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Identifying and quantifying the degradation efficiency of chitinase in *Pseudomonas*

Bachelor's thesis

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Annotation

This thesis investigated the viability of employing chitinase from *Pseudomonas* bacteria produced in bioreactors as a potential solution to control fungal crop diseases in order to minimize the demand for harmful synthetic pesticides in agriculture. Significant works related to the research were collated, providing an extensive literature review. A chitinase assay kit and fluorescent plate reader were used to analyze the efficiency of bacterial chitinase in degrading chitin from different sources and recorded fluorescent readings corresponding to endochitinase and exochitinase were quantified.

Declaration

I declare that I am the author of this qualification thesis and that in writing it I have used the sources and literature displayed in the list of used sources only.

Czech Republic, 16.8.2023

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Acknowledgement

First and foremost, I would like to extend my sincere gratitude to my supervisor doc. Ing. Jiří Bárta, PhD. The entirety of this work would not have been possible without his consideration and sharing of resources. I am also thankful for the collaboration with Mgr. Lenka Čapková, who welcomed me into her lab and assisted me with the principle measuring, and doc. Ing. Martin Halecký, PhD. and the entire Prague team, who cultivated the bacterial samples. It is my pleasure to recognize that the completion of this research is rooted in their contributions and expertise. Finally, I am forever grateful for my family and friends (especially Leen and Yura), who never failed to reassure and encourage me throughout the duration of this thesis.

Abstract

Fungal diseases are among the main contributors to crop deterioration and harvest loss within the agricultural industry. Today, it is well-known that synthetic pesticides traditionally used to treat fungal plant pathogen outbreaks not only compromise the surrounding habitats and ecological communities, but also adversely affect human health. To minimize many of these risks, biological derivatives are being investigated as potential replacements for chemical fungicides. Thus, this research aimed to determine the feasibility of using chitinolytic bacterial enzymes as a biofungicide. The chitinases synthesized by a bacterial strain from the genus *Pseudomonas*, cultivated in bioreactors by a research team in Prague, were analyzed. Chitin inducers were used to stimulate the bacteria to produce chitinases, which were quantified using fluorometric assays to measure enzyme activity. Depending on factors such as incubation time, enzyme location, and applied inducer, both endochitinase and exochitinase were discovered to be secreted by the bacteria, with endochitinase, the most crucial enzyme for chitin degradation, being present across all samples. Bacterial cultures without exposure to chitin primarily displayed elevated levels of chitobiosidase activity. The results suggest that this *Pseudomonas* species is biologically equipped to recognize and decompose chitin. Further, the chitinases exhibited significant enzyme activity indicating this strain could be cultivated as a biocontrol agent, providing a promising eco-friendly alternative to synthetic pesticides in agriculture.

List of abbreviations

4-MU	4-Methylumbelliferyl
CHIB	chitobiosidase
ENDO	endochitinase
FP	fluorescent pseudomonads
FPB-00	classified *Pseudomonas* bacterial strain
GlcNAc	N-acetylglucosamine
GH	glycoside hydrolase
NAG	N-acetyl glucosaminidase
OD	optical density
PVD	pyoverdine

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1 Introduction

1.1 Chitin in nature

Chitin is the second most abundant organic polysaccharide present in nature after cellulose. A linear amide derivative of glucose, chitin is found ubiquitously in nature but most notably in the exoskeletons of crustaceans, the cuticles and guts of insects, and within the cell walls of fungi [1]. It is synthesized by the enzyme chitin synthase from the activated precursor uridine diphosphate and belongs to family 18 and 19 glycoside hydrolase (GH) as per the CAZy database [2,3]. Combined, there are an estimated 10 to 100 billion tons of chitin produced on Earth annually [4]. Depending on its origin, chitin exists as two main polymeric forms of mainly of β-(1 → 4)-linked 2-acetamido-2-deoxy-β-D-glucopyranose units (Fig. 1) and partially of β-(1 → 4)-linked 2-amino-2-deoxy-β-D-glucopyranose [5,6]. The most stable and common polymorph, α-chitin, is associated with monoclinic cells and corresponds to anti-parallel arrangements of polymer chains while another form, β-chitin, is arranged according to orthorhombic cells and is characterized by parallel arrangements. The third variation called γ-chitin is made up of alternating arrangements of both α- and β-chitin [6,7,8].

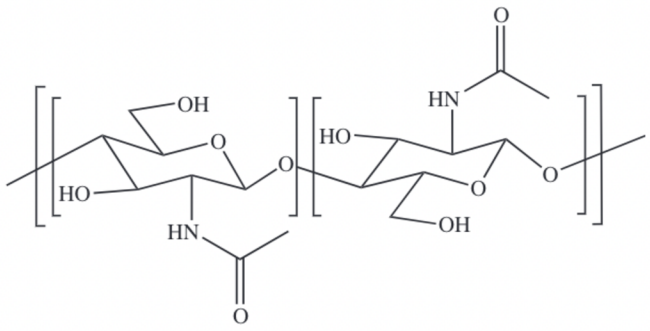


Figure 1: Chemical structure of chitin [10].

The molecular structure of chitin includes intra- and intermolecular hydrogen bonding, making it highly insoluble in many organic and inorganic solvents. It is, however, soluble in highly concentrated inorganic acids such as hydrochloric, sulfuric, and phosphoric acids [9]. The solubility is determined by the acetylation degree defined by the number of N-acetyl amino groups. The degree of N-acetylation is additionally used as a parameter to differentiate chitin from chitosan [10], which is an amino-polysaccharide derived from chitin and has an acetylation degree of less than 50% [11].

When chitin chains link together, they form strong micro/nanofibers with highly ordered symmetry. The nanofibrils are a planar woven and branched network arrangement of 18-25 polysaccharide chains and are typically 2-7 nm in diameter and 300 nm in length [12].

Chitin is a biopolymer of increasingly high interest due to its versatility and presence in a variety of global industries. In seafood manufacturing sectors, approximately 75% of the total shellfish biomass is discarded as waste of which the dry weight is 20 to 58% chitin [13]. It is also considered as a potential target in industries such as agriculture, where chitinous pests like crop-damaging nematodes and pathogenic fungi reduce viable harvests and therefore cause farmers negative economic impacts. Fungal diseases are especially prevalent and significantly problematic in the cultivation of potatoes, fruits, vegetables, vines, and cereals [14].

Pathogens that are equipped with chitin have the advantage of immunogenicity, as the chitin which is of self-origin (own body component) protects the organism while posing a threat to other life forms. In fungi particularly, the chitinous cell wall acts as the extracellular armor of the cell. Although the biosynthesis of the polysaccharide layer is not thoroughly understood, it is now clear that the cell wall undergoes composition and localization modifications that not only support in the defense against non-self-origin (other organism) immune systems but may also function as true virulence factors [15]. This implies that in pathogenic fungi, the cell wall is employed as a tool for host invasion and as a shield against host defense mechanisms.

1.1.1 Pathogenic role of fungi in plants

Although chitin makes up only a small percentage of the cell wall composition in fungi (up to 10-20% of the dry weight of the cell wall in filamentous fungi) [16], its presence can elicit strong responses from plant immune systems. Plants that produce chitinases will degrade foreign chitin from invader organisms into chitin oligomers or oligosaccharides (CTOS) [17] that trigger the activation of cascading defense mechanisms. Some pathogens, however, have evolved to evade the classical chitin stress response via accumulation of short N-acetylglucosamine (GlcNAc) units in the apoplastic regions. These small oligosaccharides can compete with noncovalent interactions in the apoplast, conditionally altering plant cell wall architecture and accessibility to enzymes, yet are not long enough to stabilize the homodimerization of plant pattern recognition receptors (PRRs), therefore do not activate defense signaling. [17,18].

There are two major categories of pathogenic fungi: biotrophs, which preserve living tissues while simultaneously persisting in and feeding on them, and necrotrophs, which kill healthy cells and then extract nutrients from the dead tissues. Taxonomically, plant pathogen species are found mainly in the phyla *Ascomycota* and *Basidiomycota* [19].

Nearly all the species follow the same general steps to successful host invasion. First, fungal spores must be spontaneously dispersed by wind, water, or insect vectors so that initial contact between host and pathogen can occur. The spores then secrete an adhesive extracellular matrix [19] onto the surface of the plant, ensuring attachment and reducing the chance of being swept away by external forces. When environmental conditions are favorable, the spore begins performing the infiltration of host tissues. This is achieved by various species-dependent mechanisms, including spore germination and subsequent polarized cell growth of runner hyphae along the surface of the plant. Other methods include direct perforation of the plant cuticle via a specialized appressorium cell. This cell differentiates at the tip of the germ tube that extends from the spore and is unique in that its cell wall is enriched with not only chitin, but also densely packed melanin. Due to this highly impermeable melanin layer, the cell can reach enormous turgor pressures of up to 8.0 MPa, which provide the force necessary to drive the hyphal peg through the cuticle and into the underlying epidermal and mesophyll tissue [20,21]. Some fungi use plant anatomy to their advantage and can orient themselves on leaf surfaces, identifying stomatal guard cells and inducing appressorium cell formation directly over the stomata [22]. Infection is then achieved when the penetration peg begins to differentiate into invasive hyphae and substomatal vesicles that aggressively spread within host tissues (Fig. 2) [19,23].

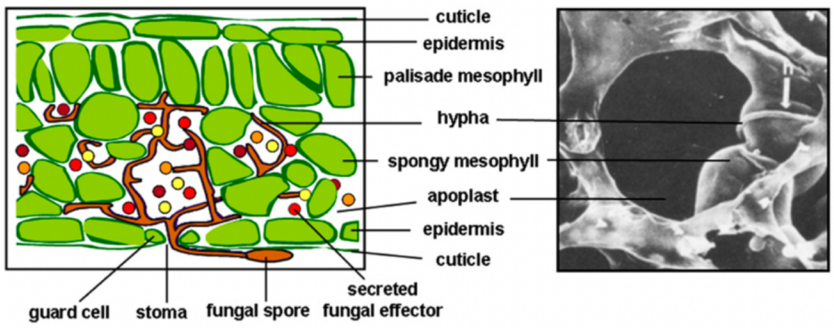


Figure 2: Pathogenic fungus *Cladosporium fulvum* colonizing a tomato leaf. Schematic sketch of fungal growth within leaf cross section (left) and scanning electron microscope image of leaf interior (right). After penetration of an open stomata, the fungus invades the apoplastic space surrounding the mesophyll cells, impairing the host by secreting effectors [23].

Primary hyphae originating from substomatal vesicles are the fundamental structures required for occupying the organs of the plant. Once a biotrophic fungus has established itself within the plant, it can begin to produce haustoria: specialized infection structures that form behind the cell wall, invaginate the cell membrane, and act as a sustainable "cell within cell" complex [24]. As a result of this fungus-plant hybrid structure, the pathogen has a means of nutrient acquisition (using transporters) while simultaneously releasing effectors that aid in the evasion of the host's immune system (Fig. 3) and support the survival of the afflicted cells [23,25].

On the other hand, necrotrophic fungi induce plant cell death to subsequently colonize and feed on the dead tissue. The pathogen must first infect the living plant cells at the initial infection zone, whereupon it comes into direct contact with the host's immune system. For the fungus to survive and progress to the next stages of infection, it is necessary to overcome the plant's defense mechanisms [19]. This is mainly accomplished by the immediate production of necrosis-inducing effectors and manipulation of the host cell death pathways [26], leading to the establishment of a local apoptotic region protecting the pathogen at the zone of infection. The necrotic area can then continue to spread via the exploitation of the plant's immune responses, such as oxidative burst and programmed cell death (PCD), in addition to the production of virulence factors, reactive oxygen species (ROS), host-specific toxins (in some necrotrophs), and hydrolytic enzymes [19,27].

Given the wide range of lifestyles and plethora of host colonization strategies, fungi are an abundantly diverse group of plant pathogens. The reproductive and virulent nature of these organisms enables them to rapidly infect hosts and outmaneuver host defense mechanisms, while their high genetic flexibility is responsible for their ability to develop resistance to fungicides [19]. As a result, plant susceptibility to fungal pathogens is a growing concern and suggests that novel solutions are required to control advancing fungal diseases.

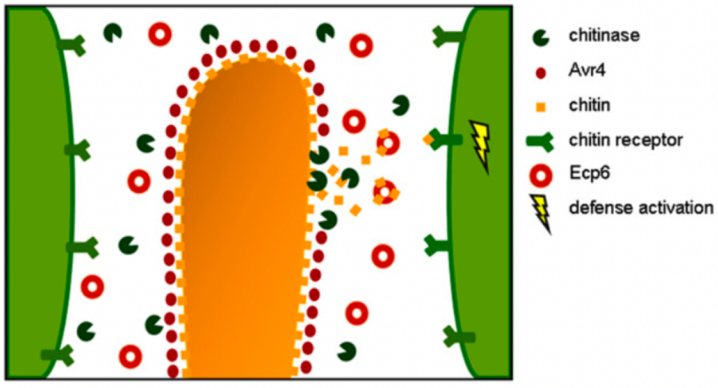


Figure 3: Contribution of chitin-binding effectors to the virulence of fungi. The Avr4 effector protects chitin in the fungus cell wall from being hydrolyzed by chitinases. Chitin fragments that are released trigger the host's defense mechanisms via surface receptors. To avoid provoking the host's immune response the Ecp6 effector sequesters them [23].

1.2 Chitinolytic bacteria

Chitinases are produced by numerous bacterial species of phyla including *Actinobacteria*, *Firmicutes*, *Proteobacteria*, *Pseudomonadota*, green non-sulfur bacteria, and purple bacteria [28]. The enzyme provides bacteria with nutrition and in some cases, enables parasitic behavior [29] or plays a role in nitrogen metabolism [30,31]. The mass of bacterial chitinases ranges from 20 to 60 kDa [31], with most belonging to the GH18 family which is divided into three subfamilies A, B, and C [32]. Several bacteria synthesize chitinases that are classified under the GH19 family [33]. Evidence established by molecular chronometers suggests that bacterial species from different phyla received their chitinase through horizontal gene transfer [34,35]. Although this indicates that chitinolytic bacteria are related through some common ancestral genomes, the phenotypic characteristics of their chitinases are vastly diverse. The functional variety of the enzymes may correspond to different physiological functions of the enzymes, as they have different structures and catalytic mechanisms [36]. Additionally, bacterial chitinases can operate over a broad scope of temperature, pH, and isoelectric points depending on the species and environment [31,37], and are thus regarded as highly versatile proteins of considerable scientific and industrial interest.

1.2.1 Genus *Pseudomonas*

Pseudomonads are taxonomically classified as *Gammaproteobacteria* under the phylum *Pseudomonadota*. They are ubiquitous rod-shaped, Gram-negative, and non-sporulating bacteria that are polar-flagellated (with one or more flagella), which enables their motility. These bacteria survive only under aerobic conditions, metabolizing a wide range of organic compounds as energy sources, and are both catalase- and oxidase-positive [38,39]. In nature, *Pseudomonas* bacteria can colonize and thrive in diverse ecological niches such as soil, plants, freshwater, seawater, and higher organisms (e.g., as pathogens of insects, fish, and humans) [40]. They are commonly regarded as key constituents of the bacterial community and therefore serve important ecological functions in their environment [41,42].

The genus *Pseudomonas* is one of the largest among Gram-negative bacteria and has become a model organism known to exhibit high genetic diversity. Due to the growing availability of *Pseudomonas* genome sequences, the number of newly discovered species within the genus is

steadily rising. Historically, the genus had been assigned an estimated 800 species by the middle of the twentieth century as a result of misannotation and limited genomic analyses [43]. Now, there are approximately 259 confirmed *Pseudomonas* species, excluding subspecies and synonymous species, with over thirty new species being characterized from March 2020 to March 2021 alone [44-46]. Emerging technologies such as next-generation high throughput sequencing (NGS), multilocus sequence analysis (MLSA), matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry, siderophore typing, and single-copy protein-encoding gene homologies (as an accompaniment to 16S rRNA sequence analysis) have revolutionized the standards of species classification [47,48]. Still, approximately 10% of the sequenced *Pseudomonas* genomes available at the National Center for Biotechnology Information (NCBI) cannot be confidently assigned to a particular species. Additionally, other isolates (referred to as "orphan" species) are exceptionally heterogeneous to the point that they cannot be categorized into established groups and are therefore placed within "enigmatic taxa" [46,49].

A study published in 2018 revealed detailed genomic characteristics based on the analysis of 166 *Pseudomonas* type strains isolated from different sources originating from around the world. It established the large variability present across the genus in aspects such as genome size and GC content (Fig. 4). According to the research, the genomic size of the 166 type strains ranged from 3.03 Mbp (*Pseudomonas caeni*) to 7.38 Mbp (*P. saponiphila*) with an average of 5.63 Mbp, while the GC content and number of predicted coding sequences (CDSs) resulted in an average of 61.2% and 5261, respectively. The core genome analyses of the isolates indicated the presence of 794 orthologous CDSs consisting primarily of housekeeping functions. This discovery further supports the plurality of the genus as the core genome contributes to only 11-28% of the gene inventory of any type strain. However, in comparison to the phylogenetic analysis of 1224 *Pseudomonas* genomes available in the US Department of Energy, Joint Genome Institute, Integrated Microbial Genome with Metagenome samples (IMG/M) system [50], the findings of this limited-sample study do not entirely reflect the phylogenetic diversity of *Pseudomonas* spp. as a whole [40]. A more in-depth study from 2016 validated this conclusion by determining that 1224 protein-coding gene families were shared among 1073 multifarious genomes that aggregated to an increasing pangenome size totaling 200,839 protein-coding gene families and 99,176 singleton protein-coding gene families [49-51].

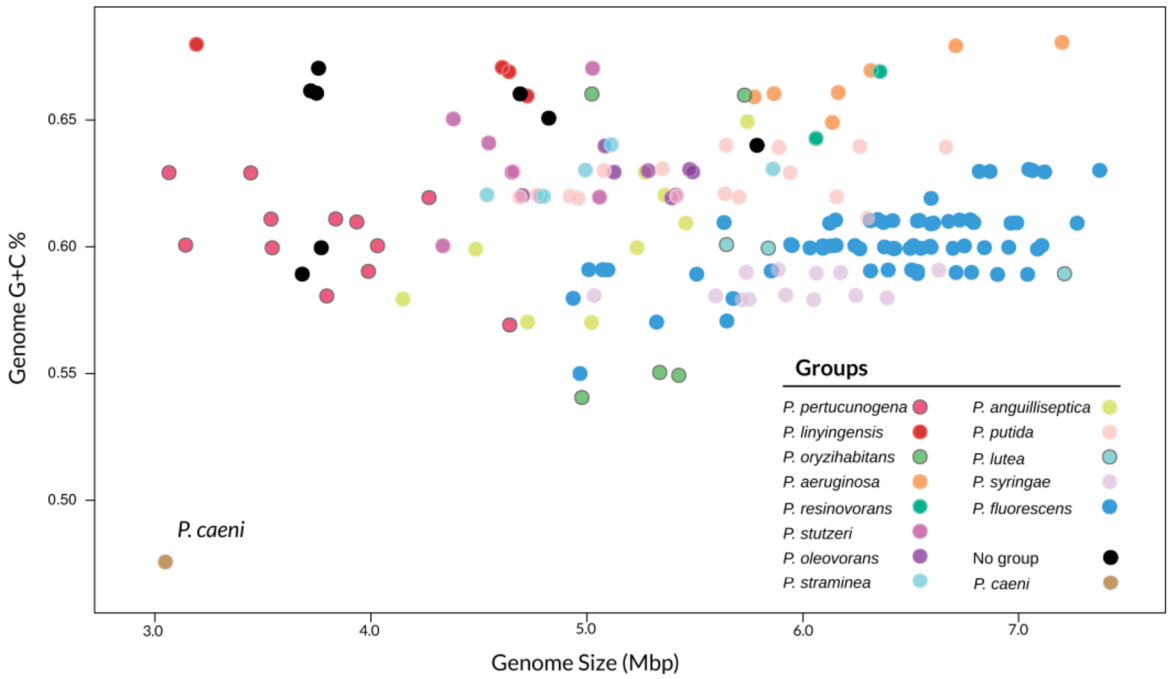


Figure 4: Correlation between genome size and GC content for the various color-coded *Pseudomonas* groups within the 166 type strains [40].

Accumulative analyses have determined that the *Pseudomonas* species are divided into thirteen groups and three newly described groups, with an additional eight orphan groups. The three main lineages include *P. aeruginosa*, *P. fluorescens*, and *P. pertucinogena*. One of the largest groups, *P. fluorescens*, is further subdivided into eleven groups which amount to a total of 134 species [46]. In addition to this group, there are seven other clusters (namely *P. chlororaphis*, *P. putida*, *P. protegens sp. nov.*, *P. syringae*, *P. putida*/*P. aeruginosa*, *P. pertucinogena*, and *P. stutzeri*) in which all fluorescent species can be found [52].

The fluorescence in pseudomonads is associated with their production of secondary metabolites such as siderophores, iron-chelating molecules that facilitate the acquisition of iron by scavenging them from the environment. Under iron-deficient circumstances, the bacteria secrete these agents that capture ferric iron (Fe^{3+}) and deliver them to the bacterial cell via express transporters located at the cell surface [53,54]. The major siderophore produced by all FPs is pyoverdine (PVD), to which the bacteria's yellow-green fluorescence is attributed. To date, there have been nearly 100 unique PVDs characterized in a variety of different *Pseudomonas* species and strains [55]. PVDs have three general components that make up their structure, including: a

dihydroquinoline-type chromophore, a side chain, and a strain-specific peptide moiety [53]. The (*1S*)-5-amino-2,3-dihydro-8,9-dihydroxy-1H-pyrimido-[1,2-a]quinolone-1-carboxylic acid chromophore is identical in all PVDs, exhibiting a 400 nm absorbance (at neutral pH) and a 447 nm fluorescent emission when in the apo forms (as the ferric form of the molecule is non-fluorescent) [53,56].

In nearly all FP species, secondary siderophores alongside PVD are synthesized. This includes metal-chelating molecules such as pyochelin (PCH), pseudomonin, quinolobactin (QB), and thioquinolobactin (TQB), an unstable siderophore which, when hydrolyzed, results in QB. Some of these molecules (i.e., TQB) have been shown to exhibit antimicrobial and antifungal properties, allowing specific strains of FPs to act as plant growth promoting rhizobacteria (PGPR) [57,58]. The root-associated strain *P. aeruginosa* 7NSK2 synthesizes both PCH and pyocyanin (a blue phenazine pigment) that behave in a synergistic manner to induce resistance in tomatoes against the aerial pathogen *Botrytis cinerea* [59]. This suggests that in FPs, the hydrolytic enzymes are not the only contributors to *Pseudomonas* spp. ability to antagonize and suppress phytopathogens while concurrently facilitating plant growth by induced systemic resistance (ISR) [60].

1.2.2 Catalytic mechanism of chitin degradation

As previously mentioned, most bacterial chitinases belong to the family GH18 and catalyze the release of chitobiose, although a few are additionally classified in the GH20 family, such as β-N-acetylhexosaminidase [35,61-63]. All chitinases in the GH18 family are regularly identified by their catalytic region consisting of a triosephosphate isomerase (TIM) barrel (β/α)8 domain [64,65]. In bacteria specifically, the majority of chitinases fall into the subfamilies ChiA and ChiB that are distinct from each other only in the presence of a chitin insertion domain (CID) found in group A [66]. This CID is an (α+β) domain located between the seventh and eighth β-strands of the TIM barrel fold of the catalytic site [67]. In extensively studied organisms like *Serratia marcescens* it is hypothesized that the CID, found in the TIM barrel catalytic domain, is attributed to the tunnel-like deep clefts and processivity in chitin degradation [68]. These topological features are common in GH18 chitinases and define substrate specificities in addition to the lengths of the substrate and cleavage subsites in chitinases [69,70]. The following

information will be focused primarily on chitinases in the GH family 18, as they are more relevant to the content of this thesis.

Chitinases are also regarded as either retaining or inverting depending on the stereochemical outcome of products of chitin hydrolysis. GH18 chitinases are retaining which indicates that they maintain the configuration of the β-anomeric carbon of substrates in the products [66]. This process is achieved by a substrate-assisted type of double displacement hydrolytic mechanism involving a DXDXE [71] (d-aspartic acid, e-glutamic acid, and X-any other amino acid) motif in the catalytic site. In the acid-catalyzed glycosyl hydrolysis performed by GH18 chitinases, the substrate and potentially a second carboxylic acid residue play the nucleophilic role, while the highly conserved glutamate residue fixed above the TIM barrel acts as the general acid/base, protonating the glycosidic oxygen and triggering the cleavage of the glycosidic bond [62,72]. Then, the first phase, glycosylation, is followed by the second phase, deglycosylation, completing the hydrolysis process. The glycosylation stage is described as the transfer of a proton to the glycosidic oxygen (the leaving group) and the parallel nucleophilic assistance of the N-acetyl oxygen to the anomeric carbon leading to the cleavage of the glycosidic bond and the formation of the oxazolinium intermediate. The deglycosylation is then initiated, and the intermediate is hydrolyzed by a nucleophilic water molecule in the immediate vicinity of the anomeric carbon. As a result, the overall configuration of the anomeric center is retained [72-74].

1.2.3 Endochitinase and exochitinase

According to the hydrolysis mechanism and the method of chitin chain cleavage, chitinases are additionally separated into endochitinase and exochitinase (Fig. 5). Endochitinases arbitrarily hydrolyze the bonds at internal sites along the chain generating low molecular mass GlcNAc oligomers, while exochitinases degrade the products of endochitinase including chitobiosidase, which continuously liberates di-acetylchitobiose from the non-reducing end of the chitin, and β-1,4-N-acetylglucosaminidase, which produces monomers of N-acetylglucosamine by cleaving oligomers of chitin also from the non-reducing terminal [1,75,76].

One study determined that ChiA produced by the strain *Pseudomonas* sp. YHS-A2 is made up of three domains, including two putative microbial chitin-binding domains and one catalytic

domain consistent with family 18 chitinases. The analysis of the final reaction products ($(GlcNAc)_2$ from $(GlcNAc)_6$) when ChiA was treated with chitooligosaccharides corresponded to the mode of action typical of chitobiosidase [77]. Another study investigated the ChiA and ChiC chitinases secreted by *P. aeruginosa* and concluded that due to ChiC being active on the artificial substrates carboxymethyl-chitin-Remazol Brilliant Violet and p-nitrophenyl-β-d-N,N′,N"-triacetylchitotriose, but not on p-nitrophenyl-β-d-N-acetylglucosamine, it is an endochitinase [78].

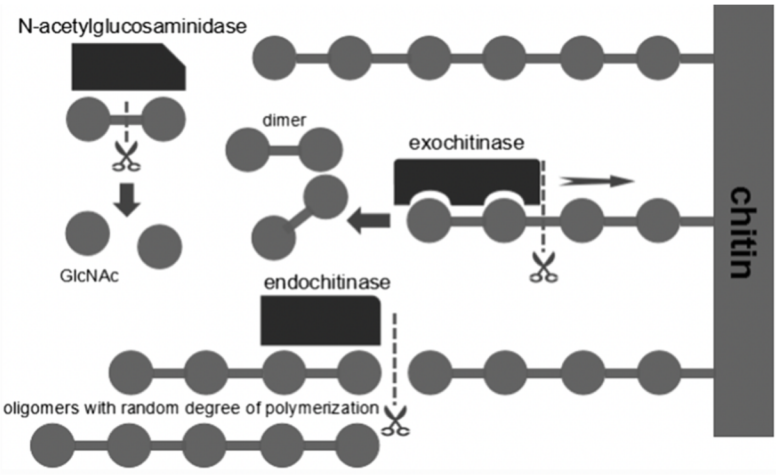


Figure 5: Illustrative representation of the complete hydrolyzation of long-chain chitin into GlcNAc monomers accomplished by endochitinase, exochitinase, and subsequently, NAG [76].

Furthermore, some novel chitinolytic enzymes are being newly described as "broad specificity" chitinases. These obscure enzymes possess two or three various catalytic activities among endochitinases, exochitinases, and NAG either with a single-catalytic domain or multi-catalytic domain. The former is characterized by a single catalytic domain that simultaneously exhibits both exo-type and N-acetylaminoglycosidase functions. This is exemplified in the isolated PbChi74 enzyme of the bacterium *Paenibacillus barengoltzii*. The latter is distinguished by separate catalytic domains that can perform multi-catalytic activities, as seen in ChiB isolated from the marine bacterial strain *Microbulbifer degradans* 2-40, that have both exo- and endo-type catalytic activity [76,79,80].

1.3 Applications in agricultural biocontrol

In order to meet the demands of the steadily rising world population's food consumption, the agricultural industry has historically adopted numerous methods for maximizing crop harvest and minimizing crop mortality. The most widely utilized practice for combating the 40%

preharvest- and additional 10% postharvest-loss caused by over 65,000 different crop pest species in crop production [81] is the application of chemical pesticides. Due to their high success rate and overall availability, an estimated 2.3 billion kg of pesticides are used worldwide per year (totaling almost $56 billion in 2012), with a 1900% between the 1940s and the 1980s [82,83]. However, studies have revealed that these agents have major detrimental disadvantages. This includes the toxicity of the chemicals accumulating and persisting in the environment and ecosystem food chains, as well as posing a threat to human health, fostering the evolution of pest resistance, and exposing non-target organisms to severe health risks or even death [81,84,85]. Consequently, researchers have been exploring the viability of employing biological organisms as a safe and effective antifungal/insecticidal solution in the form of biocontrol agents.

Bacteria that produce chitinases are one example of a proposed biocontrol against phytopathogens in regard to antagonistically targeting the cell walls of fungi and encouraging transgenic fungal resistance in crops carrying a chitinase transgene [86,87]. Chitin-containing pestiferous organisms have been shown to be regulated by chitinases of chitinolytic bacteria under the genera *Bacillus*, *Streptomyces*, *Serratia*, and *Pseudomonas*, among others [88]. A greenhouse experiment carried out on the chitinase of *Bacillus cereus* strain CH2 demonstrated its effectiveness in inhibiting spore germination of the fungus *Verticillium dahliae* (causal agent of Verticillium wilt of eggplants) by up to 95.67%. Additionally, the general severity of Verticillium wilt was lowered by 69.69% in cell suspension of the CH2 strain [89]. In 2007, a study conducted by Arora et al. documented the synergistic effect of chitinase and β-1,3-glucanase produced by fluorescent *Pseudomonas* in suppressing the mycelial growth of fungal plant pathogens [90]. Regarding transgenics, a chitinase gene-engineered strain P5-1 of *P. fluorescens* P5 exhibited a large impact on the repression of wheat take-all induced by *Gaeumannomyces graminis* var. *tritici* as well as increasing the defense of rice and cotton pots against rice sheath blight and cotton damping-off caused by *Rhizoctonia solani*, respectively [91].

1.4 Aims and hypothesis of the thesis

The principal objectives of this thesis were to provide an extensive literature review of chitin and chitinolytic bacteria (mainly in the genus *Pseudomonas*) and bacterial chitinolytic enzymes with respect to taxonomy, chemical mechanisms, and suitability as a biocontrol alternative to synthetic pesticides and the additional identification and quantification of endo- and exochitinase produced by the *Pseudomonas* sp. FPB-00. A fluorometric assay was performed to determine the chitinolytic activity of the preselected *Pseudomonas* strain, focusing on the analysis of the chitinase activity. The experiments were conducted via applying downstream processing techniques for enzyme separation, using chitin inducers to stimulate bacterial enzyme production, and utilizing fluorescent markers to quantify the enzyme activity.

The hypothesis proposed that this *Pseudomonas* strain may be capable of being utilized as an agricultural biofungicide with the intention of commercial-scale manufacturing as a synthetic fungicide alternative. Further, it was postulated that the strain produces enzymes that are biologically equipped to recognize and degrade chitin, and that a significant portion of this ability will be attributed to endochitinase activity.

2 Materials and Methods

2.1 Bacterial strain and cultivation conditions

The bacterial strain FPB-00 in this study was identified as belonging to the genus *Pseudomonas*. For patent protection of the bacterial strain, the experimental strain will be referred to as FPB-00 throughout the text. The bacterial strain FPB-00 was cultivated in 250 mL Erlenmeyer flasks with 50 mL of a basal salt medium (BSM) with a pH of 7. They were inoculated with glycerol as the primary carbon energy source with an initial treatment of 800 mg/L and a second day addition of 1500 g/mL. The start OD was 0.2 and a temperature of 22ºC was maintained throughout. The bioreactors were shaken on a rotary shaker at 150 rpm. Samples were collected after 24h, 48h, and 72h of incubation and stored at -20°C for later analysis.

2.2 Chitin induction

The bacterial colonies from the bioreactors described above were stimulated with two inducers to promote chitinase synthesis and secretion. These two chitin inductors included: shrimp shells and pure chitin (from Sigma Aldrich). No inductor was added for the control and for the samples subjected to downstream processing.

2.3 Chemicals and reagents

Fluorometric chitinase assay kit was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Additional chemicals used were of high quality and laboratory grade.

2.4 Sample preparations for analysis of extracellular, bound, and intracellular enzymes

Bacterial colony samples taken directly from the bioreactors (under control conditions) were processed using the downstream processing methodology in preparation for the fluorometric assay. Ultrapure water (18 MΩ·cm) was used to prepare a 0.9% (wt/vol) NaCl solution. The samples (24h and 48h colonies) were shortly vortexed and 1 mL of each was pipetted into sterile Eppendorfs which were then centrifuged at 10,000 rpm for 10 min. The supernatant above the

pellet was removed and added into a new sterile Eppendorf, now consisting of extracellular enzymes in solution. Then, 1 mL of the prepared saline solution was added to the remaining pellet and quickly vortexed to produce a solution with the enzymes bound to the cells. Subsequently, 0.5 mL of this solution were pipetted into a BeadBeater tube, which was then placed into a BeadBeater grinder from MP Biomedicals (Irvine, CA, USA) and the samples were homogenized for approximately 30 sec. After the cell lysis, the tubes were centrifuged at 10,000 rpm for 10 min and the supernatant was again separated into a sterile Eppendorf, containing intracellular enzymes.

2.5 Fluorometric assay

The activity of three different chitinases, endochitinase and two exochitinases (chitobiosidase and β-N-acetylglucosaminidase) were quantified for the intracellular, bound, and extracellular enzymes and the 24h, 48h, and 72h samples. To achieve this, corresponding substrates with an attached 4-Methylumbelliferyl (4-MU) fluorescent marker were used. The substrates were prepared by adding 0.25 mL of dimethyl sulfoxide (DMSO) to each of the substrate bottles: 4-MU N-acetyl-β-D-glucosaminide [4-MU-GlcNAc], 4-MU β-D-N,N′-diacetylchitobioside hydrate [4-MU-$(GlcNAc)_2$], and 4-MU β-D-N,N′,N″-triacetylchitotrioside [4-MU-$(GlcNAc)_3$] and vortexed until dissolved. These Substrate Working Solutions were then diluted 40-fold with Assay Buffer to a concentration of 0.5 mg/mL just before the assay. A pure Chitinase Control Enzyme isolated from *Trichoderma viride* was dissolved in 5 mL of PBS to a final concentration of 0.2 mg/mL. All measurements were performed in a flat-bottom Nunc-Immuno™ MicroWell™ 96 well black plate (Fig. 6) and analyzed with a Spark® Multimode Microplate Reader from Tecan (Männedorf, CH). The chitinase hydrolysis occurred in an acidic environment (pH ~5.0) at temperatures ranging from 35 - 37ºC. The fluorescence of the liberated 4-MU as a result of the enzymatic hydrolysis was detected by the fluorimeter with an excitation of 360 nm and emission of 450 nm. Each reaction was independently replicated in three consecutive wells.

The endochitinase (ENDO) activity was determined using the substrate 4-MU β-D-N,N′,N″- triacetylchitotrioside and exochitinase (CHIB and NAG) activity was determined using the substrates 4-MU N,N′-diacetylchitobioside hydrate and 4-MU N-acetyl-β-D-glucosaminide. For each sample well, 5 µL of the previously prepared sample solutions were mixed with 95 µL

of Substrate Working Solutions. Additionally included in the well plate was a Blank reaction (Substrate Solution without enzyme), a Positive Control (*T. viride* chitinase), and an assay of Standards made up of the diluted sample solutions and Assay Buffer that yielded concentrations of 10, 100, 500, and 1000 ng/assay Standard.

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1S	S1S	S1S	S1B	S1B	S1B	S1I	S1I	S1I	blank	blank	blank
B	S1S	S1S	S1S	S1B	S1B	S1B	S1I	S1I	S1I	Assay buffer	Assay buffer	Assay buffer
C	S1S	S1S	S1S	S1B	S1B	S1B	S1I	S1I	S1I	PC	PC	PC
D	S2S	S2S	S2S	S2B	S2B	S2B	S2I	S2I	S2I	ST10	ST10	ST10
E	S2S	S2S	S2S	S2B	S2B	S2B	S2I	S2I	S2I	ST100	ST100	ST100
F	S2S	S2S	S2S	S2B	S2B	S2B	S2I	S2I	S2I	ST250	ST250	ST250
G										ST500	ST500	ST500
H										ST1000	ST1000	ST1000
		PC	positive control		ST	standard	B	bound to cell				
		S1/S2	sample		S	extracellular	I	intracellular				

Figure 6: Arrangement of reactions prepared on the well plate for the measurement of extracellular, bound, and intracellular enzymes of 24h and 48h samples.

2.6 Statistical analysis

The data from the fluorometric assay measurements was processed using Python (Python 3.10.12). Several libraries were imported to produce the bar graphs. The raw data was first extracted from the Excel (Microsoft 16.75) sheets, generated by the microplate reader, with **import pandas**. It was then categorized based on fluorescent marker type and enzyme location and/or duration of cultivation. Groupings were labeled by their position on the well plate and clustered into the three replicates (i.e., A1-A3, A4-A6, etc.). **import numpy** was used to convert each measurement from fluorescence into nmol/mL/min according to the equation in Fig. 7. Additionally, the mean of the three replicate values (at 15 min) was calculated and standard deviation was determined to assign error bars to the graphs. The results were plotted into bar graphs with **import matplotlib.pyplot**.

$$Units/mL = \frac{(FLU_{sample} - FLU_{blank}) \times 1.9 \times 0.1 \times DF}{FLU_{std} \times time \times V_{enz}}$$

Where:

Units/mL — nmol MU/mL/min
FLU_{sample} — fluorescence of the sample
FLU_{blank} — fluorescence of the Blank (containing only Substrate Working Solution)
1.9 — concentration of the 100 ng Standard in nmol/mL
0.1 — final reaction volume in milliters
DF — enzyme dilution factor (20)
FLU_{std} — fluorescence of Standard Solution minus fluorescence of Standard Blank
time — minutes (15)
V_{enz} — volume of the sample in milliters (0.005)

Figure 7: Equation extracted from the technical bulletin (CS1030) of the fluorometric chitinase assay kit along with a guide to the equation variables.

3 Results

3.1 Chitinase quantification

Two trials were performed for the quantification of extracellular, (cell membrane) bound, and intracellular chitinases of the 24h and 48h samples, while one trial was performed for the quantification of total chitinases in the pure chitin induction, shrimp shell induction, and control samples after three 24h-interval collections. Results from all trials were analyzed at the 15-minute mark of the reactions to establish comparability. The results supported the hypothesis by showing that the bacterial strain FPB-00's array of chitinases are capable of degrading various chitin substrates.

3.1.1 Growth phase-dependence of chitinase production

To investigate if bacterial incubation time influenced chitinase production, bacterial samples were collected at intervals of 24h and sampled after 24h, 48, and 72h. When considering extracellular, bound, and intracellular chitinases, the highest enzyme production occurred after 48h of induction. For Trial 1, the largest differences were observed for NAG, CHIB, and ENDO enzymes in the extracellular region, with higher levels occurring after 48h of incubation in increases of 231%, 127%, and 188%, respectively (Fig. 8A,8B).

The total amount of each type of enzyme present across all regions was numerically the highest for CHIB after 48h, at 71.0 nmol MU/mL/min in Trial 2, and 103.0 nmol MU/mL/min in Trial 1. The largest percentage increase was observed for total amount of NAG in Trial 2, in which the bacteria produced 303% more enzymes after 48h in comparison to the 24h. Additionally, the total amount of enzymes detected in the extracellular and intracellular regions increased by 76% and 96%, respectively (Fig. 9A,9B).

Trial 1 exhibited a higher overall amount of enzymes in each region in the 48h samples. The exception is the concentration of enzymes present in the bound region, which measured about 4x higher in the 48h sample of Trial 2 and exhibited an 11x higher increase in Trial 2 compared to Trial 1. Similarly, the bacteria produced 11.5 nmol MU/mL/min more NAG in the bound region and 9.9 nmol MU/mL/min more NAG in the intracellular region after 48h of incubation in Trial

2 than in Trial 1. In contrast, the increase of CHIB produced after 48h in Trial 2 measured 3.25x less in comparison to the increase in Trial 1.

In Trial 3, there was either a positive or negative linear change depending on inducer for the 24h, 48h, and 72h samples. Bacteria with shrimp shell induction had the maximum level of enzyme production across all chitinase types after 24h of incubation, and the lowest enzyme production after 72h of incubation (Fig. 10C). The opposite trend was observed for pure chitin induction and no induction, where the maximum level of enzyme production across all chitinase types was after 72h of incubation, and the lowest enzyme production was after 24h of incubation (Fig. 10A,10B).

No NAG enzymes were detected after 72h in the shrimp shell samples. On the other hand, the control and bacteria induced with pure chitin only began producing enzymes after 48h of incubation. For the pure chitin induction, a subsequent increase of 95%, 82%, and 149% of NAG, CHIB, and ENDO, respectively, was observed as a result of an additional twenty-four hours of incubation from 48h until a total of 72h.

3.1.2 Effect of enzyme location on chitinase production

The type of enzyme produced also varied among the enzyme locations extracellular, bound, and intracellular. Extracellular enzymes consisted of the highest concentration of all chitinase types. The intracellular enzymes were made up of high levels of CHIB, 1.9-7.5 nmol MU/mL/min of ENDO, and 11.5 nmol MU/mL/min of NAG, only as seen in Fig. 9B. The predominant chitinase found across all bound enzyme samples was low levels of CHIB, with less than 10 nmol MU/mL/min of activity (Fig. 8,9).

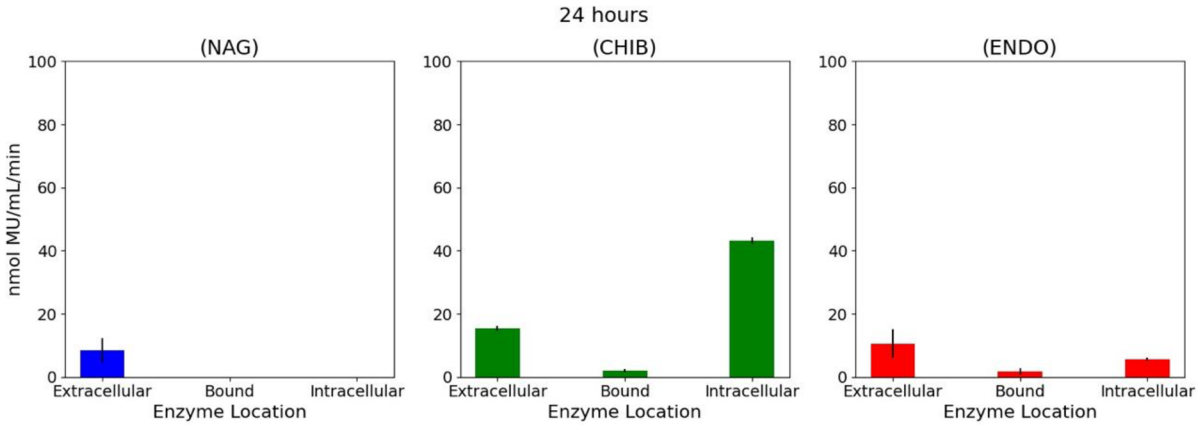
3.1.3 Effect of inductor on chitinase production

The control in Trial 3 showed elevated levels of all chitinases, which were slightly different from those of the samples in Trials 1 and 2 (these 24h and 48h samples also had no inducer added) but followed the same trend. The shrimp shell samples displayed the opposite trend, however, and there was a considerably steep decline of NAG and ENDO between the 48h and 72h samples. When induced by shrimp shells, FPB-00 produced the largest amounts of all chitinases out of all the samples. After 24h of incubation, levels of ENDO reached 120 nmol

MU/mL/min while levels of CHIB reached nearly 125 nmol MU/mL/min. The measured NAG resulted in approximately 64 nmol MU/mL/min more in the 24h sample, a 90% increase in the 48h sample, and 37 nmol MU/mL/min less in the 72h sample in comparison to the control.

A positive linear increase occurred in the pure chitin induced sample. All levels of chitinases steadily increased with increasing incubation time. The amount of chitinase present after 48h of incubation was similar in both the control and the pure chitin induced sample, whereas the amount of NAG in the 72h sample was approximately 65% higher in the control. In the 24h sample, there was over 40 nmol MU/mL/min more NAG produced in the control FPB-00 as there was after 24h of incubation of the pure chitin induced (Fig. 10).

A



B

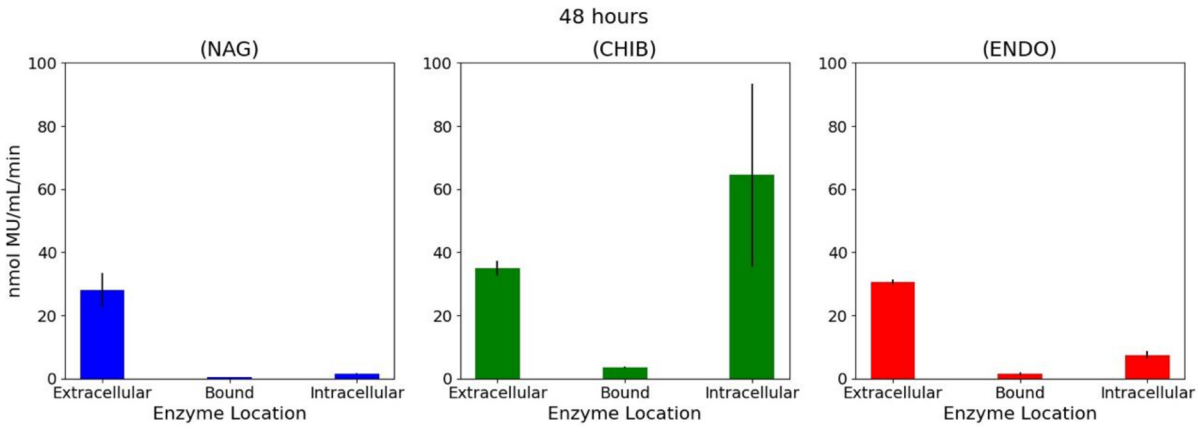
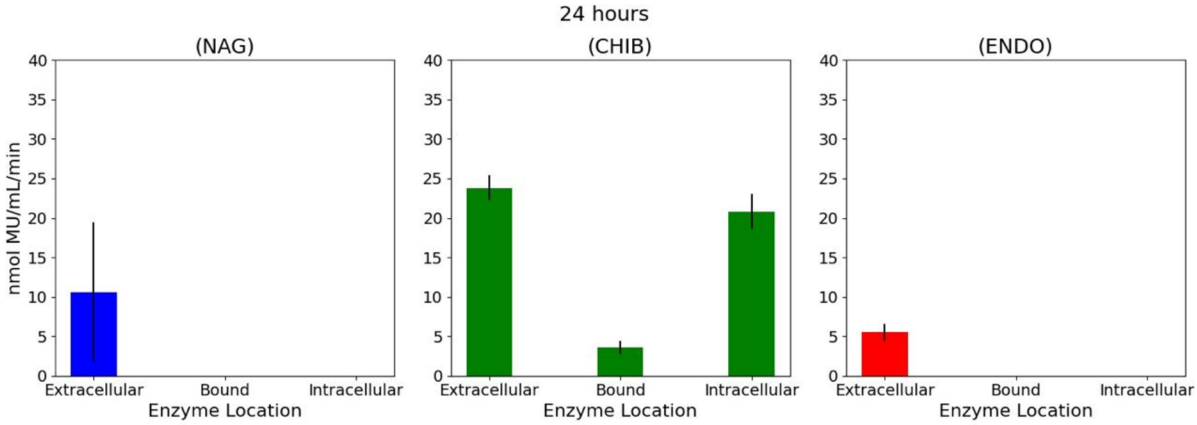


Figure 8: Trial 1 histograms of chitinase activity of extracellular, bound, and intracellular enzymes for 24h and 48h samples. **A** 24h sample after 15 min of reaction. **B** 48h sample after 15 min of reaction.

A



B

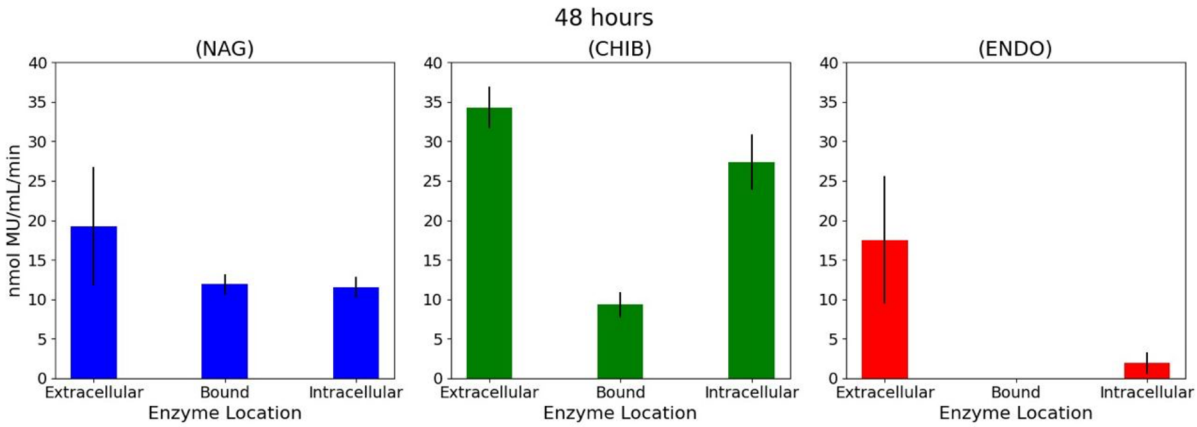
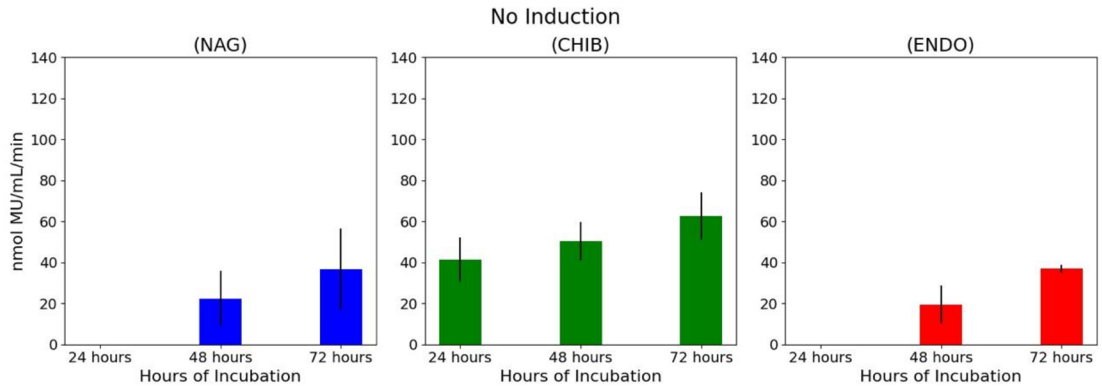
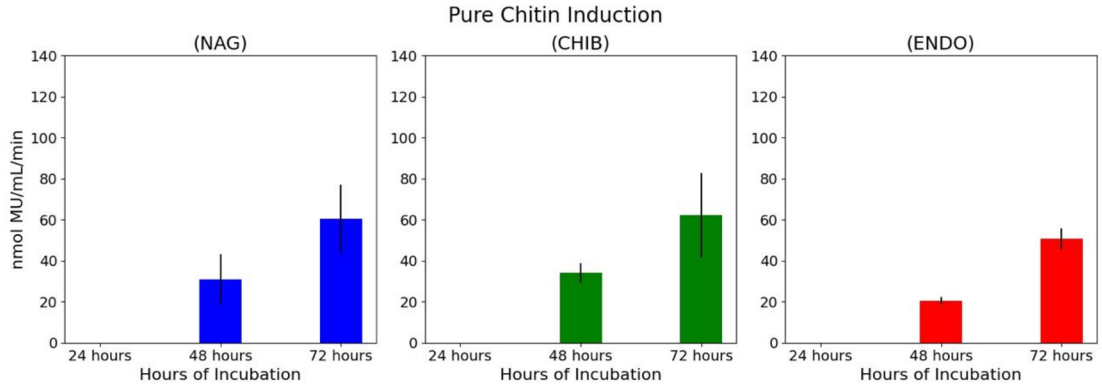


Figure 9: Trial 2 histograms of chitinase activity of extracellular, bound, and intracellular enzymes for 24h and 48h samples. **A** 24h sample after 15 min of reaction. **B** 48h sample after 15 min of reaction.

A



B



C

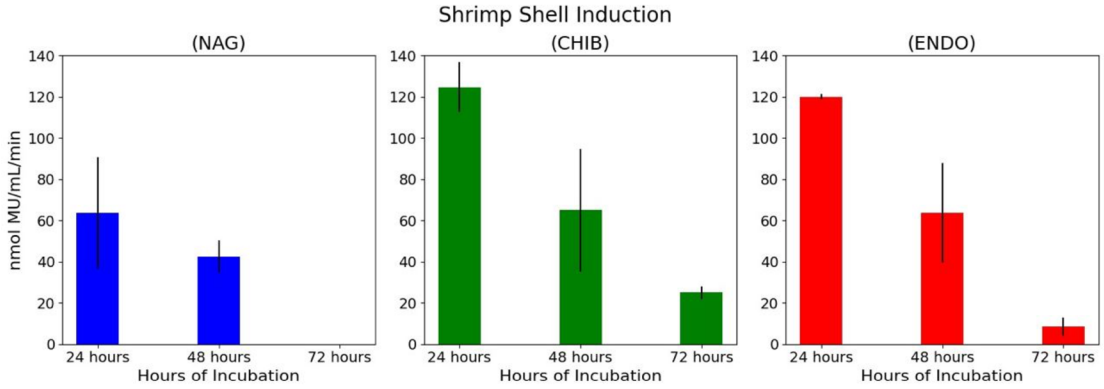


Figure 10: Trial 3 histograms of chitinase activity of no induction, pure chitinase induction, and shrimp shell induction for 24h, 48h, and 72h samples. **A** No induction sample after 15 min of reaction. **B** Pure chitin induction sample after 15 min of reaction. **C** Shrimp shell induction sample after 15 min of reaction.

4 Discussion

4.1 Presence of chitinases in samples

The chitinases produced by pseudomonads are of increasing importance regarding targeting the cell walls of plant pathogenic fungi and are subsequently being considered as biological components capable of being cultivated as biopesticides. Detection of chitinolytic enzymes in these bacteria is useful in determining their potential while characterizing the methods and conditions for maximum chitinase production can aid in developing solutions for large-scale agricultural applications. The hypothesis postulating the chitinolytic abilities of the FPB-00 bacterial strain was supported by the data which identified chitinase activity in all the analyzed samples, with some variations likely due to incubation time and applied inducer. However, the enzyme with the largest overall quantity was CHIB.

It has been observed that extracellular chitinases are influenced by growth media components such as carbon and nitrogen sources [92]. Many bacteria are commonly grown in glucose-containing media, which some studies reported caused an enhancing effect, while others showed a suppressing effect [92,93]. In this study, glycerol was added to the growth medium. Although no direct comparison can be made between different types of energy sources and their effects on chitinase production in this study, it was still proven that the bacteria are able produce relatively substantial levels of chitinase with glycerol as the supporting primary energy source. The growth media used for two of the samples in Trial 3 was also amended with two types of chitins: pure chitin (Sigma Aldrich) and shrimp shell chitin. These samples generally showed higher total chitinase activity than the control sample; the same was observed in a study done by Ajit et al. (2005) [94], suggesting that including the addition of exogenous chitin with already available nutrients, the bacteria is enabled to produce more metabolites rather than exhibit growth.

The fluorometric enzymatic assay in this experiment showed that NAG was present in levels similar to ENDO primarily in the extracellular region, with seldom present in the periplasm, and a negligible amount found in the intracellular fraction (with the exception of 48h in Trial 2). A study by Neiendam Nielsen and Sørensen (1999) also discovered a relatively low level of extracellular NAG activity and no observed detectable activity in the cell-bound fraction of *P. fluorescens* isolates, which was consistently reported in past experiments [95,96]. Furthermore,

their data showed that isolates of *P. fluorescens* cultivated in growth media without chitin inducers showed significant production of chitinolytic activity, reporting that the production of chitinolytic enzymes in bacteria is both constitutive and inducible [95]. The same was true for this study, where chitinolytic activity occurred in both control samples, with only glycerol, and induced samples, with both glycerol and chitin. This may be an ecological advantage for chitinolytic bacteria as chitinases could be produced without the necessity of chitinous inducers.

The data suggested that the majority of endochitinase was released by FPB-00 as extracellular enzymes in levels 2-8x higher than those found in the intracellular region. Nearly no endochitinase activity was detected in the cell-bound fraction. The similarity in enzyme activity distribution of NAG and ENDO could indicate that the two chitinases are either coupled or one single enzyme. The present work disproves the latter, as slight differences in quantity were observed including more ENDO than NAG produced in the intracellular region. Differences were also revealed with more ENDO than NAG present in the shrimp shell samples, and more NAG than ENDO produced in the pure chitin sample.

In addition, the FPB-00 culture induced by shrimp shells displayed the highest levels of endochitinase and chitobiosidase activity across all samples. This could be due to the presence of α-chitin in crustacean shells, the most stable crystalline form of chitin with antiparallel polymer chains that result in strong hydrogen bonding (two per unit cell) [97-99], requiring intensive hydrolysis to be broken down. The analysis therefore suggested that this FPB-00 bacterial strain can successfully recognize and adequately degrade α-chitin by responding with a significantly high production of ENDO and CHIB enzymes. Given that α-chitin is common in beetle shells and fungal cell walls, the strong metabolic reaction elicited from the bacteria further establishes FPB-00 as a potential candidate for a bacterial biofungicide.

In all samples, most notably the shrimp shell induced, sufficient amounts of chitinase were produced by the bacteria within 48h of cultivation, providing efficiency that may prove useful for biopesticide development and further biotechnological upscaling. Distinctions were seen concerning the effect of incubation time and chitinase production, however complementary studies investigating the role of other parameters such as incubation temperature, media composition, and pH could be carried out to optimize efficiency and enzyme quantity. Moreover, the molecular characterization of chitinase expression patterns coupled to the occurrence of chitinase genes would improve the decipherment of degradation mechanisms and allow for

increased control over the whole underlying process, according to Beier and Bertilsson (2013) [4].

Nonetheless, the overall results of this study determined that the FPB-00 bacterial strain produces significant amounts of three main chitinases, both induced and not induced, when treated with glycerol over the period of three days. Future studies may additionally consider focusing on increasing endochitinase productivity specifically, as this is the most crucial enzyme for chitin degradation. Different methods of chitinase quantification should also be explored for comparison, considering that there were several substantial values of error and a limited number of data trials.

5 Conclusion

The present study provides a comprehensive summation detailing the behavior of chitinous pathogens in phytological environments and the hydrolytic activities of three chitinolytic bacterial enzyme types produced by a selected fluorescent *Pseudomonas* species. The quantification of these enzymes indicates that the bacteria is capable of significant extracellular protein secretion of NAG, CHIB, and ENDO. The highest total enzyme production was induced by shrimp shells, where the stability of the chitin likely necessitated the high levels of ENDO and CHIB, offering a model of how the bacteria might act when targeting fungal cells. Furthermore, enzyme productivity showed either increasing or decreasing trends over time depending on the inducer or lack of inducer applied. Additional research could clarify the effects of growth media, temperature, and pH on chitinase production and determine the antifungal properties of this bacterial strain. Nevertheless, the use of a fluorometric assay confirmed the chitinolytic performance of FPB-00 and established its potential in being considered as a biofungicide for agricultural purposes.

6 Literature

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