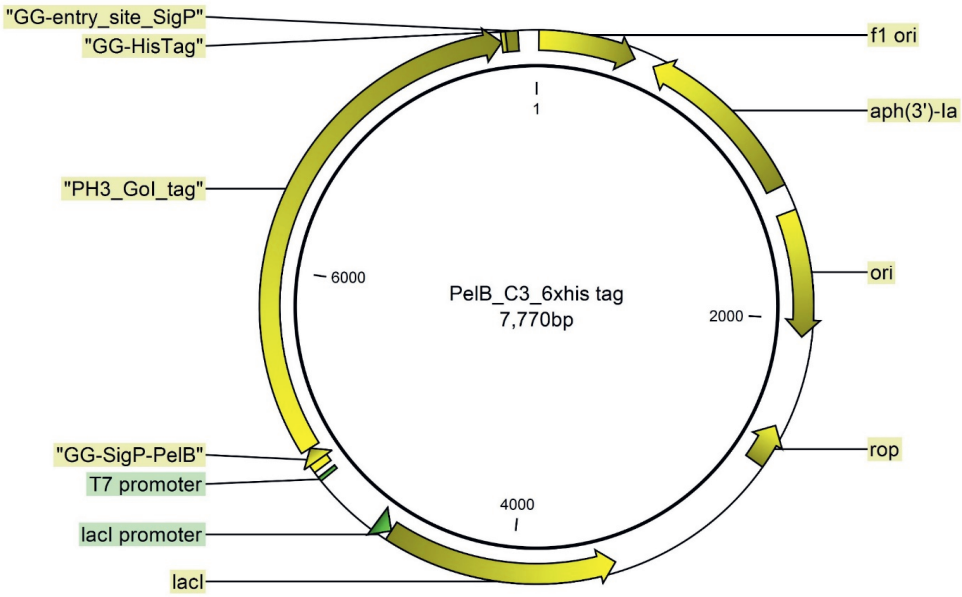

Figure A2.6. Exemplary annotated vector map of assembled construct comprising the genes for PelB signal peptide (GG-SigP-PelB), chitinase 3 (PH3_GoI_tag) and 6xHis tag (GG-HisTag) after golden gate cloning into pGR_SigP. All positive constructs were selected using the kanamycin resistance gene introduced by the vector backbone (pGR_SigP). T7 promoter: promoter for bacteriophage T7 RNA polymerase. LacI: The lac repressor binds to the lac operon to inhibit expression in *E. coli*. Addition of lactose or IPTG can relieve this repression and initiate expression; Rop: The repressor of primer, regulates the replication of the plasmid inside the bacterium; ori: origin of replication; aph(3´)-Ia: confers resistance to kanamycin; f1 ori: bacteriophage origin of replication.

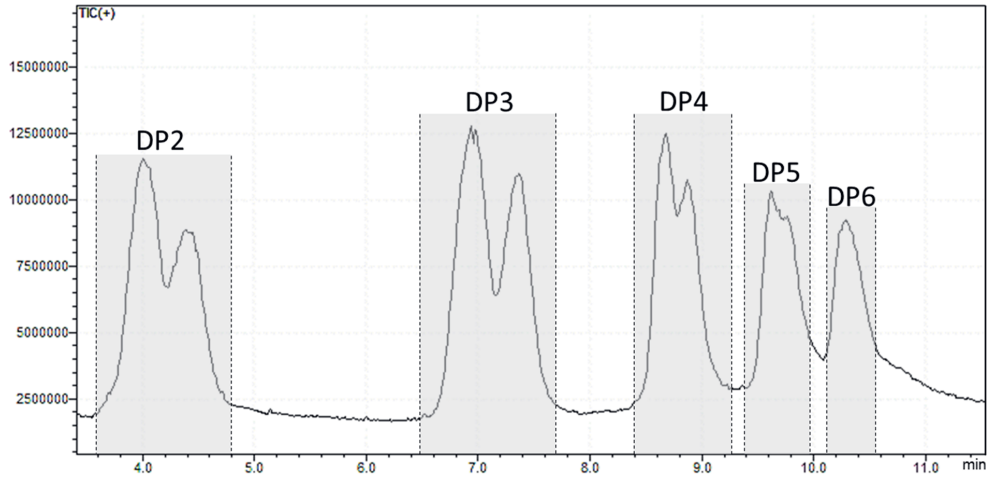

Figure A2.7. Chromatogram of pure chitin oligomers separated by LC-MS using an Amide BEH column. A mixture containing 0.5 mg/ml of chitin dimers (DP2), trimers (DP3), tetramers (DP4) pentamers (DP5) and hexamers (DP6) was prepared freshly in dH_2O. Two µl of the mixture was injected using an autosampler. Single compounds tend to generate two peaks due to anomerization to α- and β- chitin oligomers.

List of publications

This thesis resulted in the following publications:

Schmitz, C.; Lijnen P.; Schillberg, S.; Fischer, R.; Rasche, S.; Bortesi, L., Towards a fully enzymatic conversion process for the generation of partially deacetylated chitosan oligomers from chitin. Prepared for submission. Journal: Bioprocess and Biosystems Engineering.

Schmitz, C.; Schillberg, S.; Fischer, R.; Rasche, S.; Bortesi, L., Recombinant production and characterization of five novel chitin-deacetylases isolated from a marine *Photobacterium* strain. Prepared for submission. Journal: Scientific reports.

Schmitz, C.; Jablonka, N.; Schillberg, S.; Fischer, R.; Rasche, S.; Bortesi, L., Recombinant production and characterization of five novel chitinolytic enzymes isolated from a marine *Photobacterium* strain for the *in vitro* degradation of chitin. Prepared for submission. Journal: International Journal of Biological Macromolecules.

Schmitz, C.; Agdour, S.; Jablonka, N.; Fritsch, L.; Fischer, R.; Schillberg, S.; Bortesi, L.; Rasche, S., *Photobacterium orarium* sp. nov., a bacterium isolated from coastal seawater. Prepared for submission. Journal: Journal of Systematic and Evolutionary Microbiology.

Schmitz, C.; Gonzales-Auza, L.; Koberidze, D.; Rasche, S.; Fischer, R.; Bortesi, L. Conversion of chitin to defined chitosan oligomers: current state and future prospects. *Marine Drugs* 2019, 17(8), doi: 10.3390/md17080452.

Other publications by the author:

Jmel, M. A.; Anders, N.; Yahmed, N. B.; **Schmitz, C.**; Marzouki M. N.; Spiess, A.; Smaali, I., Variations in physicochemical properties and bioconversion efficiency of *Ulva lactuca* polysaccharides after different biomass pretreatment techniques. *Applied Biochemistry and Biotechnology* 2018, 184(3), 777-793.

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Rasche, S.; **Schmitz, C.**; Jablonka, N.; Schillberg, S., LED-Beleuchtung von Fußballrasen – Möglichkeiten, Chancen und Grenzen. *European Journal of Turfgrass Science*, Ausgabe 4-2016.

Vasilev, N.; **Schmitz, C.**; Grömping, U.; Fischer, R.; Schillberg, S., Assessment of cultivation factors that affect biomass and geraniol production in transgenic tobacco cell suspension cultures. *PLOS ONE* 2014, 9(8).

Vasilev, N.; **Schmitz, C.**; Dong, L.; Ritala, A.; Imseng, N.; Häkkinen, S. T.; van der Krol, S.; Eibl, R.; Oksman-Caldentey, K-M.; Bouwmeester, H.; Fischer, R.; Schillberg, S., Comparison of plant-based expression platforms for the heterologous production of geraniol. *Plant Cell Tissue and Organ Culture* 2014*,* 117, 373–380.

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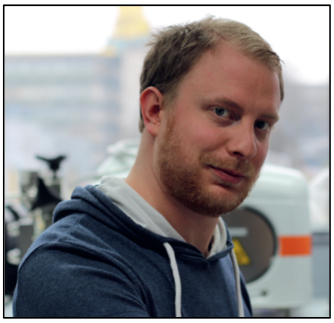
Curriculum vitae



Christian Schmitz was born on the 1st of March 1988 in Simmerath, Germany. In 2008 he started studying biotechnology at the Fachhochschule Aachen, Campus Jülich in Germany where he obtained a Bachelor of Science degree in 2012. The same year, he continued his education in molecular biotechnology at the Rheinisch Westfälische Technische Hochschule (RWTH) Aachen. During his study he worked as a student assistant and intern on multiple projects at the Fraunhofer Institute for molecular biology and applied ecology IME, Aachen broadening is knowledge in the design-of-experiments methodology and bioprocess engineering. At the same location he also carried out his Master thesis in the group of Prof. Stefan Schillberg under the supervision of Dr. Stefan Rasche on the development of a screening method for the assessment of protein purification conditions. In 2016, he graduated as Master of Science with a specialization in bioprocess engineering.

The same year he received the opportunity to pursue his PhD initially under the supervision of Prof. Rainer Fischer and followed by Assoc. Prof. Luisa Bortesi as well as Dr. Stefan Rasche at the Aachen-Maastricht institute for biobased materials (AMIBM). During his PhD, he investigated chitinolytic enzymes for the establishment of a fully enzymatic conversion process from marine chitin to chitosan oligomers. The most essential results of his work are summarized in this dissertation.