Technische Universität München
TUM School of Natural Sciences

Unlocking a Potent Chitin Saccharification System in a Novel Bacterium Through Omics and Bioinformatics

Nathanael David Arnold

Vollständiger Abdruck der von der TUM School of Natural Sciences der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften (Dr. rer. nat.)

genehmigten Dissertation.

Vorsitz: apl. Prof. Dr. Wolfgang Eisenreich

Prüfer*innen der Dissertation:

1. Prof. Dr. Thomas B. Brück
2. Prof. Dr. Volker Sieber

Die Dissertation wurde am 25.10.2023 bei der Technischen Universität München eingereicht und durch die TUM School of Natural Sciences am 31.01.2024 angenommen.

I. Abstracts

Manuscript I

The works described in this thesis are part of the BMBF funded project ChitoMat, which was concerned with the biotechnological extraction of crab shell chitin and its conversion into tailored chitooligosaccharides (COS) using new chitinolytic enzyme systems. In the quest for novel chitinolytic activities, soil samples were enriched with chitin to boost the abundance of chitin assimilating organisms. This led to the isolation of two highly potent candidate strains, both of which shared high 16S rRNA sequence similarity with *Jeongeupia naejangsanensis* BIO-TAS4-2. Biochemical characterization regarding the substrate utilization capabilities revealed profound differences between the candidates and the type strain. Subsequently, high-quality genomes were sequenced with a PacBio Sequel device to confirm or reject the novelty of the discovered organisms and to enable recombinant expression of chitinolytic enzymes. Circular chromosomes of 2.8 Mbps each were assembled and annotated. Whole genome inferred phylogenetics enabled by the type strain genome server (TYGS) confirmed earlier suggestions at the discovery of novel species, provisionally named *J. wiesaeckerbachi*. The strains exhibited an extraordinarily rich chitinolytic machinery, comprising 22 glycoside hydrolases and a lytic polysaccharide monooxygenase. Sequence alignments and phylogenetic analysis demonstrated conservation of the chitin-active enzymes within the *Jeongeupia* genus.

Manuscript II

In the second manuscript, the chitinolytic response system of *J. wiesaeckerbachi* was examined, utilizing a comprehensive three-way systems biology approach. First, exported enzymes under chitin conditions were analyzed. Second and third, intracellular proteomes and transcriptomes were compared to glucose controls. Data sets derived from intracellular proteomics indicated a shift in biosynthesis toward glucosamine transportation and utilization in addition to cell maintenance proteins. In synergy, differential transcriptome analysis highlighted the upregulation of stress and pili associated genes. While the temporal separation between late growth phase transcription and protein biosynthesis led to low correlation between the two intracellular system biology datasets, our research demonstrated the involvement of approximately 550 proteins and genes in the response of *Jeongeupia wiesaeckerbachi* to a chitin-rich environment. Our study proposed a holistic model, advancing the understanding and appreciation of complex natural chitin saccharification systems and allowed identification of potential enzymes for biotechnological chitin valorization in the process.

II. Zusammenfassungen

Manuskript I

Die in dieser Dissertation beschriebenen Arbeiten sind im Rahmen des BMBF geförderten Projektes ChitoMat entstanden, das sich mit der biotechnologischen Gewinnung von Krabbenschalenchitin und dessen Umwandlung in maßgeschneiderte Chitooligosaccharide (COS) mittels neuer chitinolytischer Enzymsysteme befasste. In diesem Zuge wurde zwei vielversprechende Kandidatenstämme isoliert, welche beide eine hohe 16S rRNA-Sequenzähnlichkeit mit *Jeongeupia naejangsanensis* BIO-TAS4-2 aufwiesen. Die biochemische Charakterisierung hinsichtlich der Substratverwertung zeigte deutliche Unterschiede zwischen den Kandidaten und dem *type strain* BIO-TAS4-2 auf. Anschließend wurden hochwertige Genome mit einem PacBio Sequel-Gerät sequenziert, um die Neuartigkeit der entdeckten Organismen zu bestätigen oder zu verwerfen und um die rekombinante Expression chitinolytischer Enzyme zu ermöglichen. Zirkuläre Chromosomen à 2,8 Mbps wurden assembliert und annotiert. Die Genom-abgeleitete Phylogenetik mit Hilfe des Type Strain Genome Servers (TYGS) bestätigte Hinweise auf die Entdeckung einer neuen Art, der wir den vorläufigen Namen *Jeongeupia wiesaeckerbachi* vergaben. Die Stämme wiesen eine äußerst reichhaltige chitinolytische Maschinerie auf, die 22 Glykosidhydrolasen und eine LPMO umfasst. Sequenzvergleiche und phylogenetische Analysen zeigten weiterhin, dass die Chitin-aktiven Enzyme innerhalb der Gattung *Jeongeupia* konserviert sind.

Manuskript II

Im dem zweiten Manuskript wurde das chitinolytische Reaktionssystem von *J. wiesaeckerbachi* mit Hilfe eines umfassenden systembiologischen Ansatzes in drei Schritten untersucht. Zunächst wurden die unter Chitinbedingungen exportierten Enzyme analysiert. Zweitens und drittens wurden die intrazellulären Proteome und Transkriptome mit Glukosekontrollen verglichen. Die intrazelluläre Proteomanalyse zeigte eine Verschiebung der Biosynthese in Richtung Glucosamintransport, -verwertung und Zellerhaltung. Die Transkriptomanalyse zeigte eine Hochregulierung von Stress- und Pili-assoziierten Genen. Obwohl aufgrund der zeitlichen Trennung von Transkription und Proteinbiosynthese nur eine geringe Korrelation zwischen den beiden intrazellulären Datensätzen bestand, zeigten unsere Untersuchungen die Beteiligung von etwa 550 Proteinen und Genen an der Reaktion von *Jeongeupia wiesaeckerbachi* auf eine chininhaltige Umgebung. Die Studie schlug ein ganzheitliches Modell vor, welches das Verständnis und die Wertschätzung von natürlichen Chitinverzuckerungssystemen verbessert und weiterhin geeignete Enzyme für die biotechnologische Chitinverwertung identifizierte.

III. Acknowledgements

I would like to express my sincere gratitude to Professor Thomas Brück for his invaluable guidance, unwavering support and mentorship throughout my journey towards this PhD. His wisdom and expertise have been instrumental in shaping my research and academic development.

I am deeply grateful to my supervisor, Daniel Garbe, for his exceptional dedication, patience, and encouragement. His insightful feedback and constructive criticism have been instrumental in refining the quality of my work and research.

I would also like to give special thanks to my colleagues who have shared this academic adventure with me (in alphabetical order): Felix, Kevin, Linda, Manfred, Melania, Michi, Nadim, Niko, Pariya, Petra, Philipp, Selina, Sophia, Tobi, and Zora.

In particular, my immediate fellow sufferers and friends from the SFZ during the pandemic, and later my (best) office colleagues, have provided me with many unforgettable moments that I will always cherish.

It is no exaggeration to say that I could not have done the PhD without you. Your advice, encouragement and our community have (almost) always made me look forward to going to work and made this challenging time a truly special experience. Thank you all.

I would also like to express my gratitude for Gülnaz, Gabi and especially Martina, who kept the institute alive through their hard work and always had an open ear for the problems of the PhD students.

From the bottom of my heart, I would like to thank all my friends and family who have always supported me no matter what I set out to do. Your emotional support, encouragement, patience, and sometimes metaphorical kicks in the ass over the past few years have been invaluable to me.

Mum, Emi, Josua and Wolfgang, your love and encouragement have been the foundation of my success and words can't express how much you mean to me.

I truly appreciate everyone who has been a part of my academic pursuits as your collective support and guidance has led me to where I am now and shaped me as a person.

IV. Contents

V. List of Abbreviations

% w/w	percent weight per weight
°C	degree Celcius
µg	microgram
µl	microliter
AA	auxiliary activity
CBM	carbohydrate binding module
CC	colloidal chitin
CCR	carbon catabolite repression
CDA	chitin deacetylase
CID	chitinase insertion domain
COD	chitooligosaccharide
COS	chitooligosaccharides
CSM	chitinase screening medium
DA	degree of acetylation
DD	degree of deacetylation
diH_2O	deionized water
DP	degree of polymerization
FA	fraction of acetylation
g	gram
GH	glycoside hydrolases
GlcN	$_D$-glucosamine
GlcNAc	*N*-Acetyl-$_D$-glucosamine
HMW	high molecular weight
Kbps	kilo base pairs
L	liters

LPMO	lytic polysaccharide monooxygenase
Mbps	mega base pairs
M_w	molecular weight
PA	pattern of acetylation
paCOS	partially acetylated chitooligosaccharides
pM	picomole
ppm	parts per million
TG	transglycosylation
TIM	triosephosphate isomerase

1 Introduction

1.1 Chitin – A Biopolymer of Undisputed Potential

Chitin, the most abundant marine biopolymer, is estimated to be biosynthesized in the magnitude of 10^{10}–10^{11} tons annually [1,2]. Approximately 630 thousand tons originate from industrial crustacean shell waste for seafood production from shrimps, prawns, crabs or lobsters, which amounted to 5.6 million tons in 2022 worldwide [3].As commonly no controlled, value adding disposal is feasible, shells are burned, landfilled, dried for chicken feed or discarded into the sea [4]. These byproducts encompass a chitin matrix hardened by calcium carbonate and interspersed with residual meat, carotenoids (primarily astaxanthin), and small amounts of lipids [5,6]. Adhesive proteins need to be separated from the shells, which are then decalcified and the extracted chitin deacetylated to chitosan for applications in various industries [1,5,7,8].

Present-day industrial crustacean shell waste processing to yield purified chitin or chitosan is not sustainable due to deployment of hydrochloric acid and hot alkali with negative implications on physio-chemical properties of the obtained products, hazardous wastewater generation and treatment thereof, which results in increased costs [5]. Improper disposal of these waste streams poses a threat to ecosystems, biodiversity and ultimately human health [9]. Therefore, there is a pressing need to explore biotechnological approaches, that utilize enzymes to process crustacean shells [107]. Not only does this address value adding applications of residues streams for seafood processing companies, but it also unlocks the untapped potential of readily available industrial feedstock to produce specialty chemical products in a more sustainable and higher-quality manner [4,5].

1.2 Structures and Physio-Chemical Characteristics of Chitin, Chitosan and Chitooligosaccharides

1.2.1 Chitin

The homo-polymer chitin is constructed through the covalent linkage of *N*-Acetyl-$_D$-glucosamine (GlcNAc) units, which are connected via β-(1-4)-glycosidic bonds, resulting in a high molecular weight (M_w). This polysaccharide exhibits structural similarities with cellulose, except for the presence of acetamido groups replacing hydroxyl groups at position 2 of the 2-deoxy-$_D$-glucopyranose subunits [11]. Chitin is naturally found as a co-polymer containing randomly distributed GlcNAc and $_D$-glucosamine (GlcN) subunits, with the acetylated $_D$-glucosamines being predominant [12]. Its high hydrophobicity renders it insoluble in aqueous

solutions, and it possesses a rigid, inflexible crystalline structure. This rigidity is a result of strong hydrogen bonding between adjacent polymer chains facilitated by the acetamido

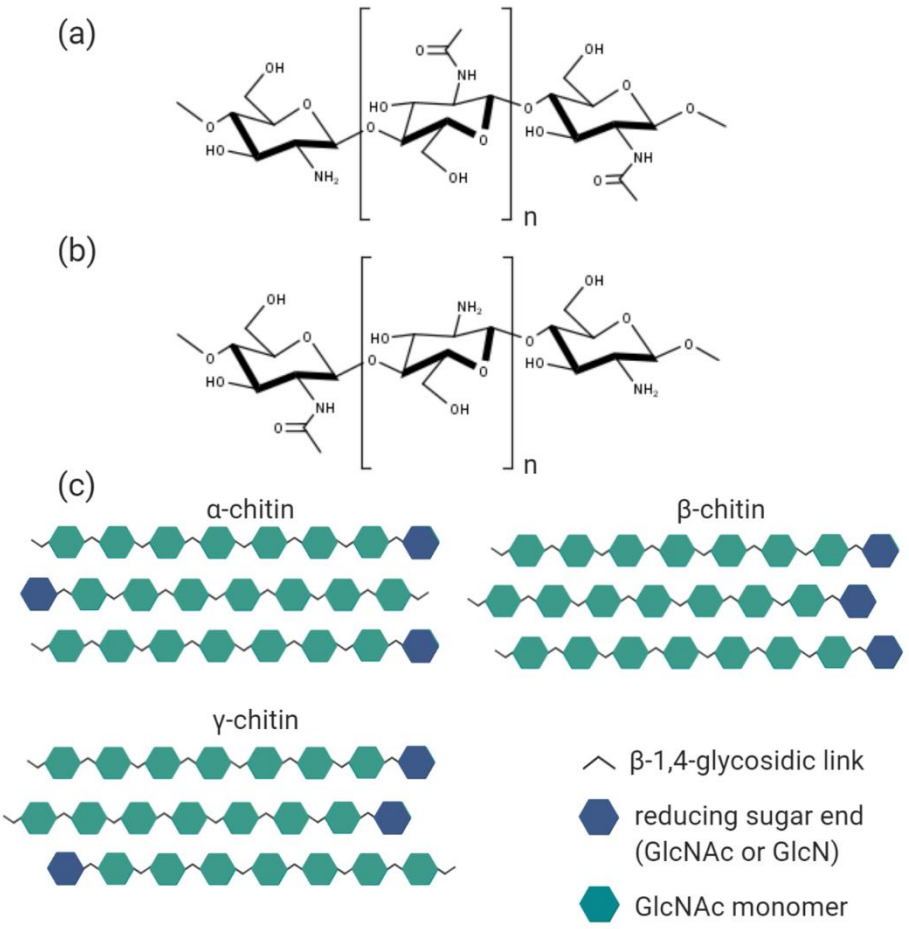

Figure 1: Chemical structures of the polysaccharides (a) Chitin and (b) Chitosan, consisting of different ratios of *N*-acetyl-glucosamine and glucosamine monomers. (c) The three chitin allomers, diverging in the antiparallel (α), parallel (β) or mixed (γ) alignment of chitin microfibrils.

groups, leading to an arrangement in microfibrils.

Chitin primarily exists as part of composite materials, acting as a stabilizing scaffold in conjunction with minerals like calcium carbonate, proteins, or both. The strength of inter- and intramolecular interactions within chitin microfibrils depends on the orientation of the polymer chains, giving rise to three naturally occurring allomorphs: α-, β-, and γ-chitin [1,13]. The most abundant and robust α-chitin features antiparallel alignment of polymer chains and is present in the exoskeletons of arthropods. Conversely, β-chitin showcases a parallel arrangement of polymer chains, resulting in weaker intramolecular interactions and greater accessibility for enzymes. Squid pens represent a notable source of β-chitin, though it is also found in other mollusks like krill. Structurally, γ-chitin differs from other chitin forms, as it comprises a combination of parallel and antiparallel alignment of interspersed polymer chains. This leads

to fractions with varying levels of crystallinity. This allomorph occurs naturally in beetle larvae and cephalopods.

1.2.2 Chitosan

Chitosan, the deacetylated form of the abundant chitin polysaccharide, offers a plethora of applications. It is characterized by a GlcN to GlcNAc units' ratio > 1.0 in the random copolymer, which corresponds to the molar fraction of N-acetylated glucosamine monomers, defining the %DA. In research, chitosan encompasses hetero-polymer chains with a minimum DD of about 10%, whereas fully acetylated chains composed solely of GlcNAc are referred to as chitin. Unlike chitin, chitosan is less commonly found in nature and is limited to cell walls of *Zygomycetes* fungi species [14].

The conventional conversion of chitin to chitosan involves treating it with 50% w/w NaOH at high temperatures (80 – 120 °C) using a 1:10 solid to liquid ratio to hydrolyze the acetamido groups [15]. This process yields chitosan with a low DP, an unspecified DA, and an undefined M_w, all of which significantly influence the physio-chemical properties and bioactivity of chitosan.

Chitosan exhibits solubility in weakly acetic solutions and demonstrates various beneficial activities, including antibacterial, antifungal, antioxidant, anti-inflammatory, and antitumoral effects [16–18]. Remarkably, it possesses a unique cationic nature in acetic solution, setting it apart from other polysaccharides. This property allows it to bind to negatively charged surfaces effectively. This particular feature is believed to be responsible for its antibacterial properties, as it can either bind to the surface of bacteria [12,19], blocking their metabolism, or attach to negatively charged DNA, leading to the inhibition of RNA translation [18,20]. Additionally, chitosan is physiologically inert and biodegradable, further enhancing its appeal for various applications.

1.2.3 Chitooligosaccharides

Similar to chitin, the high molecular weight (M_w) and viscosity of chitosan, coupled with its low water solubility, pose challenges for easy industrial processing. Therefore, chitin and chitosan are typically depolymerized through chemical, mechanical, or enzymatic degradation to obtain smaller fragments known as chitooligosaccharides (COS) or partially acetylated chitooligosaccharides (paCOS). These COS are soluble in water and retain the positive properties of their highly polymerized source materials [13]. They have a DP ranging from 2 to 20 and exhibit varying DA, pattern of acetylation (PA), and fraction of acetylation (FA), which determine their specific biological activity. The solubility of COS increases with the DD, with lower DP and M_w up to 3.9 kDa resulting in higher solubility[6].

Chitooligosaccharides with higher DP but lower Mw are believed to be more biologically active than those with lower DP and higher M_w. Like all sugars, chitooligosaccharides are susceptible to autooxidation and should be stored at -20 °C under dry and inert conditions. Their shelf life can be extended significantly by adding antioxidants like vitamin C before storage [21].

1.3 Applications of Chitin Derivates

The inherent chemical and physical characteristics of chitin limit its current commercial applications in its native form. It is commonly utilized as fertilizer in agriculture [22], due to its nitrogen content and as an inducer of plant defense mechanisms, enhancing resistance against fungi by promoting chitinase production *in-planta* [8,23]. Furthermore, chitin finds applications as a food additive [24] and as a packaging material for affinity chromatography columns to purify proteins with a carbohydrate-binding domain [25]. Additionally, it serves as a sorbent to pre-concentrate phenols and chlorophenols using solid-phase high-performance liquid chromatography (HPLC)[26].

In contrast, chitosan is a highly attractive biomolecule for various industry segments, from cosmetics and biomedicine to wastewater treatment, textile and paper production, biotechnology as well as food and agricultural industry [23,27–31]. In agriculture, chitosan has been demonstrated to increase fruit yields [32,33] , plant growth [34], and enhance protection against plant pathogens by soil treatment [35] or in the form of nanoparticles [36,37]. The-coating of fruits helped mitigate fungal postharvest diseases in pomegranate fruits [38]. Additionally, ripening has been demonstrated to be delayed and shelf life extended in red kiwis and mangos [39,40]. To that end, through a combined treatment of mangos with chitosan and spermidine, delay of fruit softening, accumulation of phenolic compounds and induction of defence enzyme activities could be effected [41].

Owed to its antimicrobial [6] and antioxidant characteristics, chitosan can also serve as additive to cosmetics [29,42–44]. Based on its anti-inflammatory [17], low toxicity and biocompatibility [45], chitosan has been applied in wound treatment [46,47], drug delivery [48,49], tissue regeneration [50–53] and as anti-tumoral therapeutic [54–56] in the biomedical field [13]. Most biomedical applications originate in chitosan's feasibility as component for 3D printing applications [57], creating hydrogel scaffolds with great potential for drug delivery or implants [58].

By virtue of its characteristic to adsorb metal ions [59,60], dyes [61,62], bacteria cells, humic acids and xenobiotics it is commonly deployed as coagulation agent in the wastewater treatment [63,64] and paper industry [65]. More specifically, chitosan increases the mechanical properties of paper [66], and can act as drainage or retention additive [67] or dye fixative [61]. Several studies demonstrated, that chitosan and derived paCOS supplementation of swine and poultry feed

has positive implications on their growth performance, nutrient digestibility and immune function [24].

1.4 Chitin-Active Enzymes

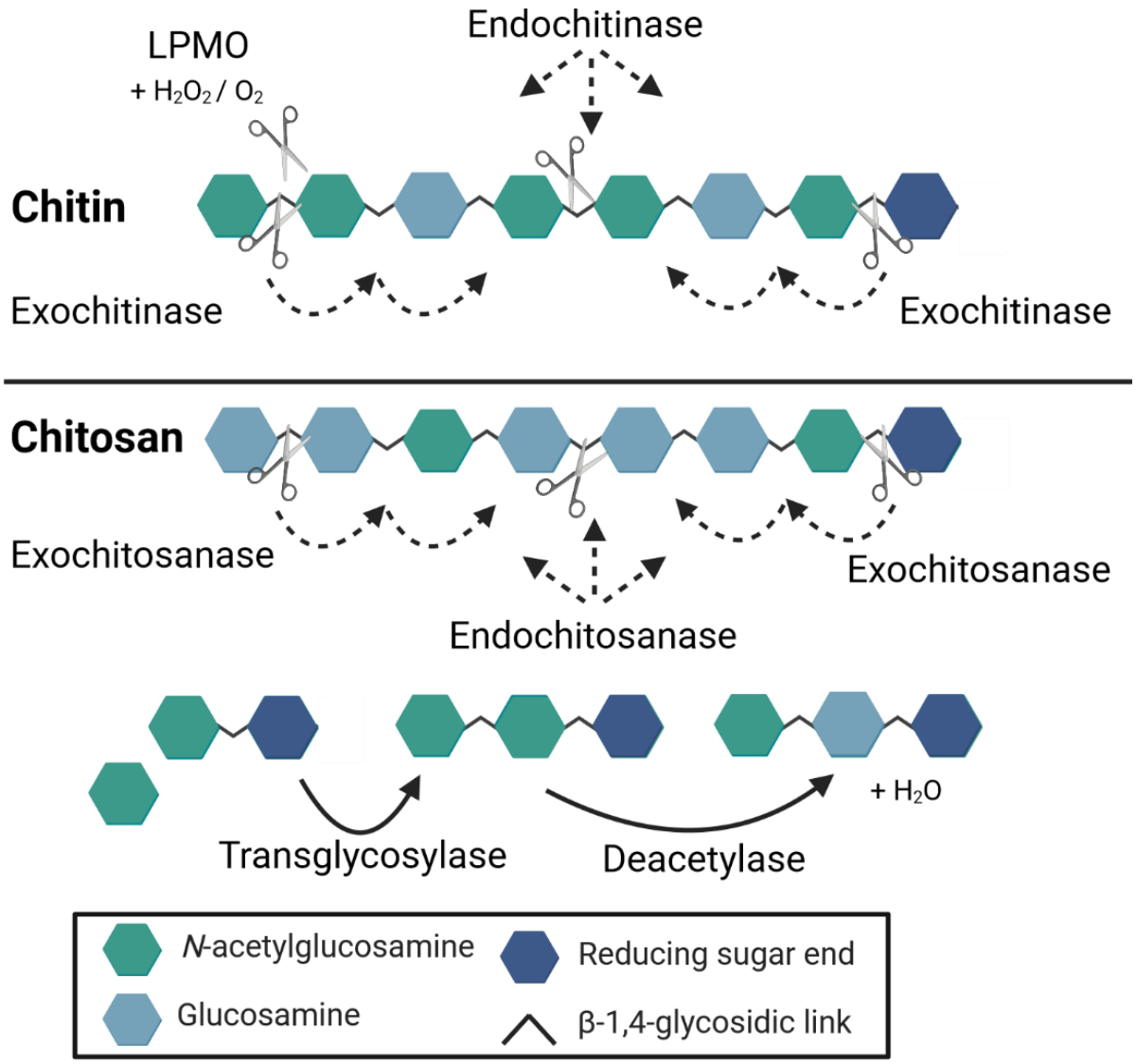

Figure 2: Schematic overview of chitin- and chitosan active enzymes. Exo-acting enzymes hydrolyze the polysaccharide chain processively from the reducing or non-reducing sugar end. Endo-acting enzymes cleave the β-1,4-glycosidic links randomly along the chain. The lytic polysaccharide monooxygenase (LPMO) hydrolyzes the β-1,4-glycosidic links with O_2 or H_2O_2 as reducing agents. Enzymes with transglycosylase activity extend the polysaccharide chain through addition of monomers. (Chitin-) Deacetylases remove the acetate residue from *N*-acetylglucosamine molecules, yielding glucosamine and a water molecule in the process.

1.4.1 Chitinases

Chitinases (EC 3.2.1.14) are part of the glycosyl hydrolase family and can be found in GH families 18 and 19, with additional chitinolytic enzymes in GH20 [68,69]. Among these, β-*N*-acetylhexosaminidases (EC 3.2.1.52), also known as chitobiases, play a crucial role [70]. They catalyze the breakdown of chitobiose (dimeric GlcNAc-units) from terminal reducing or non-reducing ends of chitin or chitindextrin. Although they all hydrolyze acetylated D-glucosamine

units via glycosidic β(1-4)-links, there are notable differences in their mode of action, amino acid sequences, and catalytic sites.

GH18 and GH20 glycosidases share a conserved DxDxE motif on the 4th β-strand and possess catalytic regions with a triosephosphate isomerase (TIM) barrel (β/α)8 domain [71]. In contrast, chitinases from GH19 have a lysozyme-like domain rich in α-helices. The GH18 family can be further divided into subfamilies A and B, with subfamily A containing an additional chitinase insertion domain (α+β) domain (CID) localized between the 7th α and β-strand of the TIM barrel. Both enzyme families employ a substrate-assisted mechanism for the hydrolytic reaction.

Chitinases can be categorized as processive or non-processive based on their behavior during chitin hydrolysis. Processive chitinases remain attached to the solid substrate and catalyze multiple hydrolysis cycles without releasing the substrate, threading single chitin chains through their tunnel-like catalytic cleft while cleaving off disaccharides simultaneously [72]. On the other hand, non-processive chitinases detach after each hydrolysis round, reattaching to other GlcNAc-GlcNAc links located elsewhere in a random fashion [69,73]. Processivity is particularly crucial for hydrolyzation of crystalline chitin, as it enables the enzyme to operate in proximity to a free polymer chain end at the cost of a reaction deceleration [74]. Additionally, through constant attachment of the enzyme and substrate, reattachment of cleaved off glucosamine-dimers is sterically prevented, thus enhancing overall efficiency.

Chitinases can also be distinguished as endo- or exo-chitinases based on their mode of action. Endo-chitinases cleave glycosidic bonds randomly and non-processively along the polymer chain, generating principally higher M_w COS in a time-dependent manner. Longer reaction times ultimately lead to predominant $(GlcNAc)_{2\text{-}5}$ production. In contrast, exo-chitinases degrade crystalline chitin from the reducing or non-reducing end in a processive manner, releasing *N, N′*-diacetylchitobioses successively while remaining attached to the substrate. Another type of processively or exo-acting enzyme is the β-*N*-acetylhexosaminidase [70], which targets the non-reducing end of low M_w *N*-acetylglucosides, often products of endo-chitinases in natural systems, converting them into glucosamine-monomers [75]. The specific substrate accessibility, rather than the enzyme architecture, seems to determine whether chitinases act in an endo or exo manner, potentially making the definition based on product length obsolete [76,77].

Some chitinases of family 18 have been discovered to possess transglycosylation (TG) capabilities, enabling them to not only break glycosidic bonds in chitin but also to create new links between small polysaccharide fragments, resulting in longer COS with a DP of up to 13 [75]. Enzymatically producing COS with DPs >4 is currently limited, as the main products of chitinases are typically dimeric or monomeric, given a sufficiently long incubation time.

However, higher polymeric COS hold greater value for advanced applications in various fields. The TG reaction occurs when a retaining enzyme transfers a glycosidic residue from a donor to an acceptor sugar molecule instead of using a nucleophilic water molecule [78]. Crystal structure studies have provided insights into the structural characteristics responsible for TG aptitude in certain chitinases [79,80]. The mechanism of TG events is not fully understood, but it has been demonstrated that the TG reaction is autocondensation-driven and must occur faster than glycosidic hydrolysis [81]. The transfer of COS depends on factors such as substrate amount and length, as well as the enzyme's proportion and nature. An excess of substrate appears to be beneficial for TG activity, while an excess of glycosidase promotes predominant hydrolysis [82].

1.4.2 Chitosanases

Chitosanases (EC 3.2.1.132) are widely distributed across diverse enzyme families, including GH5, GH7, GH8, GH46, GH75, and GH80, reflecting their extensive sequence variations. Another pertinent family in the context of chitosan-active enzymes is GH2, housing exo-1,4-β-$_D$-glucosaminidases, that catalyze the successive hydrolysis of $_D$-glucosamine residues from chitosan and chitosan-oligosaccharides at the non-reducing ends [83,84]. Among the chitosanases, GH46 family members have received thorough investigation, with four crystal structures available [85–88], and various expression and site-directed mutagenesis studies have elucidated their catalytic mechanisms. GH46 chitosanases predominantly adopt an α-helix architecture, comprising two globular domains, a minor and major lobe, separated by a deep substrate-binding cleft [85,89]. Remarkably, their core structure exhibits similarities to the well-studied *E. coli* bacteriophage T4 lysozyme (GH24) and, to a lesser extent, lysozymes from GH22, GH23 families, and barley chitinases from GH19, despite limited sequence homology. This catalytic and substrate-binding site consists of two α-helices and a three-stranded β-sheet, suggesting its evolution through divergent pathways [90], leading to these families being collectively referred to as the 'lysozyme superfamily.'

Within GH5, GH7, and GH8 families, enzymes with diverse activities, such as cellulases, xylanases, and glucanases are found, while GH46, GH75, and GH80 families exclusively encompass chitosanases, which likely operate via an inverting mechanism [91,92]. The inverting reaction mechanism entails a one-step, single-displacement hydrolysis of a glycosidic bond, resulting in the anomeric configuration's net inversion. Amino acid side chains act as general base and general acid [93], facilitating this reaction. Conversely, the retaining reaction mechanism involves a two-step, double-displacement hydrolysis of a glycosidic bond, leading to the retention of the anomeric configuration. In this mechanism, amino acid side chains serve as both acid/base and nucleophiles to catalyze the process [94]. Both pathways involve

oxocarbenium ion-like transition states, with the retaining reaction mechanism additionally featuring an intermediate covalent glycosyl-enzyme bond.

Chitosanases are further classified into subclasses I, III, and IV based on their substrate specificity [95]. While all chitosanases can hydrolyze glycosidic β-(1-4) linked GlcN-GlcN bonds, most of them can recognize either GlcN-GlcNAc (subclass III) or GlcNAc-GlcN (subclass I) bonds, but not both [96,97]. Subclass II chitosanases exclusively target and cleave GlcN-GlcN links. Chitosanases of subclass IV, however, possess the ability to cleave both GlcNAc-GlcN and GlcN-GlcNAc, as well as GlcN-GlcN [98]. Additionally, chitosanases share a common feature of not recognizing GlcNAc-GlcNAc links in partially acetylated chitosan, although some evidence challenges the strict distinction between chitinases and chitosanases. To that end, in *Streptomyces coelicolor* A3(2) a glycoside hydrolyzing enzyme ScCsn46A was identified [99], capable of cleaving GlcN-GlcN and GlcNAc-GlcN links and, uncommon for chitosanases, GlcNAc-GlcNAc links, albeit at a much slower rate.

1.4.3 Chitin-Deacetylases

Chitin deacetylases (CDAs, EC 3.5.1.41) and chitooligosaccharide deacetylases (CODs, EC 3.5.1.105) are enzymes capable of de-*N*-acetylating chitin and COS, respectively, by hydrolyzing their acetamido groups. They belong to carbohydrate esterase family 4 (CE4), which also includes acetyl xylan esterase, peptidoglycan GlcNAc deacetylase, and peptidoglycan *N*-acetylmuramic acid deacetylase. These enzymes share a common structural feature known as the NodB homology domain, named after the NodB oligosaccharide esterase found in *Rhizobium* bacteria involved in nod factor biosynthesis. The current hypothesis regarding the de-*N*-acetylation reaction mechanism proposes a nucleophile attack on the carbonyl carbon realized by the Asp-His-His catalytic triad of the CDA. To date, five CDA catalytic motifs are documented, all of them sharing conserved histidine and aspartic acid residues [100].

CDAs are utilized by living organisms both intracellularly, such as in fungi like *Mucor rouxii* for cell wall morphogenesis and defense against chitinases [101], and extracellularly, as observed in marine bacteria that secrete these enzymes to convert and hydrolyze crustacean shell wastes [102]. CDAs and CODs are highly substrate-specific, relying on different recognition patterns, and generate products with specific DA and PA [100]. The DA is influenced by the mechanism of the respective CDA, which can be categorized into three types:

i) *Multiple attack mechanism:* Enzymes bind to the recognition site in the polysaccharide chain and perform several sequential deacetylations before detaching and binding to another region. This leads to block-copolymer structures in HMW substrates, while shorter polymer chains or COS are entirely deacetylated [103].

ii) *Multiple chain mechanism*: Enzymes tightly bind to the recognition site, forming an enzyme-polymer complex. After a single deacetylation reaction, the complex dissociates, and the enzyme binds to another recognition site [104]. For polymeric substrates, this results in a random distribution of deacetylated subunits and PA, while for COS, the pattern depends on the specific substrate length and involved enzyme.

iii) *Single chain acting mechanism*: Processive enzymes deacetylate a single substrate molecule sequentially through multiple catalytic events. Some bacterial CODs, specific for a single position, belong to this group [105], leading to the production of mono-deacetylated products.

Carbohydrate esterases are less active on crystalline substrates, preferring soluble derivatives like glycol chitin, chitosan, or COS/paCOS. However, this limitation can be overcome by the co-application of lytic chitin monooxygenases (LPMOs), which cleave polymer chain ends on the surface of crystalline chitin, thereby enhancing the activity of CDAs [106]. Additionally, some CDAs and CODs have a carbohydrate-binding module (CBM) fused to their catalytic domain, thus increasing the accessibility of the recalcitrant substrate to the active site [107].

1.4.4 Lytic Polysaccharide Monooxygenases

Lytic polysaccharide monooxygenases (LPMOs, EC 1.14.99.53) fall under the classification of nine auxiliary activity enzyme (AA) families, numbered 9-17, based on their sequence. Those involved in chitin processing belong to groups 10, 11, and 15. These copper-dependent enzymes possess a unique ability to disrupt the crystalline structure of polymeric polysaccharide substrates through the oxidation of glycosidic links [108]. Therefore, new chain ends are generated, enabling common hydrolases to act on otherwise recalcitrant carbohydrates. LPMOs hold a distinctive position as the sole enzymes known to target recalcitrant polysaccharides, making them pivotal in utilizing crustacean shell wastes as a chitin source.

A prominent feature among all LPMOs is the presence of a histidine-brace motif within the catalytic region, situated close to the enzyme's surface, which facilitates the tight binding of a single copper ion by two conserved histidine residues [109,110]. The flat, solvent-exposed architecture of the active site allows certain LPMOs to oxidize the crystalline lattice structure of insoluble substrates such as chitin or cellulose. The structural arrangement of the catalytic region varies between different LPMOs, as specific loops determine substrate specificity and regioselectivity. This versatility is reflected in the discovery of both single catalytic domain and multidomain LPMO proteins.

Similar to CDAs, some LPMOs are known to possess a C-terminal carbohydrate-binding module (CBM) fused to their catalytic domain via a short linker. This further enhances substrate

specificity and binding affinity [111,112]. The nomenclature "polysaccharide monooxygenases" (PMOs) has been proposed by some researchers, as enzymes of that class do not actively cleave glycosidic linkages within polysaccharide chains. Instead, the cleavage of glycosidic bonds occurs spontaneously, following the deprivation of one hydrogen atom from the strong C-H bond of either C1 or C4, followed by hydroxylation by the LPMO with O_2 or H_2O_2 as a cosubstrate. To enhance binding affinity to the polysaccharide and activate the cosubstrate, reduction of the active site Cu(II) to Cu(I) is required [113]. Different reaction mechanisms occur depending on the cosubstrate and the carbon atom attacked. C1-positioned carbon atom oxidation leads to aldonolactone products, which subsequently hydrate to aldonic acids, while C4 oxidation results in the formation of 4-ketoaldose products that hydrate to gem-diol [114–116].

1.5 Chitin Metabolism in Bacteria

Chitin utilization in bacteria typically involves the following steps [117]: (1) chemotaxis and subsequently (2) adherence to the chitinous substrate, (3) hydrolysis of the polysaccharide chain into smaller COS, (4) import into the periplasm, (5) and lastly conversion of the transport products to fructose-6-P, acetate and NH_3. To facilitate the aforementioned processes, Keyhani and Roseman [118] postulated the necessity of a minimum of one extracellular chitinase; COS specific chemotaxis systems; a nutrient sensor responsible for adherence and gene regulation; a specific chitoporin in the outer membrane [119]; two or more hydrolases in the periplasmatic space which generate $(GlcNAc)_2$ and (GlcNAc), respectively [120,121]; three transport complexes situated in the inner membrane; and at least six cytoplasmatic enzymes that catabolize the imported sugar molecules into fructose-6-P, acetate and NH_3 [118,122,123]. It is hypothesized that 50 – 100 genes are directly involved in the orchestration and execution of the sophisticated chitin utilization pathway [118,124]. Since the chitin monomer *N*-acetylglucosamine can be derived from the hydrolyzation of glycosaminoglycans, glycoproteins or glycolipids, it would represent a poor signal molecule for the expression of chitinolytic proteins [118]. Instead, the disaccharide $(GlcNAc)_2$, coincidentally the predominant product of most chitinase activities [76], seems to act as focal point for bacterial chemotaxis and chitinase expression induction [125].

Hydrolysis, the third step in chitin utilization, is a multi-step process, mediated through an enzyme cocktail also referred to as the chitinolytic machinery. The process of complete degradation of the insoluble chitin polymer typically involves three key sequential steps: (1) enzymatic breakdown of the polymer into water-soluble oligomers, (2) division of these oligomers into dimers, and (3) enzymatic cleavage of the dimers into monomers [126]. To this end, the chitinolytic machinery comprises synergistic endo- and exochitinases, *N*-acetylhexosaminidases and a lytic polysaccharide monooxygenase, the functions, and modes of actions

of which have been extensively described above. Several studies investigated bacterial chitinolytic machineries, thus elucidating the enzymatic key components of *Serratia marcescens*, [127,128], *Enterococcus faecalis* [129], *Andreprevotia ripae* [130], *Cellvibrio japonicus* [131–133], *Streptomyces* sp. F-3 [134] or *Vibrio* spp. [122,135,136], among others.

Based on the effects of gene disruption experiments on chitinase expression, some proteins or genes could be identified as chitin response regulators. For example, the removal of the pleiotropic *reg1* gene in *Streptomyces lividans* resulted in the synthesis of chitinases even without the presence of chitin, while also eliminating glucose catabolite repression [137]. Disruption of the *dasR* gene in *Streptomyces coelicolor* had far-reaching implications not only on chitinase expression, but also non-canonical targets, affecting the expression of 1200 genes [138,139]. In *Vibrio* spp., a two component-system consisting of a hybrid sensor kinase ChiS and a chitooligosaccharides binding protein (CBP), which keeps the former in an inactive state when no $(GlcNAc)_n$ ($n > 1$) is present, regulate the chitin response cascade [118,140].

1.6 Systems Biology or -Omics Approaches

The current facility with which genomes are sequenced has designated genomics as one of the foremost stages in the realm of microbial systems biology. Irrespective of the employed methodology, the processes of assembly and annotation customarily ensue genome sequencing, furnishing an almost comprehensive portrayal of the genetic reservoir inherent to a given microorganism. Conversely, a genome sequence solely presents a momentary glimpse into the authentic phenotypic potentialities of an organism, affording scant indications pertaining to other pivotal facets of the underlying life cycle, including reactions to perturbations of environmental and genetic origins, temporal variations, gene indispensability, and so on.

To attain a holistic and all-encompassing elucidation of biological entities, the static insights derived from genome sequences prove inadequate, necessitating the inclusion of supplementary tiers of knowledge [141]. This is where -omics come into play, characterized as the comprehensive investigation and examination of extensive datasets embodying the complete configuration and operational dynamics of a specific biological entity [142].

Highly multivariate dataset analysis of biological -omics samples such as genomics, transcriptomics, proteomics, and metabolomics (the “big four -omics”) offer elucidation of previously hidden features, and at the same time immense challenges due to sheer data amounts and differing nomenclature [142].

In that context, Genomics focuses on the comprehensive study of an organism's entire genetic material, encompassing all its genes and their specific sequences. This field delves into understanding the structure, function, and variations within an organism's DNA [143].

Transcriptomics investigates the complete set of RNA transcripts produced by an organism's genome at a given time. It plays a pivotal role in revealing gene expression levels and patterns, facilitating an understanding of which genes are actively involved and their roles in various cellular processes [143,144].

Proteomics revolves around the exploration of the entire complement of proteins within a cell, tissue, or organism. This field involves the identification, quantification, and characterization of proteins. It offers valuable insights into protein functions, interactions, post-translational modifications, and their roles in diseases [143,145].

Metabolomics examines the comprehensive array of small molecules, known as metabolites, present within biological samples like cells, tissues, or biofluids. It provides a window into an organism's metabolic pathways, biochemical processes, and the influence of external factors on metabolism. Metabolomics plays a crucial role in disease diagnosis and personalized medicine by uncovering metabolic signatures [143].

Especially the integration of more than two -omics datasets for a systems-level understanding proved difficult, requiring to consider statistical behavior, non-obvious relationships between datasets and exploiting time-resolved measurements [146].

To that end, systems biology approaches have previously demonstrated their utility in identifying bacterial proteins involved in chitin or cellulose degradation, respectively, by the example of *Cellvibrio japonicus* [132,147].

In conclusion, the biopolymer chitin is a readily available raw material. Sea food industry derived crustacean shell waste is so abundant in fact, that its appropriate disposal can pose a financial burden for the producers, while incorrect disposal threatens the environment and human health. On the upside, a growing demand for chitin and chitosan persists with a myriad of suitable high value applications. However, large-scale biotechnological processes must be developed for sustainable chitin valorization first. Therefore, exploration of novel chitinolytic machineries and their encompassing enzymes is imperative for advancement in this field.

1.7 Project Overview ChitoMat - Holistic Chitin Conversion to Materials for 3D Printing Application and Performance Animal Feed Additives

The research presented in this thesis was carried out in the framework of the international project ChitoMat, funded by the German Federal Ministry of Education and Research (BMBF).

It involved the Werner-Siemens Chair for Synthetic Biotechnology (WSSB) as part of the Technical University of Munich (TUM) and Fraunhofer Institute for Interfacial Engineering and Biotechnology (IGB) on the German side. Partners from Canada comprised CigaIaTech

(formerly BBSI), in addition to the Atlantic Veterinary College (AVC) and the Atlantic Biofabrication Laboratory (ABFL) of the Prince Edward Island University (UPEI) (Figure 3).

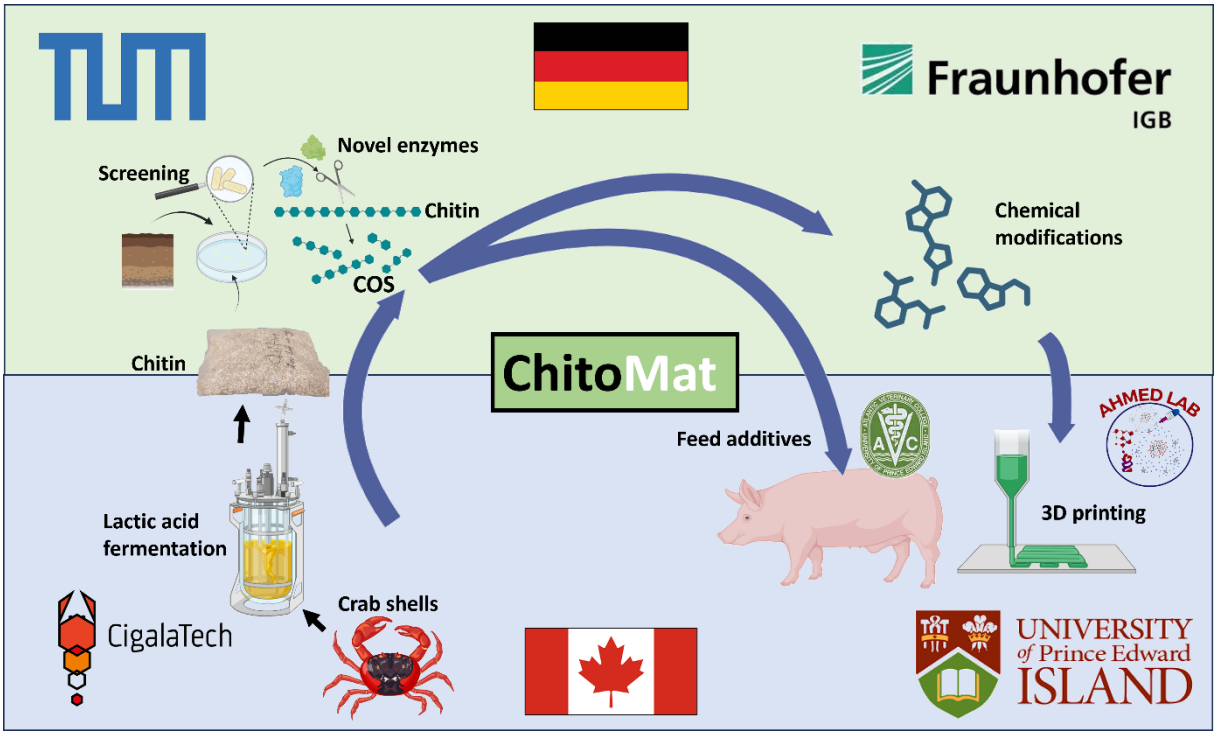

Figure 3: Flow chart of the ChitoMat (Holistic **chi**tin conversion to **mat**erials for 3D printing application and performance animal feed additives) project. It was a BMBF international funded joint effort involving the German partners TUM and Fraunhofer IGB in addition to the Canadian partners CigalaTech and UPEI.

Goal of ChitoMat was to extract chitin from Canadian crab shell waste biotechnologically without the utilization of harsh chemicals (CigalaTech) and then functionalize the polysaccharide through enzymatic hydrolysis and potentially deacetylation by means of novel enzyme activities or cocktails thereof (WSSB). A pilot scale fermentation was intended, given that feasible conversion processes were discovered.

Resulting COS of defined length should be transferred to the cooperation partners of IGB and UPEI, which would carry out chemical modifications or create blends for 3D printing (AFBL) applications or experiment with COS as antimicrobial feed additives (AVC), respectively.

Unfortunately, the cooperation partner CigalaTech, had to file for insolvency during the project due to the strict closure of the Canadian state in the first year of the corona pandemic. As a result, the project consortium lost a central project partner with expertise in the areas of fermentation scaling, chitin production from pilot to industrial scale and accompanying quality assurance.

In addition, a complete lockdown of approximately 1.5 months was imposed on the TUM in spring 2020 due to the global corona pandemic, after which only half-day laboratory work was permitted under very difficult conditions as part of the hygiene concept, which had a lasting negative impact on and delayed the scientific progress of the project.

2 Materials and Methods

All chemicals were supplied from Sigma-Aldrich (Darmstadt, Germany), general consumables were obtained from VWR (Darmstadt, Germany). All necessary buffers and enzymes for next generation genome sequencing were shipped from Pacific Biosciences (Menlo Park, CA, USA). High molecular weight DNA was extracted with the Quick-DNA™ HMW MagBead Kit from Zymo Research (Freiburg, Germany) and HMW gDNA shearing was conducted with g-TUBEs (Covaris, Woburn, MA, USA) according to the manufacturer's manual.

2.1 Buffers and Media

Chitinase Screening Medium

Table 1: Chitinase screening medium, adapted and modified from Lee et al. and Singh et al.[148,149]:

Ingredient	Amount (g/L)
Colloidal chitin	20
K_2HPO_4	0.7
KH_2PO_4	0.3
$FeSO_4$ x $7H_2O$	0.01
$MgSO_4$ x 5 H_2O	5
Agar (for plates)	20
diH_2O	Add 1 L

After autoclaving, 0.001 g/L $ZnSO_4$ and $MnCl_2$, respectively, were added from sterile filtrated stock solutions prior to pouring of agar plates/inoculation of liquid media.

2.2 Bacterial Strains

The bacterial strains B2 ("*J.n.*") and A1 ("*J. sp.*") are both members of a novel species *Jeongeupia wiesaeckerbachi*, which was isolated, biochemically characterized, and whole genome sequenced during this dissertation. Their genomes can be accessed at NCBI under the BioSample IDs SAMN35557021 and SAMN35557022, respectively. *Jeongeupia* is a genus of gram-negative, rod-shaped soil bacteria, belonging to the family of *Chromobacteriaceae*, in the order of Neisseriales as part of the Betaproteobacteria phylum. By virtue of their cellulose[150] and chitin[151] hydrolyzation capabilities, *Jeongeupia* spp. bacteria are promising research subjects for waste stream valorization studies.

2.3 Colloidal Chitin Preparation

Colloidal chitin (CC) was synthesized following the methodology of Murthy and Bleakly[152] with slight adjustments. In this process, 20 g of crab shell chitin powder (Sigma-Aldrich, Darmstadt, Germany) was gradually introduced into 150 ml of 37% HCl under gentle agitation, leading to an increase in solution viscosity. Upon achieving a suitable viscosity reduction, additional chitin was cautiously added. The resulting mixture was then subjected to incubation at room temperature for 2-3 hours with moderate stirring, ensuring the avoidance of bubble formation. Subsequently, the non-viscous chitin solution, displaying a deep brown colour, was slowly poured into a 5 L glass beaker containing 2 L of ice-cold diH_2O, and vigorous stirring ensued. This led to the swift transformation of the solution into white colloidal chitin, which underwent overnight incubation at 4°C without stirring. The next day, the colloidal chitin was neutralized by adding abundant quantities of deionized water and subjected to centrifugation using a Beckman JLA8.1000 rotor for 15 minutes at 10,000 g until the supernatant reached a pH of 5. The resulting CC was harvested, autoclaved, and stored in a refrigerated environment until its utilization in liquid chitinase screening media (CSM) or agar plates.

2.4 Soil Screening for Chitinolytic Organisms

Soil samples were gathered in sterile 50 ml falcon tubes and standardized to a mass of 60 g before being transferred into 250 ml glass beakers. In the case of completely desiccated collected soil, tap water was introduced. Following this, the samples were supplemented with either 1% or 10% w/w colloidal chitin or crab shell chitin powder (Sigma-Aldrich, Darmstadt, Germany) and covered with tin foil. Subsequently, the samples were subjected to incubation at ambient temperature for a duration of two weeks. Sub-portions of the amended soil samples were then transferred to sterile 50 ml falcon tubes and adjusted to a volume of 50 ml with sterile 1x PBS. The soil samples were subjected to further incubation in a thermal shaker operating at 30°C and 600 rpm for 30 minutes. Supernatants were subsequently streaked out on CSM agar plates with varying pH levels (5.5, 6, 6.5, 7, 8) using inoculation loops and then incubated at 28°C for a period of 2-3 days. Colonies exhibiting halo formation were streaked onto separate CSM agar plates corresponding to the respective pH until axenic strains were successfully isolated.

2.5 Genomics

2.5.1 HMW genomic DNA extraction, HiFi (High-Fidelity) Library Preparation and Genome Sequencing with PacBio Sequel IIe

Individual bacterial colonies were carefully selected from CSM agar (pH 6.5) and then cultivated in 20 ml of Tryptic Soy Broth medium within 150 ml baffled shaking flasks. The

incubation was carried out overnight at 28°C with agitation at 120 rpm. Subsequently, high molecular weight genomic DNA (HMW gDNA) extraction was performed using the Quick-DNA HMW MagBead Kit (Zymo Research, Freiburg, Germany) as per the provided instructions. To evaluate the quantity and purity of the obtained DNA, measurements of the 260/280 nm absorption ratios and concentrations were conducted using a photometer (Nano Photometer NP80, IMPLEN, Munich, Germany) and a Qubit 4 fluorometer with the Qubit 1X dsDNA HS Assay-Kit (Thermo Fisher Scientific, Waltham, MA, USA), respectively. The high molarity of the gDNA was confirmed by analyzing fragment sizes through a Femto Pulse capillary electrophoresis instrument (Agilent Technologies, Santa Clara, CA, USA).

After passing the quality control, 8 µg of gDNA in 150 µl Elution Buffer was subjected to shearing using g-TUBEs (Covaris, Woburn, MA, USA) at 1,700 g in a tabletop centrifuge, resulting in DNA fragments with an approximate size of 12 kbps, as verified by Femto Pulse analysis. Following this, HiFi libraries were generated following the SMRTbell prep kit 3.0 manual, where barcoded adapters were fused to the samples (Pacific Biosciences, Menlo Park, CA; USA). The prepared libraries were stored at -20°C until the day of sequencing, during which the primers and polymerase were employed, and the samples were bound with the Sequel II Binding Kit 3.2 (Pacific Biosciences, Menlo Park, CA, USA), following the manufacturer's recommendations closely.

Whole genome sequencing was executed on a Sequel IIe platform (Pacific Biosciences, Menlo Park, CA, USA) using a single SMRT cell. The sequencing process involved the following specific parameters: two hours of pre-extension, followed by two hours of adaptive loading with a target of p1 + p2 = 0.95 to achieve a final on-plate concentration of 85 pM. The signal detection was performed for a continuous period of thirty hours during the movie window [153].

2.5.2 Genome Assembly, Annotation and Quality Control

After demultiplexing the raw read files using the SMRT link software (version 11.0.0.144466), thus separating the barcoded reads, the resulting FASTQ files were subjected to genome assembly using Canu v2.0 [154]. The assembly process considered an estimated genome size of 3.8 mega base pairs (mbps), and the *-pacbio* parameter was applied while keeping other settings at their standard values.

Subsequently, the assembled genome was annotated using NCBI's Prokaryotic Genome Annotation Pipeline (PGAP) [155–158]. Within this pipeline, gene prediction was performed using GeneMarkS-2+ [159], and the functional identification of proteins was accomplished using the TIGRFAMs databases [160–163].Genome qualities were assessed through CheckM [164] and BUSCO v.5.3.2 [165], based on near-universal single-copy orthologs. The tool CheckM was executed through Protologger, which is part of the Galaxy network [166].

2.5.3 Biocomputational Genome Analyses

All bioinformatic analysis tools deployed for genome and encoded protein evaluation can be found in Table 2.

Table 2: Biocomputational software utilized for genome evaluation with the utilized environment (and website if browser-based), respective application and reference. GO = gene ontology, COG = Cluster of Orthologous Proteins, KO = KEGG Orthology, CAZyme = carbohydrate-active enzyme, ORI = origin of replication.

Software	Environment/ Website	Application	Ref.
BlastKOALA	KEGG server https://www.kegg.jp/blastkoala/	Function annotation (KO terms)	[167]
ClustalW	GenomeNET https://www.genome.jp/tools-bin/clustalw	Protein alignment and integrated phylogenetic tree creation with ETE3 3.1.2 and FastTree or PhyML 3.0	[168–171]
dbCAN 3.0	https://bcb.unl.edu/dbCAN2/blast.php	CAZyme prediction	[172]
DoriC 12.0	http://tubic.tju.edu.cn/doric/	ORI finder	[173]
eggNOG Mapper 5.0	Galaxy web platform https://usegalaxy.eu/	Functional annotation (GO terms, COG terms)	[174]
FastTree 2.1.8	GenomeNET	Phylogenetic tree	[168,175]
LipoP 1.0	https://services.healthtech.dtu.dk/services/LipoP-1.0/	Protein localization prediction	[176]
PHASTER	http://phaster.ca/	Phage region prediction	[177]
progressiveMauve	Geneious Prime 2022.0.1	Whole genome alignment	[178]
SecretomeP 2.0	https://services.healthtech.dtu.dk/services/SecretomeP-2.0/	Non-classical protein secretion prediction	[179]
SignalP 6.0	https://dtu.biolib.com/SignalP-6	Signal peptide prediction (classical)	[180]
SWISS MODEL	https://swissmodel.expasy.org/	Protein modelling and functional prediction	[181,182]
TYGS	https://tygs.dsmz.de	Whole genome and 16s rRNA sequence-based bacteria species identification	[183–191]

2.6 Proteomics

2.6.1 Cultivation conditions

Initially, the organism was streaked out onto CSM-agar (pH 6.5, 2% CC (w/v)) from axenic cryostocks containing 50% v/v of 50% v/v glycerol solution. For precultures, single colonies were selected and inoculated into 150 ml baffled shaking flasks containing 20 ml of Tryptic Soy Broth. Incubation was carried out at 28°C and 120 rpm overnight, and cell densities were measured spectrophotometrically at 600 nm using 2 ml cuvettes (Nano Photometer NP80, IMPLEN, Munich, Germany).

Intracellular protein investigation involved preparing main cultures in 500 ml baffled shaking flasks with 50 ml of either CSM (pH 7, 2% CC (w/v)) or modified CSM with 0.5% (w/v) glucose and 1% (w/v) $(NH_4)_2Cl_2$ instead of colloidal chitin. Bacterial cells were washed twice in sterile phosphate-buffered saline (PBS) before inoculating the media to an OD_{600} of 0.05. Incubation conditions were maintained at 28°C and 120 rpm for one day (glucose-fed) or three days (chitin-fed) to obtain sufficient cell mass. Both carbon sources (glucose or colloidal chitin) were tested in biological quadruplicates.

2.6.2 Extracellular Proteome Preparation

For extracellular protein investigation, 500 ml CSM in 5 L baffled shaking flasks were inoculated with *J. w.* to an OD_{600} of 0.05 in biological triplicates. Additionally, one flask contained CSM with 2% (w/v) processed crab shell chitin (unbleached) as the sole C and N source. After three days of incubation at 28°C and 120 rpm, cultures were centrifuged at 10,000 g for 10 min, and supernatants were filtered with a 0.22 µm syringe filter and concentrated using a tangential flow filter membrane (MWCO 10 kDa; Omega 10K Membrane, Pall Cooperation, New York, USA) and a peristaltic pump (Masterflex P/S Model 910-0025, Thermo Scientific, Menlo Park, USA) to a volume of 10-15 ml. Subsequently, 10 kDa MWCO centrifugal filter units (Centriprep, Merck Millipore, Darmstadt, Germany) were applied to further concentrate the secreted crude enzyme mixtures to a final volume of approximately 1 ml per sample. Protein concentrations were measured with a photometer based on 260/280 nm absorption ratios (Nano Photometer NP80, IMPLEN, Munich, Germany). Of these protein extracts, 15 µl was transferred into a new reaction tube, mixed with 5 µl 4x SDS-sample buffer, and boiled for 5 min at 95°C.

2.6.3 Intracellular Proteome Preparation

Whole cell protein extraction was based on an adapted protocol from Engelhart-Straub and Cavelius et al. [192]. It involved harvesting bacterial cultures by centrifugation at 8,000 g for 10 min, washing the cells with 5 ml of sterile PBS, and inducing cell lysis by horizontal vigorous

shaking (Vortex Genie 2, Scientific Industries, Bohemia, NY; USA) for 30 min with fine glass beads, supported by 1:3 (v/v) Protein Extraction Reagent Type 4 (Sigma-Aldrich, St. Louis, MO, USA). After centrifugation at 14,000 g for 30 min, protein precipitation was achieved by addition of 1:1 (v/v) 20% trichloroacetic acid in HPLC-grade acetone (w/v) solution, supplemented with 10 mM DL-1,4-Dithiothreitol (DTT). Samples were vigorously vortexed and then incubated at -20°C for one hour. Following centrifugation at 14,000 g for 10 min at 4°C, protein pellets were washed twice with HPLC-grade acetone supplemented with 10 mM DTT and air-dried under a sterile bench. Lastly, protein pellets were resuspended in 450 µl 8 M urea with 10 mM DTT and homogenized with a micro pestle suitable for 2 ml micro reaction tubes. Of this protein extract, 15 µl was transferred into a new reaction tube, mixed with 5 µl 4x SDS-sample buffer, and boiled for 5 min at 95°C.

2.6.4 Tryptic In-Gel Digestion and LC-MS/MS Analysis

Tryptic in-gel digestion and LC-MS/MS analysis were carried out following the protocol from Fuchs et al. and Engelhart-Straub and Cavelius et al. [192,193], using a timsTOF Pro mass spectrometer coupled with a NanoElute LC System (Bruker Daltonik GmbH, Bremen, Germany) equipped with an Aurora column (250 × 0.075 mm, 1.6 µm; IonOpticks, Hanover St., Australia). One-dimensional 12% SDS PAGEs with short stacking gels were deployed to transfer 10 µl of each whole cell protein extract into the resolving gel matrix. Leaving several wells empty between the different samples was pivotal to prevent sample migration.

The mobile phase consisted of two solvents for reverse-phase chromatography: (A) 0.1% formic acid, 2% acetonitrile in water and (B) 0.1% formic acid in acetonitrile, which was added linearly with a constant flow rate 0.4 µl/min. Both separation cycles started at 2% of B (v/v). For the less complex, extracellular protein mixtures, a short gradient was carried out: t = 25 min & 17% B (v/v), t = 27 min & 25% B, t = 30 min & 37% B, with t = 33 min and a hold at 95% B for ten more minutes. In case of the more complex, intracellular protein compositions, a longer separation cycle of 100 min was selected: t = 60 min & 17% B, t = 90 min & 25% B, t = 100 min & 37% B, t = 110 min and 95% B with a hold at 95% B for ten more minutes. The oven temperature was kept at a constant 50°C during measurements.

2.6.5 Biocomputational Evaluation of Proteomic Datasets

Refer to the publication “Proteomic and transcriptomic analyses to decipher the chitinolytic response of *Jeongeupia* spp.” [194] for an in-detail description of the biocomputational evaluation of the proteomic datasets, revolving around the PEAKS studio software 10.6 and our in-house sequenced, assembled and annotated *J. wiesaeckerbachi* genomes. Additional analyses involved the browser-based dbCAN 3.0 [172] for carbohydrate-active enzyme identification and the browser-based functional annotation BlastKOALA on the Kegg server [167].

2.7 Transcriptomics

2.7.1 RNA-Extraction

For RNA extraction and quality control, bacterial cells were harvested by centrifugation at 6,800 g for 10 minutes. Total cell RNA was isolated following the recommendations of the SV total RNA Isolation System Kit (Promega, Madison, WI, USA). The purity and quantity of the obtained RNA were assessed using a photometer based on 260/280 nm absorption ratios (Nano Photometer NP80, IMPLEN, Munich, Germany). Additionally, quality assessment was performed using the Qubit 4 fluorometer and the Qubit RNA IQ Assay-Kit (Thermo Fisher Scientific, Waltham, MA, USA). The experiment was conducted in biological triplicates, but only two samples per condition (chitin or glucose-containing media) were analyzed. Samples were selected based on Qubit derived RNA quality numbers and RNA concentrations.

2.7.2 cDNA Library Preparation, Next-Generation Sequencing and Evaluation

Next-generation sequencing and bioinformatic analysis were carried out by EuroFins Genomics Europe Sequencing GmbH (Konstanz, Germany). The process included rRNA depletion, cDNA library construction, next-generation sequencing using the Illumina NovaSeq platform (6000 S4 PE150 XP mode), and subsequent bioinformatic analyses. Raw sequencing data were first purified of rRNA reads using RiboDetector [195]. Subsequently, adapter trimming, quality filtering, and per-read quality pruning were performed using fastp [196]. High-quality reads were aligned to the provided *J. wiesaeckerbachi* genome with STAR [197]. Gene-wise quantification was achieved by evaluating transcriptome alignments using the software featureCounts [198]. Differential gene expression analysis between the glucose-fed and chitin-fed sample groups was conducted using the R/Bioconductor package edgeR [199]. Variant calling for SNP and InDel assessment was carried out using Sentieon's Haplotype-Caller [200].

3 Research

3.1 Summaries of Included Publications

Isolation, biochemical characterization, and sequencing of two high-quality genomes of a novel chitinolytic *Jeongeupia* species

The article “Isolation, biochemical characterization, and sequencing of two high-quality genomes of a novel chitinolytic *Jeongeupia* species” was published in MicrobiologyOpen in July 2023 (DOI: 10.1002/mbo3.1372). The author of this thesis, Nathanael David Arnold, designed and carried out the experiments, evaluated the experimental data and wrote the manuscript.

The aim of the study was to screen for and identify novel and efficient chitinases as part of the “ChitoMat” project in cooperation with the Fraunhofer Institute for Interfacial Engineering and Biotechnology IGB, the Atlantic Veterinary College and Atlantic Biofabrication Laboratory at the University of Prince Edward Island and CigalaTech (Charlottetown, Canada).

The polysaccharide chitin is the second most abundant biopolymer on earth after cellulose, with which it shares a highly similar chemical structure. Although accumulation in marine and soil sediments is prevented by saccharification through fungi or bacteria, the excessive amounts of crustacean shell waste from fisheries often being disposed into landfills or coastal areas can damage ecosystems.

With the myriad of possible applications for chitin and its derivative, chitosan, exhibiting antitumoral, antifungal, antibacterial and anti-inflammatory characteristics, the rising global demand for these raw materials becomes evident. However, commonly applied chitin extraction and conversion methods rely on HCl and NaOH, with hazardous by-products and low product specificity. Therefore, exploration of novel chitin-enacting enzymes is critical to drive biotechnological chitin valorization forward.

In this study, to isolate novel chitinolytic organisms, soil samples were amended with varying amounts of chitin and incubated for a week. Thus, the abundance of species with the capability to hydrolyze, import and accumulate chitin could be enhanced. Modulated soil samples were sequentially streaked out onto colloidal chitin containing agar plates, with halos around colony forming units indicating chitinolytic activities. in addition to reported chitin decomposers, this approach yielded two strains assigned with the names “*J.n.”* and *“J.sp*.” based on 16S rRNA informed identification as *Jeongeupia naejangsanensis* BIO-TAS4-2.

Biochemical characterization of the two strains revealed several differences in regard to substrate utilization capabilities compared to the type strain BIO-TAS4-2.

With the long-term goal of recombinant expression in mind, high quality genomes were required, allowing for reliable primer development and enzyme characteristics prediction. Therefore, genomes of the investigated strains were sequenced with the long-read platform PacBio Sequel IIe, resulting in 40-fold genome coverages for both bacteria. Assembly with Canu 2.0 and analysis with the orthologue based bio-computational tools BUSCO 5.3.2 and CheckM confirmed the high completeness and low contamination of the obtained circular genomes, approximately 3.8 Mbps in size. Annotation was performed by NCBI's Prokaryotic Genome Annotation Pipeline (PGAP), which implements a combination of sequence homology and functional prediction of proteins informed by TIGRFAM databases. Further, it assesses the average nucleotide identity (ANI) as standard procedure, a widely accepted method to identify bacteria with, which suggested for the strains to be BIO-TAS4-2 with inconclusive results.

Earlier assumptions, that the novel strains were indeed a novel species, were further supported by whole-genome sequence-based phylogeny at the Type (Strain) Genome Server (TYGS).

In-silico genome analysis with dbCAN3.0 revealed the unusually rich chitinolytic machinery of the novel strains, including 13 glycoside hydrolases of family 18 (GH18), three GH19 and GH23 each, and a lytic polysaccharide monooxygenase of auxiliary activity family 10 (AA10). A ClustalW inferred phylogenetic tree could demonstrate the conserved nature of this chitinolytic system within the genus of *Jeongeupia*.

Based on these results, the discovered strains were proposed to represent members of a novel species *Jeongeupia wiesaeckerbachi*.

Proteomic and transcriptomic analyses to decipher the chitinolytic response of *Jeongeupia* spp.

The article “Proteomic and transcriptomic analyses to decipher the chitinolytic response of *Jeongeupia* spp.” was published in Marine Drugs in August 2023 (DOI: 10.3390/md21080448). The author of this thesis, Nathanael David Arnold, designed and carried out the experiments, evaluated the experimental data and wrote the manuscript.

As a follow-up on the previous publication, in which the novel chitinolytic gram-negative bacterium *Jeongeupia wiesaeckerbachi* was isolated, genome-sequenced, and biochemically characterized, this study had the aim to decipher its chitinolytic machinery.

To illuminate the underlying cellular and extracellular processes during chitin metabolism, a three-way systems biology approach was chosen. First, exported enzymes during cultivation in colloidal chitin minimal medium were harvested, concentrated, and subsequently analyzed with MS/MS tandem spectrometry. Second, intracellular proteomic analyses were conducted with cells offered either glucose- or colloidal chitin containing media. Third, differential carbon source growth conditions were repeated, followed by RNA extraction and next generation sequencing for transcriptomic analyses.

Secretome analysis of the tryptic digested proteins uncovered 386 common proteins, that were detected among all four samples, comprising of colloidal chitin triplicates and a singular sample grown on unbleached crab shell flakes. On the background of potential false-positive proteins through cell lysis, *in silico* signal peptide and cellular localization prediction were performed, resulting in 192 confirmed translocated proteins, some of which through non-classical secretion pathways. Interestingly, most of the previously predicted chitinolytic enzymes could be confirmed extracellularly.

Data of the intracellular proteome suggested enhanced biosynthesis of glucosamine utilization and general cell maintenance proteins over hydrolysis involved enzymes as observed in the secretome. Hereby, a total of 203 proteins were upregulated in colloidal chitin provided bacterial cells compared to glucose-fed samples.

Lastly, evaluation of the differential transcriptomes revealed the upregulation of 600 transcripts and distinct mRNA expression patterns, with a seeming emphasis on stress- and pili-related genes. Comparison with the corresponding intracellular proteomics dataset showed a relatively low correlation, most likely due to the temporal separation between transcription and protein biosynthesis. Furthermore, other studies demonstrated that chitin metabolism related mRNAs are transcribed in early cell growth phases, as soon as 4 hours, which was significantly earlier than the sample time of this study.

Overall, the upregulation or involvement of over 350 unique proteins and 570 unique genes in the chitinolytic system of *Jeongeupia wiesaeckerbachi* was supported by the synergistic systems biology approach, which is far more complex and extensive than previously assumed. Based on the cumulative data, a holistic model for the chitinolytic system of *Jeongeupia* spp. was proposed, advancing our knowledge of natural chitin saccharification systems. Additionally, promising candidate enzymes for expression and characterization studies were uncovered, expanding the tool kit for biotechnological chitin valorization.

3.2 Full Length Publications

Isolation, biochemical characterization, and sequencing of two high-quality genomes of a novel chitinolytic *Jeongeupia* species

Proteomic and transcriptomic analyses to decipher the chitinolytic response of *Jeongeupia* spp.

4 Discussion and Outlook

4.1 Insights into the Chitin Utilization Program of *Jeongeupia*

In our studies regarding the novel chitinolytic bacterium *Jeongeupia wiesaeckerbachi*, we could reproduce the promotion of chitinolytic bacteria growth through chitin amendment of soil [201], which has led to discovery of rare (actinomycetes) species previously [202]. Contrarily, decrease of chitinase copy numbers and even diversity in actinomycetes populations has also been reported when supplementing soils with chitin or sludge [203–205].

As mentioned previously (1.5), Keyhani and Roseman [117] proposed five general steps of bacterial chitin assimilation: (1) sensing and chemotaxis towards the chitinous substrate, (2) pili-mediated attachment to the polysaccharide's surface, (3) hydrolysis of the polymer chain into smaller COS through secretion of chitinases, with the disaccharide $(GlcNAc)_2$ as main product, (4) uptake of the soluble chitin fragments and lastly, (5) cleavage of the disaccharide

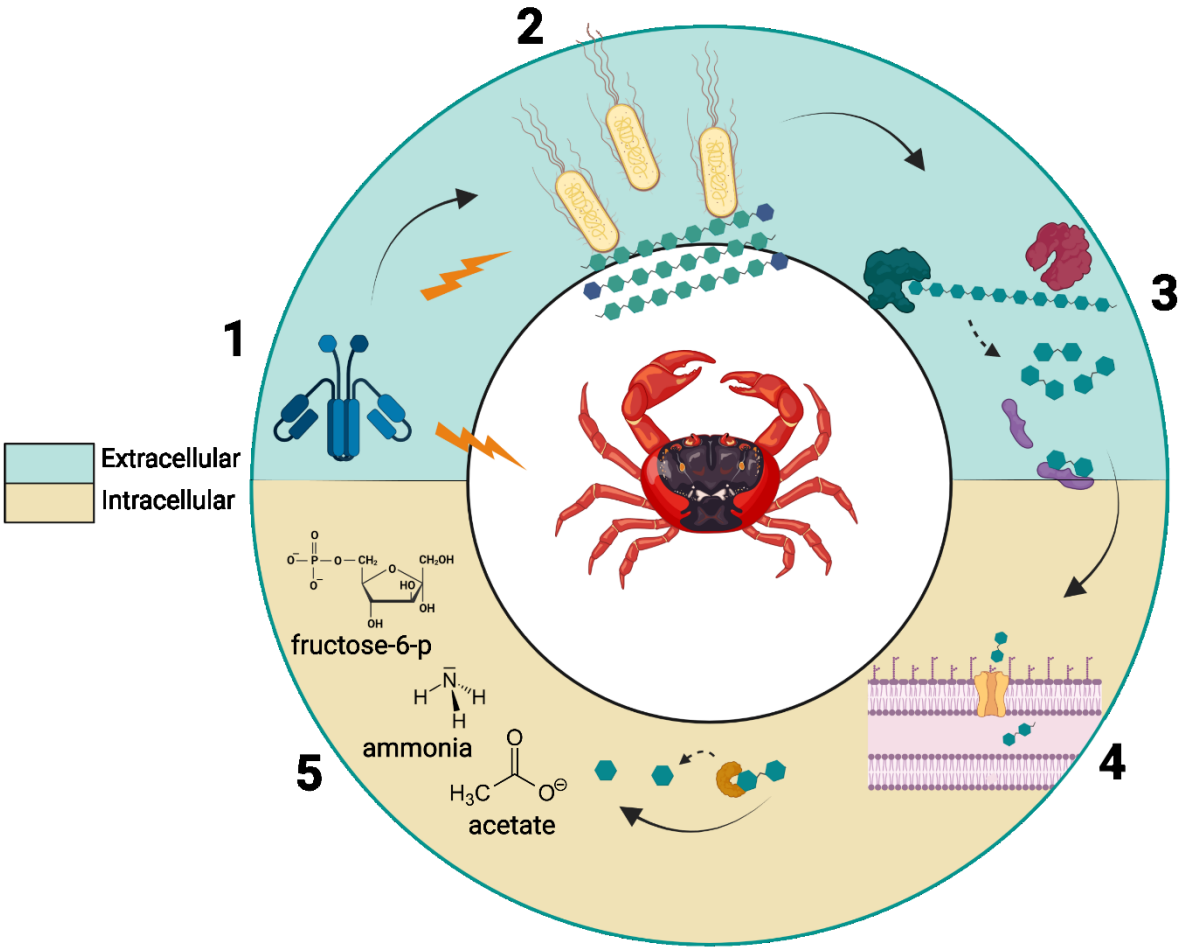

Figure 4: Schematic flow-chart of bacterial chitin utilization. (1) Sensing and chemotaxis, (2) attachment, (3) hydrolysis, (4) uptake and (5) metabolism of N-acetylglucosamine to fructose-6-phosphate, acetate and NH_3. Figure created with BioRender.

to monomers in the periplasm and conversion of GlcNAc to fructose-6-P, acetate and NH_3 in the cytoplasm (Figure 1). Our differential transcriptome and proteome investigations were generally in alignment with this hypothesis. Refer to Table 3 for an overview.

To that end, (1) could be confirmed by upregulation of several (methyl-accepting) chemotaxis-related proteins, presumably responsible for chitin sensing, flagellum biosynthesis and consequently movement towards the substrate [206]. Moreover, step (2) was apparent both under the microscope, revealing attachment of *J. wiesaeckerbachi* cells to the colloidal chitin particles, and by means of highly upregulated pili-associated proteins, which were shown to confer a significant growth advantage to *V. cholerae* cells on chitin surface [136]. Hydrolysis (3) was initially observed through lysis zones on the chitin agar plates, in shaking flasks, and furthermore supported by detection of glycosidase hydrolases in the secretome and intracellular proteome data. Breakdown of the polysaccharide chain into soluble COS represents a continuous multistage process, requiring secretion of synergistic endochitinases, exochitinases, and a lytic polysaccharide monooxygenase (LPMO) as well as the expression of hexosaminidases for monomer production intracellularly [126]. Expectedly, all aforementioned enzymes could be detected in the secretome of *J. wiesaeckerbachi,* with few hexosaminidases and a single chitinase present inside the bacteria cells [194].

Upregulation of putative chitobiose ABC transporters, their respective substrate-binding proteins and permeases were strong indicators for the COS import-system (4) of *J. wiesaeckerbachi*. Additional upregulated and uncharacterized porins or ABC transporters and associated substrate-binding proteins represent promising candidates as putative COS importers, as reported before [119,207,208]. Atypically, in actinomycetes, the phosphotransferase system (PTS), utilizing phosphoenolpyruvate (PEP) as phosphate donor, plays a crucial role for carbon sensing and import of amino sugars and fructose [209], instead of glucose. While the PTS-associated glucose transporter unit IIBC (gene ID 1296) was upregulated 1.6-fold intracellularly under chitin in our dataset (1.3-fold for GlcNAc IIBC, gene ID 3368), it remains unclear, whether the PTS in *Jeongeupia* spp. is equally biased towards GlcNAc as reported for *Streptomyces* spp. [209,210], or rather specialized for deacetylated GlcN uptake as seen in *Vibrio* spp.[211]. Detection of the 1.4-fold upregulated phosphoenolpyruvate-protein phosphotransferase (gene ID 982) and the 2-fold upregulated protein HPr (**h**istidine-containing **pr**otein; gene ID 1703), which donates phosphoryl to the membrane-associated EII transporters, underpins the impression, that the PTS might be involved in $(GlcNAc)_2$ import in *Jeongeupia* spp., similarly to *E. coli* [212].

Although no metabolomic analyses were conducted, which could have provided unequivocal evidence for the presence of certain chitin metabolites, the conversion of GlcNAc to fructose-6-P, acetate and NH_3 [117,210] (5) were indirectly inferred by upregulation of several proteins. In

this regard, 25-fold upregulation of a glutamine amidotransferase (gene ID 419) and 9-fold upregulation of an isomerizing glutamine-fructose-6-phosphate transaminase (gene ID 2381), support conversion of GlcNAc to fructose-6-P. Glucosamine monomer phosphorylation may be executed by the 2.5-fold upregulated glucosamine kinase (gene ID 444) intracellularly. Deacetylation of *N*-acetylglucosamine to glucosamine, releasing acetate (Ac^-) in the process, was indicated by detection of an 8-fold intracellularly upregulated polysaccharide deacetylase family protein (gene ID 281). Additionally, likewise 8-fold upregulation of an acetate CoA ligase (gene ID 2139) hints at implementation of the generated acetate into the pyruvate and ultimately citrate acid cycle (TCA) in the form of acetyl CoA.

Table 3: Overview *of J. wieseackerbachi* genes involved in chitin metabolism. The combined results were obtained from intracellular transcriptomics and proteomics, as well as extracellular proteomics under chitin (vs. glucose if applicable).

↓ Process	Upregulated genes under chitin		
	Gene IDs		Functions
1. Chemotaxis	36, 134, 135, 138, 246, 602, 1506, 1614, 1841, 2218, 3352, 3077, 3576		Methyl-accepting chemotaxis proteins (mcp), receptor-modulating/coupling enzymes, flagellar motor proteins, sensor histidine kinase
2. Adherence	1589, 1590		Flp family type IVb pilin
3. Hydrolysis	148, 269, 302,306, 366, 371, 372, 389, 635, 680, 836, 837, 1077, 1323, 1504, 1731, 1732, 1746, 1841, 2137, 3083		Glycoside hydrolases of families 3, 18, 19 and 20, and a lytic polysaccharide/chitin monooxygenase
4. Import	440 – 442, 982, 1703, 2871, 3368		Chitobiose binding proteins and permeases, PTS associated proteins, porins
5. Assimilation	**Glucose-6-P**	419, 444, 2381	Glutamine amidotransferase, glucose-6-P transaminase, glucosamine kinase
	Acetate	281, 2139	Polysaccharide deacetylase, acetate CoA ligase
	NH_3	337, 1335, 1757, 2225, 3265	Nitrogen regulators, nitrate reductase subunit b, nitrogen stress response genes

Regarding the chitin derived NH_3 production and concomitant nitrogen metabolism, we found the nitrogen regulation protein NR(I) (gene ID 1757) to be upregulated above 2-fold, which induces the transcription and ultimately formation of glutamine synthetase [213]. The latter was upregulated 6.5-fold and is a telltale sign of a low nitrogen regiment in *E. coli*, responsible for the ammonium assimilation into amino acids [214,215]. Glutamine synthetase activity level enhancement is modulated by adenylylation, which is mediated by P_{II} family nitrogen regulator proteins (upregulated approx. 3-fold, gene ID 1335), due to their function as 2-oxoglutarate master regulator metabolite sensors [216,217]. In this process, P_{II} are first activated by uridylylation

through a P_{II}-uridylyltransferase (upregulated 2-fold, gene ID 3265), before the glutamine synthetase is then adenylated. Evidence for this process was provided by 1.6-fold upregulation of a bifunctional [glutamate-ammonia ligase]-adenylyl-L-tyrosine phosphorylase/ adenylyltransferase (gene ID 2225). Further evidence for nitrogen starvation could be inferred from the detection of the 10-fold upregulated serine/threonine kinase PrkA, whose orthologue's role in *E. coli*, yeaG, is well understood in the nitrogen stress response [214]. Fifty-fold upregulation of a nitrate reductase beta subunit (gene ID 337) indicates how *Jeongeupia* spp. appears to seek out nitrate as preferred nitrogen source, when no glucose is present. These observations are in agreement with transcriptome investigations of a pathogenic *Serratia marcescens* strain [218], which revealed the significant upregulation of a nitrate reductase and other nitrogen metabolism related proteins.

Moreover, a study regarding a marine *Chitinibacter* sp. SCUT-21 isolate's chitin utilization program [219] demonstrated N-selectivity towards nitrate (NO_3^-) when offered chitin as sole carbon source. When provided with glucose in addition to chitin, the effects of carbon catabolite repression (CCR) were so profound, that cells could not grow without the supplementation of another nitrogen source, which also held true for our investigated *J. wiesaeckerbachi* strain [219].

Inspection of underlying metabolic pathways unraveled the involvement of the citrate (first carbon oxidation) and glyoxylate cycles, in addition to the Entner-Doudoroff and pentose phosphate pathways in chitin metabolism of *Jeongeupia wiesaeckerbachi,* according to the KEGG mapper reconstruction on our intracellular proteome data [167].

Future implementation of differential metabolomic analyses between glucose and chitin supplied bacterial cells could provide critical context of metabolic fluxes and gene expression [141]. Subtle shifts in gene transcription in response to changing nutrient availability evaded our experimental design, which only distinguished between chitin and glucose, or on and off states.

In doing so, nitrogen or glucose starvation cell states can be directly linked to the biosynthesis of functional transcripts and proteins. Furthermore, monitoring of glucosamine levels might provide important data on optimal chitinous substrate concentrations and respective fermentation durations for maximum chitinolytic enzyme expression in the context of industrial applications.

Multiplicity of bacterial chitinases and their origins

GH18 [220] and GH19 [221] are believed to have evolved independently, with little to no sequence homology and diverging catalytic mechanisms (retaining vs. inverting, respectively) [69,75]. Occurrence of multiple chitinase copies within a given genome, as is the case in our

investigated bacterium *Jeongeupia wiesaeckerbachi,* is deemed as customizable and synergistic enzyme tool kit for varying chitin allomorphs [222]. Observation of a reduced chitinolytic machinery in the secretome in response to crude crab shells (eight GH18) opposed to colloidal chitin (eleven GH18) further supports this notion [194].

In terms of their origins, strong sequence homologies of glycoside hydrolases within a single organism suggest, that these enzymes resulted from gene duplication events [135]. Conversely, when there are weaker similarities in the internal sequences or when these enzymes are found in organisms that are not closely related taxonomically, it implies that the genes were transferred laterally between different organisms [223–225]. CLUSTALW protein sequence homology alignment [170] inferred phylogenetic trees with PhyML [171] revealed both presumably from lateral gene transfer as well as gene duplication events acquired chitinases (Figure 2).

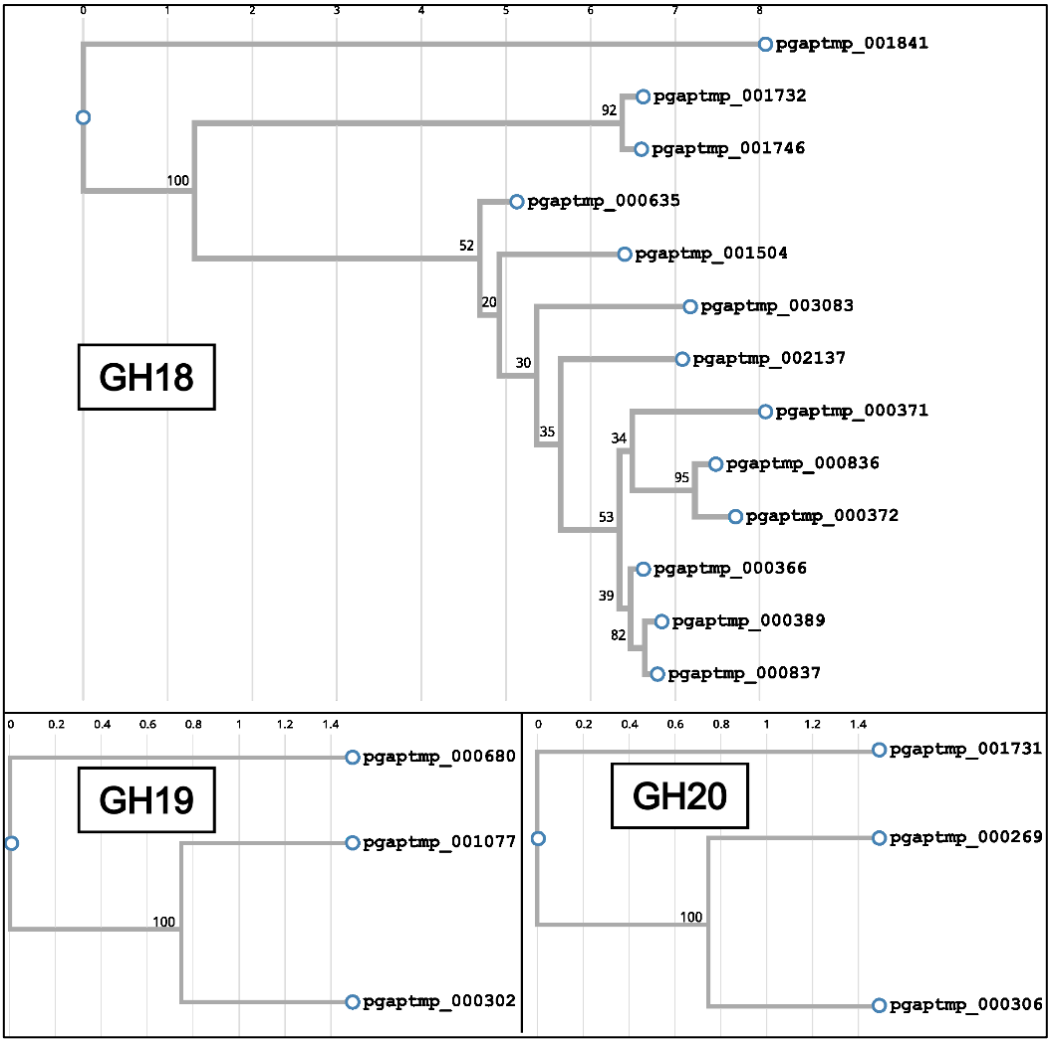

Figure 5: Amino acid sequence homologies and inferred rootless phylogenetic trees of *Jeongeupia wiesaeckerbachi*'s glycoside hydrolases (GH) of families, 18, 19 and 20. Sequence alignment was performed with CLUSTALW [138] and phylogenetic reconstructions were performed using the function "build" of ETE3 3.1.2 as implemented on the GenomeNet [168]. Tree was inferred using PhyML v20160115 [171]. Values at nodes are branch supports in %, computed out of 100 bootstrapped trees.

To that end, it is likely that glycoside hydrolase 1841 within family 18 resulted from an inter-species gene transfer, a phenomenon potentially applicable to the 1732 and 1746 pair as well. Among these, a probable gene duplication event within the genome of Jeongeupia appears more plausible. Except for the strongly branch supported GH18 pairs 389/837 and 372/836, no other dependable indications of gene duplication were discernible. Despite this, due to the shared branch encompassing all GH18 chitinases except the distinct 1841 and the separate branch of 1732/1746, the concept of a common ancestral enzyme holds merit. This view is further supported by the relatively short branch lengths (high protein sequence homologies). In the instances of chitinase families within both GH19 and GH20, comprising three hydrolases each, one enzyme's origin is linked to lateral gene transfer, while the remaining two enzymes appear to have originated from gene duplication.

Although whole-genome sequence alignments with progressiveMauve [178] uncovered several gene rearrangements between the genomes of *Jeongeupia wiesaeckerbachi* and the closely related *J. naejangsanensis* BIO-TAS4-2 [150], the chitinolytic machinery itself was highly conserved.

Implications Regarding the Complexity of the Bacterial Chitin Metabolism and Regulation

Strikingly, we could demonstrate the upregulation of unique 570 genes during chitin metabolism, opposed to the previously estimated 50-100 involved genes [118,124]. However, a distinction between direct involvement of proteins and mere interference or manipulation by the adapted metabolic pathways to a chitin-rich environment must be made. Comparable to our observations, 360 genes were reported to be significantly regulated (up- or down-) in *V. cholerae* on crab shell surface with microarray expression in 2004 [136]. Similarly, the *N*-acetylglucosamine-responsive regulator protein DasR in *Streptomyces coelicolor* was reported to additionally affect the expression of around 1200 genes [138], which aligns with our data on the differential gene recruitment during chitin metabolism [194]. Another common feature is the growth phase dependent regulatory function of DasR, as our transcriptomic analyses of *J. wiesaeckerbachi* also suggested a strong time-dependence of chitinolytic transcript biosynthesis. Hydrolysis related transcripts were largely absent at the late growth phase sampling time of the study, while respective protein levels were abundant [194]. Surprisingly, a follow up study on chitinase transcript expression levels of Δ*dasR* strains and wildtype strains demonstrated only minor differences, indicating the involvement of other forms of gene regulation [226,227].

Promising candidates for protein expression and characterization studies

While the presence and identity of a central chitin-response regulator in *Jeongeupia* remain uncertain, intracellular proteomics data suggest several potential candidates: A response regulator (gene ID 1712) was upregulated 7-fold, a YbhB/YbcL family Raf kinase inhibitor-like protein (gene ID 1299) 5.5-fold, and a HAMP domain-containing histidine kinase (gene ID 264) 4-fold on colloidal chitin. Additionally, a GntR family transcriptional regulator (gene ID 443) could be of interest, due to its proximity to a glucosamine-related gene cluster, encompassing putative chitobiase transporter proteins, a GlcNAc-kinase, and a polysaccharide deacetylase.

Further interesting candidates for protein expression or knockout experiments besides the regulatory proteins are the as intracellularly upregulated or secreted identified porins and transporter proteins (gene IDs 439 – 443, 1471, 2871, 3368), oxidoreductases (gene IDs 1157, 1215,1929, 2996), methyl-accepting chemotaxis proteins (gene IDs 246, 602, 1841, 2218 3077, 3352) and pili-associated proteins (gene IDs 1589 & 1590).

Various chitinoplastic (chitin structure-altering) proteins were detected in the novel *J. wiesaeckerbachi* genome, among them a multiplicity of chitinases, a LPMO and potentially two chitin deacetylases (CE4) (Table 4). Despite the more obvious connection between some of these enzymes and the chitin metabolism based on our findings [194], other interesting candidates exist among the 159 carbohydrate-active enzymes (CAZymes) in the genome.

Table 4: Promising carbohydrate-active enzymes (CAZymes) in the genome of *Jeongeupia wiesaeckerbachi*, detected by dbCAN 3.0. CAZy classifications marked with an asterisk (*) contain proteins that have been detected in proteomic or transcriptomic analyses under exclusive colloidal chitin feed. CBM = carbohydrate binding motif, CE = carbohydrate esterase, GH = glycosyl hydrolase, GT = glycosyltransferase. While some enzymes exhibit CBMs, they are not included in separately, but in their respective enzyme classification to avoid duplicates.

CAZy classification	Relevant included activities	Gene count
AA10*	Lytic polysaccharide monooxygenase	1
CBM 12	Chitin binding	3
CBM 32	LacNAc-binding	1
CBM 5	Chitin binding	6
CBM 5/12	Chitin binding	1
CBM 50	Chitin- or peptidoglycan- binding	4
CE4	Chitin oligosaccharide deacetylase	2
CE9	N-acetylglucosamine-6-phosphate deacetylase	1
GH18*	Chitinase	13
GH19*	Chitinase	3
GH20*	β-N-acetylhexosaminidase	3

GH23*	Chitinase	6
GH3	Chitosanase, β-N-acetylhexosaminidase	3
GH9	Exo-β-1,4-glucosaminidase	1
GT2	Chitin synthase	19

Recombinant expression and more importantly, elucidation of their modes of action, will prove their utility for tailored COS production as envisioned in the ChitoMat project.

Especially endo-processing glycoside hydrolases, but also multi-modal enzymes such as the chitosanase ScCsn46A in *Streptomyces coelicolor* A3(2) [228] are of great interest. Furthermore, novel binding specificities outside the common GlcNAc/GlcN moieties at the -1 or +1 polymer subsites for chitinases or chitinases, respectively, would expand the biotechnological toolkit immensely. An example of this would be the self-proclaimed chitinosanase of the fungus *Alternaria alternata*, with the novel subsite specificity DA/XX [229].

Additionally, the two carbohydrate esterases of family 4 with potential COS deacetylase activities are highly interesting candidates for wet lab characterization studies. Currently, enzymatic chitin deacetylation to yield defined chitosan or paCOS is hard to realize due to low conversion rates and more importantly, their inability to act on crystalline substrates, with the acetyl moieties tightly packed in the chitin structure [105]. Supplementation with LPMOs, which oxidize crystalline chitin and create free polymer chain ends on the substrate surface has been demonstrated to enhance deacetylase activities [106]. Furthermore, fusions with the chitin binding modules CBM5/12 might increase their adherence to crystalline chitin and thus enhance deacetylation rates of non-processed chitin [107].

In summary, novel enzymes with potentially unknown mechanisms or deacetylation patterns are highly desirable in the hope of understanding protein-ligand interactions and substrate specificities at a more sophisticated level, thus enabling tailor-made paCOS production.

4.2 Prospects of Chitin Valorization

The trajectory of chitin valorization in the coming years will be shaped not only by the advancement of ecologically sound techniques for its extraction and processing, but also by the emergence of innovative applications and technologies integrating chitin-derived materials. Moreover, shifts in consumer behavior, particularly the growing demand for bio-based products, will serve as a catalyst compelling the cosmetics and food industries to contemplate the incorporation of chitin or chitosan as fundamental constituents.

Green Chitin Extraction Methods - Lactic Acid Fermentation and NADEs

At this point in time, it is well established that lactic acid fermentation is an excellent sustainable method to demineralize and deproteinate high quality chitin from crustacean shell waste [10,230–234], suitable for the industrial scale [10]. Advantages over conventional chemical methods include low costs, no requirement for wastewater treatment and consequently the feasibility of waste stream (mainly peptides, free amino acids and astaxanthin) valorization [5,235] e.g. for the food [236], cosmetics [237] or medical industries [238].

Natural deep eutectic solvents (NADEs), composed of natural primary metabolites (amino acids, organic acids, sugars, or choline derivatives) represent another promising, innovative and green path to extract chitin from crustacean shell waste [239–248]. They arise from the intricate interaction between a hydrogen acceptor (mostly quaternary ammonium salts) and a hydrogen-bond donor (amines, amides, polyols, or acids). Subsequent charge distribution is accountable for the observed reduction in the mixture's melting point in comparison to the individual melting points of the constituent raw materials [240,249]. Functionally, NADEs fulfill the role of biocatalytic solvents and can be utilized as multipurpose media for carbohydrate processing [244], including pretreatment, chemical modification or extraction.

Due to their reusable, non-toxic, economical, and often biodegradable characteristics [244], NADEs are interesting chemicals to consider for industrial crustacean shell waste processing, possibly complementary to lactic acid fermentation demineralization and deproteination.

Huang et al. demonstrated shrimp shell demineralization and deproteinization with a NADE composed of choline chloride and malic acid combined with microwave radiation. Optimal yields chitin yields of 20% [248] were obtained with an 1:20 shell to NADE ratio and 9 min of radiation [239]. Interestingly, the resulting chitin was of higher crystallinity compared to alkali treated shrimp shells. Additionally, they could reuse the NADE up to three times without negatively affecting the chitin yields when supplementing malic acid in between extraction rounds, which was diminished during demineralization [239]. At that point in time, the deproteinization rates decreased significantly. After 5 cycles, the sheer extracted protein amounts rendered the solution too viscous for further deployment in chitin extraction. It might however represent an interesting feed source for subsequent biotechnological conversion into lipids by oleaginous yeasts [250] or serve as high value feed additive in livestock or fish farms. There is an urgent need to reduce the use of soybean meal in feed formulations in order to free up arable lands for human food production, thereby increasing food security [251].

This report exemplifies the potential as chemical but environmentally friendly crustacean shell waste method, without the necessity of large fermenters in the case of lactic acid fermentation. On the other hand, the requirement for microwave radiation assistance in successful shell

demineralization could render this method impracticable and highly energy consuming for the industrial scale.

Biotechnological COS production

Physiochemical attributes play a pivotal role in the biological functionalities of chitosan oligosaccharides (paCOS). Consequently, meticulous control over the manufacturing procedure is imperative. Enzymatic methodologies excel in this regard, exhibiting significantly augmented reaction specificity in comparison to traditional chemical approaches. Nonetheless, the feasibility of enzymatic methods vis-à-vis chemical routes hinges on surmounting the obstacles of elevated production expenses and protracted reaction durations [75,252,253]. Furthermore, a universal strategy is untenable due to the necessity of tailored enzyme combinations contingent on the desired product length, deacetylation extent, and related factors.

Mitigating the cost of enzyme expression can be achieved through strategies such as enzyme immobilization onto beads [254], enzyme fusion with carbohydrate-binding modules (CBM) [255], or integration into columns [256] to enable continuous production. Alternatively, streamlined approaches like one-pot methods [257] prove beneficial.

Addressing the issue of reaction speed entails optimizing enzyme cocktails [258] or refining reaction conditions, exemplified by solvent-less techniques [259]. Molecular engineering of enzymes [260], including fusion with CBMs [261,262] or employing mutagenesis (random [263] or site-directed [79]), can enhance catalytic efficiency.

An alternate avenue is biosynthesis, particularly through transglycosidases [79], offering greater adjustability albeit potentially higher costs due to an initial GlcNAc byproduct, followed by subsequent synthesis into larger fragments [264,265]. This option remains intriguing, particularly for advanced applications, which require defined COS.

Advances in Biomedical and Material Sciences

Medical research is generally of high public interest, reflected in the sizable funding proportion in respect to that of other fields [266,267], and thus represents one of the main driving forces for the continued success of chitin valorization. Due to chitosan's antimicrobial, anti-inflammatory, antitumoral, and hemostatic characteristics, it is a highly attractive molecule for biomedical applications, particularly drug delivery [13,16,23,268]. Successful chitosan-based drug delivery systems include microspheres [269], tablets [270], nanoparticles [271–274], nanofibers [275], hydrogels [58,276,277], membranes [278] and microgranules/dry powder [279].

Moreover, chitosan also proved useful in tissue engineering (e.g., cardiac tissue [280,281], bone regeneration [52,53], periodontal tissue regeneration [282], and corneal regeneration [51,283]), wound healing [46,284], cancer diagnosis [285] , anti-thrombogenic and hemostatic materials [286], bioimaging [287–289], antiaging cosmetics [29,290], or as antitumor therapeutic and vaccine adjuvant [27,291–293].

Through chemical modification and functionalization [268,274,294], the scope of application for chitosan-based materials can be significantly expanded and diversified. A particularly promising application are chitosan hydrogels for 3D-printing [57,58], serving for instance as biocompatible synthetic organ scaffolds.

Concluding remarks

The biopolymer chitin and its derivative chitosan are abundant and versatile biomaterials, originating as waste products from the seafood industry, with diverse potential applications. They have gained increasing attention due to their versatile nature. Chitin and chitosan can be enzymatically hydrolyzed to produce chitosan oligosaccharides (COS) and partially acetylated chitosan oligosaccharides (paCOS), respectively. These hydrolysis products exhibit remarkable properties for various applications, particularly in the field of biomedicine, which is a major area of interest, often supported by substantial research funding.

Current chitin valorization methods primarily involve the use of harsh chemicals, resulting in the generation of unspecific products and necessitating the treatment of hazardous waste streams to mitigate harm to both human health and the environment. While biotechnological chitin extraction has made considerable progress, notably through lactic acid fermentation, the production of chitosan oligosaccharides (COS) has not advanced at the same pace. There is an imperative need for substantial efforts to optimize enzyme production processes, reduce associated costs, and explore novel enzymes, that could enhance the efficiency of customized COS production. Key milestones include the development of synergistic hydrolase formulations and research into chitin or COS deacetylating enzymes capable of processing crystalline substrates. These steps are vital for the successful biotechnological valorization of chitin. Not only do they enable the production of well-defined chitin-derived products that only enzymes can provide, but they also allow us to move away from harsh chemicals and the detrimental environmental impact associated with them.

In this context, *Jeongeupia wiesaeckerbachi* emerges as a promising candidate for identifying novel enzymes with chitin-processing capabilities. Moreover, it holds the potential to serve as a model organism for expanding our understanding of bacterial chitin metabolism, complementing the knowledge we already possess about well-established genera, such as *Vibrio* and *Serratia*, both of which comprise many S2 organisms. It's crucial to acknowledge that research involving these microorganisms demands compliance with rigorous S2 laboratory safety protocols. These safety requirements can pose logistical challenges, underlining the potential benefits of exploring alternative model organisms, such as *Jeongeupia wiesaeckerbachi*.

The emerging field of omics, encompassing various "-omics" technologies, is poised to accelerate the pace of enzyme discovery by providing comprehensive insights into biological systems. Furthermore, the integration of AI technologies has the potential to further expedite the molecular engineering of chitinases, thereby enabling the development of enhanced enzyme variants for a wide range of applications.

5 List of Publications

1. Enzymatic Modification of Native Chitin and Conversion to Specialty Chemical Products

Arnold ND, Brück WM, Garbe D, Brück TB.

Mar Drugs. 2020 Jan 30;18(2):93. doi: 10.3390/md18020093. PMID: 32019265; PMCID: PMC7073968.

2. A Newly Designed Automatically Controlled, Sterilizable Flat Panel Photobioreactor for Axenic Algae Culture

Fuchs T, **Arnold ND**, Garbe D, Deimel S, Lorenzen J, Masri M, Mehlmer N, Weuster-Botz D, Brück TB.

Front Bioeng Biotechnol. 2021 Jul 1;9:697354. doi: 10.3389/fbioe.2021.697354. PMID: 34277591; PMCID: PMC8280782.

3. Metabolic stress constrains microbial L-cysteine production in Escherichia coli by accelerating transposition through mobile genetic elements

Heieck K, **Arnold ND**, Brück TB.

Microb Cell Fact. 2023 Jan 16;22(1):10. doi: 10.1186/s12934-023-02021-5. PMID: 36642733; PMCID: PMC9841684.

4. Isolation, biochemical characterization, and sequencing of two high-quality genomes of a novel chitinolytic *Jeongeupia* species

Arnold ND, Garbe D, Brück TB.

MicrobiologyOpen, 2023, 12, e1372. https://doi.org/10.1002/mbo3.1372

5. Proteomic and transcriptomic analyses to decipher the chitinolytic response of *Jeongeupia* spp.

Arnold, ND; Garbe, D; Brück, TB

Mar. Drugs 2023, 21, 448. https://doi.org/10.3390/md21080448

6. Algal-based Biopolymers. In: Abomohra, A., Ende, S. (eds) Value-added Products from Algae

Younes, S., **Arnold, N.**, Paper, M., Keil, L., Awad, D., Brueck, T. (2024).

Springer, Cham. https://doi.org/10.1007/978-3-031-42026-9_13.

7. High-Quality Genome of a novel *Thermostichus* species from Namibia and characterization of its adaptive protein expression patterns to elevated temperatures

Arnold ND[1], Paper M[1], Wortmann D[1], Ahmad N, Fuchs T, Qoura F, Mehlmer N, Kandawa-Schulz M, Brück TB

[1]Equally contributed.

MicrobiologyOpen, in publication.

6 Reprint Permissions

Isolation, biochemical characterization, and sequencing of two high-quality genomes of a novel chitinolytic *Jeongeupia* species

Published in MicrobiologyOpen (*MicrobiologyOpen*, 2023, 12, e1372)

Proteomic and transcriptomic analyses to decipher the chitinolytic response of *Jeongeupia* spp.

Published in Marine Drugs (Mar. Drugs 2023, 21, 448)

7 Figures & Tables

Figure 1: Chemical structures of the polysaccharides (a) Chitin and (b) Chitosan, consisting of different ratios of N-acetyl-glucosamine and glucosamine monomers. (c) The three chitin allomers, diverging in the antiparallel (α), parallel (β) or mixed (γ) alignment of chitin microfibrils.

Figure 2: Schematic overview of chitin- and chitosan active enzymes. Exo-acting enzymes hydrolyze the polysaccharide chain processively from the reducing or non-reducing sugar end. Endo-acting enzymes cleave the β-1,4-glycosidic links randomly along the chain. The lytic polysaccharide monooxygenase (LPMO) hydrolyzes the β-1,4-glycosidic links with O2 or H2O2 as reducing agents. Enzymes with transglycosylase activity extend the polysaccharide chain through addition of monomers. (Chitin-) Deacetylases remove the acetate residue from N-acetylglucosamine molecules, yielding glucosamine and a water molecule in the process.

Figure 3: Flow chart of the ChitoMat (Holistic **chitin** conversion to **materials** for 3D printing application and
performance animal feed additives) project. It was a *BMBF* international *funded joint effort* involving the German partners TUM and Fraunhofer IGB *in addition to* the Canadian partners CigalaTech and UPEI.

Figure 4: Schematic flow-chart of bacterial chitin utilization. (1) Sensing and chemotaxis, (2) attachment, (3) hydrolysis, (4) uptake and (5) metabolism of N-acetylglucosamine to fructose-6-phosphate, acetate and NH3. Figure created with BioRender.

Figure 5: Amino acid sequence homologies and inferred rootless phylogenetic trees of Jeongeupia wiesaeckerbachi's glycoside hydrolases (GH) of families, 18, 19 and 20. Sequence alignment was performed with CLUSTALW [138] and phylogenetic reconstructions were performed using the function "build" of ETE3 3.1.2 as implemented on the GenomeNet [168.] Tree was inferred using PhyML v20160115 [171.] Values at nodes are branch supports in %, computed out of 100 bootstrapped trees.

Table 1: Chitinase screening medium, adapted and modified from Lee et al. and Singh et al.[148,149]:

Table 2: Biocomputational software utilized for genome evaluation with the utilized environment (and website if browser-based), respective application and reference. GO = gene ontology, COG = Cluster of Orthologous Proteins, KO = KEGG Orthology, CAZyme = carbohydrate-active enzyme, ORI = origin of replication.

Table 3: Overview of J. wieseackerbachi genes involved in chitin metabolism. The combined results were obtained from intracellular transcriptomics and proteomics, as well as extracellular proteomics under chitin (vs. glucose if applicable).

Table 4: Promising carbohydrate-active enzymes (CAZymes) in the genome of Jeongeupia wiesaeckerbachi, detected by dbCAN 3.0. CAZy classifications marked with an asterisk (*) contain proteins that have been detected in proteomic or transcriptomic analyses under exclusive colloidal chitin feed. CBM = carbohydrate binding motif, CE = carbohydrate esterase, GH = glycosyl hydrolase, GT = glycosyltransferase. While some enzymes exhibit CBMs, they are not included in separately, but in their respective enzyme classification to avoid duplicates.

8 References

[1] Tharanathan RN, Kittur FS. Chitin — The Undisputed Biomolecule of Great Potential. *Crit Rev Food Sci Nutr*. 2003;43(1):61-87.

[2] Gooday GW. The Ecology of Chitin Degradation BT - Advances in Microbial Ecology. In: Marshall KC, ed. Springer US; 1990:387-430. doi:10.1007/978-1-4684-7612-5_10

[3] FAO. *The State of World Fisheries and Aquaculture 2022*.; 2022. doi:10.4060/cc0463en

[4] Xu Y, Bajaj M, Schneider R, Grage SL, Ulrich AS, Winter J, et al. Transformation of the matrix structure of shrimp shells during bacterial deproteination and demineralization. *Microb Cell Fact*. 2013;12(1):1. doi:10.1186/1475-2859-12-90

[5] Kaur S, Dhillon GS. Recent trends in biological extraction of chitin from marine shell wastes: A review. *Crit Rev Biotechnol*. 2013;35(1):44-61. doi:10.3109/07388551.2013.798256

[6] Kim SK, Rajapakse N. Enzymatic production and biological activities of chitosan oligosaccharides (COS): A review. *Carbohydr Polym*. 2005;62(4):357-368. doi:10.1016/j.carbpol.2005.08.012

[7] Healy M, Green A, Healy A. Bioprocessing of Marine Crustacean Shell Waste. *Acta Biotechnol*. 2003;23(23):151-160. doi:10.1002/abio.200390023

[8] Dutta PK, Duta J, Tripathi VS. Chitin and Chitosan: Chemistry, properties and applications. *J Sci Ind Res (India)*. 2004;63(1):20-31.

[9] Yadav M, Goswami P, Paritosh K, Kumar M, Pareek N, Vivekanand V. Seafood waste: a source for preparation of commercially employable chitin/chitosan materials. *Bioresour Bioprocess*. 2019;6(1). doi:10.1186/s40643-019-0243-y

[10] Bajaj M, Freiberg A, Winter J, Xu Y, Gallert C. Pilot-scale chitin extraction from shrimp shell waste by deproteination and decalcification with bacterial enrichment cultures. *Appl Microbiol Biotechnol*. 2015;99(22):9835-9846. doi:10.1007/s00253-015-6841-5

[11] Kurita K. Chitin and chitosan: functional biopolymers from marine crustaceans. *Mar Biotechnol (NY)*. 2006;8(3):203-226. doi:10.1007/s10126-005-0097-5

[12] Rinaudo M. Chitin and chitosan: Properties and applications. *Prog Polym Sci*. 2006;31(7):603-632. doi:10.1016/j.progpolymsci.2006.06.001

[13] Aam BB, Heggset EB, Norberg AL, Sørlie M, Vårum KM, Eijsink VGH. Production of chitooligosaccharides and their potential applications in medicine. *Mar Drugs*. 2010;8(5):1482-1517. doi:10.3390/md8051482

[14] Bartnicki-Garcia S, Nickerson WJ. Isolation, composition, and structure of cell walls of filamentous and yeast-like forms of Mucor rouxii. *BBA - Biochim Biophys Acta*. 1962;58(1):102-119. doi:10.1016/0006-3002(62)90822-3

[15] Blair HS, Ho TC. Studies in the adsorption and diffusion of ions in chitosan. *J Chem Technol Biotechnol*. 2007;31(1):6-10. doi:10.1002/jctb.503310103

[16] Younes I, Hajji S, Frachet V, Rinaudo M, Jellouli K, Nasri M. Chitin extraction from shrimp shell using enzymatic treatment. Antitumor, antioxidant and antimicrobial activities of chitosan. *Int J Biol Macromol*. 2014;69:489-498. doi:10.1016/j.ijbiomac.2014.06.013

[17] Xia W, Liu P, Zhang J, Chen J. Biological activities of chitosan and chitooligosaccharides. *Food*

Hydrocoll. 2011;25(2):170-179. doi:10.1016/j.foodhyd.2010.03.003

[18] Goy RC, De Britto D, Assis OBG. A review of the antimicrobial activity of chitosan. *Polimeros*. 2009;19(3):241-247. doi:10.1590/S0104-14282009000300013

[19] Sorlier P, Denuzière A, Viton C, Domard A. Relation between the degree of acetylation and the electrostatic properties of chitin and chitosan. *Biomacromolecules*. 2001;2(3):765-772. doi:10.1021/bm015531+

[20] Chung YC, Su YP, Chen CC, Jia G, Wang HL, Wu JCG, et al. Relationship between antibacterial activity of chitosan and surface characteristics of cell wall. *Acta Pharmacol Sin*. 2004;25(7):932-936.

[21] Bahrke S. Mass Spectrometric Analysis of Chitooligosachharides and their Interaction with proteins. Published online 2008:1-232.

[22] Winkler AJ, Dominguez-Nuñez JA, Aranaz I, Poza-Carrión C, Ramonell K, Somerville S, et al. Short-chain chitin oligomers: Promoters of plant growth. *Mar Drugs*. 2017;15(2):1-21. doi:10.3390/md15020040

[23] Park BK, Kim MM. Applications of chitin and its derivatives in biological medicine. *Int J Mol Sci*. 2010;11(12):5152-5164. doi:10.3390/ijms11125152

[24] Swiatkiewicz S, Swiatkiewicz M, Arczewska-Wlosek A, Jozefiak D. Chitosan and its oligosaccharide derivatives (chito-oligosaccharides) as feed supplements in poultry and swine nutrition. *J Anim Physiol Anim Nutr (Berl)*. 2015;99(1):1-12. doi:10.1111/jpn.12222

[25] Bloch R, Burger MM. Purification of wheat germ agglutinin using affinity chromatography on chitin. *Biochem Biophys Res Commun*. 1974;58(1):13-19. doi:10.1016/0006-291X(74)90884-5

[26] Rhee JS, Jung MW, Paeng KJ. Evaluation of Chitin and Chitosan as a Sorbent for the Preconcentration of Phenol and Chlorophenols in Water. *Anal Sci*. 1998;14(6):1089-1092. doi:10.2116/analsci.14.1089

[27] Elieh-Ali-Komi D, Hamblin MR, Daniel EAK. Chitin and Chitosan: Production and Application of Versatile Biomedical Nanomaterials. *Int J Adv Res*. 2016;4(3):411-427.

[28] Hamed I, Özogul F, Regenstein JM. Industrial applications of crustacean by-products (chitin, chitosan, and chitooligosaccharides): A review. *Trends Food Sci Technol*. 2016;48:40-50. doi:10.1016/j.tifs.2015.11.007

[29] Casadidio C, Peregrina DV, Gigliobianco MR, Deng S, Censi R, Di Martino P. Chitin and chitosans: Characteristics, eco-friendly processes, and applications in cosmetic science. *Mar Drugs*. 2019;17(6). doi:10.3390/md17060369

[30] Malerba M, Cerana R. Recent advances of chitosan applications in plants. *Polymers (Basel)*. 2018;10(2):1-10. doi:10.3390/polym10020118

[31] Thadathil N, Velappan SP. Recent developments in chitosanase research and its biotechnological applications: A review. *Food Chem*. 2014;150:392-399. doi:10.1016/j.foodchem.2013.10.083

[32] Rahman M, Mukta JA, Sabir AA, Gupta DR, Mohi-Ud-Din M, Hasanuzzaman M, et al. Chitosan biopolymer promotes yield and stimulates accumulation of antioxidants in strawberry fruit. Huang Y, ed. *PLoS One*. 2018;13(9):e0203769. doi:10.1371/journal.pone.0203769

[33] Falcón-Rodríguez AB, Costales D, Gónzalez-Peña D, Morales D, Mederos Y, Jerez E, et al. Chitosans of different molecular weight enhance potato (Solanum tuberosum L.) yield in a field trial. *Spanish J Agric Res*. 2017;15(1 SE-Plant production (Field and horticultural crops)):e0902.

doi:10.5424/sjar/2017151-9288

[34] Phothi R, Theerakarunwong CD. Effect of chitosan on physiology, photosynthesis and biomass of rice (Oryza sativa L.) under elevated ozone. *Aust J Crop Sci*. 2017;11(5):624-630. doi:10.21475/ajcs.17.11.05.p578

[35] Mwaheb MAMA, Hussain M, Tian J, Zhang X, Hamid MI, El-Kassim NA, et al. Synergetic suppression of soybean cyst nematodes by chitosan and Hirsutella minnesotensis via the assembly of the soybean rhizosphere microbial communities. *Biol Control*. 2017;115:85-94. doi:10.1016/j.biocontrol.2017.09.011

[36] Choudhary RC, Kumaraswamy R V, Kumari S, Sharma SS, Pal A, Raliya R, et al. Cu-chitosan nanoparticle boost defense responses and plant growth in maize (Zea mays L.). *Sci Rep*. 2017;7(1):9754. doi:10.1038/s41598-017-08571-0

[37] Kheiri A, Moosawi Jorf SA, Malihipour A, Saremi H, Nikkhah M. Synthesis and characterization of chitosan nanoparticles and their effect on Fusarium head blight and oxidative activity in wheat. *Int J Biol Macromol*. 2017;102:526-538. doi:10.1016/j.ijbiomac.2017.04.034

[38] Munhuweyi K, Lennox CL, Meitz-Hopkins JC, Caleb OJ, Sigge GO, Opara UL. Investigating the effects of crab shell chitosan on fungal mycelial growth and postharvest quality attributes of pomegranate whole fruit and arils. *Sci Hortic (Amsterdam)*. 2017;220:78-89. doi:10.1016/j.scienta.2017.03.038

[39] Jongsri P, Wangsomboondee T, Rojsitthisak P, Seraypheap K. Effect of molecular weights of chitosan coating on postharvest quality and physicochemical characteristics of mango fruit. *LWT*. 2016;73:28-36. doi:10.1016/j.lwt.2016.05.038

[40] Kaya M, Česonienė L, Daubaras R, Leskauskaitė D, Zabulionė D. Chitosan coating of red kiwifruit (Actinidia melanandra) for extending of the shelf life. *Int J Biol Macromol*. 2016;85:355-360. doi:10.1016/j.ijbiomac.2016.01.012

[41] Jongsri P, Rojsitthisak P, Wangsomboondee T, Seraypheap K. Influence of chitosan coating combined with spermidine on anthracnose disease and qualities of 'Nam Dok Mai' mango after harvest. *Sci Hortic (Amsterdam)*. 2017;224:180-187. doi:10.1016/j.scienta.2017.06.011

[42] Sun T, Zhou D, Xie J, Mao F. Preparation of chitosan oligomers and their antioxidant activity. *Eur Food Res Technol*. 2007;225(3-4):451-456. doi:10.1007/s00217-006-0439-1

[43] Mahdy Samar M, El-Kalyoubi MH, Khalaf MM, Abd El-Razik MM. Physicochemical, functional, antioxidant and antibacterial properties of chitosan extracted from shrimp wastes by microwave technique. *Ann Agric Sci*. 2013;58(1):33-41. doi:10.1016/j.aoas.2013.01.006

[44] Juliano C, Magrini G. Methylglyoxal, the Major Antibacterial Factor in Manuka Honey: An Alternative to Preserve Natural Cosmetics? *Cosmetics*. 2018;6(1):1. doi:10.3390/cosmetics6010001

[45] Muzzarelli RAA, Muzzarelli C. Chitosan chemistry: Relevance to the biomedical sciences. *Adv Polym Sci*. 2005;186(August):151-209. doi:10.1007/b136820

[46] Ribeiro MP, Espiga A, Silva D, Baptista P, Henriques J, Ferreira C, et al. Development of a new chitosan hydrogel for wound dressing. *Wound Repair Regen*. 2009;17(6):817-824. doi:10.1111/j.1524-475X.2009.00538.x

[47] Dai T, Tanaka M, Huang YY, Hamblin MR. *Chitosan Preparations for Wounds and Burns: Antimicrobial and Wound-Healing Effects*. Vol 9.; 2011. doi:10.1586/eri.11.59.Chitosan

[48] Trapani A, Cometa S, De Giglio E, Corbo F, Cassano R, Di Gioia ML, et al. Novel Nanoparticles

Based on N,O-Carboxymethyl Chitosan-Dopamine Amide Conjugate for Nose-to-Brain Delivery. *Pharmaceutics*. 2022;14(1):147. doi:10.3390/pharmaceutics14010147

[49] Qi XJ, Liu XY, Tang LMY, Li PF, Qiu F, Yang AH. Anti-depressant effect of curcumin-loaded guanidine-chitosan thermo-sensitive hydrogel by nasal delivery. *Pharm Dev Technol*. 2020;25(3):316-325. doi:10.1080/10837450.2019.1686524

[50] Liu J, Yang B, Li M, Li J, Wan Y. Enhanced dual network hydrogels consisting of thiolated chitosan and silk fibroin for cartilage tissue engineering. *Carbohydr Polym*. 2020;227:115335. doi:10.1016/j.carbpol.2019.115335

[51] Xu W, Liu K, Li T, Zhang W, Dong Y, Lv J, et al. An in situ hydrogel based on carboxymethyl chitosan and sodium alginate dialdehyde for corneal wound healing after alkali burn. *J Biomed Mater Res Part A*. 2019;107(4):742-754. doi:10.1002/jbm.a.36589

[52] Dasgupta S, Maji K, Nandi SK. Investigating the mechanical, physiochemical and osteogenic properties in gelatin-chitosan-bioactive nanoceramic composite scaffolds for bone tissue regeneration: In vitro and in vivo. *Mater Sci Eng C*. 2019;94:713-728. doi:10.1016/j.msec.2018.10.022

[53] Ghosh P, Rameshbabu AP, Das D, Francis NK, Pawar HS, Subramanian B, et al. Covalent cross-links in polyampholytic chitosan fibers enhances bone regeneration in a rabbit model. *Colloids Surfaces B Biointerfaces*. 2015;125:160-169. doi:10.1016/j.colsurfb.2014.11.031

[54] Lima B V., Oliveira MJ, Barbosa MA, Gonçalves RM, Castro F. Immunomodulatory potential of chitosan-based materials for cancer therapy: a systematic review of in vitro , in vivo and clinical studies. *Biomater Sci*. 2021;9(9):3209-3227. doi:10.1039/D0BM01984D

[55] Huang R, Mendis E, Rajapakse N, Kim SK. Strong electronic charge as an important factor for anticancer activity of chitooligosaccharides (COS). *Life Sci*. 2006;78(20):2399-2408. doi:https://doi.org/10.1016/j.lfs.2005.09.039

[56] Maeda Y, Kimura Y. Antitumor Effects of Various Low-Molecular-Weight Chitosans Are Due to Increased Natural Killer Activity of Intestinal Intraepithelial Lymphocytes in Sarcoma 180–Bearing Mice. *J Nutr*. 2004;134(4):945-950. doi:10.1093/jn/134.4.945

[57] Zhou L, Ramezani H, Sun M, Xie M, Nie J, Lv S, et al. 3D printing of high-strength chitosan hydrogel scaffolds without any organic solvents. *Biomater Sci*. 2020;8(18):5020-5028. doi:10.1039/D0BM00896F

[58] Rajabi M, McConnell M, Cabral J, Ali MA. Chitosan hydrogels in 3D printing for biomedical applications. *Carbohydr Polym*. 2021;260:117768. doi:10.1016/j.carbpol.2021.117768

[59] Varma A., Deshpande S., Kennedy J. Metal complexation by chitosan and its derivatives: a review. *Carbohydr Polym*. 2004;55(1):77-93. doi:10.1016/j.carbpol.2003.08.005

[60] Wan MW, Wang CC, Chien-Min Chen CM. The Adsorption Study of Copper Removal by Chitosan-Coated Sludge Derived from Water Treatment Plant. *Int J Environ Sci Dev*. Published online 2013:545-551. doi:10.7763/IJESD.2013.V4.411

[61] Ali NF, El-Mohamedy RSR. Microbial decolourization of textile waste water. *J Saudi Chem Soc*. 2012;16(2):117-123. doi:10.1016/j.jscs.2010.11.005

[62] Khalili M, Abedi M, Amoli HS, Mozaffari SA. Comparison of chitosan and chitosan nanoparticles on the performance and charge recombination of water-based gel electrolyte in dye sensitized solar cells. *Carbohydr Polym*. 2017;175:1-6. doi:10.1016/j.carbpol.2017.07.061

[63] Abu Hassan MA, Tan PL, Zainon Noor Z. Coagulation and Flocculation Treatment of Wastewater

in Textile Industry Using Chitosan. *J Chem Nat Resour Eng*. 2009;4(1):43-53.

[64] Pokhrel D, Viraraghavan T. Treatment of pulp and paper mill wastewater—a review. *Sci Total Environ*. 2004;333(1-3):37-58. doi:10.1016/j.scitotenv.2004.05.017

[65] Song Z, Li G, Guan F, Liu W. Application of chitin/chitosan and their derivatives in the papermaking industry. *Polymers (Basel)*. 2018;10(4). doi:10.3390/polym10040389

[66] S H, M H, M A, E K. The effect of chitosan on physical and mechanical properties of paper. *J Chem Eng Mater Sci*. 2016;7(1):1-10. doi:10.5897/JCEMS2015.0235

[67] Chi H, Li H, Liu W, Zhan H. The retention- and drainage-aid behavior of quaternary chitosan in papermaking system. *Colloids Surfaces A Physicochem Eng Asp*. 2007;297(1-3):147-153. doi:10.1016/j.colsurfa.2006.10.039

[68] Cantarel BL, Coutinho PM, Rancurel C, Bernard T, Lombard V, Henrissat B. The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics. *Nucleic Acids Res*. 2009;37(Database issue):D233-8. doi:10.1093/nar/gkn663

[69] Davies G, Henrissat B. Structures and mechanisms of glycosyl hydrolases. *Structure*. 1995;3(9):853-859. doi:10.1016/S0969-2126(01)00220-9

[70] Slámová K, Bojarová P, Petrásková L, Křen V. B-N-Acetylhexosaminidase: What's in a name...? *Biotechnol Adv*. 2010;28(6):682-693. doi:10.1016/j.biotechadv.2010.04.004

[71] Van Aalten DMF, Komander D, Synstad B, Gåseidnes S, Peter MG, Eijsink VGH. Structural insights into the catalytic mechanism of a family 18 exo-chitinase. *PNAS*. 2001;98(16):8979-8984. doi:10.1038/nsb927

[72] Hamre AG, Frøberg EE, Eijsink VGH, Sørlie M. Thermodynamics of tunnel formation upon substrate binding in a processive glycoside hydrolase. *Arch Biochem Biophys*. 2017;620:35-42. doi:10.1016/j.abb.2017.03.011

[73] Sakai K, Yokota A, Kurokawa H, Wakayama M, Moriguchi M. Purification and characterization of three thermostable endochitinases of a noble Bacillus strain, MH-1, isolated from chitin-containing compost. *Appl Environ Microbiol*. 1998;64(9):3397-3402. doi:10.1128/aem.64.9.3397-3402.1998

[74] Zakariassen H, Aam BB, Hom SJ, Vårum KM, Sørlie M, Eijsink VGH. Aromatic residues in the catalytic center of chitinase A from Serratia marcescens affect processivity, enzyme activity, and biomass converting efficiency. *J Biol Chem*. 2009;284(16):10610-10617. doi:10.1074/jbc.M900092200

[75] Oyeleye A, Normi YM. Chitinase: Diversity, limitations, and trends in Engineering for suitable applications. *Biosci Rep*. 2018;38(4):1-21. doi:10.1042/BSR20180323

[76] Horn SJ, Sørbotten A, Synstad B, Sikorski P, Sørlie M, Vårum KM, et al. Endo/exo mechanism and processivity of family 18 chitinases produced by Serratia marcescens. *FEBS J*. 2006;273(3):491-503. doi:10.1111/J.1742-4658.2005.05079.X

[77] Sikorski P, Sørbotten A, Horn SJ, Eijsink VGH, Vårum KM. Serratia marcescens chitinases with tunnel-shaped substrate-binding grooves show endo activity and different degrees of processivity during enzymatic hydrolysis of chitosan. *Biochemistry*. 2006;45(31):9566-9574. doi:10.1021/bi060370l

[78] Purushotham P, Podile AR. Synthesis of long-chain chitooligosaccharides by a hypertransglycosylating processive endochitinase of Serratia proteamaculans 568. *J Bacteriol*. 2012;194(16):4260-4271. doi:10.1128/JB.06473-11

[79] Madhuprakash J, El Gueddari NE, Moerschbacher BM, Podile AR. Production of bioactive chitosan oligosaccharides using the hypertransglycosylating chitinase-D from Serratia proteamaculans. *Bioresour Technol*. 2015;198:503-509. doi:10.1016/j.biortech.2015.09.052

[80] Mallakuntla MK, Vaikuntapu PR, Bhuvanachandra B, Das SN, Podile AR. Transglycosylation by a chitinase from Enterobacter cloacae subsp. cloacae generates longer chitin oligosaccharides. *Sci Rep*. 2017;7(1):1-12. doi:10.1038/s41598-017-05140-3

[81] Bojarová P, Křen V. Glycosidases: a key to tailored carbohydrates. *Trends Biotechnol*. 2009;27(4):199-209. doi:10.1016/j.tibtech.2008.12.003

[82] Mangas-Sánchez J, Adlercreutz P. Enzymatic preparation of oligosaccharides by transglycosylation: A comparative study of glucosidases. *J Mol Catal B Enzym*. 2015;122:51-55. doi:10.1016/j.molcatb.2015.08.014

[83] Fukamizo T, Fleury A, Côté N, Mitsutomi M, Brzezinski R. Exo-β-D-glucosaminidase from Amycolatopsis orientalis: Catalytic residues, sugar recognition specificity, kinetics, and synergism. *Glycobiology*. 2006;16(11):1064-1072. doi:10.1093/glycob/cwl026

[84] Tanaka T, Fukui T, Atomi H, Imanaka T. Characterization of an Exo-ß-D-Glucosaminidase Involved in a Novel Chitnolytic Pathway from the Hyperthermophilic Archaeon Thermococcus kodakaraensis KOD1. *J Bacteriol*. 2003;185(17):5175-5181. doi:10.1128/JB.185.17.5175

[85] Fukamizo T, Amano S, Yamaguchi K, Yoshikawa T, Katsumi T, Saito JI, et al. Bacillus circulans MH-K1 chitosanase: Amino acid residues responsible for substrate binding. *J Biochem*. 2005;138(5):563-569. doi:10.1093/jb/mvi156

[86] Saito JI, Kita A, Higuchi Y, Nagata Y, Ando A, Miki K. Crystal structure of chitosanase from Bacillus circulans MH-K1 at 1.6-Å resolution and its substrate recognition mechanism. *J Biol Chem*. 1999;274(43):30818-30825. doi:10.1074/jbc.274.43.30818

[87] Marcotte EM, Monzingo AF, Ernst SR, Brzezinski R, Robertus JD. X-ray structure of an anti-fungal chitosanase from streptomyces N174. *Nat Struct Biol*. 1996;3(2):155-162. doi:10.1038/nsb0296-155

[88] Tremblay H, Yamaguchi T, Fukamizo T, Brzezinski R. Mechanism of chitosanase-oligosaccharide interaction: Subsite structure of Streptomyces sp. N174 chitosanase and the role of Asp57 carboxylate. *J Biochem*. 2001;130(5):679-686. doi:10.1093/oxfordjournals.jbchem.a003034

[89] Viens P, Lacombe-Harvey MÈ, Brzezinski R. Chitosanases from family 46 of glycoside hydrolases: From proteins to phenotypes. *Mar Drugs*. 2015;13(11):6566-6587. doi:10.3390/md13116566

[90] Monzingo AF, Marcotte EM, John Hart P, Robertus JD. Chitinases, chitosanases, and lysozymes can be divided into procaryotic and eucaryotic families sharing a conserved core. *Nat Struct Biol*. 1996;3(2):133-140. doi:10.1038/nsb0296-133

[91] Cheng CY, Chang CH, Wu YJ, Li YK. Exploration of glycosyl hydrolase family 75, a chitosanase from Aspergillus fumigatus. *J Biol Chem*. 2006;281(6):3137-3144. doi:10.1074/jbc.M512506200

[92] Hoell IA, Vaaje-Kolstad G, Eijsink VGH. Structure and function of enzymes acting on chitin and chitosan. *Biotechnol Genet Eng Rev*. 2010;27(1):331-366. doi:10.1080/02648725.2010.10648156

[93] Mccarter JD, Withers SG. Mechanisms of enzymatic glycoside hydrolysis. *Curr Opin Struct Biol*. 1994;4:885-892. doi:10.1016/0959-440X(94)90271-2

[94] McIntosh LP, Hand G, Johnson PE, Joshi MD, Korner M, Plesniak LA, et al. The pK(a) of the general acid/base carboxyl group of a glycosidase cycles during catalysis: A 13C-NMR study of

Bacillus circulans xylanase. *Biochemistry*. 1996;35(31):9958-9966. doi:10.1021/bi9613234

[95] Fukamizo T, Ohkawa T, Ikeda Y, Goto S. Specificity of chitosanase from Bacillus pumilus. *Biochim Biophys Acta (BBA)/Protein Struct Mol*. 1994;1205(2):183-188. doi:10.1016/0167-4838(94)90232-1

[96] Mitsutomi M, Hata T, Kuwahara T. Purification and characterization of novel chitinases from Streptomyces griseus HUT 6037. *J Ferment Bioeng*. 1995;80(2):153-158. doi:10.1016/0922-338X(95)93211-2

[97] Fenton DM, Eveleigh DE. Purification and mode of action of a chitosanase from Penicillium islandicum. *J Gen Microbiol*. 1981;126(1):151-165. doi:10.1099/00221287-126-1-151

[98] Hirano K, Watanabe M, Seki K, Ando A, Saito A, Mitsutomi M. Classification of chitosanases by hydrolytic specificity toward N1,N4-diacetylchitohexaose. *Biosci Biotechnol Biochem*. 2012;76(10):1932-1937. doi:10.1271/bbb.120408

[99] Heggset EB, Dybvik AI, Hoell IA, Norberg AL, Sørlie M, Eijsink VGH, et al. Degradation of chitosans with a family 46 chitosanase from streptomyces coelicolor A3(2). *Biomacromolecules*. 2010;11(9):2487-2497. doi:10.1021/bm1006745

[100] Zhao Y, Park RD, Muzzarelli RAA. Chitin deacetylases: Properties and applications. *Mar Drugs*. 2010;8(1):24-46. doi:10.3390/md8010024

[101] Araki Y, Ito E. A Pathway of Chitosan Formation in Mucor rouxii Enzymatic Deacetylation of Chitin. *Eur J Biochem*. 1975;55(1):71-78. doi:10.1111/j.1432-1033.1975.tb02139.x

[102] Tsigos I, Martinou A, Kafetzopoulos D, Bouriotis V. Chitin deacetylases: new, versatile tools in biotechnology. *Trends Biotechnol*. 2000;7799(1999):129-135.

[103] Martinou A, Bouriotis V, Stokke BT, Vårum KM. Mode of action of chitin deacetylase from Mucor rouxii on partially N-acetylated chitosans. *Carbohydr Res*. 1998;311(1-2):71-78. doi:10.1016/S0008-6215(98)00183-9

[104] Hekmat O, Tokuyasu K, Withers SG. Subsite structure of the endo-type chitin deacetylase from a Deuteromycete, Colletotrichum lindemuthianum: An investigation using steady-state kinetic analysis and MS. *Biochem J*. 2003;374(2):369-380. doi:10.1042/BJ20030204

[105] Grifoll-Romero L, Pascual S, Aragunde H, Biarnés X, Planas A. Chitin deacetylases: Structures, specificities, and biotech applications. *Polymers (Basel)*. 2018;10(4):1-29. doi:10.3390/polym10040352

[106] Liu Z, Gay LM, Tuveng TR, Agger JW, Westereng B, Mathiesen G, et al. Structure and function of a broad-specificity chitin deacetylase from Aspergillus nidulans FGSC A4. *Sci Rep*. 2017;7(1):1-12. doi:10.1038/s41598-017-02043-1

[107] Hoßbach J, Bußwinkel F, Kranz A, Wattjes J, Cord-Landwehr S, Moerschbacher BM. A chitin deacetylase of Podospora anserina has two functional chitin binding domains and a unique mode of action. *Carbohydr Polym*. 2018;183:1-10. doi:10.1016/j.carbpol.2017.11.015

[108] Hemsworth GR, Johnston EM, Davies GJ, Walton PH. Lytic Polysaccharide Monooxygenases in Biomass Conversion. *Trends Biotechnol*. 2015;33(12):747-761. doi:10.1016/j.tibtech.2015.09.006

[109] Vaaje-Kolstad G, Forsberg Z, Loose JS, Bissaro B, Eijsink VG. Structural diversity of lytic polysaccharide monooxygenases. *Curr Opin Struct Biol*. 2017;44:67-76. doi:10.1016/j.sbi.2016.12.012

[110] Tandrup T, Frandsen KEH, Johansen KS, Berrin JG, Leggio L Lo. Recent insights into lytic

polysaccharide monooxygenases (LPMOs). *Biochem Soc Trans*. 2018;46(6):1431-1447. doi:10.1042/BST20170549

[111] Crouch LI, Labourel A, Walton PH, Davies GJ, Gilbert HJ. The contribution of non-catalytic carbohydrate binding modules to the activity of lytic polysaccharide monooxygenases. *J Biol Chem*. 2016;291(14):7439-7449. doi:10.1074/jbc.M115.702365

[112] Hansson H, Karkehabadi S, Mikkelsen N, Douglas NR, Kim S, Lam A, et al. High-resolution structure of a lytic polysaccharide monooxygenase from Hypocrea jecorina reveals a predicted linker as an integral part of the catalytic domain. *J Biol Chem*. 2017;292(46):19099-19109. doi:10.1074/jbc.M117.799767

[113] Hangasky JA, Marletta MA. A Random-Sequential Kinetic Mechanism for Polysaccharide Monooxygenases. *Biochemistry*. 2018;57(22):3191-3199. doi:10.1021/acs.biochem.8b00129

[114] Beeson WT, Phillips CM, Cate JHD, Marletta MA. Oxidative cleavage of cellulose by fungal copper-dependent polysaccharide monooxygenases. *J Am Chem Soc*. 2012;134(2):890-892. doi:10.1021/ja210657t

[115] Vaaje-Kolstad G, Westereng B, Horn SJ, Liu Z, Zhai H, Sørlie M, et al. An oxidative enzyme boosting the enzymatic conversion of recalcitrant polysaccharides. *Science (80-)*. 2010;330(6001):219-222. doi:10.1126/science.1192231

[116] Isaksen T, Westereng B, Aachmann FL, Agger JW, Kracher D, Kittl R, et al. A C4-oxidizing lytic polysaccharide monooxygenase cleaving both cellulose and cello-oligosaccharides. *J Biol Chem*. 2014;289(5):2632-2642. doi:10.1074/jbc.M113.530196

[117] Keyhani NO, Roseman S. Physiological aspects of chitin catabolism in marine bacteria. *Biochim Biophys Acta - Gen Subj*. 1999;1473(1):108-122. doi:10.1016/S0304-4165(99)00172-5

[118] Li X, Roseman S. The chitinolytic cascade in Vibrios is regulated by chitin oligosaccharides and a two-component chitin catabolic sensor/kinase. *Proc Natl Acad Sci U S A*. 2004;101(2):627-631. doi:10.1073/pnas.0307645100

[119] Suginta W, Chumjan W, Mahendran KR, Janning P, Schulte A, Winterhalter M. Molecular Uptake of Chitooligosaccharides through Chitoporin from the Marine Bacterium Vibrio harveyi. Muller DJ, ed. *PLoS One*. 2013;8(1):e55126. doi:10.1371/journal.pone.0055126

[120] Lacombe-Harvey MÈ, Brzezinski R, Beaulieu C. Chitinolytic functions in actinobacteria: ecology, enzymes, and evolution. *Appl Microbiol Biotechnol*. 2018;102(17):7219-7230. doi:10.1007/s00253-018-9149-4

[121] Saito A, Ebise H, Orihara Y, Murakami S, Sano Y, Kimura A, et al. Enzymatic and genetic characterization of the DasD protein possessing N -acetyl-β- d -glucosaminidase activity in Streptomyces coelicolor A3(2). *FEMS Microbiol Lett*. 2013;340(1):33-40. doi:10.1111/1574-6968.12069

[122] Bassler BL, Yu C, Lee YC, Roseman S. Chitin utilization by marine bacteria. Degradation and catabolism of chitin oligosaccharides by Vibrio furnissii. *J Biol Chem*. 1991;266(36):24276-24286. doi:10.1016/S0021-9258(18)54225-3

[123] COMB DG, ROSEMAN S. Glucosamine-6-phosphate deaminase. *Biochim Biophys Acta*. 1956;21(1):193-194. doi:10.1016/0006-3002(56)90124-X

[124] Noelle Gracy K. A conversation with Saul Roseman. *Biochem Biophys Res Commun*. 2003;300(1):5-8. doi:10.1016/S0006-291X(02)02805-X

[125] Saito A, Ishizaka M, Francisco J, Fujii T, Miyashita K. Transcriptional co-regulation of five

chitinase genes scattered on the Streptomyces coelicolor A3(2) chromosome. *Microbiology*. 2000;146(11):2937-2946. doi:10.1099/00221287-146-11-2937

[126] Beier S, Bertilsson S. Bacterial chitin degradation-mechanisms and ecophysiological strategies. *Front Microbiol*. 2013;4(JUN):1-12. doi:10.3389/fmicb.2013.00149

[127] Itoh T, Kimoto H. Bacterial Chitinase System as a -Model of Chitin Biodegradation. In: *Targeting Chitin-Containing Organisms*. Vol 1142. Springer Singapore; 2019:131-151. doi:10.1007/978-981-13-7318-3

[128] Vaaje-Kolstad G, Horn SJ, Sørlie M, Eijsink VGH. The chitinolytic machinery of Serratia marcescens - A model system for enzymatic degradation of recalcitrant polysaccharides. *FEBS J*. 2013;280(13):3028-3049. doi:10.1111/febs.12181

[129] Vaaje-Kolstad G, Bøhle LA, Gåseidnes S, Dalhus B, Bjørås M, Mathiesen G, et al. Characterization of the chitinolytic machinery of enterococcus faecalis V583 and high-resolution structure of its oxidative CBM33 enzyme. *J Mol Biol*. 2012;416(2):239-254. doi:10.1016/j.jmb.2011.12.033

[130] Lorentzen SB, Arntzen M, Hahn T, Tuveng TR, Sørlie M, Zibek S, et al. Genomic and Proteomic Study of Andreprevotia ripae Isolated from an Anthill Reveals an Extensive Repertoire of Chitinolytic Enzymes. *J Proteome Res*. 2021;20(8):4041-4052. doi:10.1021/acs.jproteome.1c00358

[131] Tuveng TR, Hagen LH, Mekasha S, Frank J, Arntzen MØ, Vaaje-Kolstad G, et al. Genomic, proteomic and biochemical analysis of the chitinolytic machinery of Serratia marcescens BJL200. *Biochim Biophys Acta - Proteins Proteomics*. 2017;1865(4):414-421. doi:10.1016/j.bbapap.2017.01.007

[132] Monge EC, Tuveng TR, Vaaje-Kolstad G, Eijsink VGH, Gardner JG. Systems analysis of the glycoside hydrolase family 18 enzymes from Cellvibrio japonicus characterizes essential chitin degradation functions. *J Biol Chem*. 2018;293(10):3849-3859. doi:10.1074/jbc.RA117.000849

[133] Tuveng TR, Arntzen MØ, Bengtsson O, Gardner JG, Vaaje-Kolstad G, Eijsink VGH. Proteomic investigation of the secretome of Cellvibrio japonicus during growth on chitin. *Proteomics*. 2016;16(13):1904-1914. doi:10.1002/pmic.201500419

[134] Sun X, Li Y, Tian Z, Qian Y, Zhang H, Wang L. A novel thermostable chitinolytic machinery of Streptomyces sp. F-3 consisting of chitinases with different action modes. *Biotechnol Biofuels*. 2019;12(1):1-12. doi:10.1186/s13068-019-1472-1

[135] Hunt DE, Gevers D, Vahora NM, Polz MF. Conservation of the chitin utilization pathway in the Vibrionaceae. *Appl Environ Microbiol*. 2008;74(1):44-51. doi:10.1128/AEM.01412-07

[136] Meibom KL, Li XB, Nielsen AT, Wu C yen, Roseman S, Schoolnik GK. The Vibrio cholerae chitin utilization program. *Proc Natl Acad Sci*. 2004;101(8):2524-2529. doi:10.1073/pnas.0308707101

[137] Nguyen J, Francou F, Virolle MJ, Guérineau M. Amylase and chitinase genes in Streptomyces lividans are regulated by reg1, a pleiotropic regulatory gene. *J Bacteriol*. 1997;179(20):6383-6390. doi:10.1128/jb.179.20.6383-6390.1997

[138] Świątek-Połatyńska MA, Bucca G, Laing E, Gubbens J, Titgemeyer F, Smith CP, et al. Genome-Wide Analysis of In Vivo Binding of the Master Regulator DasR in Streptomyces coelicolor Identifies Novel Non-Canonical Targets. Virolle MJ, ed. *PLoS One*. 2015;10(4):e0122479. doi:10.1371/journal.pone.0122479

[139] Colson S, Stephan J, Hertrich T, Saito A, van Wezel GP, Titgemeyer F, et al. Conserved *cis*-Acting Elements Upstream of Genes Composing the Chitinolytic System of Streptomycetes Are DasR-Responsive Elements. *Microb Physiol*. 2007;12(1-2):60-66. doi:10.1159/000096460

[140] Klancher CA, Yamamoto S, Dalia TN, Dalia AB. ChiS is a noncanonical DNA-binding hybrid sensor kinase that directly regulates the chitin utilization program in Vibrio cholerae. *Proc Natl Acad Sci*. 2020;117(33):20180-20189. doi:10.1073/pnas.2001768117

[141] Fondi M, Liò P. Multi -omics and metabolic modelling pipelines: Challenges and tools for systems microbiology. *Microbiol Res*. 2015;171:52-64. doi:10.1016/j.micres.2015.01.003

[142] Misra BB, Langefeld C, Olivier M, Cox LA. Integrated omics: Tools, advances and future approaches. *J Mol Endocrinol*. 2019;62(1):R21-R45. doi:10.1530/JME-18-0055

[143] Dai X, Shen L. Advances and Trends in Omics Technology Development. *Front Med*. 2022;9(July). doi:10.3389/fmed.2022.911861

[144] Wang Z, Gerstein M, Snyder M. RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet*. 2009;10(1):57-63. doi:10.1038/NRG2484

[145] Petricoin EF, Zoon KC, Kohn EC, Barrett JC, Liotta LA. Clinical proteomics: translating benchside promise into bedside reality. *Nat Rev Drug Discov*. 2002;1(9):683-695. doi:10.1038/NRD891

[146] Buescher JM, Driggers EM. Integration of omics: more than the sum of its parts. *Cancer Metab*. 2016;4(1):1-8. doi:10.1186/s40170-016-0143-y

[147] Gardner JG, Crouch L, Labourel A, Forsberg Z, Bukhman Y V., Vaaje-Kolstad G, et al. Systems biology defines the biological significance of redox-active proteins during cellulose degradation in an aerobic bacterium. *Mol Microbiol*. 2014;94(5):1121-1133. doi:10.1111/mmi.12821

[148] Lee HS, Lee HJ, Choi SW, Her S, Oh DH. Purification and Characterization of Antifungal Chitinase from Pseudomonas sp. YHS-A2. *J Microbiol Biotechnol*. 1997;7(2):107-113.

[149] Singh PP, Shin YC, Park CS, Chung YR. Biological Control of Fusarium Wilt of Cucumber by Chitinolytic Bacteria. *Phytopathology*. 1998;89(1):92-99. doi:10.1094/phyto.1999.89.1.92

[150] Yoon JH, Choi JH, Kang SJ, Choi NS, Lee JS, Song JJ. Jeongeupia naejangsanensis gen. nov., sp. nov., a cellulose-degrading bacterium isolated from forest soil from Naejang Mountain in Korea. *Int J Syst Evol Microbiol*. 2010;60(3):615-619. doi:10.1099/ijs.0.012591-0

[151] Chen WM, Chang RC, Cheng CY, Shiau YW, Sheu SY. Jeongeupia chitinilytica sp. nov., a chitinolytic bacterium isolated from soil. *Int J Syst Evol Microbiol*. 2013;63(Pt_3):934-938. doi:10.1099/ijs.0.043125-0

[152] Murthy N, Bleakley B. Simplified Method of Preparing Colloidal Chitin Used For Screening of Chitinase- Producing Microorganisms. *Internet J Microbiol*. 2012;10(1):1-5. doi:10.5580/2e93

[153] Ritz M, Ahmad N, Brueck T, Mehlmer N. Comparative Genome-Wide Analysis of Two Caryopteris x Clandonensis Cultivars: Insights on the Biosynthesis of Volatile Terpenoids. *Plants*. 2023;12(3). doi:10.3390/plants12030632

[154] Koren S, Walenz BP, Berlin K, Miller JR, Bergman NH, Phillippy AM. Canu: Scalable and accurate long-read assembly via adaptive κ-mer weighting and repeat separation. *Genome Res*. 2017;27(5):722-736. doi:10.1101/gr.215087.116

[155] Li W, O'Neill KR, Haft DH, DiCuccio M, Chetvernin V, Badretdin A, et al. RefSeq: expanding the Prokaryotic Genome Annotation Pipeline reach with protein family model curation. *Nucleic Acids Res*. 2021;49(D1):D1020-D1028. doi:10.1093/nar/gkaa1105

[156] Tatusova T, DiCuccio M, Badretdin A, Chetvernin V, Nawrocki EP, Zaslavsky L, et al. NCBI prokaryotic genome annotation pipeline. *Nucleic Acids Res*. 2016;44(14):6614-6624. doi:10.1093/nar/gkw569

[157] Haft DH, DiCuccio M, Badretdin A, Brover V, Chetvernin V, O'Neill K, et al. RefSeq: an update on prokaryotic genome annotation and curation. *Nucleic Acids Res*. 2018;46(D1):D851-D860. doi:10.1093/nar/gkx1068

[158] Ciufo S, Kannan S, Sharma S, Badretdin A, Clark K, Turner S, et al. Using average nucleotide identity to improve taxonomic assignments in prokaryotic genomes at the NCBI. *Int J Syst Evol Microbiol*. 2018;68(7):2386-2392. doi:10.1099/ijsem.0.002809

[159] Lomsadze A, Gemayel K, Tang S, Borodovsky M. Modeling leaderless transcription and atypical genes results in more accurate gene prediction in prokaryotes. *Genome Res*. 2018;28(7):1079-1089. doi:10.1101/gr.230615.117

[160] Haft DH, Loftus BJ, Richardson DL, Yang F, Eisen JA, Paulsen IT, et al. TIGRFAMs: a protein family resource for the functional identification of proteins. *Nucleic Acids Res*. 2001;29(1):41-43. doi:10.1093/nar/29.1.41

[161] Haft DH, Selengut JD, Richter RA, Harkins D, Basu MK, Beck E. TIGRFAMs and Genome Properties in 2013. *Nucleic Acids Res*. 2013;41(Database issue):D387-95. doi:10.1093/nar/gks1234

[162] Selengut JD, Haft DH, Davidsen T, Ganapathy A, Gwinn-Giglio M, Nelson WC, et al. TIGRFAMs and Genome Properties: tools for the assignment of molecular function and biological process in prokaryotic genomes. *Nucleic Acids Res*. 2007;35(Database issue):D260-4. doi:10.1093/nar/gkl1043

[163] Haft DH, Selengut JD, White O. The TIGRFAMs database of protein families. *Nucleic Acids Res*. 2003;31(1):371-373. doi:10.1093/nar/gkg128

[164] Parks DH, Imelfort M, Skennerton CT, Hugenholtz P, Tyson GW. CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Res*. Published online 2015:1043-1055. doi:10.1101/gr.186072.114.Freely

[165] Manni M, Berkeley MR, Seppey M, Simão FA, Zdobnov EM. BUSCO Update: Novel and Streamlined Workflows along with Broader and Deeper Phylogenetic Coverage for Scoring of Eukaryotic, Prokaryotic, and Viral Genomes. *Mol Biol Evol*. 2021;38(10):4647-4654. doi:10.1093/molbev/msab199

[166] Hitch TCA, Riedel T, Oren A, Overmann J, Lawley TD, Clavel T. Automated analysis of genomic sequences facilitates high-throughput and comprehensive description of bacteria. *ISME Commun*. 2021;1(1):16. doi:10.1038/s43705-021-00017-z

[167] Kanehisa M, Sato Y, Morishima K. BlastKOALA and GhostKOALA: KEGG Tools for Functional Characterization of Genome and Metagenome Sequences. *J Mol Biol*. 2016;428(4):726-731. doi:10.1016/j.jmb.2015.11.006

[168] Kyoto University Bioinformatics Center. GenomeNet. Published 2023. https://www.genome.jp/tools/ete/

[169] Huerta-Cepas J, Serra F, Bork P. ETE 3: Reconstruction, Analysis, and Visualization of Phylogenomic Data. *Mol Biol Evol*. 2016;33(6):1635-1638. doi:10.1093/molbev/msw046

[170] Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res*. 1994;22(22):4673-4680. doi:10.1093/nar/22.22.4673

[171] Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst Biol*. 2010;59(3):307-321. doi:10.1093/SYSBIO/SYQ010

[172] Zheng J, Ge Q, Yan Y, Zhang X, Huang L, Yin Y. dbCAN3: automated carbohydrate-active enzyme and substrate annotation. *Nucleic Acids Res*. Published online May 2023:gkad328. doi:10.1093/nar/gkad328

[173] Dong MJ, Luo H, Gao F. DoriC 12.0: an updated database of replication origins in both complete and draft prokaryotic genomes. *Nucleic Acids Res*. 2023;51(D1):D117-D120. doi:10.1093/nar/gkac964

[174] Huerta-Cepas J, Szklarczyk D, Heller D, Hernández-Plaza A, Forslund SK, Cook H, et al. eggNOG 5.0: a hierarchical, functionally and phylogenetically annotated orthology resource based on 5090 organisms and 2502 viruses. *Nucleic Acids Res*. 2019;47(D1):D309-D314. doi:10.1093/nar/gky1085

[175] Price MN, Dehal PS, Arkin AP. FastTree 2--approximately maximum-likelihood trees for large alignments. *PLoS One*. 2010;5(3):e9490. doi:10.1371/journal.pone.0009490

[176] Juncker AS, Willenbrock H, Von Heijne G, Brunak S, Nielsen H, Krogh A. Prediction of lipoprotein signal peptides in Gram-negative bacteria. *Protein Sci*. 2003;12(8):1652-1662. doi:10.1110/ps.0303703

[177] Arndt D, Grant JR, Marcu A, Sajed T, Pon A, Liang Y, et al. PHASTER: a better, faster version of the PHAST phage search tool. *Nucleic Acids Res*. 2016;44(W1):W16-21. doi:10.1093/nar/gkw387

[178] Darling AE, Mau B, Perna NT. Progressivemauve: Multiple genome alignment with gene gain, loss and rearrangement. *PLoS One*. 2010;5(6). doi:10.1371/journal.pone.0011147

[179] Bendtsen JD, Kiemer L, Fausbøll A, Brunak S. Non-classical protein secretion in bacteria. *BMC Microbiol*. 2005;5(1):58. doi:10.1186/1471-2180-5-58

[180] Teufel F, Almagro Armenteros JJ, Johansen AR, Gíslason MH, Pihl SI, Tsirigos KD, et al. SignalP 6.0 predicts all five types of signal peptides using protein language models. *Nat Biotechnol*. 2022;40(7):1023-1025. doi:10.1038/s41587-021-01156-3

[181] Waterhouse A, Bertoni M, Bienert S, Studer G, Tauriello G, Gumienny R, et al. SWISS-MODEL: homology modelling of protein structures and complexes. *Nucleic Acids Res*. 2018;46(W1):W296-W303. doi:10.1093/nar/gky427

[182] Studer G, Rempfer C, Waterhouse AM, Gumienny R, Haas J, Schwede T. QMEANDisCo-distance constraints applied on model quality estimation. *Bioinformatics*. 2020;36(6):1765-1771. doi:10.1093/bioinformatics/btz828

[183] Meier-Kolthoff JP, Carbasse JS, Peinado-Olarte RL, Göker M. TYGS and LPSN: A database tandem for fast and reliable genome-based classification and nomenclature of prokaryotes. *Nucleic Acids Res*. 2022;50(D1):D801-D807. doi:10.1093/nar/gkab902

[184] Meier-Kolthoff JP, Göker M. TYGS is an automated high-throughput platform for state-of-the-art genome-based taxonomy. *Nat Commun*. 2019;10(1). doi:10.1038/s41467-019-10210-3

[185] Ondov BD, Treangen TJ, Melsted P, Mallonee AB, Bergman NH, Koren S, et al. Mash: fast genome and metagenome distance estimation using MinHash. *Genome Biol*. 2016;17(1):132. doi:10.1186/s13059-016-0997-x

[186] Lagesen K, Hallin P, Rødland EA, Stærfeldt HH, Rognes T, Ussery DW. RNAmmer: consistent and rapid annotation of ribosomal RNA genes. *Nucleic Acids Res*. 2007;35(9):3100-3108. doi:10.1093/nar/gkm160

[187] Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, et al. BLAST+: architecture

and applications. *BMC Bioinformatics*. 2009;10(1):421. doi:10.1186/1471-2105-10-421

[188] Meier-Kolthoff JP, Auch AF, Klenk HP, Göker M. Genome sequence-based species delimitation with confidence intervals and improved distance functions. *BMC Bioinformatics*. 2013;14(1):60. doi:10.1186/1471-2105-14-60

[189] Lefort V, Desper R, Gascuel O. FastME 2.0: A Comprehensive, Accurate, and Fast Distance-Based Phylogeny Inference Program. *Mol Biol Evol*. 2015;32(10):2798-2800. doi:10.1093/molbev/msv150

[190] Farris JS. Estimating Phylogenetic Trees from Distance Matrices. *Am Nat*. 1972;106(951):645-668.

[191] Kreft Ł, Botzki A, Coppens F, Vandepoele K, Van Bel M. PhyD3: a phylogenetic tree viewer with extended phyloXML support for functional genomics data visualization. *Bioinformatics*. 2017;33(18):2946-2947. doi:10.1093/bioinformatics/btx324

[192] Engelhart-Straub S, Cavelius P, Hölzl F, Haack M, Awad D, Brueck T, et al. Effects of Light on Growth and Metabolism of Rhodococcus erythropolis. *Microorganisms*. 2022;10(8). doi:10.3390/microorganisms10081680

[193] Fuchs T, Melcher F, Rerop ZS, Lorenzen J, Shaigani P, Awad D, et al. Identifying carbohydrate-active enzymes of Cutaneotrichosporon oleaginosus using systems biology. *Microb Cell Fact*. 2021;20(1):1-18. doi:10.1186/s12934-021-01692-2

[194] Arnold ND, Garbe D, Brück TB. Proteomic and Transcriptomic Analyses to Decipher the Chitinolytic Response of Jeongeupia spp. *Mar Drugs*. 2023;21(8):448. doi:10.3390/md21080448

[195] Deng ZL, Münch PC, Mreches R, McHardy AC. Rapid and accurate identification of ribosomal RNA sequences via deep learning. *Nucleic Acids Res*. 2022;50(10):e60-e60. doi:10.1093/nar/gkac112

[196] Chen S, Zhou Y, Chen Y, Gu J. fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics*. 2018;34(17):i884-i890. doi:10.1093/bioinformatics/bty560

[197] Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*. 2013;29(1):15-21. doi:10.1093/bioinformatics/bts635

[198] Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics*. 2014;30(7):923-930. doi:10.1093/bioinformatics/btt656

[199] Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*. 2010;26(1):139-140. doi:10.1093/bioinformatics/btp616

[200] Sentieon. Sentieon provides complete solutions for secondary dna/rna analysis for a variety of sequencing platforms, including short and long reads. Published 2021. https://www.sentieon.com/

[201] Jacquiod S, Franqueville L, Cécillon S, Vogel TM, Simonet P. Soil bacterial community shifts after Chitin enrichment: An integrative metagenomic approach. *PLoS One*. 2013;8(11):1-13. doi:10.1371/journal.pone.0079699

[202] Subramani R, Aalbersberg W. Culturable rare Actinomycetes: diversity, isolation and marine natural product discovery. *Appl Microbiol Biotechnol*. 2013;97(21):9291-9321. doi:10.1007/s00253-013-5229-7

[203] Kielak AM, Cretoiu MS, Semenov A V, Sørensen SJ, van Elsas JD. Bacterial chitinolytic communities respond to chitin and pH alteration in soil. *Appl Environ Microbiol*. 2013;79(1):263-272. doi:10.1128/AEM.02546-12

[204] Cretoiu MS, Kielak AM, Schluter A, van Elsas JD. Bacterial communities in chitin-amended soil as revealed by 16S rRNA gene based pyrosequencing. *Soil Biol Biochem*. 2014;76:5-11. doi:https://doi.org/10.1016/j.soilbio.2014.04.027

[205] Metcalfe AC, Krsek M, Gooday GW, Prosser JI, Wellington EMH. Molecular Analysis of a Bacterial Chitinolytic Community in an Upland Pasture. *Appl Environ Microbiol*. 2002;68(10):5042-5050. doi:10.1128/AEM.68.10.5042-5050.2002

[206] Salah Ud-Din AIM, Roujeinikova A. Methyl-accepting chemotaxis proteins: a core sensing element in prokaryotes and archaea. *Cell Mol Life Sci*. 2017;74(18):3293-3303. doi:10.1007/s00018-017-2514-0

[207] Aunkham A, Zahn M, Kesireddy A, Pothula KR, Schulte A, Baslé A, et al. Structural basis for chitin acquisition by marine Vibrio species. *Nat Commun*. 2018;9(1):220. doi:10.1038/s41467-017-02523-y

[208] Davidson AL, Dassa E, Orelle C, Chen J. Structure, Function, and Evolution of Bacterial ATP-Binding Cassette Systems. *Microbiol Mol Biol Rev*. 2008;72(2):317-364. doi:10.1128/MMBR.00031-07

[209] Nothaft H, Dresel D, Willimek A, Mahr K, Niederweis M, Titgemeyer F. The Phosphotransferase System of Streptomyces coelicolor Is Biased for N -Acetylglucosamine Metabolism. *J Bacteriol*. 2003;185(23):7019-7023. doi:10.1128/JB.185.23.7019-7023.2003

[210] van der Meij A, Worsley SF, Hutchings MI, van Wezel GP. Chemical ecology of antibiotic production by actinomycetes. *FEMS Microbiol Rev*. 2017;41(3):392-416. doi:10.1093/femsre/fux005

[211] Berg T, Schild S, Reidl J. Regulation of the chitobiose–phosphotransferase system in Vibrio cholerae. *Arch Microbiol*. 2007;187(6):433. doi:10.1007/s00203-006-0207-4

[212] Keyhani NO, Wang LX, Lee YC, Roseman S. The Chitin Disaccharide,N,N'-Diacetylchitobiose, Is Catabolized byEscherichia coli and Is Transported/Phosphorylated by the Phosphoenolpyruvate:Glycose Phosphotransferase System. *J Biol Chem*. 2000;275(42):33084-33090. doi:10.1074/jbc.M001043200

[213] Weiss V, Magasanik B. Phosphorylation of nitrogen regulator I (NRI) of Escherichia coli. *Proc Natl Acad Sci*. 1988;85(23):8919-8923. doi:10.1073/pnas.85.23.8919

[214] Figueira R, Brown DR, Ferreira D, Eldridge MJG, Burchell L, Pan Z, et al. Adaptation to sustained nitrogen starvation by Escherichia coli requires the eukaryote-like serine/threonine kinase YeaG. *Sci Rep*. 2015;5(1):17524. doi:10.1038/srep17524

[215] Brown DR, Barton G, Pan Z, Buck M, Wigneshweraraj S. Nitrogen stress response and stringent response are coupled in Escherichia coli. *Nat Commun*. 2014;5(1):4115. doi:10.1038/ncomms5115

[216] Huergo LF, Chandra G, Merrick M. PII signal transduction proteins: Nitrogen regulation and beyond. *FEMS Microbiol Rev*. 2013;37(2):251-283. doi:10.1111/j.1574-6976.2012.00351.x

[217] Huergo LF, Dixon R. The Emergence of 2-Oxoglutarate as a Master Regulator Metabolite. *Microbiol Mol Biol Rev*. 2015;79(4):419-435. doi:10.1128/mmbr.00038-15

[218] Costa M de AA, Owen RA, Tammsalu T, Buchanan G, Palmer T, Sargent F. Controlling and co-

ordinating chitinase secretion in a Serratia marcescens population. *Microbiology*. 2019;165(11):1233-1244. doi:10.1099/mic.0.000856

[219] Yang ZD, Zhang MS, Lu DL, Li ZW, Mao HH, Wu L, et al. The chitin utilization mechanisms of a new Chitinibacter sp. isolate SCUT-21. *Green Chem*. 2023;25(14):5575-5590. doi:10.1039/D3GC00969F

[220] Karlsson M, Stenlid J. Evolution of Family 18 Glycoside Hydrolases: Diversity, Domain Structures and Phylogenetic Relationships. *Microb Physiol*. 2009;16(3-4):208-223. doi:10.1159/000151220

[221] Udaya Prakash NA, Jayanthi M, Sabarinathan R, Kangueane P, Mathew L, Sekar K. Evolution, homology conservation, and identification of unique sequence signatures in GH19 family chitinases. *J Mol Evol*. 2010;70(5):466-478. doi:10.1007/s00239-010-9345-z

[222] Svitil AL, Chadhain S, Moore JA, Kirchman DL. Chitin Degradation Proteins Produced by the Marine Bacterium Vibrio harveyi Growing on Different Forms of Chitin. *Appl Environ Microbiol*. 1997;63(2):408-413. doi:10.1128/aem.63.2.408-413.1997

[223] SAITO A, FUJII T, YONEYAMA T, REDENBACH M, OHNO T, WATANABE T, et al. High-multiplicity of Chitinase genes in Streptomyces coelicolor A3(2). *Biosci Biotechnol Biochem*. 1999;63(4):710-718. doi:10.1271/bbb.63.710

[224] Kawase T, Saito A, Sato T, Kanai R, Fujii T, Nikaidou N, et al. Distribution and Phylogenetic Analysis of Family 19 Chitinases in Actinobacteria. *Appl Environ Microbiol*. 2004;70(2):1135-1144. doi:10.1128/AEM.70.2.1135-1144.2004

[225] SUZUKI K, TAIYOJI M, SUGAWARA N, NIKAIDOU N, HENRISSAT B, WATANABE T. The third chitinase gene (chiC) of Serratia marcescens 2170 and the relationship of its product to other bacterial chitinases. *Biochem J*. 1999;343(3):587. doi:10.1042/0264-6021:3430587

[226] Urem M, Świątek-Połatyńska MA, Rigali S, van Wezel GP. Intertwining nutrient-sensory networks and the control of antibiotic production in Streptomyces. *Mol Microbiol*. 2016;102(2):183-195. doi:10.1111/mmi.13464

[227] Romero-Rodríguez A, Robledo-Casados I, Sánchez S. An overview on transcriptional regulators in Streptomyces. *Biochim Biophys Acta - Gene Regul Mech*. 2015;1849(8):1017-1039. doi:10.1016/j.bbagrm.2015.06.007

[228] Heggset EB, Dybvik AI, Hoell IA, Norberg AL, Sørlie M, Eijsink VGH, et al. Degradation of Chitosans with a Family 46 Chitosanase from Streptomyces coelicolor A3(2). *Biomacromolecules*. 2010;11(9):2487-2497. doi:10.1021/bm1006745

[229] Kohlhoff M, Niehues A, Wattjes J, Bénéteau J, Cord-Landwehr S, El Gueddari NE, et al. Chitinosanase: A fungal chitosan hydrolyzing enzyme with a new and unusually specific cleavage pattern. *Carbohydr Polym*. 2017;174:1121-1128. doi:10.1016/j.carbpol.2017.07.001

[230] Xu Y, Gallert C, Winter J. Chitin purification from shrimp wastes by microbial deproteination and decalcification. *Appl Microbiol Biotechnol*. 2008;79(4):687-697. doi:10.1007/s00253-008-1471-9

[231] Jung WJ, Jo GH, Kuk JH, Kim KY, Park RD. Extraction of chitin from red crab shell waste by cofermentation with Lactobacillus paracasei subsp. tolerans KCTC-3074 and Serratia marcescens FS-3. *Appl Microbiol Biotechnol*. 2006;71(2):234-237. doi:10.1007/s00253-005-0126-3

[232] Cira LA, Huerta S, Hall GM, Shirai K. Pilot scale lactic acid fermentation of shrimp wastes for chitin recovery. *Process Biochem*. 2002;37(12):1359-1366. doi:10.1016/S0032-9592(02)00008-0

[233] Shirai K, Guerrero I, Huerta S, Saucedo G, Castillo A, Obdulia Gonzalez R, et al. Effect of initial glucose concentration and inoculation level of lactic acid bacteria in shrimp waste ensilation. *Enzyme Microb Technol*. 2001;28(4-5):446-452. doi:10.1016/S0141-0229(00)00338-0

[234] Castro R, Guerrero-Legarreta I, Bórquez R. Chitin extraction from Allopetrolisthes punctatus crab using lactic fermentation. *Biotechnol Reports*. 2018;20:e00287. doi:10.1016/j.btre.2018.e00287

[235] Pacheco N, Garnica-Gonzalez M, Gimeno M, Bárzana E, Trombotto S, David L, et al. Structural characterization of chitin and chitosan obtained by biological and chemical methods. *Biomacromolecules*. 2011;12(9):3285-3290. doi:10.1021/bm200750t

[236] Stachowiak B, Szulc P. Astaxanthin for the Food Industry. *Molecules*. 2021;26(9). doi:10.3390/MOLECULES26092666

[237] Dutta S, Kumar SPJ, Banerjee R. A comprehensive review on astaxanthin sources, structure, biochemistry and applications in the cosmetic industry. *Algal Res*. 2023;74:103168. doi:10.1016/J.ALGAL.2023.103168

[238] Giannaccare G, Pellegrini M, Senni C, Bernabei F, Scorcia V, Cicero AFG. Clinical Applications of Astaxanthin in the Treatment of Ocular Diseases: Emerging Insights. *Mar Drugs*. 2020;18(5). doi:10.3390/MD18050239

[239] Huang WC, Zhao D, Guo N, Xue C, Mao X. Green and Facile Production of Chitin from Crustacean Shells Using a Natural Deep Eutectic Solvent. *J Agric Food Chem*. 2018;66(45):11897-11901. doi:10.1021/acs.jafc.8b03847

[240] Paiva A, Craveiro R, Aroso I, Martins M, Reis RL, Duarte ARC. Natural Deep Eutectic Solvents - Solvents for the 21st Century. *ACS Sustain Chem Eng*. 2014;45(24). doi:10.1002/chin.201424290

[241] Feng M, Lu X, Zhang J, Li Y, Shi C, Lu L, et al. Direct conversion of shrimp shells to: O -acylated chitin with antibacterial and anti-tumor effects by natural deep eutectic solvents. *Green Chem*. 2019;21(1):87-98. doi:10.1039/c8gc02506a

[242] Vanda H, Dai Y, Wilson EG, Verpoorte R, Choi YH. Green solvents from ionic liquids and deep eutectic solvents to natural deep eutectic solvents. *Comptes Rendus Chim*. 2018;21(6):628-638. doi:10.1016/j.crci.2018.04.002

[243] Yuntao D, Jaapvan S, Geert Jan W, Robert V, Young Hae C. Natural deep eutectic solvents as new potential media for green technology. *Anal Chim Acta*. 2013;766(2013):61-68. doi:10.1016/j.aca.2012.12.019

[244] Zdanowicz M, Wilpiszewska K, Spychaj T. Deep eutectic solvents for polysaccharides processing. A review. *Carbohydr Polym*. 2018;200:361-380. doi:10.1016/j.carbpol.2018.07.078

[245] Mukesh C, Mondal D, Sharma M, Prasad K. Choline chloride-thiourea, a deep eutectic solvent for the production of chitin nanofibers. *Carbohydr Polym*. 2014;103(1):466-471. doi:10.1016/j.carbpol.2013.12.082

[246] Jaworska MM, Górak A. New ionic liquids for modification of chitin particles. *Res Chem Intermed*. 2018;44(8):4841-4854. doi:10.1007/s11164-018-3337-9

[247] King C, Shamshina JL, Gurau G, Berton P, Khan NFAF, Rogers RD. A platform for more sustainable chitin films from an ionic liquid process. *Green Chem*. 2017;19(1):117-126. doi:10.1039/c6gc02201d

[248] Zhu P, Gu Z, Hong S, Lian H. One-pot production of chitin with high purity from lobster shells

using choline chloride–malonic acid deep eutectic solvent. *Carbohydr Polym*. 2017;177(August):217-223. doi:10.1016/j.carbpol.2017.09.001

[249] Roda A, Matias AA, Paiva A, Duarte ARC. Polymer science and engineering using deep eutectic solvents. *Polymers (Basel)*. 2019;11(5):1-22. doi:10.3390/polym11050912

[250] Li Q, Kamal R, Wang Q, Yu X, Zhao ZK. Lipid Production from Amino Acid Wastes by the Oleaginous Yeast Rhodosporidium toruloides. *Energies 2020, Vol 13, Page 1576*. 2020;13(7):1576. doi:10.3390/EN13071576

[251] Sturm V, Banse M, Salamon P. The role of feed-grade amino acids in the bioeconomy: Contribution from production activities and use in animal feed. *Clean Environ Syst*. 2022;4:100073. doi:10.1016/J.CESYS.2022.100073

[252] Kaczmarek MB, Struszczyk-Swita K, Li X, Szczęsna-Antczak M, Daroch M. Enzymatic Modifications of Chitin, Chitosan, and Chitooligosaccharides. *Front Bioeng Biotechnol*. 2019;7(September). doi:10.3389/fbioe.2019.00243

[253] Jung W jin, Park R dong, Sashiwa H. Bioproduction of Chitooligosaccharides: Present and Perspectives. *Mar Drugs*. 2014;12:5328-5356. doi:10.3390/md12105328

[254] Ismail SA, Hassan ME, Hashem AM. Single step hydrolysis of chitin using thermophilic immobilized exochitinase on carrageenan-guar gum gel beads. *Biocatal Agric Biotechnol*. 2019;21:101281. doi:https://doi.org/10.1016/j.bcab.2019.101281

[255] Zhou J, Harindintwali JD, Yang W, Han M, Deng B, Luan H, et al. Engineering of a chitosanase fused to a carbohydrate-binding module for continuous production of desirable chitooligosaccharides. *Carbohydr Polym*. 2021;273:118609. doi:https://doi.org/10.1016/j.carbpol.2021.118609

[256] Kidibule PE, Costa J, Atrei A, Plou FJ, Fernandez-Lobato M, Pogni R. Production and characterization of chitooligosaccharides by the fungal chitinase Chit42 immobilized on magnetic nanoparticles and chitosan beads: selectivity, specificity and improved operational utility. *RSC Adv*. 2021;11(10):5529-5536. doi:10.1039/D0RA10409D

[257] Chen Y, Ling Z, Mamtimin T, Khan A, Peng L, Yang J, et al. Chitooligosaccharides production from shrimp chaff in chitosanase cell surface display system. *Carbohydr Polym*. 2022;277:118894. doi:https://doi.org/10.1016/j.carbpol.2021.118894

[258] Mekasha S, Byman IR, Lynch C, Toupalová H, Anděra L, Næs T, et al. Development of enzyme cocktails for complete saccharification of chitin using mono-component enzymes from Serratia marcescens. *Process Biochem*. 2017;56.

[259] Therien JPD, Hammerer F, Friscic T, Auclair K. Mechanoenzymatic breakdown of chitinous material to N-acetylglucosamine: the benefits of a solvent-less environment. *ChemSusChem*. Published online 2019. doi:10.1002/cssc.201901310

[260] Pan M, Li J, Lv X, Du G, Liu L. Molecular engineering of chitinase from Bacillus sp. DAU101 for enzymatic production of chitooligosaccharides. *Enzyme Microb Technol*. 2019;124:54-62. doi:https://doi.org/10.1016/j.enzmictec.2019.01.012

[261] Su H, Gao L, Sun J, Mao X. Engineering a carbohydrate binding module to enhance chitinase catalytic efficiency on insoluble chitinous substrate. *Food Chem*. 2021;355:129462. doi:https://doi.org/10.1016/j.foodchem.2021.129462

[262] Kowsari M, Motallebi M, Zamani M. Protein engineering of chit42 towards improvement of chitinase and antifungal activities. *Curr Microbiol*. 2014;68(4):495-502. doi:10.1007/s00284-013-0494-3

[263] Fan Y, Fang W, Xiao Y, Yang X, Zhang Y, Bidochka MJ, et al. Directed evolution for increased chitinase activity. *Appl Microbiol Biotechnol*. 2007;76(1):135-139. doi:10.1007/s00253-007-0996-7

[264] Weyer R, Hellmann MJ, Hamer-Timmermann SN, Singh R, Moerschbacher BM. Customized chitooligosaccharide production—controlling their length via engineering of rhizobial chitin synthases and the choice of expression system . *Front Bioeng Biotechnol* . 2022;10.

[265] Naqvi S, Moerschbacher BM. The cell factory approach toward biotechnological production of high-value chitosan oligomers and their derivatives: an update. *Crit Rev Biotechnol*. 2017;37(1):11-25. doi:10.3109/07388551.2015.1104289

[266] Wu J. Distributions of scientific funding across universities and research disciplines. *J Informetr*. 2015;9(1):183-196. doi:10.1016/J.JOI.2014.12.007

[267] (BMBF) B für B und F. *Förderkatalog, Einzelplan 30 Nach Förderbereichen Und Förderarten (in Tausend Euro)*.; 2022.

[268] Mourya VK, Inamdar NN. Chitosan-modifications and applications: Opportunities galore. *React Funct Polym*. 2008;68(6):1013-1051. doi:10.1016/j.reactfunctpolym.2008.03.002

[269] Saranya TS, Rajan VK, Biswas R, Jayakumar R, Sathianarayanan S. Synthesis, characterisation and biomedical applications of curcumin conjugated chitosan microspheres. *Int J Biol Macromol*. 2018;110:227-233. doi:10.1016/J.IJBIOMAC.2017.12.044

[270] Badwan AA, Rashid I, Al Omari MMH, Darras FH. Chitin and Chitosan as Direct Compression Excipients in Pharmaceutical Applications. *Mar Drugs 2015, Vol 13, Pages 1519-1547*. 2015;13(3):1519-1547. doi:10.3390/MD13031519

[271] Li X, Min M, Du N, Gu Y, Hode T, Naylor M, et al. Chitin, chitosan, and glycated chitosan regulate immune responses: The novel adjuvants for cancer vaccine. *Clin Dev Immunol*. 2013;2013. doi:10.1155/2013/387023

[272] Garg U, Chauhan S, Nagaich U, Jain N. Current Advances in Chitosan Nanoparticles Based Drug Delivery and Targeting. *Adv Pharm Bull*. 2019;9(2):195-204. doi:10.15171/apb.2019.023

[273] Rizeq BR, Younes NN, Rasool K, Nasrallah GK. Synthesis, Bioapplications, and Toxicity Evaluation of Chitosan-Based Nanoparticles. *Int J Mol Sci*. 2019;20(22):5776. doi:10.3390/ijms20225776

[274] Jhaveri J, Raichura Z, Khan T, Momin M, Omri A, Stancanelli R, et al. Chitosan Nanoparticles-Insight into Properties, Functionalization and Applications in Drug Delivery and Theranostics. *Mol 2021, Vol 26, Page 272*. 2021;26(2):272. doi:10.3390/MOLECULES26020272

[275] AL-Jbour ND, Beg MD, Gimbun J, Alam AKMM. An Overview of Chitosan Nanofibers and their Applications in the Drug Delivery Process. *Curr Drug Deliv*. 2019;16(4):272-294. doi:10.2174/1567201816666190123121425

[276] Peers S, Montembault A, Ladavière C. Chitosan hydrogels for sustained drug delivery. *J Control Release*. 2020;326:150-163. doi:10.1016/J.JCONREL.2020.06.012

[277] Shen X, Shamshina JL, Berton P, Gurau G, Rogers RD. Hydrogels based on cellulose and chitin: Fabrication, properties, and applications. *Green Chem*. 2015;18(1):53-75. doi:10.1039/c5gc02396c

[278] Trombino S, Curcio F, Poerio T, Pellegrino M, Russo R, Cassano R. Chitosan Membranes Filled with Cyclosporine A as Possible Devices for Local Administration of Drugs in the Treatment of Breast Cancer. *Molecules*. 2021;26(7). doi:10.3390/MOLECULES26071889

[279] Singh DJ, Lohade AA, Parmar JJ, Hegde DD, Soni P, Samad A, et al. Development of Chitosan-

based Dry Powder Inhalation System of Cisplatin for Lung Cancer. *Indian J Pharm Sci*. 2012;74(6):521-526. doi:10.4103/0250-474X.110584

[280] Lv J, Liu W, Shi G, Zhu F, He X, Zhu Z, et al. Human cardiac extracellular matrix-chitosan-gelatin composite scaffold and its endothelialization. *Exp Ther Med*. 2020;19(2):1225-1234. doi:10.3892/ETM.2019.8349

[281] Patel B, Manne R, Patel DB, Gorityala S, Palaniappan A, Kurakula M. Chitosan as Functional Biomaterial for Designing Delivery Systems in Cardiac Therapies. *Gels 2021, Vol 7, Page 253*. 2021;7(4):253. doi:10.3390/GELS7040253

[282] Lauritano D, Limongelli L, Moreo G, Favia G, Carinci F. Nanomaterials for Periodontal Tissue Engineering: Chitosan-Based Scaffolds. A Systematic Review. *Nanomater 2020, Vol 10, Page 605*. 2020;10(4):605. doi:10.3390/NANO10040605

[283] Feng L, Liu R, Zhang X, Li J, Zhu L, Li Z, et al. Thermo-Gelling Dendronized Chitosans as Biomimetic Scaffolds for Corneal Tissue Engineering. *ACS Appl Mater Interfaces*. 2021;13(41):49369-49379. doi:10.1021/ACSAMI.1C16087/SUPPL_FILE/AM1C16087_SI_001.PDF

[284] Deng P, Jin W, Liu Z, Gao M, Zhou J. Novel multifunctional adenine-modified chitosan dressings for promoting wound healing. *Carbohydr Polym*. 2021;260:117767. doi:10.1016/J.CARBPOL.2021.117767

[285] Siddique S, Chow JCL. Application of Nanomaterials in Biomedical Imaging and Cancer Therapy. *Nanomater 2020, Vol 10, Page 1700*. 2020;10(9):1700. doi:10.3390/NANO10091700

[286] Peng X, Xu X, Deng Y, Xie X, Xu L, Xu X, et al. Ultrafast Self-Gelling and Wet Adhesive Powder for Acute Hemostasis and Wound Healing. *Adv Funct Mater*. 2021;31(33):2102583. doi:10.1002/ADFM.202102583

[287] Zu Y, Bi J, Yan H, Wang H, Song Y, Zhu BW, et al. Nanostructures Derived from Starch and Chitosan for Fluorescence Bio-Imaging. *Nanomater 2016, Vol 6, Page 130*. 2016;6(7):130. doi:10.3390/NANO6070130

[288] Wang H, Mukherjee S, Yi J, Banerjee P, Chen Q, Zhou S. Biocompatible Chitosan-Carbon Dot Hybrid Nanogels for NIR-Imaging-Guided Synergistic Photothermal-Chemo Therapy. *ACS Appl Mater Interfaces*. 2017;9(22):18639-18649. doi:10.1021/acsami.7b06062

[289] Agrawal P, Strijkers GJ, Nicolay K. Chitosan-based systems for molecular imaging. *Adv Drug Deliv Rev*. 2010;62(1):42-58. doi:10.1016/J.ADDR.2009.09.007

[290] Kulka K, Sionkowska A. Chitosan Based Materials in Cosmetic Applications: A Review. *Mol 2023, Vol 28, Page 1817*. 2023;28(4):1817. doi:10.3390/MOLECULES28041817

[291] Desai N, Rana D, Salave S, Gupta R, Patel P, Karunakaran B, et al. Chitosan: A Potential Biopolymer in Drug Delivery and Biomedical Applications. *Pharmaceutics*. 2023;15(1313). doi:10.3390/pharmaceutics15041313

[292] Wang D, Zou Y, Wang N, Wu J. Chitosan hydrochloride salt stabilized emulsion as vaccine adjuvant. *Carbohydr Polym*. 2022;296:119879. doi:10.1016/J.CARBPOL.2022.119879

[293] Dmour I, Islam N. Recent advances on chitosan as an adjuvant for vaccine delivery. *Int J Biol Macromol*. 2022;200:498-519. doi:10.1016/J.IJBIOMAC.2021.12.129

[294] Wang J, Zhuang S. Chitosan-based materials: Preparation, modification and application. *J Clean Prod*. 2022;355:131825. doi:10.1016/J.JCLEPRO.2022.131825