THE PURIFICATION, IDENTIFICATION, AND CHARACTERIZATION OF A THERMOSTABLE METALLOPROTEASE IN *SERRATIA GRIMESII* FROM CHANNEL CATFISH (*ICTALURUS PUNCTATUS*) SPOILAGE

by

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DEDICATION

In memory of my grandmother,

Alma Peay

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ABSTRACT

Food spoilage and food poisoning in fishery products is an emerging issue affecting the health of consumers throughout the world. Fish are susceptible to various specific spoilage organisms (SSOs) that produce extracellular enzymes during low temperature storage which deteriorates the tissue after fillet processing. In this study, *Serratia grimesii* was isolated from fillets of channel catfish (*Ictalurus punctatus*). The protease producing bacteria from catfish fillets where identified through 16S rDNA sequencing and categorized through a phylogenic tree where most isolated SSOs were closely neighbored except *S. grimesii*. This species was unique from the other protease producing bacteria isolated on the fillet as to its protease protein profile. The goal of this study was to investigate a thermostable metalloprotease from *S. grimesii* through purification, identification, and characterization. The growth of *S. grimesii* and its protease production were measured to examine the secretion properties. Optimal growth conditions were achieved at pH 7 and 28 ºC as psychrotrophic bacteria. The protease secretion was best during the log phase of the bacterial growth.

The extracellular crude proteins were isolated after protein induction for three days before biochemical properties were determined by enzyme assays. The crude protein was thermostable and tolerant of a wide range of pH values, solvents, and co-factors. Distinct bands from SDS-PAGE were also observed from the crude protein. The target protein migrated in the gel underneath the 50 kDa was selected because it showed the greatest protease production. The target protease was purified through Next Generation Chromatography using ion exchange and gel filtration columns. Using the ion exchange chromatography, the pH of 8.6 was optimal for found to separate the target protease. The purified protein had a 10-fold increase in specific activity compared to the crude protein preparation. Following purification, biochemical studies were performed to characterize the protease. The protease was thermostable even up to 100 ºC. The activity of the protease was highest at pH 9, however it was still stable in some acidic and alkaline ranges. Manganese was a co-factor that increased activity. Solvents decreased the activity to around 50%. Detergents showed varied results with Triton X-100 not having an effect but SDS inhibiting protease activity. The substrate casein was hydrolyzed the best. To identify the protease, peptides were sequenced using MALDI-TOF-TOF mass spectrometry. The mass spectrometry result of the target enzyme identified as a stable metalloprotease precursor. It is believed that extracellular proteases including this metalloprotease are a major cause of rapid spoilage growth in fish tissue.

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LIST OF ABBREVIATIONS

bp …… Base pairs

BSA ……... Bovine serum albumin

CFU ……... Colony forming units

CV …….. Column volume

DNA ……. Deoxyribonucleic acid

DNase ……... Deoxyribonuclease

EDTA …… Ethylenediaminetetraacetic acid

kDa ……. Kilodalton

LPSN ……..…… List of Prokaryotic Names with Standing in Nomenclature

mAU ……. Milli absorption units

NGC …….. Next Generation Chromatography

OD ……... Optical density

PBS ……. Phosphate buffered saline

PCR …… Polymerase chain reaction

rDNA ……... Ribosomal DNA

rpm ……. Revolution per minute

SDS-PAGE ……...…… Sodium dodecyl polyacrylamide gel electrophoresis

Spp. ……. Species within bacteria

SSO ……. Specific spoilage organism

TAE ……... Tris acetate-EDTA

TMAO …… Trimethylamine Oxide

TSA ……... Tryptic soy agar

TSB ……..... Tryptic soy broth

CHAPTER 1: INTRODUCTION

Maintaining the shelf-life of foods especially fresh fish is a common goal of both the producer and consumer. The shelf-life of food is the period of time at which under certain controlled conditions, the product maintains sensory, nutritional, sanitary, and other properties that make it safe for consumption (McCoy, Morrison, Cook, Johnston, Eblen, & Guo, 2011). For fish, these properties include food safety, freshness, nutrient content, eating quality, and physical attributes of the species. Unfortunately, chemical deterioration and microbial spoilage are responsible for 25% of losses in fishery products each year (Ghaly, Dave, Budge, & Brooks, 2010). Fresh fish can spoil very rapidly after it is caught. The spoilage process will start with rigor mortis causing the fish to lose flexibility due to stiffening of its muscles soon after death. Most fish species degrade as a result of digestive enzymes including lipases, microbial spoilage created by surface bacteria and oxidation (Sallam, 2007). Microbes can cause unpleasant off-flavors and other unacceptable sensory attributes and may produce toxins of multiply on the fish or in the host, causing a foodborne illness when ingested. Food poisoning and illnesses such as salmonellosis, listeriosis, and histamine fish poisoning (HSP) can occur from consuming bacterially contaminated fish (Hickey, Besong, Kalavacharla, & Lee, 2012; Sriket, 2014). These are common issues relating to microbial degradation.

In June 2008, the US Congress amended the Federal Meat Inspection Act stating that catfish is considered a species subjected to regulation by the US Department of Agriculture (USDA) Food Safety Inspection Service (FSIS) (McCoy *et al.*, 2011). This reclassification resulted in a more comprehensive look at foodborne illnesses associated with the consumption of

catfish. The use of good agricultural practices (GAPs) and the Hazard Analysis of Critical Control Point (HACCP) are important at all steps of the production. Contamination can occur during catching, on-board handling, processing, storage, and retail distribution. Worldwide, an estimated 10% of caught fish are lost because of spoilage due to lack of chilling facilities (McCoy, Morrison, Cook, Johnston, Eblen, & Guo, 2010). Furthermore, commercial processing of channel catfish spread skin and gut microflora on the work surface and processing equipment that increase contamination of the final product (Bal'a, Podolak, & Marshall, 2010).

The preservation of fish is reliant on the knowledge of microbial activity and its spoilage effects on fish tissue. In particular, many specific spoilage organisms (SSOs) secrete extracellular proteases. Proteases catalyze hydrolytic reactions breaking down proteins to peptides and amino acids. During postmortem handling and storage, fish can be degraded by these enzymes (Delbarre-Ladrat, Cheret, Taylor, Verrz-Banis, 2006). The protease may affect tenderization which is one of the most important qualities of food fish (Lerke, Farber, & Adams, 1967). An improved science-based understanding of the growth and activity of specific spoilage organisms in fishery products is necessary for the ability to reduce the economic loss due to microbial spoilages.

Problem Statement

Rapid spoilage of fresh fish products stored at low temperature is a problem to the producer and consumer causing both economic loss and foodborne illness. Microbial spoilage can cause slime, bacteria colonies, changes in texture, and off-flavors (Gram and Huss, 1996). Psychrotrophic gram-negative bacteria such as *Pseudomonas*, *Aeromonas*, and *Shewanella*

species have been commonly studied; however, *Serratia* spp. needs to be further researched. Surface degradation of muscle has been a result of bacterial colonization in fishery products (Venugopal, 1990). Since fish fillets mainly contain protein, proteases may play a role in shortened shelf life. The analysis of proteases, like the one in this study, can lead to a better elucidation of the roles they play in food spoilage.

In this study, a protease from *S. grimesii* was isolated from channel catfish fillets; the protease was purified, identified and characterized. The protease was purified using ion exchange and gel filtration columns attached to the Next Generation Chromatography system. The identification of the protease was performed through mass spectrometry matching the amino acid sequences in the protein database. The purified protease was characterized through numerous biochemical assays and storage stability tests.

Objectives

The primary objective of this study was to investigate a protease from *Serratia grimesii* through purification, identification, and characterization since *S. grimesii* belongs to SSOs found on catfish that causes a shortened shelf life of this food product. Extracellular proteases in bacteria are known to degrade the quality of fishery products. Investigating this protease will give a better understanding of catfish spoilage.

The objectives of this study were: (1) To isolate and identify protease producing spoilage bacteria from channel catfish fillets through bacterial sampling and then 16S rDNA sequencing. (2) To optimize growth conditions of *S. grimesii* and examine the enzyme secretion related to bacterial growth. (3) To extract the crude protein from the bacteria culture to determine thermal

stability, pH optimization, and other characteristics. (4) To determine and purify the most active protease. (5) To determine biochemical properties and storage stability of the purified target protease. (6) To identify the target protease through mass spectrometry and analyze it using bioinformatics tools.

CHAPTER 2: LITERATURE REVIEW

2.1 Channel Catfish (*Ictalurus punctatus*)

Catfish, is a popular freshwater fish species, ranked sixth among the United States most consumed aquatic food species in 2008 and has been commercially raised in the country since the 1960s (Norman-Lopez & Asche, 2008). United States consumption of catfish and international demand supports 115,000 acres of United States aquaculture ponds, mainly in Mississippi, Alabama, Arkansas, and Texas (Bal'a, Podolak, & Marshall, 2010). In 2009, the US exported more than 129 million pounds of catfish to several countries including Canada, China, Mexico, Peru, and Thailand (McCoy, Morrison, Cook, Johnston, Eblen, & Guo, 2010). The species of catfish that accounts for almost all commercial fish production in the US is channel catfish (*Ictalurus punctatus*), which belongs to the family Ictaluridae, order Siluriforms.

The consumption of fish has numerous health benefits. Fish meat, in particular channel catfish, contains 18.1% protein of high biological value which is more digestible than red meat, eggs, and milk (Casallas, Cassallas, & Mahecha, 2012). Fish also contains various amino acids including lysine, leucine, phenylalanine, arginine, and threonine (Usydus, Szlinder, Adamcyk, & Szatkowska, 2011). Amino acids aid in the bodily functions of regulating blood vessel flexibility and building muscle. However, it is because of these large quantities of free amino acids and instable nitrogen bases that make fish more perishable than chicken or red meat (Usydus *et al.*, 2011).

Since fish are surrounded by water, the muscle fibers require less structural support than terrestrial animals. Because of this, fish muscle contains less connective tissue resulting in a

more tender texture (Sallam, 2007). The unique movement of swimming allows for a structural arrangement of muscle different from land animals. The muscular content includes segmented muscles called myomeres that are arranged in adjacent bands, becoming more pronounced along the back of the fish separated by layers of collagen (*myocommata*) (Casallas *et al.*, 2012). Fish meat contains red and white muscle tissue. Red muscle has a high percentage of hemoprotein compounds providing great nutritional benefits, but it is known for instability during processing and storage (Veggetti, Mascarello, Scapolo, & Rowlerson, 1990). On the other hand, white muscle of catfish has stable textural characteristics because of its larger muscle fibers, making it more resilient for industrial processing.

The protein compositions in muscles vary but myofibrillar proteins are the primary food proteins of the fish comprising 66-77% of the total protein in fish meat (Davies, Bardley, Ledward, & Poulter, 1988). The myofibril protein complexes which are located in fillets contain actin and myosin. Myosin makes up 50-60% of the myofibrillar contractile proteins and actin only 15-30% (Samantha, Larroche, Pandey, 2006). ATPase of the head molecules causes myosin molecules to connect in the head region to polymerized actin molecules in the thin filaments. This complex is called actomyosin and is responsible for muscle contraction and relaxation. Actomyosin plays an important role in determining fish meat quality because it can be easily broken down during processing and storage. During frozen storage, the actomyosin becomes less soluble causing the flesh to become tougher (Kristinsson & Rasco, 2010). Aside from storage conditions, microorganisms affect the tissues of the catfish.

2.2 Fish Spoilage Bacteria

Fish are a perishable food causing problems for the producer and consumer. During fish spoilage, there is a breakdown of different components forming new compounds like biogenic amines that change the color, flavor, and texture of fish meat. Trimethylamine (TMA) levels are universally used to determine microbial deterioration leading to fish spoilage. Fish use trimethylamine oxide (TMAO) as an osmoregulant to avoid dehydration in marine environments and tissue water logging in fresh water (Gram, & Dalgaard, 2002). Bacteria reduce TMAO to TMA creating ammonia-like off flavors. Due to the lower glycogen content in fish meat, the neutral pH of the body does not become acidic in the postmortem process thereby facilitating bacteria growth and degradation.

Specific spoilage organisms (SSOs) use carbohydrates, amino acids, lactic acids, and nucleotides of the food as nutrients for growth and ultimately ruin the quality of the meat. Psychrotrophs, common SSOs that decrease the shelf-life of fish, include bacteria, yeasts, and molds that grow optimally at temperature 15-30ºC (Marth, 1998). Since the 19th century, low storage temperatures have been used for the preservation of seafood to retard the growth of microorganisms (Austin, 2002). However, the method does not necessarily reduce microbial metabolism. Some of the organisms in refrigerated foods can also be pathogenic (Venugopal, 1990). Psychrotrophs grow slowly in refrigerated temperatures (1-7 ºC) but overtime they multiply and degrade the quality of the food product leading to spoilage.

Gram-negative psychrotrophs are affected by oxygen tension, salt, water activity, and pH. In particular, gram-negative bacteria such as *Achromobacter* spp., *Pseudomonas* spp.,

Flavobacterium spp., *Shewanella* spp., and *Cytophaga* spp. frequently spoil fish under chilled storage (Gram & Huss, 1996). *Pseudomonas* spp. are aerobic organism and are among the most common spoilage agents of refrigerated foods resulting in histamines and sweet, fruity spoilage odors (Ghaly *et al.*, 2010). *Aeromonas* spp. also cause the production of an offensive odor by developing hydrosulfic acid (Venopal, 1989).

2.3 *Serratia* spp.

The *Serratia* genus was named after the Italian physicist Serafino and belongs to the family *Enterobacteriaceace* (Houldt, Moons, Jansen, Vanoirbeck, & Michiels, 2005). *Serratia* are gram negative motile rods that show good growth at several lower temperatures including 5, 20, and 30ºC (Mahlen, 2011). They grow in minimal medium without the addition of growth factors. Some bacterial colonies, such as *S. marcescens*, emit a red pigment while others such as the *S. liquefaciens* complex produce an off -white pigment.

Members of the *Serratia* genus exist in a wide range of habitats and evolved to have diverse ecological functions. The *Serratia* spp. has been isolated from water, plant surfaces, soil, animals, and humans (Grimont & Grimont, 2006). *Serratia* caused infections in humans producing bacteremia, pneumonia, urinary tract infections, and meningitis in humans (Peterson & Tisa, 2013). In particular, *S. marcescens* is recognized as an important nosocomial pathogen found in hospitals. *S. plymuthica* is another significant pathogen causing sepsis and wound infections (Romero, Garcia, Salas, Diaz, & Quiros, 2001).

Certain members have shown to be spoilage agents in foods (Mahlen, 2011). Because of their presence in soil and water like that of *S. grimesii*, they are associated with contamination of

raw food materials and spoilage of plant and animal products consumed by humans and pose a foodborne health hazard. *Serratia* spp. are known to secrete extracellular enzymes including at least one nuclease, two chitinases, one lipase, and two proteases (Hines, Saurugger, Ihler, & Benedik, 1988).

2.4 Proteases in SSOs

Proteases can be serine-, aspartic-, or metalloproteases. Serine proteases, generally active at neutral and alkaline pH ranging from 7-11, hydrolyze peptides at the carboxyl side of arginine and lysine residues. They are usually found in the intestines of some fish (Sriket, 2014). The cysteine family of proteases includes calcium activated mammalian lysosomal cathepsins as well as parasitic proteases. Aspartic proteases depend on aspartic residues for their catalytic activity. They are produced by a number of tissues and cells and are active at an acidic pH range of 2-4. Metalloproteases, enzymes from a variety of origins and organisms, have a binding domain for zinc atom which is catalytically active.

Bacteria may produce various extracellular enzymes to satisfy their physiological demands. Significant protease production does not occur until the second stage of bacterial growth. During stage I, bacterial counts are less than 10^6 colony forming units per g meat or per cm^2 skin surface (Venugopal, 1990). The proteolytic secretion is low due to the presence of amino acids and sugars during the early period of bacterial growth. The bacteria utilize those nutrients, thus multiplying. At stage II, bacterial growth exceeds 10^6 CFU/g. Protease synthesis in fish is depressed in the absence of low molecular weight products but the secreted bacterial enzymes are now able to degrade the muscle proteins. Proteases can be responsible for the

textual defects in seafood like the “gaping” and “mushiness” of bony fish and “tail meat” softening of crustaceans (Gornik, Albalat, Atkinson, & Neil, 2009).

Gram-negative psychrotrophic bacteria are known to produce extracellular proteases that catalyze reactions causing the degradation of proteins. The result of these reactions is the formation of peptides that can decrease the quality of the food. Proteolytic activity of microorganisms from fresh-water fish was found by Kazanas (1968). Proteases of several *Pseudomonas* spp. have low activation energies and are well adapted to low temperatures suggesting the possibility for the occurrence of the bacteria in refrigerated fishery products (Venugopal, 1990). Changes in the myocommata structures of the fish blue grenadier (*Macruronus novaevelandiae*) were noticed during chilled storage suggesting that the collagenous fibers that attach muscle fibers to the mycommatal connective tissue were degraded by proteases (Bremner & Hallert, 1985). In addition, the soluble or sarcoplasmic fraction of fish containing amino acids and vitamins form a medium for growth of microorganisms. The microbial growth in the sarcoplasm is also associated with protease secretion of the organisms. Several SSOs have more than one protease. *P. aeruginosa* secretes two types of proteases, whereas *Vibrio alginolyticus* secretes a colloganase and five serine alkaline proteases, and *Aeromonas hydrophila* secretes two types of proteinases and one aminopeptidase (Morrissey, Buckeley, & Daly, 1980). Also, *Serratia* spp. excretes a variety of degradative extracellular proteases like alkaline metalloproteases (Rao, Tanksale, Ghatge, & Deshpande, 1998). *S. marcescens* is capable of secreting up to four types of proteases (Peterson & Tisa, 2013).

2.5 Detection and Purification of Proteases from Bacteria

Qualitative assays are performed for screening of proteolytic activity within the bacteria. The initial assay to detect the secretion of protease from bacteria is using a substrate. The protein agar plate assay uses either skim milk, casein, gelatin, bovine serum albumin (BSA) or keratin (Gupta, Beg, Khan, & Chauhan, 2002). The enzymatic hydrolysis of the substrate creates a clear zone in the agar media. The most common protein used among substrates for selective screening is skim milk (Rajamani & Hilda, 1987). In another qualitative assay, the radial diffusion assay, the protease is detected by observing the zone of hydrolysis around small wells cut into the agar media containing the protein substrate. Sometimes a pre-stained Coomassie blue agarose gel with the substrate added in can allow direct assessment of the activity. Non-denaturing gel electrophoresis can be used to separate and estimate the size of numerous proteases in the bacteria (Ridgeway, Small, Atkinson, Brikbeck, Taylor, & Neil, 2008).

Quantitative measurements of the protease activity can show the actual extent of the proteolytic potential. Spectrophotometric methods are the preferred procedure. After the proteolytic reaction on a protein substrate has taken place (with BSA, casein, or hemoglobin), the quantity of peptides is determined in acid-soluble hydrolyzed product fractions. The peptide residues are estimated by an absorption of 280 nm or using Folin's reagent for a colorimetric result.

Once the protease is chosen it needs to be isolated from any other protease produced by the bacteria. The supernatant of the bacteria sample is obtained to remove the nucleic acids and debris. The enzyme must be stabilized against chemical and microbial degradations by adding a

high concentration of salt such as ammonium sulfate and glycerol to preserve the protein and increase shelf-life. Ammonium sulphate absorbs the water content and concentrates the proteins (Sumantha *et al.* 2006). Then, chromatography techniques are applied to purify the protein. Depending on the type of protease, the appropriate column must be used. Positively charged proteases are not bound to anion exchange columns, requiring the use of cation exchangers. The protein is absorbed to those column matrices containing ionizable function groups such as diethyl amino ethyl and carboxy methyl (Kumar, 2002). The absorbed protein is then eluted with a gradient change normally of ionic strength. Besides charge, proteases can be purified by affinity or size through a gel filtration chromatography. The chromatography method also increases specific activity of the protein.

CHAPTER 3: MATERIALS AND METHODS

3.1 Bacterial Isolation

Fresh channel catfish (*Ictalurus punctatus*) fillets, obtained from the DSU Aquaculture Research and Demonstration Facility and local retail establishments in Dover, DE were cut into 2 cm cubes, divided into 20 g portions, and placed in stomacher bags. After adding 80 ml of 0.85% saline was to the stomacher, contents were and homogenized (Stomacher 3500; Seward Inc., Bohemia, NY) for 40 sec at speed 2. The homogenized solution was centrifuged at 3,500 rpm for 3 min using a swing bucket in a Sorvall Legend X1 centrifuge (Thermo Scientific, Waltham, MA). The supernatant was diluted 10-fold in tryptic soy agar (TSB) (Carolina, Burlington, NC) and spread in triplicate onto milk-plate-count agar (OXOID, Basington, UK) to assess the growth of bacteria that exhibited protease activity (Kazanas, 1968). The plates were incubated for 3-5 days at 21 °C to ensure optimum growth. Protease-producing colonies were selected and isolated based on the unique characteristics.

3.2 16S rDNA Sequencing and Phylogenetic Tree Construction

Protease producing bacterial colonies were identified through 16S rDNA sequencing. Hypervariable regions of 16S rDNA sequences were used. DNA sequencing was performed by GENWIZ, Inc (South Plainfield, NJ). The bacteria were identified using the Nucleotide BLAST in the Genbank database. Identification criteria were defined on matches 99% or greater. Multiple gene alignments were completed using the software program Cluster W. A phylogenetic tree was created by the neighbor joining method through software MEGA6

(Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). The findings were compared to known strains and bootstrap values were calculated.

3.3 SDS-PAGE and SDS-PAGE with Gelatin

For this project, *Serratia grimesii*, *Pseudomonas lurida,* and three *Aeromonas jandaei* strains were reactivated to analyze the protein patterns of each bacteria. The five spoilage bacteria were cultured overnight in TSB and centrifuged at 13,000 rpm for 4 min. Bacteria pellets were re-suspended in Millipore water. Then, 4x final concentration Laemmli sample buffer (Bio-Rad) was used for cell disruptions. Samples were heated at 100 °C for 12 min and briefly centrifuged before being allowed to cool in ice. A 10% SDS-PAGE gel was used to run samples at 100 volts using the Owl EC-105 Compact Power Supply (Thermo Scientific) for 2 h. Gels were stained overnight using Coomassie Brilliant Blue- R250 dye (Thermo Scientific) and de-stained with a mixture of methanol and acetic acid until bands were visible. Non-denatured supernatants of the bacteria, not treated with β-mercaptoethanol and heat, were also inserted into the PAGE gel. Pictures were taken using a Gel Doc EZ Imager (Bio-Rad). Dissimilarities of the protein patterns from each of the five bacteria were observed.

SDS-PAGE with gelatin was used to determine which particular proteins expressed protease. Pellets and supernatants of the 5 bacteria were run on 1% gelatin (Fisher Scientific, Pittsburg, PA) 10% SDS-PAGE gels for 2 h at 100 volts. The method was adapted from Reddy and Venkateswerlu (1997). Non-denatured protein was used in this assay. Gels were rinsed in a 0.1 M Tris-HCl pH 8.0 and 1% Triton X-100 buffer solution for 1 h (Fisher Scientific). This buffer was replaced with new Tris buffer every 10 min for 30 min. Gels were incubated at 32 °C

and were then rinsed with Millipore water and stained with Coomassie Blue for 2 h. Gels de-stained with destaining solution for 1 h. Pictures were taken using a Gel-Doc. The bacteria were evaluated for their ability to produce protease.

3.4 Bacterial Cultivation, Growth Curve, and Enzyme Production

S. grimesii was chosen for further analysis due to its taxonomic distance and distinct protein patterns. First, bacteria growth was tested at different pH levels ranging from 3-12. The bacteria were inoculated in the TSB of different pH levels at 21 °C for a total of 12 h. Every 3 h, the optical density (Genesys, 10S UV-Vis Spectrophotometer; Thermo Scientific) was recorded at 600 nm. Next, the bacterial growth was assessed using different temperatures. Incubation temperatures varied from 4 to 44 °C. The optical density (600 nm) was documented every 3 h for a total of 12 h. Each test was done in duplicate.

Using the findings from the pH and temperature assays, a growth curve was developed and the enzyme production observed. *S. grimesii* was inoculated in TSB at 28 °C for 16 h. The optical density was read and fixed at 0.05 OD in 50 ml TSB. The sample was placed in a 28 °C incubator (MaxQ 4000; Thermo Fisher), shaking at 108 rpm. Every 3 h for a total of 72 h, the OD was measured. Every 3 h, 1 ml of the bacterial culture was centrifuged at 13,000 rpm and the supernatant filtered prior to storage at -4 °C. The protease activity was evaluated using milk agar. The agar was prepared using 1.2% milk plate count agar. Also, the DNase activity was examined using methyl green agar (Difco, Sparks, MD; Smith, Hancock, & Rhoden, 1969). For well diffusion, 85 µl of each filtered supernatant was added to the wells of the agars. The agar plates

were incubated overnight at 28°C. Two repetitions of both the bacterial growth curve and the enzyme production were conducted and averaged.

3.5 Crude Protein Sampling from Bacterial Culture

Crude protein was obtained through ammonium sulfate precipitation and dialysis procedures. *S. grimesii* was cultured overnight in 10 ml of TSB. The OD was used to set 50 ml of TSB to a 0.05 concentration. Bacteria were incubated for 48 h at 28 °C, shaking at rpm 108. Then, the culture was centrifuged at 12,000 xg for 20 min. Ammonium sulfate (Fisher Scientific) was added until the solution reached 70% saturation. The saturated solution was centrifuged at 12,000 xg for 20 min and the supernatant was discarded. The pellet was re-suspended in 25 mM Tris-HCl, pH 8.0 buffer and transferred into dialysis tube (Thermo Fisher). The sample in dialysis tubing was desalted by replacing fresh buffer occurred after every 5 to 7 hrs. Crude protein was further used for biochemical studies and protein purification.

3.6 Protein and Protease Assays

Protein and protease activity assays were performed several times during this project. Protein was quantified using the protein assay (Bio-rad). Ten µl of the appropriate sample, bacteria supernatant or protein, was added to 200 µl 5x concentration protein dye in a microplate. The control was 10 µl of media broth or buffer containing the sample added to 200 µl 5x concentration protein dye. The solutions were incubated at room temperate for 5 min and then the OD was obtained with a microplate reader (Synergy HT; BioTek, Winooski, VT). The total protein was determined comparing the OD to points on the standard curve (See Appendix A) which was developed using bovine serum albumin at different concentrations. The protein assay

was implemented using 0.05 to 0.4 mg/ml BSA in media broth or buffers TSB, Tris-NaCl, and phosphate buffer saline (PBS).

The protease assay was used to determine the activity of the enzyme. The protocol was adapted and modified from Cupp-Enyard (2008) using casein as a substrate. Four µg of protein was added to its appropriate buffer and placed into 0.65% casein in potassium phosphate dibasic buffer (Fisher Scientific). After incubation at 37 °C incubation for 30 min in a water bath (Isotemp; Fisher Scientific), 110 mM trichloroacetic acid (Fisher Scientific) was added to the samples to stop the reaction. Another 30-min incubation was performed before the samples were centrifuged for 7 min at 3,200 rpm. The supernatant was added to 500 mM Na carbonate (Fisher Scientific) and 1 N final concentration Folin & Ciocalteu's phenol reagent (MP Bio medicals, Solon, OH). This mixture was incubated for 30 min at 37 ºC. The OD at 660 nm was compared to the tyrosine concentration using the tyrosine standard curve (See Appendix B). The protease assay was performed using tyrosine in media broth or buffers TSB, Tris-NaCl, and phosphate buffer saline (PBS). 1 AU was defined as 1 µM tyrosine.

3.7 Thermal Stability

Biochemical studies were implemented on the crude protein to investigate the activity of the combination of all proteases present in *S. grimesii*. First, temperature was tested on the crude protein. A quantity of 4 µg was mixed with 25 mM final concentration Tris-HCl, pH 8.0 buffer and aliquoted into 8 tubes. The tubes were heated at 30, 40, 50, 60, 70, 80, 90, and 100 °C for 15 min (Mothe & Sultanpuram, 2016). Tubes were immediately chilled on ice following heating and 4x SDS sample loading buffer was added without β-mercaptoethanol. Samples were run on

1% gelatin 10% SDS-PAGE gel for 2 h at 100 volts. Samples were rinsed and incubated, stained and des-stained as previously stated.

3.8 Optimization of pH

Crude protein (4 µg) was incubated with buffers with a pH range of 3-13 at 37 ºC for 30 min, specifically with sodium citrate-HCl for pH 3-6, Tris-HCl for pH 7-9, and glycine-NaOH for pH 10-13 (Fisher Scientific; Doddapaneni, 2007). The final concentration of each buffer was 25 mM. Casein was used as the appropriate substrate. After 30 min incubation, the proceeding steps of the protease assay described earlier were followed. The OD (660 nm) of the supernatant was measured and the relative activity calculated. The pH that resulted in the highest activity was considered 100%. All biochemical studies completed with the protease assay were done in triplicates.

3.9 Solvent Compatibility

The effect of various solvents including acetone, ethanol, iosopropanol, and methanol (Fisher Scienctific), benzene (Alfa Aesar, Tewsbury, MA) and dimethyl sulfoxide (Thermo Scientific) were studied (Sanatan, Lomate, Giri, & Hivrale, 2013). The protease assay was initiated by mixing 4 µg crude protein, Tris-HCl buffer, and 15% final concentration solvent (v/v) before the assay was processed as previously discussed. The relative activity was determined by designating the control without solvent to be 100%.

3.10 Co-factor Compatibility

Protease activity was tested on the crude protein using different co-factors (Niyonzima & More, 2015). The metals in this study were cobalt chloride, ferrous sulfate, manganese chloride, nickel chloride, and zinc chloride (MP Biomedical), barium chloride, nickel chloride, ferric chloride, and copper sulfate (Ricca, Arlington, TX), and magnesium chloride (Thermo Fisher). Metals were mixed with Tris buffer to create a 3 mM working concentration prior to blending them with 4 µg crude protein for development of the protease assay as described. The relative activity using each metal was calculated, compared to the metal-free control sample.

3.11 Detergent Compatibility

Various detergents were selected to test the activity change of the proteases when exposed (Nadeem, Qazi, Syed, & Gulsher, 2013), including Triton X-100 (MP Biomedical), hydrogen peroxide (Equaline, Eden Prairie, MN), sodium hypochlorite in commercial bleach, Tween 80 (Fisher Scientific), Tween 20 (Thermo Fisher), and sodium dodecyl sulfate (Promega, Madison, WI). The final concentration of detergent used was 1% (v/v) which was added to 4 µg protein with Tris buffer. The protease assay was done accordingly. The relative activities were measured and the activity without any detergent was taken as 100%

3.12 Substrate Specificity

Crude protein was reacted with various substrates to examine specificity (Li et al., 2016). The five substrates explored were casein, egg albumin, and gelatin (Fisher Scientific), bovine serum albumin (Jackson ImmunoResearch, West Grove, PA), and gluten (Tokyo Chemical

Industry Co., Portland, OR). Trypsin (Fisher Scientific), although a protease, was also used as a substrate for competitive enzyme digestion. Substrates were incubated with the crude protein at 37 ºC for 30 min. The working concentrations of the substrates were 1% (w/v). After 30-min incubation, the remainder of the protease assay was completed as discussed earlier. Casein was selected as the standard of 100% relative activity.

3.13 Protein Purification: Ion Exchange and Gel Filtration

To purify the single protease, Next Generation Chromatography Quest-Plus (NGC, Bio-Rad) was used. After dialysis, the crude protein was filtered and injected into the Enrich Q High-Resolution column (BioRad) attached to the NGC. The 25 mM Tris buffers with Tris 1 M NaCl gradients were tested at pH 7.6, 8.0, 8.4, and 8.6. The target protein was confirmed through 10% SDS-PAGE. To further determine the optimal pH value, enzyme activities were measured using milk agar plates. The largest clear region at that particular pH was selected for the remaining studies.

After the target protein was separated from the anion exchange fractions, the sample was concentrated and buffer exchanged into 1x final concentration phosphate buffered saline (PBS). The sample was loaded into the Enrich SEC 70 High-Resolution column (BioRad) attached to the NGC using 1x final concentration PBS pH 7.2. To confirm that the protein was successfully purified, the fractions were loaded onto a SDS-PAGE gel. The final product was used to conduct the 6 biochemical studies (temperature, pH, solvent, co-factor, detergent, and substrate) described previously using purified protein for the protease assays. In addition, a purification table was created to show the steps that were used to completely separate the target protein. The

protein amount and protease activity were determined for every step. The data of samples from supernatant of the bacteria inoculate, dialysis, anion exchange, centricon, and gel filtration were included on the table.

3.14 Storage Stability

Storage stability studies, researching the shelf-life of the purified protease, were conducted by either freezing/thawing or refrigerating the enzyme. With freezing/thawing the protein was frozen at -20 °C, then thawed at room temperature until completely melted every 24 h, repeating this a total of five times. Each time, the protein was placed back in the freezer except for one aliquot of the sample needed to test enzyme activity. With refrigeration, purified protein was kept at 4 °C for two months and the activity was tested every week. The protease assay was applied to both.

3.15 Mass Spectrometry and Protein Informatics

Mass Spectrometry was the used to determine and match peptide sequences for identification of the target protein. Protein bands of interest were excised from SDS-PAGE. The bands were then stored at -20°C overnight. After thawing, staining was removed from the samples before a reducing solution was applied. Samples were then alkylated and digested resulting in peptides. Peptides from each sample were separated and individual masses determined by the 4700 Proteomics Analyzer MALDI-TOF-TOF (Applied Biosystems, Foster City, CA). Proteins were identified by matching the resulting peptide masses and corresponding amino acid sequences to the NCBI non-redundant (nr) mass spectra database (Johnson, Davis, Taylor, & Patterson, 2005).

The identified target protease was examined using several informatics tools. The hypothetical protease structure was constructed with the ExPASy ProtParam tool. In addition, the identified protease was used as a reference to search for other bacteria in the BLAST to gain similar protease originating from different bacteria species. Protein sequences were represented through a FASTA format. The proteases were compared by multiple sequence alignment using similarities of the Clustal Omega algorithm before a protein neighbor joining tree was constructed.

Statistical analysis

All bacteria growth curves were repeated at least two times. Three independent studies were performed for each biochemical assay (pH, solvent, co-factor, detergent, and substrate). Standard deviation and standard error were calculated using MS-Excel 2016. The One Sample T-test was run on each storage test using SPSS 23.

CHAPTER 4: RESULTS

4.1 Bacterial Identification

Following isolation of bacteria expressing protease from store and aquaculture catfish, bacteria were identified through 16S rDNA sequencing. Four genera were matched: *Aeromonas* spp., *Pseudomonas* spp., *Serratia* spp., and *Shewanella* spp.

Isolates were characterized in Table 1. All the matched sequences were 99% similarity. Their respective phylogeny was shown in Figure 1. *Serratia grimesii* displayed the largest taxonomical distance from the other protease producing bacteria.

Table 1: *Gene alinement of protease-producing bacteria isolates after DNA sequencing*

Bacteria Identification 16S rDNA sequencing	**Similarity** (%)	**Length** (bp)	**GenBank** Accession #	**Origin**
Aeromonas jandaei	99	843	NR037013	Aquaculture
Aeromonas hydrophila	99	843	NR074847	Aquaculture
Aeromonas jandaei	99	880	NR037013	Aquaculture
Aeromonas hydrophila	99	899	NR074841	Aquaculture
Aeromonas jandaei	99	833	NR037013	Aquaculture
Pseudomonas lurida	99	886	NR042199	Aquaculture
Serratia grimesii	99	888	NR025340	Store-bought
Serratia proteamaculans	99	885	NR074820	Store-bought
Serratia grimesii	99	856	NR025340	Store-bought
Serratia proteamaculans	99	856	NR074820	Store-bought
Aeromonas sobria	99	869	NR037012	Store-bought
Shewanella baltica	99	856	NR074843	Store-bought
Pseudomonas gessardii	99	868	NR024928	Store-bought

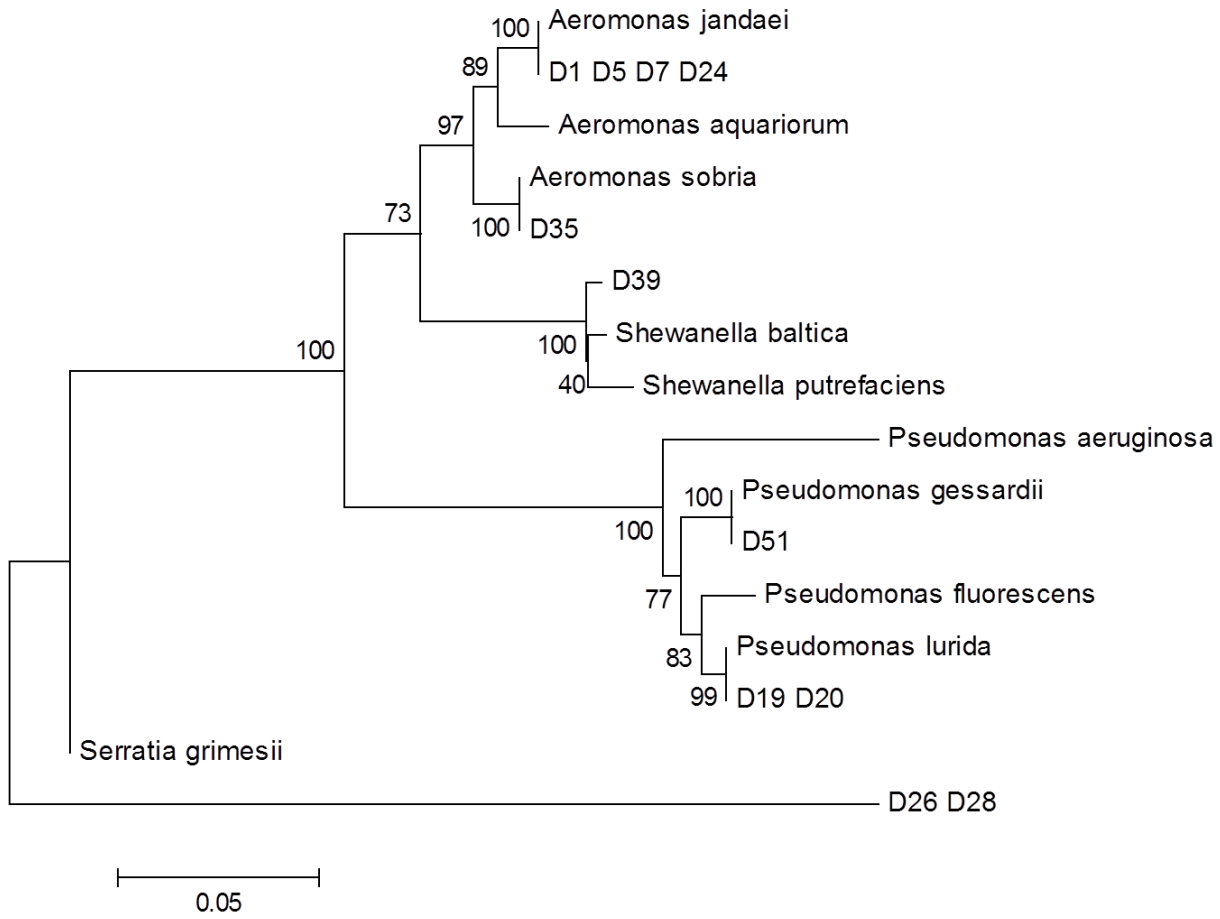

Figure 1: *Phylogenetic tree of protease-producing bacteria*
Construction based on partial 16S rDNA identification of extracellular-producing bacteria isolated from catfish tissues

4.2 Protein Pattern of Chosen Bacteria Strains

The extracellular enzyme patterns of five strains were observed to determine most unique strain. Studying both supernatant and whole cell protein, *S. grimesii* showed a different protein band pattern than the other four bacteria (Figure 2). The supernatant of *S. grimesii* only displayed bands around 50, 75 and 100 kDa while the supernatants of *P. lurida* and the three *A. jandaei* all

showed similar band patterns. In relationship to the marker bands, the four bacteria strains showed thicker bands a little after 20 and 25, and between 37 and 50 kDa. The thickest band was before 37 kDa. The whole cell protein of all five bacteria had two major bands between 37 and 50 kDa. *S. grimesii* had two other major bands around 37 kDa. *P*. lurida and the three *A.* jandaei strains had a major band between 25 and 37 and another between 37 and 50 kDa.

Supernatants of the five strains were also separated on SDS-PAGE gels with gelatin to view the patterns of protease producing proteins (Figure 3). Three distinct bands around 50 and 100 kDa were visible from *S. grimesii*. The two proteins expressed protease activity on the SDS-PAGE with gelatin; the protein with a band slightly below 50 kDa appeared to have the most protease activity. The proteolytic band pattern of *S.* grimesii was vastly different from the other bacteria. The *P. lurida* and *A. jandaei* strains all had two bands between 37 and 50 kDa and one band at 100 kDa. Bands between 37 and 60 kDa and over were expressing protease activity.

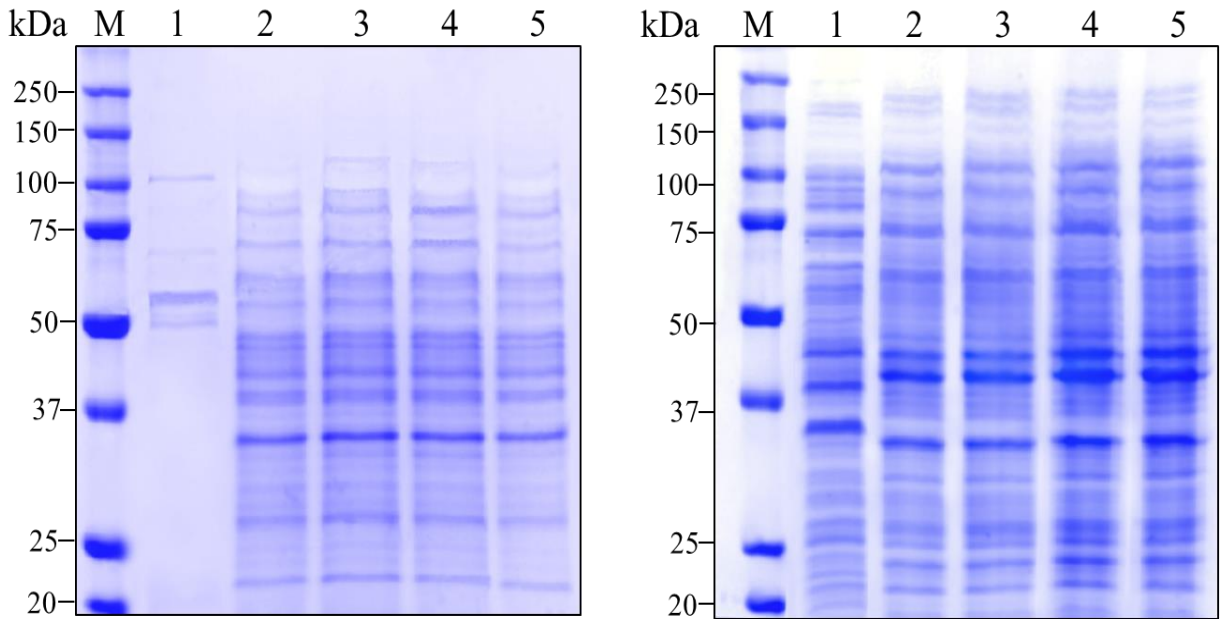

Figure 2: *Denatured protein patterns of the five bacterial strains*
Five strains denatured in 10% SDS-PAGE and stained with Coomassie blue. Left: Supernatants. Right: Whole protein. M = Marker; 1 = *S. grimesii* (D28); 2 = *P. lurida (*D19); 3 = *A. jandaei* (D24); 4 = *A. jandaei* (D60); 5 = *A. jandaei* (D61)

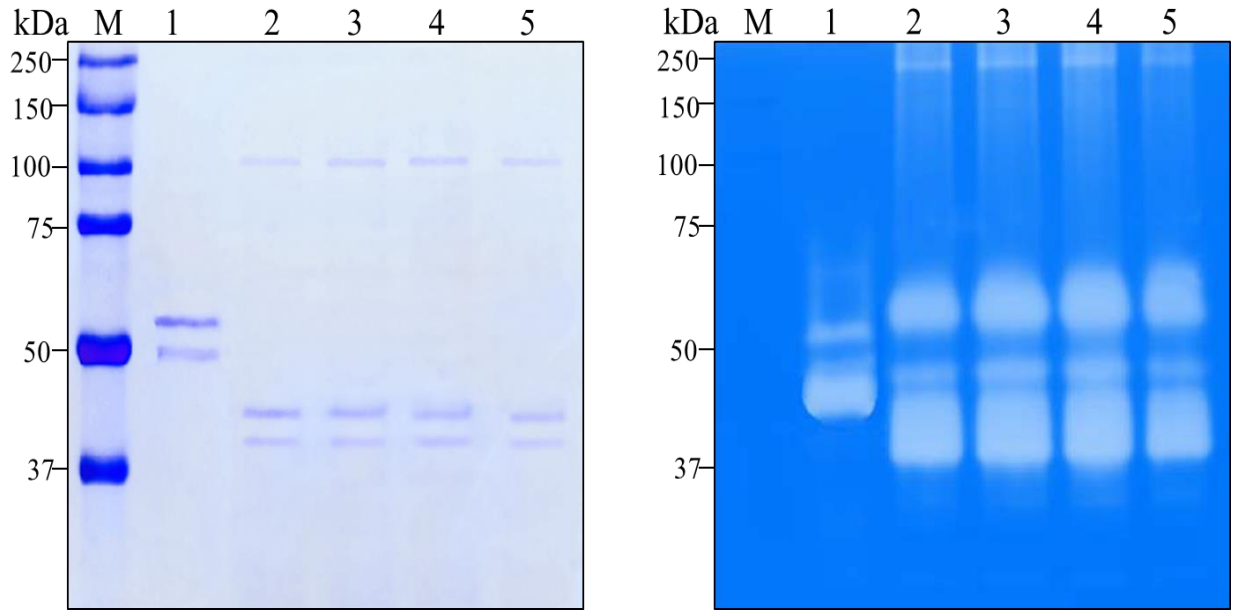

Figure 3: *Nondenatured supernatants of the five bacterial strains*
Supernatants of 5 strains nondenatured in SDS-PAGE and stained with Coomassie blue. Left: 10% SDS-PAGE. Right: SDS-PAGE with gelatin. M = Marker; 1 = *S. grimesii* (D28); 2 = *P. lurida (*D19); 3 = *A. jandaei* (D24); 4 = *A. jandaei* (D60); 5 = *A. jandaei* (D61)

4.3 Optimization of *S. grimesii* growth conditions and extracellular enzyme production

Due to the strain's uniqueness compared to the other strains isolated from catfish, *S. grimesii* was selected to be the bacteria of focus for this research. To optimize the pH value of *S. grimesii* growth, it was incubated for 15 h in TSB with pH ranging from 3-12 (Figure 4). Optical density displayed the bacterial growth through an increase in turbidity. Most growth occurred at pH range from 6-9 while very little growth took place at pH 3 and 12. The highest growth had an OD reading of 0.941 (600 nm) at pH 7 after 15 h.

Figure 5 showed the growth of *S. grimesii* incubated in TSB at temperatures 4-44 ºC over 15 h. Temperatures 4 ºC and 10 ºC had the least growth and followed by 44 ºC. After incubation of 3 h, the optical density increased with increasing temperature from 0.01 OD at 4 ºC to 0.1 OD at 36 ºC. Incubation of 6 and 9 h resulted in a gradual increase from 4 ºC to 36 ºC. Optimal growth occurred at 28 ºC after 15 h, creating a 0.483 OD.

To determine the effect of time on the bacteria growth and total enzyme production of *S. grimesii*, bacteria were incubated in TSB at optimal pH 7 and temperature 28 ºC and then evaluated every 3 h for 72 h. After the first 3 h interval, the bacteria had the optical density of 0.5 OD (600 nm) which doubled after 6 h to 1.0 and continued to increase until 39 h at 2.41 (Figure 6). In the period between 39 and 60 h, bacteria growth slowed until reaching its peak growth of 2.76. From then, the OD started decreasing back to 2.44 at 72 h.

The enzyme production was visible on the selectable media in the form of clear regions. As Figure 7 displayed, there was no protease activity at the original fixation within the first three hours. At 6 h, a clear region was visible. Between 9 and 36 h, each three-hour increment led to an increase in protease production. From 39 through 72 h, the activity remained visible as prominent clear regions. Initially, DNase production was faint, but it started increasing after 21 h with production remaining apparent throughout 72 h (Figure 8).

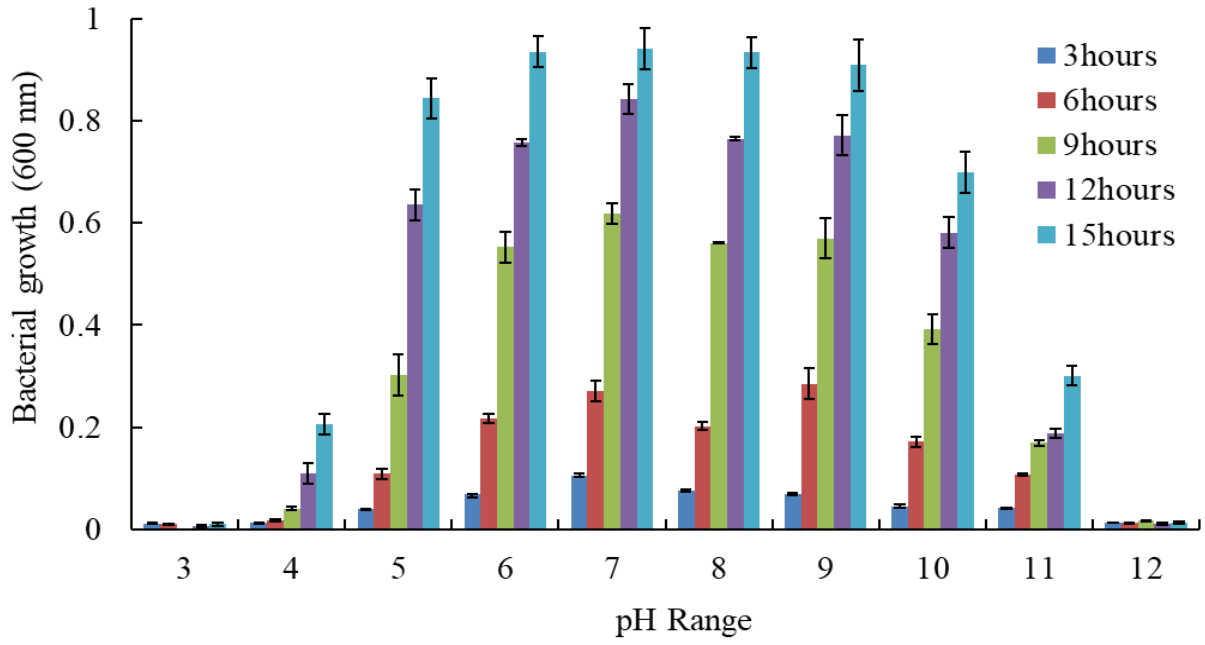

Figure 4: *Bacterial growth at different pH ranges*
The TSB was adjusted ranging from pH 3-12 and *S. grimesii* was incubated over 15 h. The growth was measured through optical density (600 nm) every 3 h

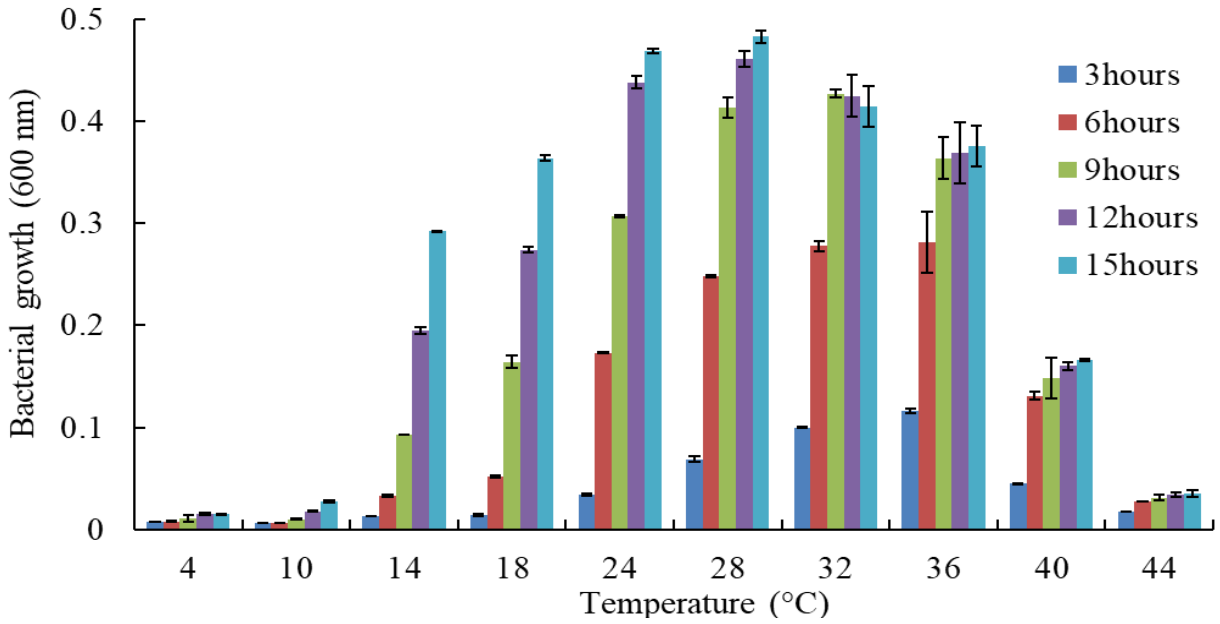

Figure 5: *Bacterial growth at different temperatures*
S. grimesii was incubated over 15 h at temperateure ranging from 4-44 ºC. Growth was measured through optical density (600 nm) every 3 h

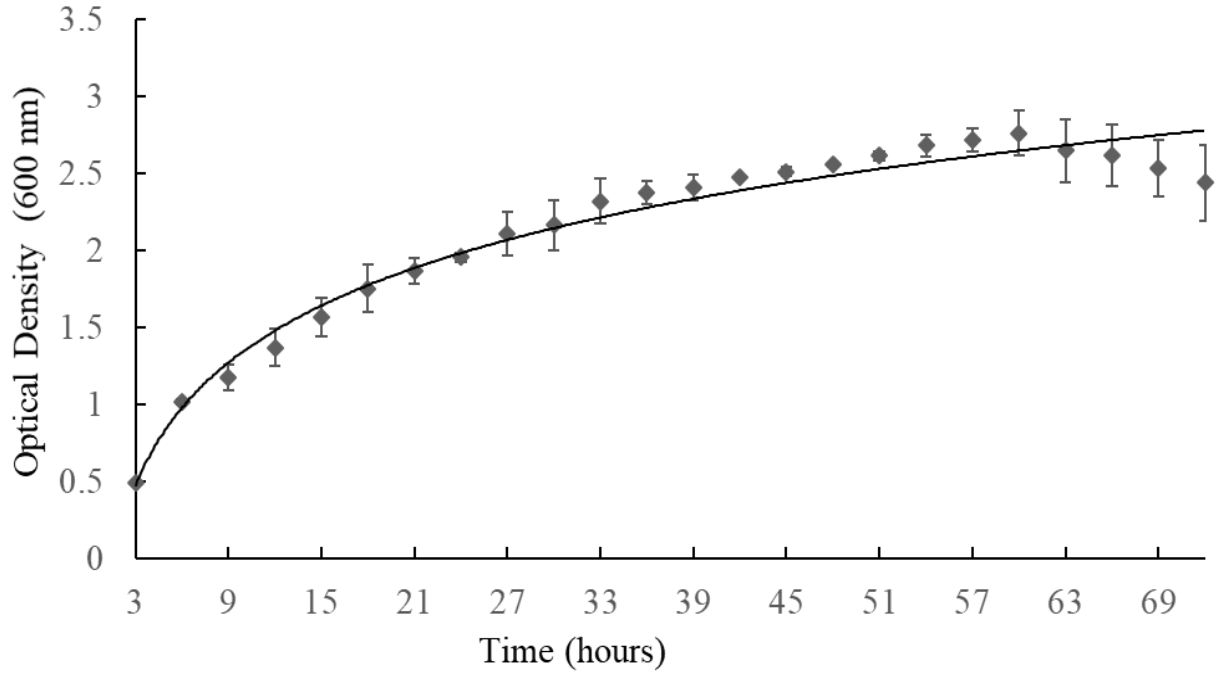

Figure 6: *Bacterial growth measured over three days*
S. grimesii was incubated at 28 ºC over 72 h. Optical density (600 nm) was evaluated every 3 h

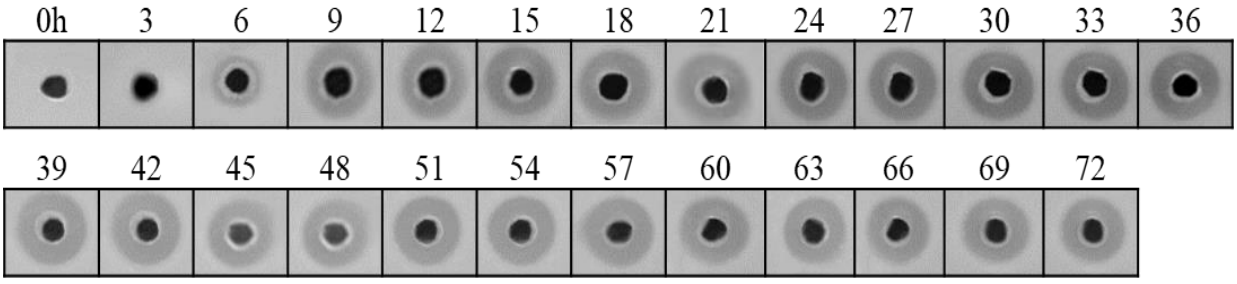

Figure 7: *Protease production over three days*
S. grimesii was incubated at 28 ºC over 72 h. The supernatant was plated into wells in 1.2% milk agar plates every 3h

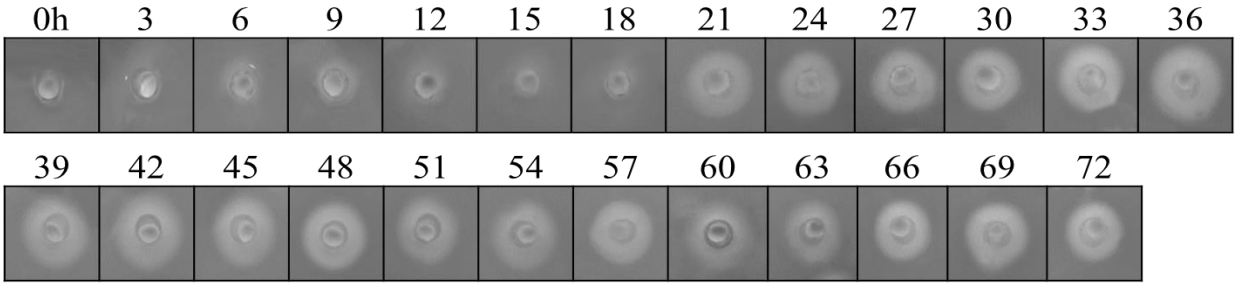

Figure 8: *DNase production over three days*
S. grimesii was incubated at 28 ºC over 72 h. The supernatant was loaded into wells in methyl green agar plates every 3h

4.4 Crude Protein Sampling

The isolation of crude protein was obtained through the filtered supernatant of S. *grimesii* culture by adding ammonium sulfate saturation of 70%. Ammonium sulfate was added to the bacteria supernatant, and the pellet was re-suspended in Tris-HCl then desalted; 2 ml of crude protein was retrieved. The protein activity was 0.32 mg/ml and total activity of the 2 ml of crude protein was 0.64 mg.

SDS-PAGE was conducted on *S. grimesii* throughout the study. After dialysis, a total of nine different bands of crude protein were present. Around 37 kDa, three weak bands were visible. Another faint band was observed above 250 kDa and two bands around 100 kDa while a more prominent band was visible below 75 kDa. The two strongest bands were observed near 50 kDa. In addition, SDS-PAGE with gelatin was performed to examine the protease activity of the crude protein. Protease activity was visible on the blue gel in the form of clear regions. The weakly stained protein weighing less than 37 kDa had some protease activity. A band at 75 kDa

and another a little below showed stronger activity. The protein greater than 50 kDa possessed slight protease activity. The largest clear region was represented by the band right below 50 kDa. This was chosen as the protease of interest.

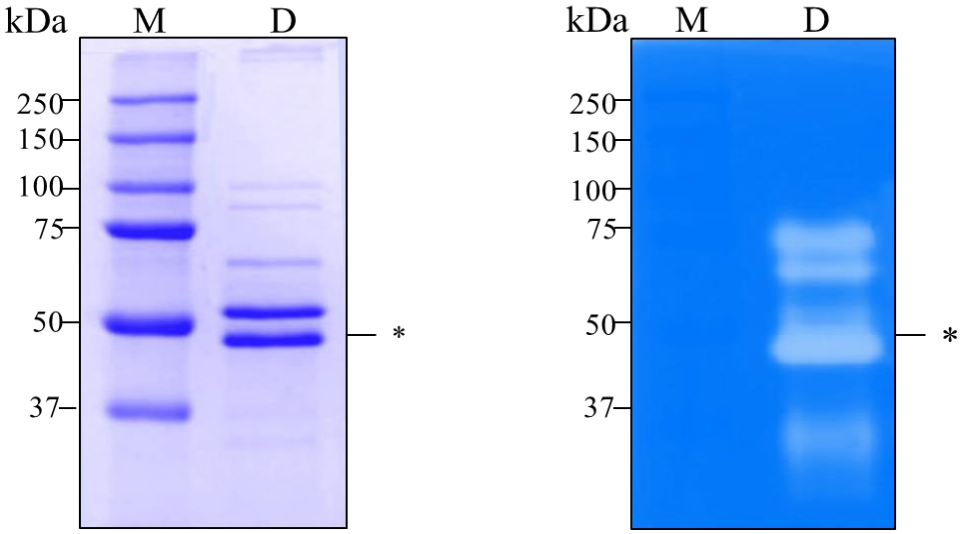

Figure 9: *Protein of interest*
After dialysis, the nondenatured crude protein of *S. grimesii* was ran in SDS-PAGE and stained with Coomassie blue. Left: 10% SDS-PAGE. Right: SDS-PAGE with gelatin. M = Marker; D = Dialysis sample; * = protease of interest

4.5 The Effect of Biochemical Assays on the Combined Proteases in Crude Protein

Numerous biochemical assays were performed on the proteases of the crude protein after dialysis of *S. grimesii*. First, temperature stability was examined using a range from 30-100 ºC (Figure 10). Samples were heated, cooled, and then loaded into SDS-PAGE gels with gelatin. At

temperatures 30 ºC and 40 ºC, the greatest number of bands and most protease activity was observed. The two bands around 50 kDa were producing the most activity, in addition to the presence of a band below 75 kDa. At 50 ºC and above, only the two bands around 50 kDa were exerting activity; however, some protease activity was observed even at 100 ºC.

Next, the pH of the proteases of the crude protein was optimized (Figure 11). Three different 25mM buffers were used at different pH values ranging from 3-13. The protease assay was done to calculate the relative activity. The measurement for pH 9 was used as the standard of 100% relative activity because it exhibited the most activity. Proteases also performed well with 78.5% relative activity at pH 8 and 10. The activity decreased to 57.13% at the neutral pH 7 and to around 40% at pH 4, 5, 6, and 11. At the extreme values of pH 3 and 13, relative activity fell to 2.28%.

Solvent (15% final concentration) compatibility was shown on Table 2. The control protease sample, tested without any added solvents, was regarded as the 100% relative activity standard. The addition of methanol increased the activity to 106.80%. The proteases were still high with DMSO at 84.70%. Isopropanol, acetone, and ethanol decreased the activity to around 65%. Benzene lowered the activity the most to 48.35%.

The effect of nine co-factors on the activity of proteases from the crude protein after dialysis was tested and reported in Table 3. Manganese chloride, magnesium chloride, cobalt chloride, and iron sulfate promoted the highest protease activity, exceeding the 100% relative activity of the control which had no metals added. The addition of manganese chloride resulted

with 115.80% in the highest protease activity. None of the added metal ions weakened the relative activity to below 50% with the exception of copper sulfate which fell to 49.30%.

The effect of 1% final concentration detergents on the proteases in crude protein were determined (Table 4). Sodium hypochlorite found in bleach promoted the highest relative activity of 179.11%. Tween 20 was closest to the control with 93.70% relative activity while Tween 80 and hydrogen peroxide resulted in an activity of around 80%. Triton X-100 caused the activity to decrease to 60.80%, but the lowest activity was that of SDS at 14.80%.

The relative activity of the proteases in the crude protein was observed when 1% final concentration of substrates were applied. As control the relative protease activity with 1% casein substrate was selected and regarded as 100%. With the other substrates, the relative decreased to less than 20% with trypsin and BSA and less than 10% with egg albumin and gluten. Gelatin showed the least relative activity of 1.90%.

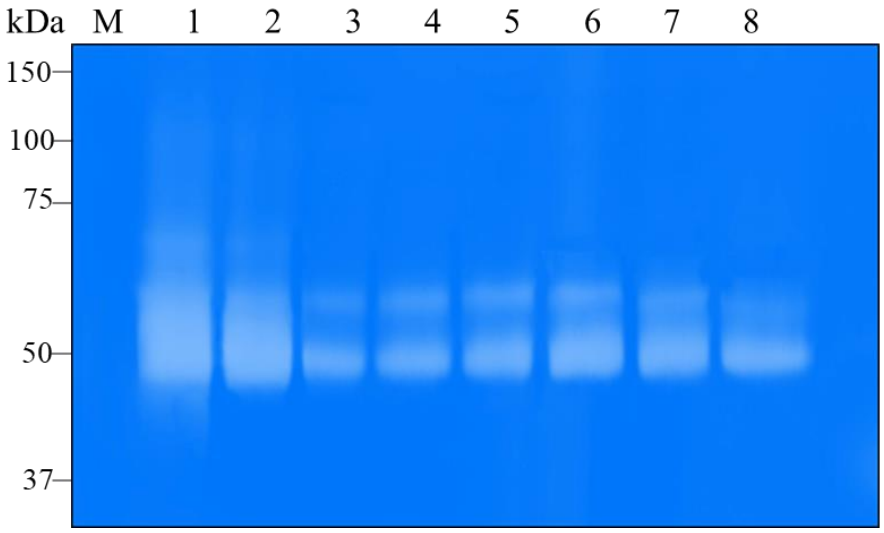

Figure 10: *Temperature stability on protease activity of crude protein*
Dialysis sample of *S. grimesii* was incubated in temperatures ranging 30-100 ºC and ran, nondenatured, in 10% SDS-PAGE with gelatin and stained in Coomassie blue. M = Marker; 1 = 30 ºC; 2 = 40 ºC; 3 = 50 ºC; 4 = 60 ºC; 5 = 70 ºC; 6 = 80 ºC; 7 = 90 ºC; 8 = 100 ºC

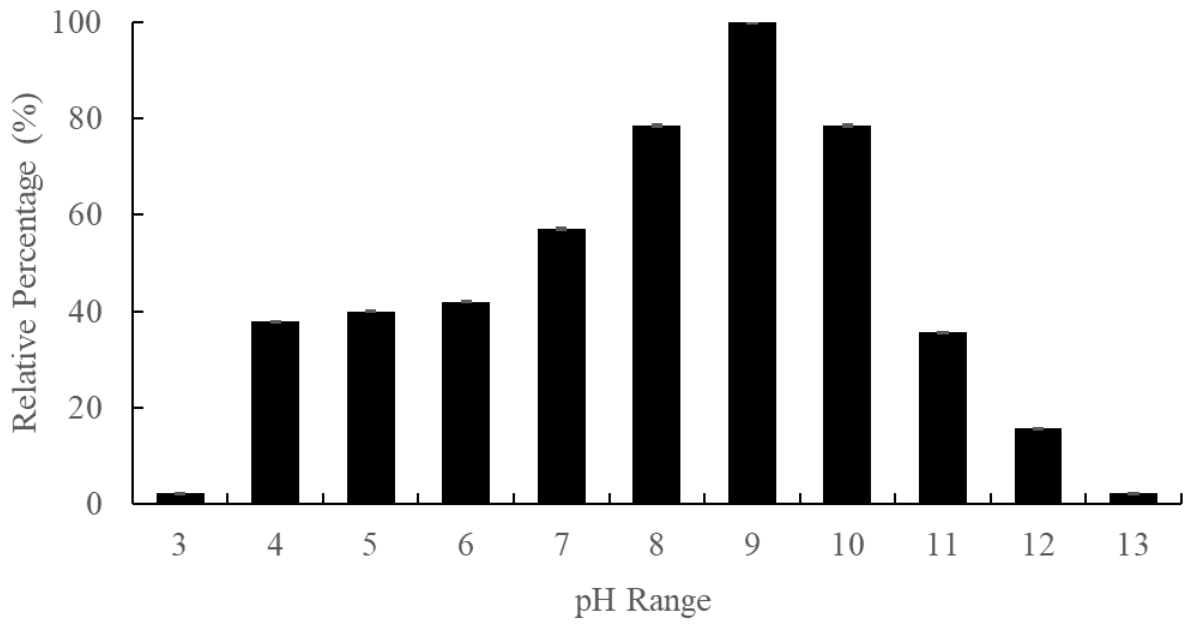

Figure 11: Effect of *pH on protease activity of crude protein*
Proteases in dialysis sample was incubated in different pH ranging from 3-13 during the protease assay. The pH with the highest activity was regarded as 100%

Table 2: *Solvent compatibility on protease activity of crude protein*

Solvent (15%)	Relative Activity (%)
Methanol	106.80 ± 5.0
None	100.00 ± 0.0
DMSO	84.70 ± 4.4
Isopropanol	66.80 ± 7.8
Acetone	65.33 ± 4.3
Ethanol	65.23 ± 4.2
Benzene	48.40 ± 5.6

Table 3: *Co-factor compatibility on protease activity of crude protein*

Co-factor (3 mM)	Relative Activity (%)
$MnCl_2$	115.80 ± 7.7
$MgCl_2$	111.10 ± 7.0
$CoCl_2$	110.80 ± 5.8
$FeSO_4$	105.21 ± 4.5
None	100.00 ± 0.0
$BaCl_2$	96.80 ± 7.0
$NiCl_2$	85.72 ± 2.4
$ZnCl_2$	79.03 ± 5.5
$FeCl_3$	69.30 ± 2.0
$CuSO_4$	49.30 ± 5.5

Table 4: *Detergent compatibility on protease activity of crude protein*

Detergent (1%)	Relative Activity (%)
NaClO	179.11 ± 5.8
None	100.00 ± 0.0
Tween 20	93.70 ± 3.5
Tween 80	84.30 ± 7.7
H_2O_2	81.40 ± 4.7
Triton X-100	60.80 ± 7.3
SDS	14.80 ± 4.4

Table 5: *Substrate compatibility on protease activity of crude protein*

Substrate (1%)	Relative Activity (%)
Casein	100.00 ± 0.0
Trypsin	17.41 ± 3.4
BSA	13.61 ± 3.2
Egg Albumin	8.80 ± 0.3
Gluten	4.10 ± 4.8
Gelatin	1.90 ± 6.3

4.6 Optimization of Protein Separation and Protease Production with Ion Exchange Chromatography

The optimum pH of the 25 mM Tris buffers used for the anion exchange chromatography was found comparing the pH of 7.6, 8.0, 8.4, and 8.6. At each run, the protein sample was injected into the NGC. At pH 7.6, the chromatogram showed a peak at fraction 18 in the 16.5 CV and 93 mAU. The second peak formed at fraction 19 in 17.3 CV with 78 mAU. The SDS-PAGE gel displayed the target protein labelled 1-O was not separated from the band more than 50 kDa labelled 2-O (Figure 12a) in both fractions 18 and 19. The pH values 8.0, 8.4, and 8.6 caused the target band to elute separately in fraction 18. At pH 8.0, there was a 98 mAU peak in 16.8 CV holding only the target protein and a 77 mAU peak in 17.5 CV holding 2-O. The pH value 8.4 allowed the target to elute from fraction 18 at 126 mAU and 16.9 CV while 2-O was 91 mAU in 17.6 CV. At pH 8.6, the target protein band formed in 16.8 CV, 96 mAU and the 2-O band formed in 17.6 CV, 81 mAU.

After using the chromatograms and PAGE gels to confirm that the separation occurred at pH 8.0-8.6, the eluted samples from the four pH values were incubated into milk agar plates for protease activity analysis. Figure 13 demonstrated that fractions 18 and 19 of pH 7.6 had the same clear regions supporting that the SDS-PAGE showed two protein bands in both fractions. At the other pH values, a larger clear region was displayed on the agar containing fraction 18 since only the target band was eluted. The separated protein greater than 50 kDa in fraction 19 produced a smaller clear region for the three pH values.

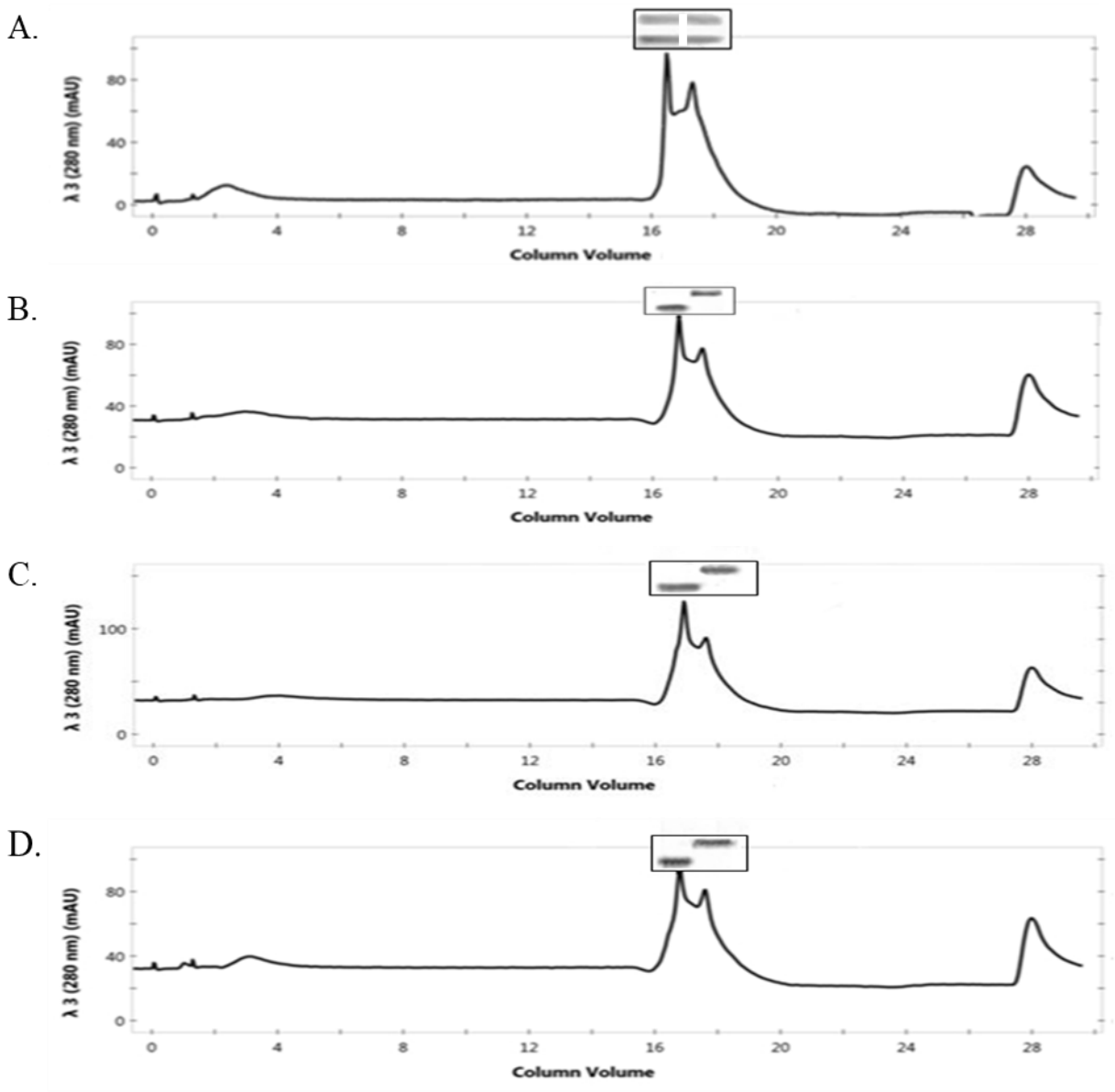

Figure 12: *Band separation with ion exchange at different pH values*
Dialysis protein of *S. grimesii* was injected into the ion exchange column. The bottom band is the target protein. A = pH 7.6; B = pH 8.0; C = pH 8.4; D = pH 8.6

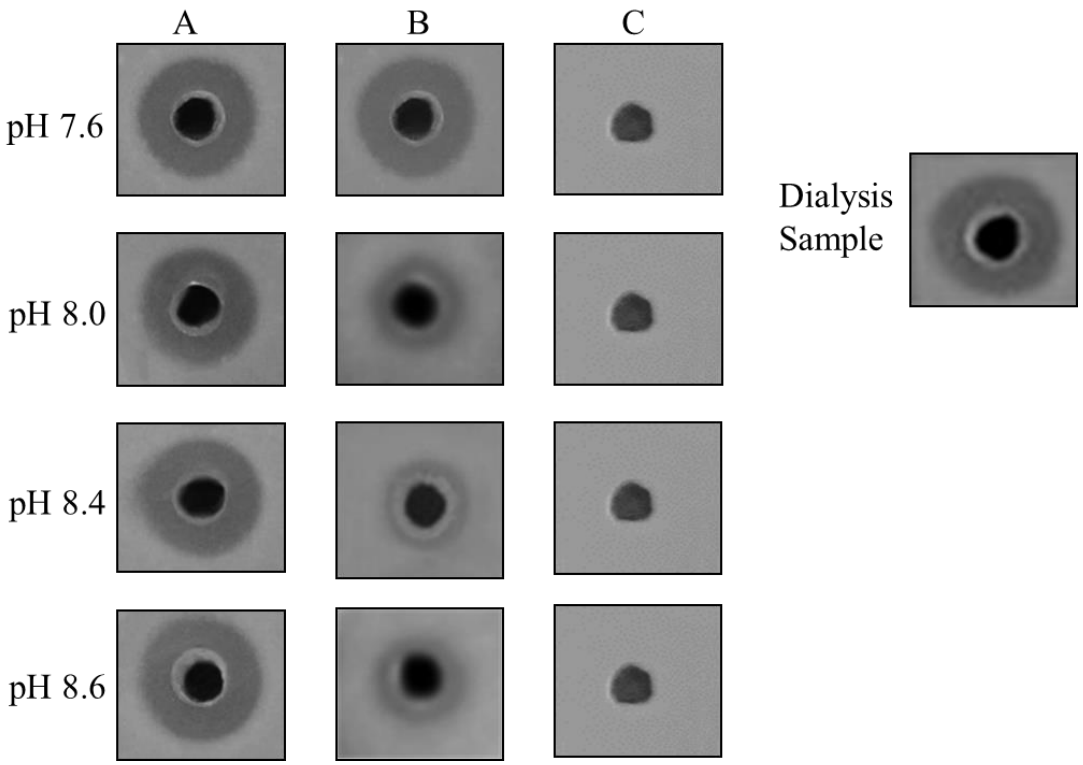

Figure 13: *Protease activity with ion exchange*
Ion exchange products of the protein eluted in fractions 18 and 19 at different pH values.
Successful separation of the target protein is shown in column A of pH ranges 8.0-8.6.
A = Fraction 18; B = Fraction 19; C = Control (Tris-HCl buffer without ion exchange product)

4.7 Purification of Target Protein from *S. grimesii*

Figure 14 is the chromatogram of the gel filtration. The NGC was injected with the pooled protein sample containing the separated target enzyme. After the run, the target protein had a peak of 31 mAU in 0.42 CV at 280 nm. The purified protein was confirmed in SDS-PAGE.

The results of the purification of the protease from *S. grimesii* were summarized in Table 6. A nearly 10-fold purification of the crude enzyme was attained with a recovery of approximately 6% and specific activity 6.0 AU/mg. Ammonium sulfate precipitation yielded of 55% recovered protease with a purification fold of 2.63. Ion exchange resulted in a 12.09% yield with 1.71 purification fold. Both fractions 18 and 19 containing the target and protein above 50 kDa were collected to obtain a greater concentration of the target.

The centricon produced a 10.87% recovery and 3.38 purification fold. The initial specific activity of the supernatant was 0.603 AU/mg which increased to 6.0 after gel filtration. As shown in figure 15, the target protease was purified successfully. After ion exchange chromatography, the two proteins around 50 kDa were eluted and the target band separated at slightly below 50 kDa.

As demonstrated in Figure 15, each step in the purification process showed the separation of bands through SDS-PAGE. The whole cell sample contained the greatest number of protein bands while the supernatant contained only the bands of extracellular enzymes. Supernatant and dialysis displayed the same number of bands. Bands were eluted with the application of anion exchange resulting in fewer protein bands. After gel filtration, only one single target band remained.

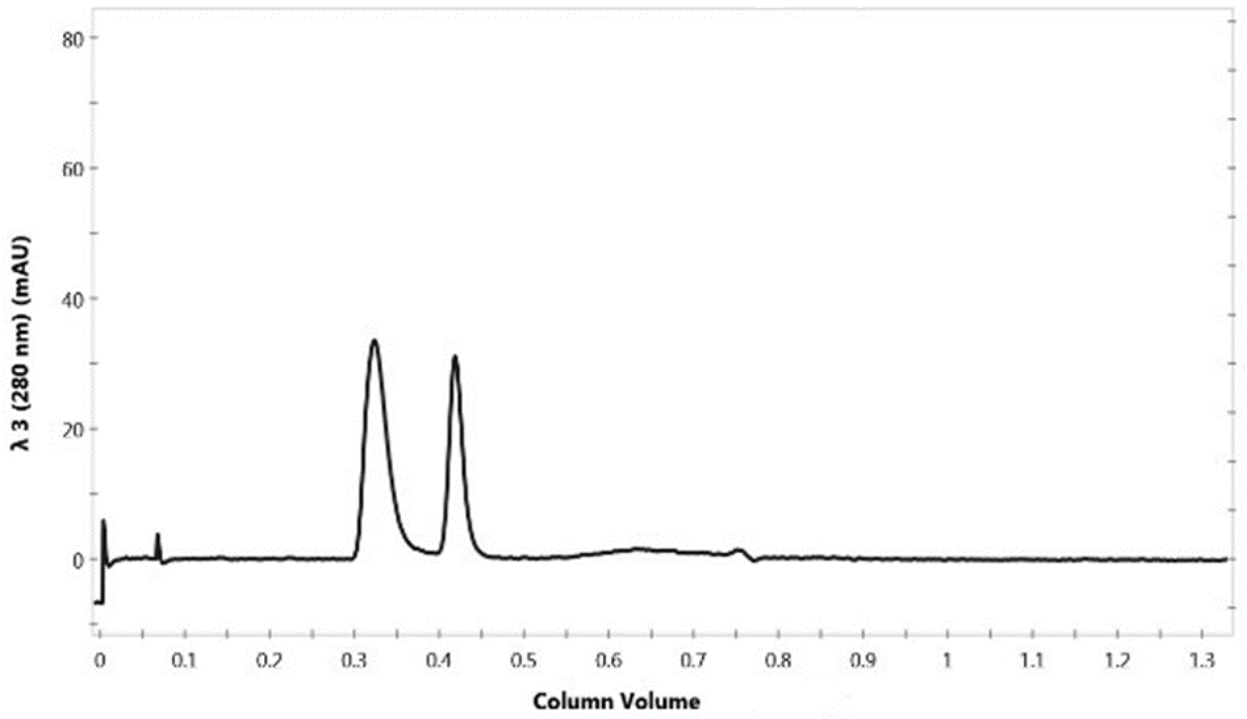

Figure 14: *Gel filtration chromatogram*
Ion exchange product containing target protein injected into the gel filtration column with PBS buffer

Table 6: *Purification summary of the target protease*

Purification Step	**Volume** (ml)	**Activity** (AU/ml)	**Total Activity** (AU)	**Total Protein** (mg)	**Specific Activity** (AU/mg)	**Recovery** (%)	**Purification Fold**
Supernatant	55.24	0.0458	2.53	4.198	0.603	100	1
Ammonium Sulfate Ppt.	2	0.698	1.396	0.88	1.586	55.18	2.63
Ion Exchange	4	0.0764	0.306	0.296	1.034	12.09	1.17
Centricon	0.54	0.509	0.275	0.135	2.037	10.87	3.38
Size Exclusion	2	0.075	0.150	0.025	6.0	5.90	9.95

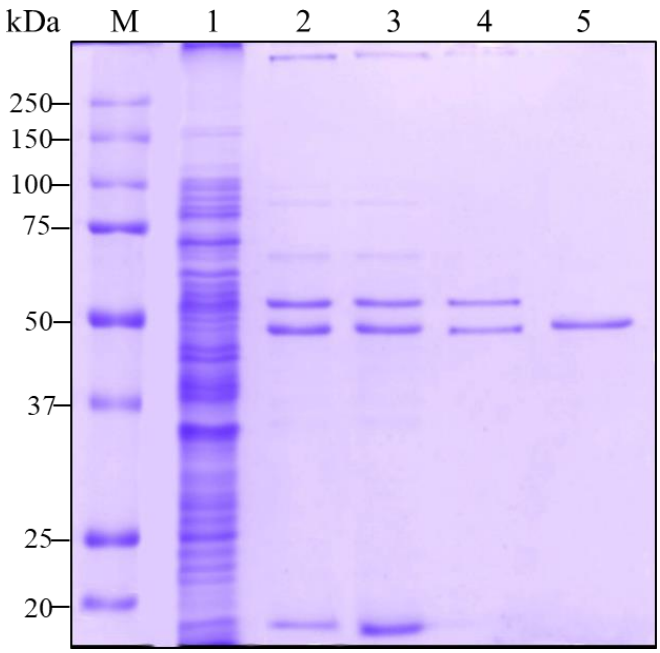

Figure 15: *Purification steps of the target protease*
Denatured extracellular protein of each purification step in SDS-PAGE. M = Marker; 1 = Whole cell; 2 = Supernatant; 3 = Dialysis; 4 = Ion exchange; 5 = Gel filtration

4.8 Biochemical Properties of the Protease of Interest

The temperature stability of the target protease found in *S. grimesii* was investigated in temperatures ranging from 30-100 °C (Figure 6). Temperatures closest to the regular *S. grimesii* optimal incubation temperature of 28 °C (30 and 40°C) exhibited the greatest protease activity on the gel. At temperatures 60-100 °C, the band was not as thick but still remained strong. Even at 100 °C, protease activity remained very visible.

The activity of the purified protease was examined at pH values ranging from 3-13 (Figure 17). The activity was highest at pH 9 thus set as the relative activity standard of 100%.

Relative activity of approximately 80% was observed at pH 7, 8, and 10. At pH 4-6, relative activity declined to around 45%. The lowest activity was observed at extreme values of pH 3 and pHs 12 and 13.

Table 7 showed the effect of solvents on the protease activity of the target protein. The control, solvent-free protein was used as the standard, 100%, in the protease assay. The majority of the solvents reduced the protease activity to about 50%. Acetone had the least effect on the protease with 57.22% relative activity followed by isopropanol with 54.54%. Benzene reduced the protease activity the most to 36.73%.

Table 8, showed the effect of co-factors on the protease activity of the purified protein. Manganese chloride actually increased the relative activity of the protease to 112.25%. Nickel chloride reduced activity to 77.31%. With all the metals used, the activity did not decrease below 50%. The lowest activity of 51.4% was observed when adding copper sulfate.

Table 9 showed the detergents tested on the protease activity. Protease was most active with Triton X-100 producing 78.7% relative activity compared to the control. Hydrogen peroxide lowered the activity to 77.91%. Most of the other detergents only decreased the activity to around 50% excepting SDS with 9.1% protease activity.

The substrate that promoted the highest relative activity was casein which was selected as the control (Table 10). The protease hydrolyzed the other substrates listed in in descending order: BSA, gelatin, gluten, trypsin and egg albumin. Egg albumin, the least hydrolyzed, showed relative activity of only 8.06%.

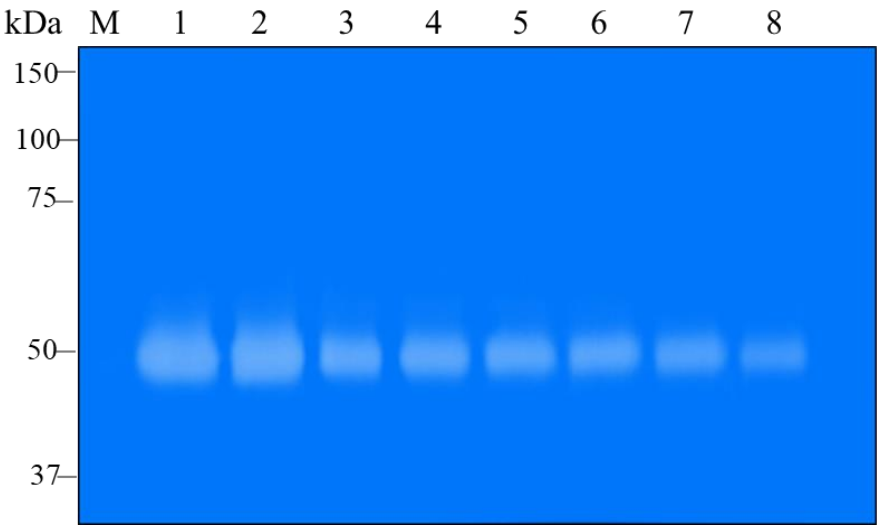

Figure 16: *Temperature stability of the target protease*
Protease was incubated in temperatures ranging from 30-100 ºC. M = Marker; 1 = 30 ºC; 2 = 40 ºC; 3 = 50 ºC; 4 = 60 ºC; 5 = 70 ºC; 6 = 80 ºC; 7 = 90 ºC; 8 = 100 ºC

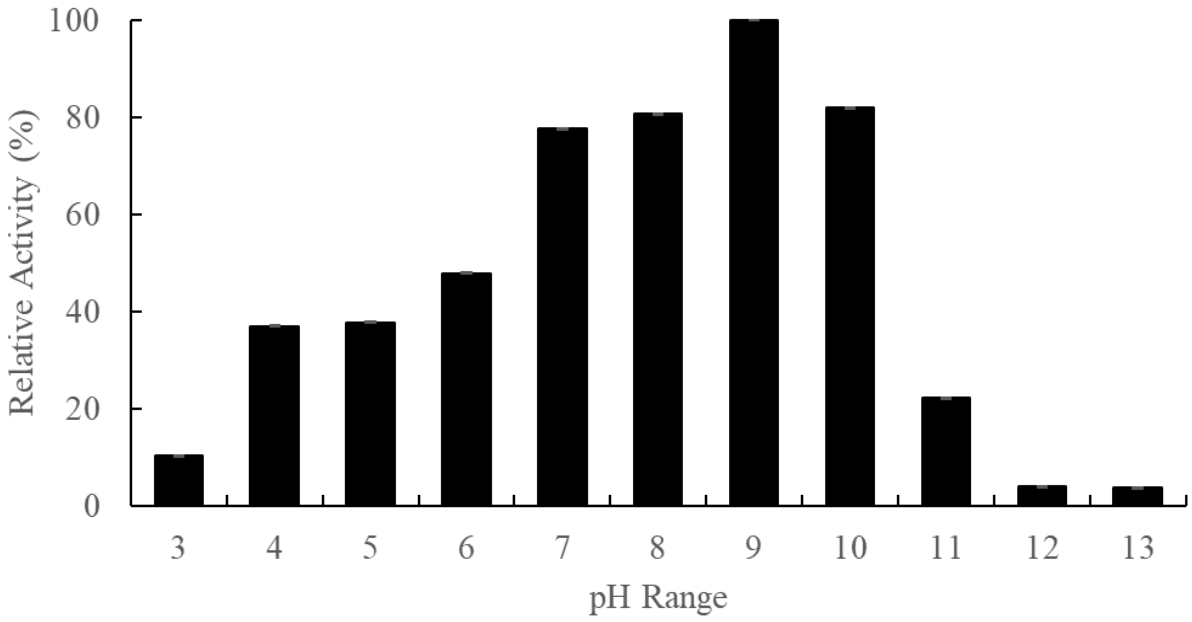

Figure 17: *pH optimization of the target protease*
Protease was incubated at pH levels ranging from 3-13 during the protease assay. The pH with the highest protease activity was regarded as 100%

Table 7: *Solvent compatibility on activity of the target protease*

Solvent (15%)	Relative Activity (%)
None	100.00 ± 0.0
Acetone	57.22 ± 4.4
Isopropanol	54.54 ± 5.0
Methanol	58.00 ± 3.4
DMSO	45.60 ± 3.5
Ethanol	43.40 ± 3.7
Benzene	36.73 ± 4.8

Table 8: *Co-factor compatibility on activity of the target protease*

Co-factor (3 mM)	Relative Activity (%)
$MnCl_2$	112.25 ± 4.3
None	100.00 ± 0.0
$NiCl_2$	77.31 ±4.8
$ZnCl_2$	70.43 ± 3.7
$BaCl_2$	69.10 ± 6.0
$MgCl_2$	64.70 ± 7.2
$FeSO_4$	56.20 ± 3.4
$CoCl_2$	54.50 ± 2.7
$FeCl_3$	54.50 ± 8.4
$CuSO_4$	51.40 ± 4.5

Table 9: *Detergent compatibility on activity of the target protease*

Detergent (1%)	Relative Activity (%)
None	100.00 ± 0.0
Triton X-100	78.70 ± 3.4
H_2O_2	77.91 ± 3.4
NaClO	69.84 ± 4.2
Tween 20	58.70 ± 6.3
Tween 80	57.90 ± 3.5
SDS	9.10 ± 0.6

Table 10: *Substrate compatibility on activity of the target protease*

Substrate (1%)	Relative Activity (%)
Casein	100.00 ± 0.0
BSA	77.33 ± 3.4
Gelatin	46.90 ± 3.4
Gluten	22.21 ± 4.2
Trypsin	9.32 ± 6.3
Egg Albumen	8.10 ± 0.6

4.9 Storage Stability of the Protease of Interest

The stability of the purified protein from *S. grimesii* was assessed using two storage techniques. Protease activity immediate after purification was the relative activity standard of 100%. Freezing at -20 ºC for 24 h and thawing at room temperature resulted in decreased activity (Figure 18). After the first freeze and thaw, the relative activity decreased to 53.04%. After the second to fourth repetition, protease activity measured at around 40%. The fifth freeze and thaw cycle resulted in an activity decline to 31.30%. Values after the third time freezing and thawing showed no significant difference ($P>0.05$).

The other storage test was refrigeration at 4 ºC for two months and protease activity measured weekly (Figure 19). Protease activity immediately after purification was again the relative activity standard. After week one, the activity only fell to 82.12%. At the end of week two, the activity reduced to 65.50% and from week three to eight, activity declined more slowly to a relative activity of 32.50% after the eighth week. Values after the week four do not differ significantly ($P>0.05$).

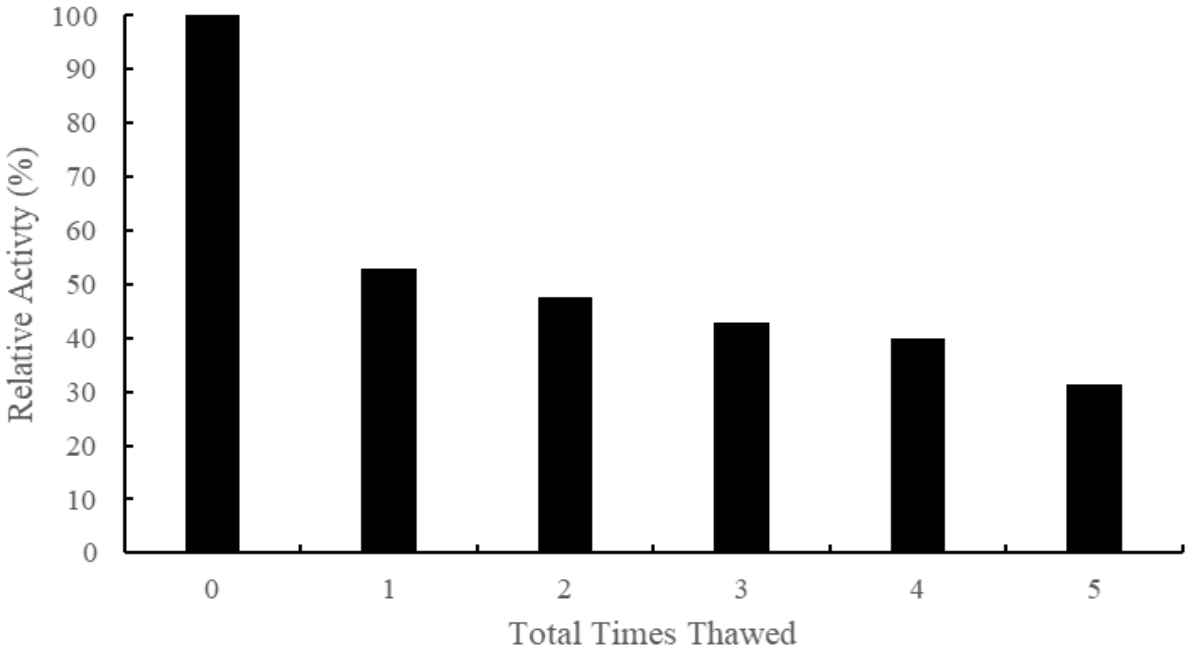

Figure 18: *Protease stability after freezing and thawing*
Protease was frozen at -20 ºC and thawed at room temperature 5 times. Purified protease without temperature treatment was regarded as 100%

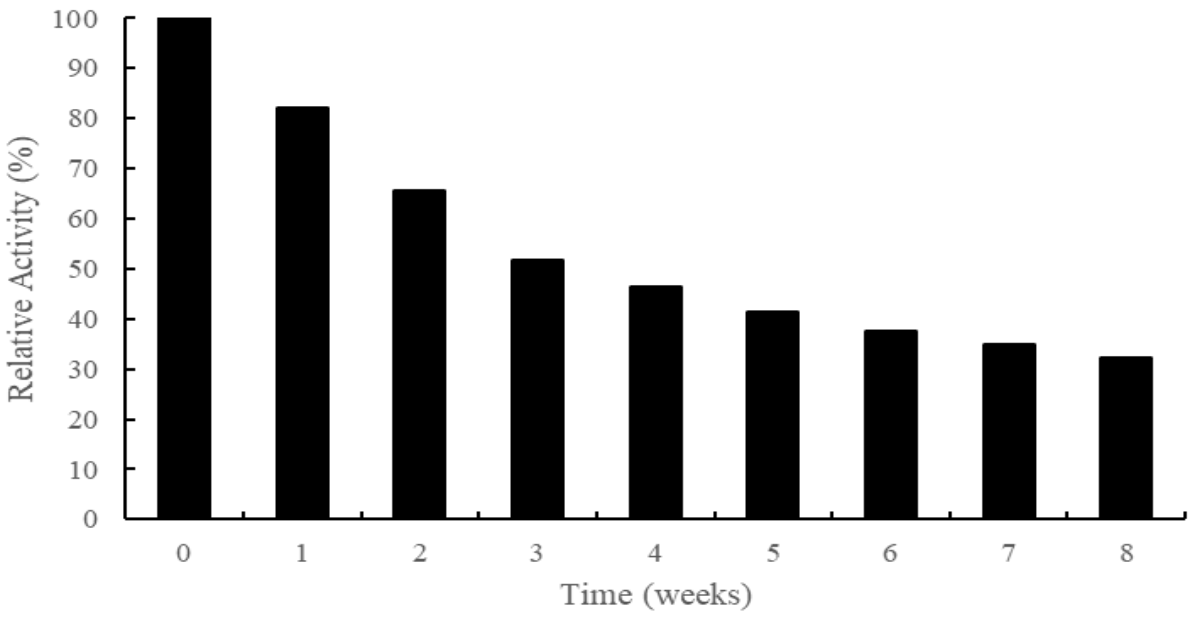

Figure 19: *Protease stability after storage at 4 ºC*
Protease was stored at 4 ºC for 2 months. The activity was tested every 7 days. Purified protease prior to temperature treatment was regarded as 100%

4.10 Protease Identification and Comparison with Similar Amino Acid Sequences in Other Microbes

Three bands labelled 1-O, 2-O, and 3-O excised from the SDS-PAGE (see Appendix C) were identified using the MALDI TOF-TOF mass spectrometer to determine the peptide masses and to match them using the NCBI database. All proteins were classified with enzymes expressed from bacteria belonging to the *Serratia* genus as shown in Table 11. The target band, 1-O, was matched with two proteins, both metalloproteases: ABN13866 and AAX21094. ABN13866 originated from *Serratia* spp. *KCK*. The peptides present in this protein matched

30% of all the amino acid residues in 1-O. AAX21094, secreted by *S. proteamaculans*, was almost identical to the other match but had an additional 11 amino acids at the C-terminal. Peptides matching AAX21094 covered 29% of all amino acid residues that compromised 1-O. The target protein could be either of these or a very similar metalloprotease.

The second band, 2-O, slightly above 50 kDa on the SDS-PAGE had mass matching scores with one protein. It was identified as a serralysin-like metalloprotease with the accession number BAK39733. This protease is produced by *S. liquefaciens*. The peptides corresponding to those in the protease covered 19% of all amino acid residues that made 2-O, making it very likely that 2-O is BAK39733 or a similar serralysin-like metalloprotease.

The 3-O band located below 75 kDa also had a mass matching only one protein. The protein was identified as a lipase produced by *S. liquefaciens* with the accession number AB04234. The peptides matching to those in AB04234 covered 15% of all the amino acid residues that compromised 3-O. Since coverage is well distributed throughout the length of the protein sequence, it is very probable that this specific lipase is the 3-O enzyme.

To better understand the metalloprotease matched with O-1, the hypothetical structure of ABN13866 was determined using ExPASy ProtParam (Figure 20). The protein derived from *Serratia* spp. *KCK* has 493 total amino acids and a molecular weight of 52,593.03 Da. The theoretical isoelectric point is 4.61 with an instability index of 25.3 meaning the protein is very stable. The structure has calcium ion and zinc ion binding ligands. Then, similar proteases to ABN13866 were found by multiple sequence alignment using the Clustal Omega algorithm for constructing a neighbor joining tree (Figure 21). These metalloproteases had similar protein

sequences like ABN13866 but were derived from different genera of bacteria. Considering the first 240 residues of each sequence (see Appendix D), the protease expressed by *Rahnella* spp. is 78% similar to that of ABN13866. The protease found in *Yersinia nurmi* is 64% similar, and the proteases in *Erwinia chrysanthemi* and *Dickeya chrysanthemi* both are 61% similar to ABN13866.

Table 11: *MALDI-TOF-TOF results of the three proteins of interest*

Band	Enzyme Identification	Accession #	Bacterial Origin	Type
1-O*	Metalloprotease	ABN13866	*Serratia spp. KCK*	Protease
	Metalloprotease	AAX21094	*S. proteamaculans*	Protease
2-O	Serralysin-like Metalloprotease	BAK39733	*S. liquefaciens*	Protease
3-O	Lipase	ABP04234	*S. liquefaciens*	Lipase

* Target protein

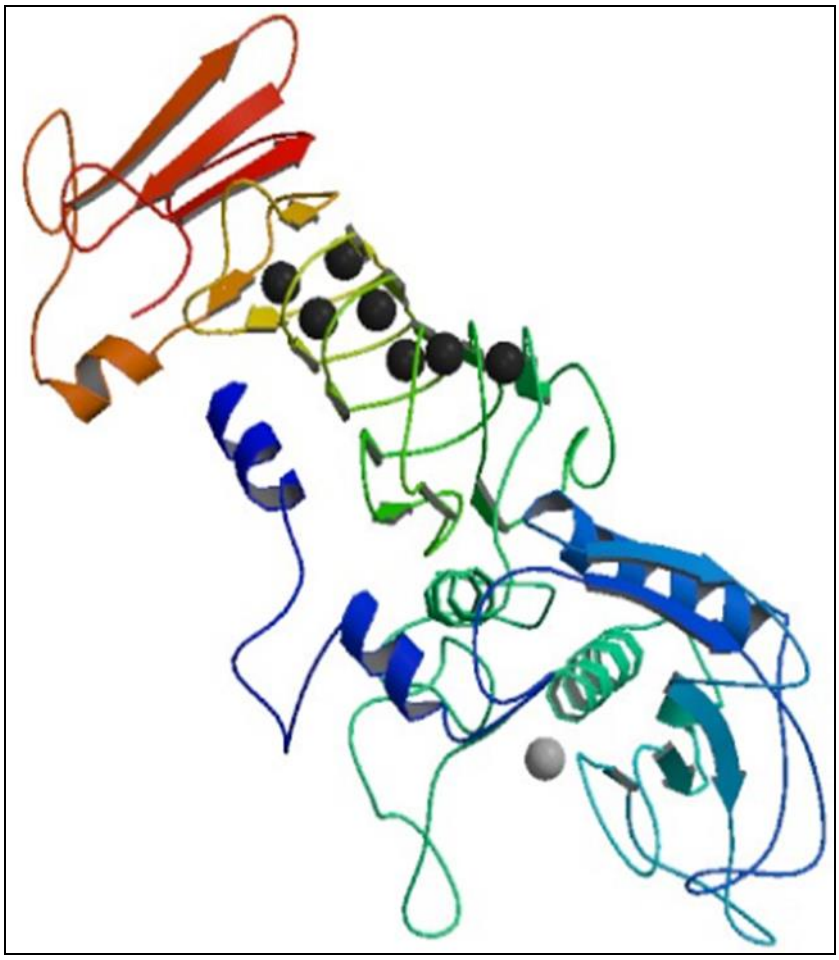

Figure 20: *Protease structure of ABN13866*
Metalloprotease *ABN13866* derived from *Serratia* spp. *KCK* is the protease most similar to the target protease

Figure 21: *Protein neighbor joining tree of proteases from different bacteria*
Similar proteases to *Serratia spp. KCK* derived *ABN13866* from different bacteria were found and compared by a multiple sequence alignment using Clustal Omega

CHAPTER 5: DISCUSSION

All the protease producing bacteria identified by 16S rDNA sequencing, including *S. grimesii*, *P. lurida*, and *A. jandaei*, have been classified as known specific spoilage organisms of freshwater fish (Gram & Dalgaad, 2002; Boulares, Mankai, Aouadhi, Moussa, & Hassouna, 2013). *Aeromonas*, *Pseudomonas*, *Shewanella*, and *Serratia* have been found on catfish fillets at 4 ºC in past studies (Gram & Huss, 1996; Hickey *et al*., 2013; Maull *et al*., 2012). A study by Lerke, Farber and Adams (1965) named all the *Aeromonas* spp. isolated from English sole (*Paaraphrys vetulus*) fillets as strong spoilers. *Shewanella baltica* was classified as a stronger spoilage species of species than *Shewanella putrefaciens* since the species produces high levels of hydrogen sulfide (Williams *et al*, 2010). While these three genera, excluding *Serratia*, had similar phylogeny and are frequently studied spoilage bacteria. *Serratia grimesii* has not been adequately researched.

In the study, the strains isolated from catfish fillets were examined for their protein band patterns through SDS-PAGE. Of the five strains, *S. grimesii*, *P. lurida*, and three *A. jandei* strains, the supernatant and whole cell protein of S. *grimesii* had the most unique band patterns. The thickest bands from the supernatant of S. *grimesii* were the two located in close proximity to 50 kDa. The other bacteria had one thick band below 37 kDa and others between 37 and 50 kDa. Singh, Tripath, Khare, and Garg (2011) found a thick band in the crude extract of *Pseudomonas putida* at 53 kDa and a lesser around 25 kDa. *Pseudomonas fluorescens* showed major bands at 20, 25, 35, 50, and 60 kDa according to Alves, Salgado, Eller, Vidigal, and de Carvalho (2016).

Because the band pattern of *S. grimesii* were different from that of the other strains, it was chosen as the main focus in the study.

With *S. grimesii*, the pattern of protein bands that showed protease activity on the gelatin SDS-PAGE was also different from the other strains. *Serratia* strains are known to show positive results in gelatin hydrolysis assays (Mahlen, 2011). The two clear regions around 50 kDa showed the most apparent protease activity. On regular SDS-PAGE, those two bands were the thickest with the band underneath the 50 kDa marker was the most predominant showing the biggest clear region. This protease selected for further study.

Optimum growth conditions for *S. grimesii* included the most growth in TSB with neutral pH 7 and at 28 ºC. The optimal incubation temperature supports the condition of psychotropic bacteria classified as SSOs. Temperature and pH allowed the performance of a 72 h long bacterial growth curve. The log phase ended at 39 h followed by the stationary phase until 57 h before reaching the decline or death phase. Low protease activity began to show at 6 h and increased after 9 h. Protease activity remained constant from 39 h through 72 h. According to Salarizadeh, Hasannia, Noghabi, and Sajedi (2014), total protease expression from *Serratia* spp. ZF03 remained constant between 48 h and 78 h of bacteria growth. Others suggested that protease secretion increases with a decrease in nutrients and reaches a maximum towards the late log or early stationary phase (Glenn, 1976; Priest, 1977). *Pseudoalteramonas paragogicola* isolated from crustaceans also started expressing protease at 6 h of growth (Ridgeway *et al.*, 2008). The protease attacked myosin and thus tissue structure was lost. *Pseudomonas stutzeri* had a shorter log phase of 5-25 h than found in this study and its highest protease activity was

recorded at 17 h which was towards the end of the exponential phase of the bacteria (Siti, 2015). In this study, *S. grimesii* was found to produce DNase. Nucleotide degradation in fish tissue affects fish quality. *Sadovski* and *Levin* (1969) found DNase and ribonuclease activity among marine and dairy isolates from *Pseudomonas putrefaciens*. *S. marcescens* contain a nuclease that non-specifically degrades RNA and DNA (Ball, Saurugger, & Benedik, 1987).

Crude protein was sampled by first using 70% supernatant saturation of ammonium sulfate. The precipitant produced a purification fold increase to 2.63. This is similar to an apricot seed β-galactosidase which had a 2.92-fold increase with 70% ammonium sulfate (Yossef & El Beltagery, 2014). Proceeding dialysis, biochemical assays were conducted on the crude protein. Proteases in *S. grimesii* had a strong affinity to temperature. Temperature allows extracellular enzyme secretion by changing the structural properties of the bacterial plasma membrane (Rehman, Ahmed, Siddique, Hasan, Hameed, & Jamal, 2017). The crude protein was not completely destroyed at any temperatures between 30 and 100 °C. The optimal temperatures for protease production in S. *grimesii* ranged from 30-40 °C. Femi-Ola, Akinsanmi, and Bamidele (2014) observed 40 °C as the optimal temperature of a protease produced by *Serratia marcescens. S. grimesii* even showed stability at temperatures 70-100 ºC. In addition, the target protease below 50 kDa was still very active to those high temperatures. The protease of *Bacillus thuringinsis* showed temperature stability from 30-60 ºC but lost some activity at 70 ºC and lost it completely at 80 °C. Wilson and Remigio (2012) suggested that thermostable enzymes typically show a more resistance to protein denaturants including detergents, organic solvents, and extreme pH as compared to mesophilic enzymes.

The optimum pH for the crude protein protease activity was 9. At pH 8 and 10, activity remained around 80%. However, the activity declined to around 40% at pH values of 4-6 and 11 suggesting that activity is apparent at fairly acidic and alkaline conditions. A metalloprotease from *Serratia* spp. ZF03 showed the same trend (Salarizadeh *et al.*, 2014) but the *Pseudomonas* genus are known to produce extracellular alkaline proteases that prefer an optimum pH near 10 (Singh, Singh, Tripathi, Khare, & Garg, 2011). A solvent stable protease from *P. stutzeri* BK AB-12 had the highest activity at pH 9 and fairly high activity at 8 and 10 (Siti & Hertadi, 2015).

The crude protein tolerated the addition of solvents. Benzene lowered the protease activity the most to at 48.35% while methanol actually heightened activity to 106.79%. DMSO resulted in an activity of 84.69% which is similar to that of alkaline proteases in *Serratia* spp. ZF03, *S. marcescens* MH6, and *Pseudomonas aeuginosa* (Morihara, Tsuzuki, & Oka, 1973; Salarizadeh *et al.*, 2014; Wan, Ren, & He, 2010).

None of the co-factors strongly inhibited the crude protein. Manganese-, magnesium-, and cobalt chlorides and iron sulfate greatly increased the protease activity. Earlier these four metal ions are used for stabilizing alkaline proteases and protecting it against thermal denaturation (Johnvesly & Naik, 2001; Rattray, Bockelmann, & Fox, 1995). According to Kwak *et al.* (2007), extracellular metalloprotease, Arazyme, from *Serratia proteamaculans* HY-3 also demonstrated increased activity with those four co-factors as well. Copper sulfate was the only co-factor limiting protease activity to the relative lever of 49.28%.

The addition of detergents affected protease activity from the crude protein had various effects on the crude protein. Sodium hypochlorite increased the activity to 179.11%. Tween-20 and -80 had relative activities between 85 and 95%. The only detergent that inhibited protease activity below 60% was the strong anionic surfactant SDS which lowered it to 14.78%. An alkaline serine protease from *Bacillus pumilus* TMS55 retained activities around 80% with nonionic detergents Tween-20 and -80 of the same concentration, 1%, but experienced a decrease to the activity of 35% with SDS (Ibrahim, Muniyandi, & Pandian, 2011).

Substrate hydrolysis by both the crude and the purified target protein were very similar. The highest protease hydrolysis occurred in casein which was also reported by others working with alkaline proteases who found greater hydrolysis in casein than other substrates such as azocasein, hemoglobin, or BSA (Gupta, Beg, Khan, & Chauhan, 2002). Milk, rich in casein micelles, is known to be degraded by heat resistant proteases excreted by psychrotrophic bacteria. Machado *et al.* (2016) reported that *Serratia liquefaciens* produces two heat stable serralysin proteases, Ser1 and Ser2. The protease Ser2 was shown to break down sodium caseinate after 95 ºC heat treatment.

In the process of protease purification, the specific activity increased from the initial 0.603 AU/mg with just the supernatant to 6.0 AU/mg at the end of gel filtration. During passage through the gel filtration column, the enzyme of interest eluted as an unbound fraction. The final purification fold was 9.95, a number similar to a the 10.27-fold of the 56 kDa extracellular protease separated from *Serratia marcescens* (Femi-Ola et al, 2014). A lower recovery yield of

5.9% reported by Yossan, Reungsang, and Yasuda (2006) was attributed to partial autolysis by molecular unfolding of the enzyme.

The target protease had the highest activity in an alkaline environment, specifically at pH 9. Other bacteria such as *P. Aeruginosa* (Baumann, Wu, Flaherty, & McKay,1993; Gambello, Kaye, & Iglewski, 1993), and several *Bacillus* spp. (Adinarayana, Jyothi, & Ellaiah, 2005) also showed strong alkaline protease activity. By far, the most prominent source for alkaline proteases is the *Bacillus* spp. (Gupta, Beg, Khan, & Chauhan, 2002).

The protease was not stable in organic solvents. The highest relative activity occurred in acetone with 57.22% while the lowest activity appeared in benzene with 36.73%. Annamalai, Rajeswari, and Balasubramanian (2014) suggested that enzymes are usually inactivated by the addition of organic solvents in a reaction. More specifically, polar solvents including DMSO and ethanol damage the enzymes by interrupting the intramolecular hydrogen bonds of the protein (Karbalaei-Heidari, Shahbazi, & Absalan, 2013).

Metal ions showed different effects on the activity of the protease activity. $MnCl_2$ was the only metal to increase relative activity. Other proteases from *Bacillus* spp. (Yossan, Reungsang, & Yasuda, 2006) also showed increased activity in the presence of manganese chloride. Magnesium chloride exhibited the same relative activity of 64% as in a *S. marcescens* metalloprotease that was grown in whey (Romero, Garcia, Salas, Diaz, & Quiros, (2001). Protease activity was inhibited to about 50% by cobalt chloride, iron chloride, and copper sulfate. In another study, cobalt actually increased the activity of a protease produced by *Bacillus*

megaterium (Yossan, Reungsang &Yasuda, 2006). Zinc, copper and iron addition have been reported to reduce enzyme activity (Li, Yang, Lv, Liu, Xia, & Chen, 2016; Wu & Chen, 2011). According to McCoy *et al.* (2011), copper sulfate is under review by the FDA for approved use in aquaculture. Right now, studies are being conducted to determine the potential use of copper sulfate for controlling external parasitic infections and the growth of algae. The effect of metal ions on enzyme activity may be caused by binding to amino acid residues important for catalysis. The metal may also affect the charge distribution within the enzyme molecules. Metal ions can maintain metalloenzymes by binding to negatively charged amino acid residues at the active site or the structure of the protein (Karbalaei-Heidari, Shahbazi, & Absalan, 2013).

Detergents lowered protease activity to various percentages. Triton X-100, hydrogen peroxide, and sodium hypochlorite all had relative activities around 70%. Alkaline protease from *Bacillus pumilus* was reported to have an activity around 79% with Triton X-100 (Ibrahim *et al*, 2011). A protease of *Gamma-proteobacterium* isolated from a marine environment was tolerant with hydrogen peroxide at 72% (Sana, Ghosh, Saha, & Mukherjee, 2006). The addition of Tween 20 and 80 lowered the protease activity to about 50% while SDS caused a decrease to 9.1%. SDS stable enzymes are not common except for few *Bacillus* spp. and *Vibrio* spp. (Doddapaneni *et al*., 2007).

To identify chosen proteins that were most prominent in the *S. grimesii*, mass spectrometry was used. Of the three samples tested using MALDI TOF-TOF, the target protein O-1 was best matched with two metalloproteases, ABN13866 metalloprotease precursor from *Serratia* sp. KCK and AAX21094 ProA Arazyme seen in *Serratia proteamaculans*. O-2, the

other protein at 50 kDa, was a match for serralysin-like metalloprotease BAK39733 from *Serratia* liquefaciens. The larger sized protein O-3 was similar to lipase ABP04234 from *Serratia liquefaciens*. Hines, Saurugger, Ihler, and Benediki, (1988) reported that members of the *Serratia* spp. produce at least two extracellular proteases, at least one nuclease, two chitinases, and a lipase.

The target metalloprotease was similar to ProA Arazyme, which was commonly seen in *Serratia proteamaculans* and shown to have high proteolytic activity and stability at a wide range of pH and temperatures (Kwak *et al.*, 2007). The metalloprotease precursor from *Serratia sp.* KCK was secreted by *Serratia* spp. when in the presence of chitin (Kim, Golyshin, & Timmis, 2007). This protease was shown to be active at pHs 5-8 and at temperatures up to 40 °C. More specifically, the optimum conditions were at a pH 7 and a temperature of 30 °C. Both matches exhibited a strong zinc-binding sequence HEXXH in the N-terminal region (Bozhokina, Khaitlina, & Adam, 2008; Maeda & Morihara, 1995). This sequence is signature in Zinc-dependent metalloproteases. Furthermore, GGXGCD was the assumed calcium-binding sites in the C-terminal region (Buamman, Wu, Flaherty, & McKay, 1993).

The protease at O-2 that matched BAK39733 was in a similar kDa size on the SDS-PAGE gel as serralysin or Prot A produced by *S. marsescens* and was only seen in the final stages of growth. According to a phylogenetic tree construction generated by others, this protease showed 100% similarity for serralysin produced by *S. liquefaciens* (Karbalaei-Heidari, Shahbazi, & Absalan, 2013). This protease was one of four produced by *S. marcescens* but was attributed to producing the most activity. Serralysin proteases, a subfamily of metalloproteases,

have been reported showing caseinolytic activity in broad pH and temperature ranges (Maeda & Morihara, 1995).

The third band on the SDS gel labeled O-3 had a probable match for the lipase (ABP04234) produced by *Serratia liquefaciens.* Although *S. marcescens* was usually associated with lipase production, it has also been reported in *S. liquefaciens* (Legakis, Nicolas, Xilinas, & Ppapvassiliou, 1978; Yao *et al.*, 2008). *S. liquefaciens* S33 DB-1 showed an extracellular alkaline lipase activity in lipolytic olive oil with similar kDa size to the one in this study and an optimal pH range of 7-9 at temperatures below 45 ºC (Yao *et al.*, 2008). The health benefits associated with long chain fatty acids were jeopardized by the extracellular lipase activity (Usydus, Szlinder-Richert, Adamczyk, & Szatkowska, 2011; Casallas, Casallas, & Mahecha, 2012).

The hypothetical structure of ABN13866 from *Serratia* spp. *KCK* provided a better understanding of the target protease. Kim, Timmis, and Golyshin (2007) conducted a BLAST search to identify the closest neighbor to ABN13866 and discovered that AY789560 from *S. grimesii* had a 97% homology. It was concluded that strain *KCK* was considered a new species of *Serratia.* The optimum temperature of the enzyme was 40 ºC. The structure includes both calcium and zinc ligands. *Serratia* spp. strain E-15 contained a protease with three zinc ligands and an active site revealed by nucleotide sequence analysis (Nakahama, 1986). ABN13866 has the isoelectric point of 4.61. It is said that protein side chains gain a net positive charge in extreme acidic pH ranges or negative charge toward more alkaline ranges if the isoelectric point is 5-6 (Hamm, 1994; Kristinsson, Theodore, Demir, & Ingadottir, 2005). This would cause the

proteins to repel each other and increase solubility. This may explain why the target protein was best eluted from ion exchange chromatography at pH 8.4 and 8.6.

Metalloproteases similar to ABN13866 were found in other genera. Proteases most like the one from *Serratia* spp. *KCK* were found in *Raahnella* spp., *Yersinia nurmi*, *Erwinia chrysanthemi*, and *Dickeya chrysanthemi*. In a previous study, *Rahnella* spp. was isolated and identified from North American catfish (*Ictalurus punctatus*) fillets (Hickey *et al.*, 2013). *Rahnella* spp. is known for unsafe levels of histamine production on fish tissue (Tash, 2005). *Yersinia* spp. have also been identified as pathogens and contaminants. *Y. ruckeri* is the cause of enteric redmouth disease in channel catfish (Danley, Goodwin, & Killian, 1999). *Y. enteroclitica* is most often associated with foodborne illness, although rarely in catfish and other fish species (MacMillan & Santucci, 1990; Nedoluha, Owens, Russek-Cohen, & Westhoff, 2001). *Erwinia* and *Dickeya* spp. are known to soft rot vegetables (Wevers, Moons, Van Hodt, Lurquin, Aersten, & Michiels, 2009).

Conclusions

The purpose of this study was to identify the most unique protease producing spoilage bacteria strain isolated from channel catfish fillets and then examine the protease that showed the greatest activity. Proteases degrade proteins, including actin and myosin. The bacteria showing protease secretion were identified through 16S rDNA sequencing as species belonging to *Aeromonas*, *Pseudomonas*, *Serratia*, and *Shewanella* genera. Of those species, *Serratia grimesii* showed taxonomical distance and had the most unique extracellular protein pattern. Although

S. grimesii exhibited fewer protein bands on the SDS-PAGE gel than the other bacteria, two bands around 50 kDa revealed substantial protease secretion. Therefore, *S. grimesii* was chosen as the focus spoilage bacteria of the study.

Preferred conditions for growth and enzyme production in *S. grimesii* after 15 h incubation included an optimal pH 7 of TSB and an optimal temperature of 28 ºC. A 72 h growth curve using these ideal conditions revealed that the log phase ended at 39 h and the stationary phase continued until 60 h. Protease activity started after 6 h and increased through hour 39 before remaining constant. The DNase production was faint initially but increased after 21 h.

Crude protein was extracted from *S. grimesii* for biochemical assays and for further purification processes. The crude protein proved thermostable after a temperature assay. Protease activity was highest at 30 and 40 ºC but remained strong to 100 ºC. The proteases preferred an alkaline range of pH 8-10 but was also active at acidic pH levels. The crude protein tolerated the addition of solvents and co-factors relatively well with the most substantial decrease in protease activity being 50%. Methanol increased the activity while benzene decreased it. The protease activity was increased by manganese, magnesium, cobalt, and iron sulfate. The co-factor that decreased the activity most was copper. Sodium hypochlorite of the detergents increased protease activity while SDS inhibited it. Casein was the preferred protease substrate. The other substrates were not hydrolyzed as effectively which was especially true for gelatin.

A single protease selected from the crude protein of *S. grimesii* was successfully purified. At pH values of 8.4 and 8.6, the target protein was separated and produced the greatest protease

activity. The gel filtration column was used to obtain the purified protein. The specific activity of increased up to a 10-fold after purification.

The purified protease was characterized using the same biochemical assays as the crude protein. The optimal temperature for the protease was 30 and 40 ºC yet the enzyme remained stable to 100 ºC, demonstrating thermostability. The target protease also tolerated a range of pH values. Protease activity was highest at pH 7-10 with an optimal pH of 9. The protease remained active in acidic in acidic conditions. The protease was sensitive to solvents. The highest relative activity occurred in acetone at 57%, the lowest in benzene. Some co-factors, especially manganese, increased protease activity while others decreased it with copper showing the most reduction. The protease was most tolerant of the detergent Triton X-100 and most inhibited by the addition of SDS. Casein was the substrate best hydrolyzed and egg albumin the least hydrolyzed. The protease remained active in repeated freezing and thawing and two months of refrigeration.

The target protease was identified as a metalloprotease. The match, protease in *Serratia* spp. *KCK*, was 30% most like amino acid sequence of the target. By using bioinformatics tools, the protease was researched to be very stable and had a molecular weight close to the target. This study evidently shows the strength and stability of the target metalloprotease in *S. grimesii* can play a role in catfish spoilage. EDTA, a metal ionic chelator and 1, 10-phenanthroline, a specific inhibitor of metalloprotease is known to suppress tenderization of flounder muscle (Sriket, 2003). The results of this study will provide significant background to control the protease for prevention of spoilage in fish products.

Suggestions for Future Research

In this study, the investigation of biochemical properties was limited due to inability to purify a large amount of enzyme through bacteria cultivation and liquid chromatography. By cloning the target metalloprotease using an *Escherichia coli* system, more in-depth molecular studies could be possible. Enzyme productions can increase approximately 50-fold through cloning the responsible genes into expression vector (Sakokamoto, Terada, Iijima, Matsuzawa, & Ohto, 1994; Sumantha, Larroche, & Pandey, 2006).

Another overexpression system can be developed in yeast for industry purposes. Microbial proteases account for almost 40% of the total enzyme sales worldwide (Rao *et al.*, 1998). Some alkaline proteases are employed in dehairing hides for leather, meat tenderizing, cheese flavor development and laundry detergents (Singh *et al.*, 2011). In addition, the target protein can be studied for pharmaceutical uses. For example, a metalloprotease from *Serratia* spp. E-15 isolated from the gut of a larval silkworm is used in in drugs to treat breast engorgement, relieve swelling, and degrade plaque inside arteries (Salarizadeh *et al*., 2014).

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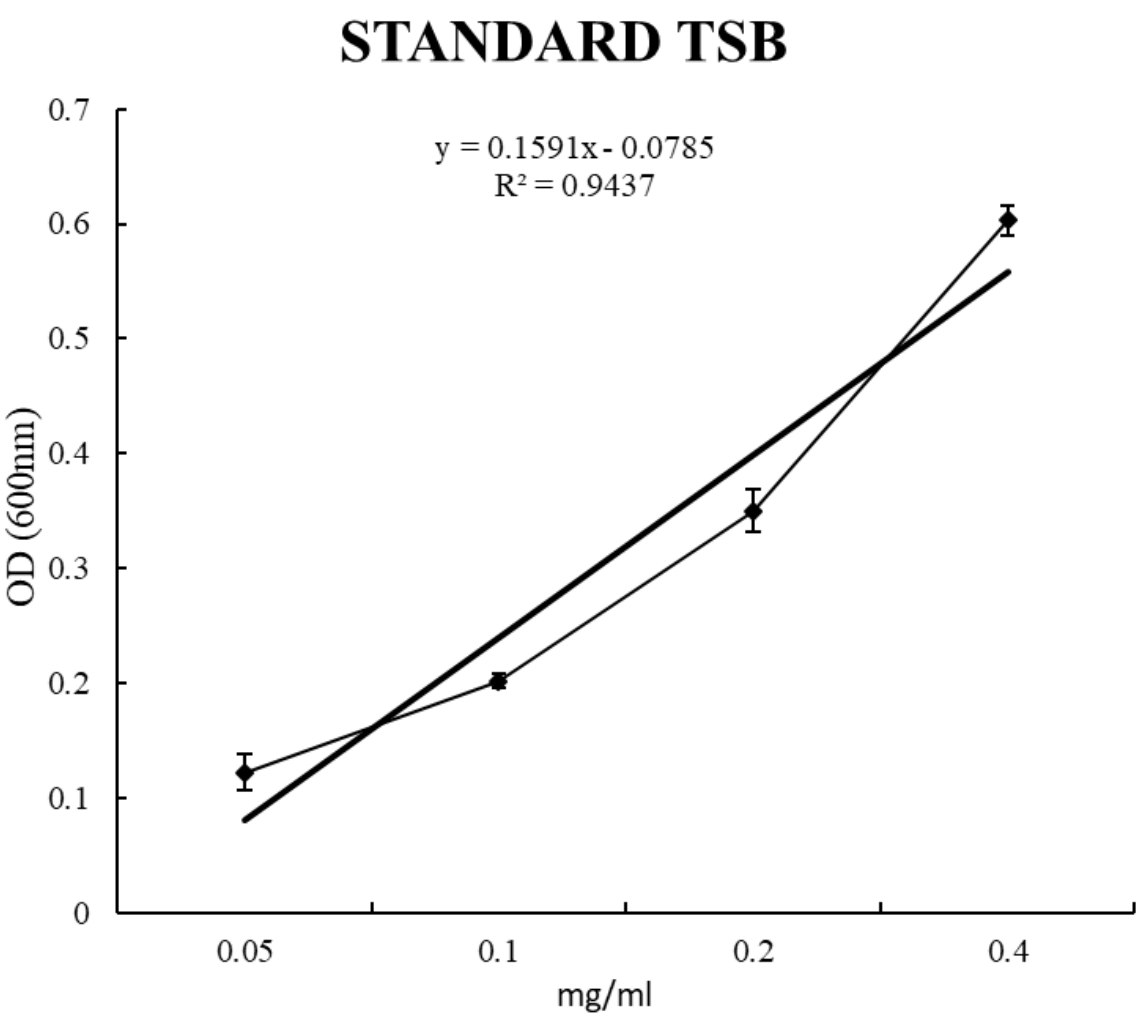
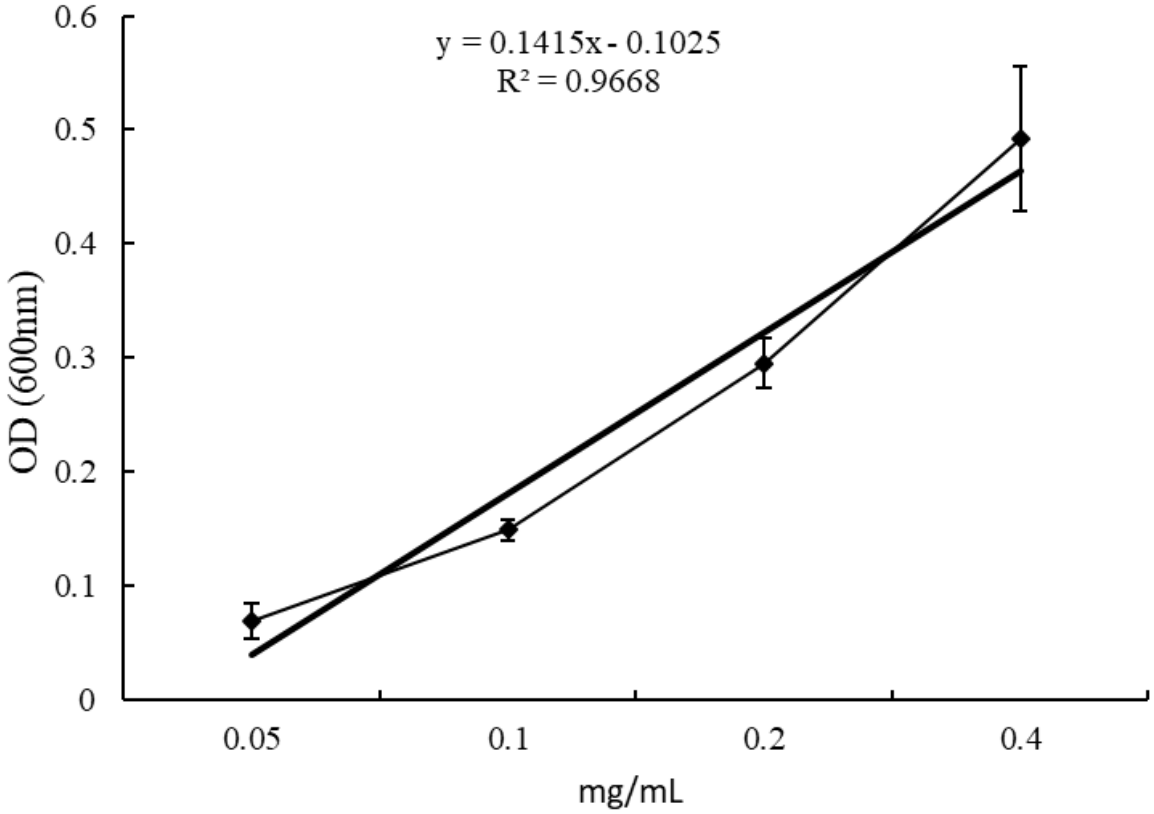
Appendix A

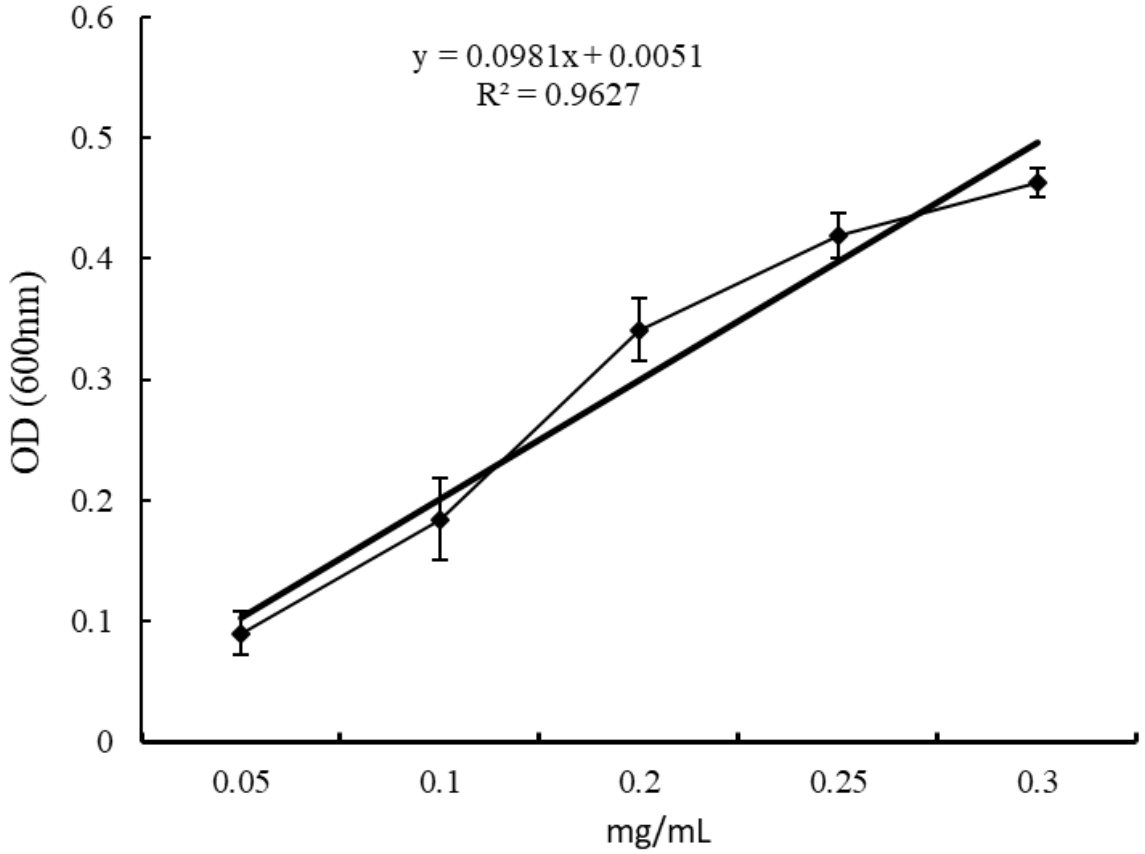
BSA Standard Curves for the Protein Assay


STANDARD TSB
y = 0.1591x - 0.0785
R² = 0.9437
OD (600nm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.05
0.1
0.2
0.4
mg/ml

STANDARD Tris-NaCl

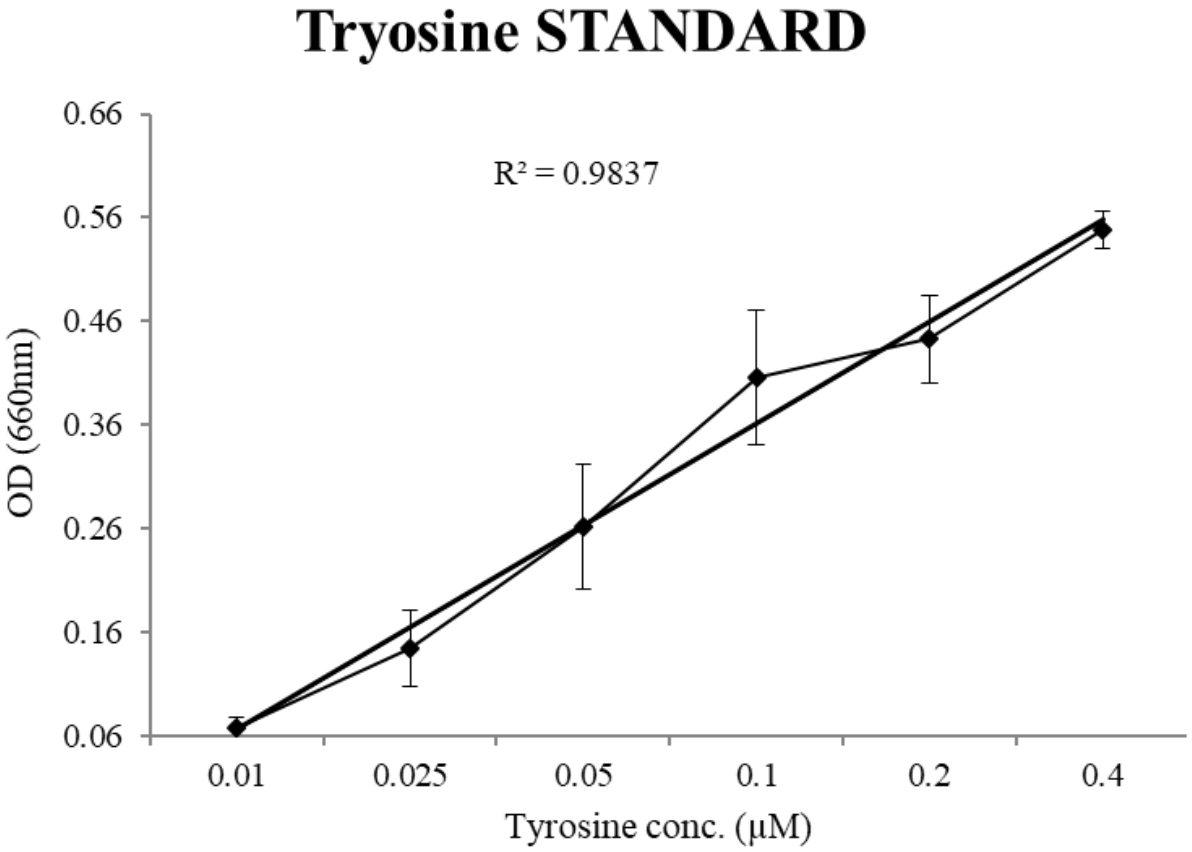


STANDARD PBS



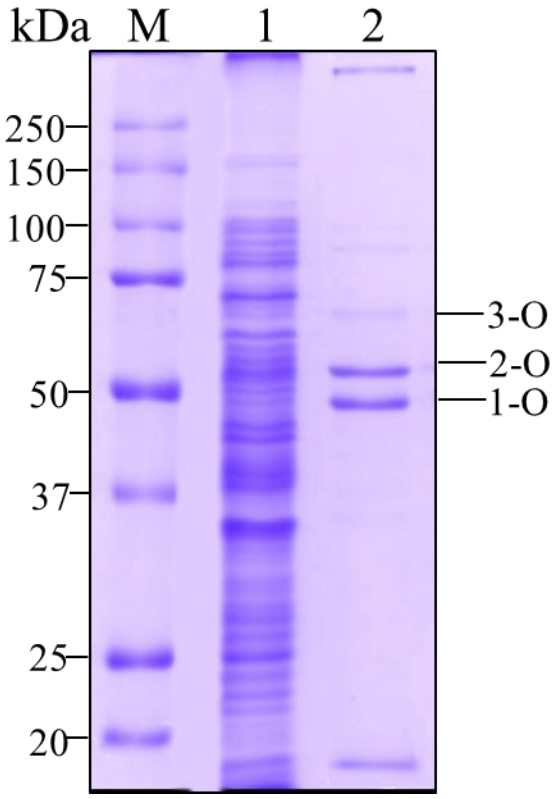
Appendix B

Tyrosine Standard Curve for the Protease Assay


Tryosine STANDARD
R² = 0.9837
OD (660nm)
0.66
0.56
0.46
0.36
0.26
0.16
0.06
0.01
0.025
0.05
0.1
0.2
0.4
Tyrosine conc. (µM)

Appendix C

Bands Chosen for Identification and Amino Acid Sequences


kDa
M
1
2
250
150
100
75
50
37
25
20
3-O
2-O
1-O

ABN13866.1 metalloprotease precursor [*Serratia* sp. *KCK*]
MSGIEPMQSTKKAIEITESSLAAASSAYNAVDDLLHYHERGNGIQVNGKDSFSTEQAGLF
ITRENQTWNGYKVFGQPVKLTFSFPDYKFSSTNVAGDTGLSKFSAEQQQQAKLSLQSWS
DVANITFTEVGAGQKANITFGNYSQDRPGHYDYDTQAYAFLPNTIYQGQNLGGQTWYN
VNQSNVKHPASEDYGRQTFTHEIGHALGLSHPGDYNAGEGNPTYRDASYAEDTREFSL
MSYWSETNTGGDNGGHYAAAPLLDDISAIQHLYGANQTTRTGDTVYGFNSNTGRDFLS
TTSNSQKVIFAAWDAGGNDTFDFSGYTANQRINLNEKSFSDVGGLKGNVSIAAGVTI
ENAIGGSGNDVIVGNAANNVLKGGAGNDVLFGGGGADELWGGAGKDTFVFSAVSDSA
PGASDWIKDFQKGIDKIDLSFFNQGAQGGDQIHFVDHFSGAAGEALLSYNASNNVSDLA
LNIGGHQAPDFLVKIVGQVDVATDFIV

AAX21094.1 ProA [*Serratia proteamaculans*]
MSICLIENNQLMSGIEPMQSTKKAIEITESSLAAASSAYNAVDDLLHYHERGNGIQVNGK
DSFSTEQAGLFITRENQTWNGYKVFGQPVKLTFSFPDYKFSSTNVAGDTGLSKFSAEQQ
QQAKLSLQSWSDVANITFTEVGAGQKANITFGNYSQDRPGHYDYDTQAYAFLPNTIYQ
GQNLGGQTWYNVNQSNVKHPASEDYGRQTFTHEIGHALGLSHPGDYNAGEGNPTYRD
ASYAEDTREFSLMSYWSETNTGGDNGGHYAAAPLLDDISAIQHLYGANQTTRTGDTVY
GFNSNTGRDFLSTTSNSQKVIFAAWDAGGNDTFDFSGYTANQRINLNEKSFSDVGGLK
GNVSIAAGVTIENAIGGSGNDVIVGNAANNVLKGGAGNDVLFGGGGADELWGGAGKD
TFVFSAVSDSAPGASDWIKDFQKGIDKIDLSFFNQGAQGGDQIHFVDHFSGAAGEALLSY
NASNNVSDLALNIGGHQAPDFLVKIVGQVDVATDFIV

BAK39733.1 serralysin-like metalloprotease [*Serratia liquefaciens*]
MDNSLNGKTNGWDSVNDLLNYHNRGNGLTINNKPSFDIEAAGEQIARSEQTWNGTHVI
GQGATVTYSFPDWDYNKSNLNGRFESQDTGLSAFTAAQKAQAKLSLQSWADVANLNF
VEVAPGQKSNITFGNYEGDGQAYAIKPFTGAGKDYRGHNTDGQSWFNINYDYADPRDG
VYANLHPELGNYGRLSITHELGHTLGLDHPGVYNAGQSPSYAKATYAEDTRMFSLMSY
WDESVTGGDHGGYYAAAPLVDDIAAIQYLYGANTTTRTGDTVYGFNSNSGRDFYTATD
SSQKLIFSVWDAGGNDTLDFSGYTQDQRINLTEGSFSDVGGLTGNISIAVGAVIENAIGGS
GNDVIVGNDAANILQGGAGNDVIYGGGGQDQLSGGSGSDIFVFSAVSDSPFKAPDKILD
FETGIDKIDLSFFNQGENGAGFIHFVDSFSGQAGEATLTYHAQNDFSELALNISGHATPDF
LVNIVGQANTATDFIV

ABP04234.1 lipase [*Serratia liquefaciens*]
MGIFNYQGLDEAKSKTLFTDAMAISTYAYHNIDNGFDEGYHSSGFGLGLPFTLVTALIGS
TQSQGGLPGIPWNPDSEQAALAAVNNAGWSLISADQLGYQGKTDARGTYYGETFGYTT
AQAEVLGKYDSEGNLTGIGIAFRGTSGPRESLITDTIGDLVNDLLAGFGPNGYADNYSLK
AFGTLLEDVAKFAQAHGLSGDDITISGHSLGGLAVNSMAALSDGNWGGFYAQSNYVAF
ASPTQYETGGKVINVGYENDPVFRALDGTTRTSATLGVHDAPQDSATNNIVNFNDHYA
SAAWNILPFSILNIPTWLSHLPFFYQDGLMRVLNSEFYSLTSKDSTVIVSNLSDVTRGNT
WVEDLNRNAEQHSGPTFIVGSDGNDLIKGGTGNDYLEGRAGNDTFRDDGGFNIISGGEG
HNTLDLQHALKNTEVAYDGNTLYLRDADGGITLANSIGTLKSKESSLLIFTKEVDHQVT
DNGLLSTKGLTAYASSANGTAADDVLTAKESGSWLFGLEGNDQLFGGKGNDVFVGGA
GNDVMHSQGGSNTFLFSGDFGQDLIYGYQAQDKLVFIGTEGSSSGGNYRDFVSEVNDN
LVFNFGGSTVTLVGIGFDSLSDGQVVLA

Appendix D

Multiple Sequence Aliment


Serretia MSGIEPMQSTKKAIEITESSLAAASSAYNAVDDLLHYHERGNGIQVNGKDSFSTEQAGLF
Rahnella ------MQSTKEIIEVSGSSLS-AITGYGAVEALLHYHERGNGIQINGKDSFSTEQAGLF
Yersenia ---MK--ASS--NKTVDQFDAAVARNGYNAVNELLQYHARGDGLTINGKPSYSTEDAGLQ
Erwinia ---MEKNLSS--RDDDALHSLSAPSSSYNSIYDLLHYHERGNGLTINGKPSYSIEEAGDQ
Dickeya -------MSS--RDDDALHSLSAPSSSYNSIYDLLHYHERGNGLTINGKPSYSIEEAGDQ
: . : ...:: **:** **:*: :*** *:* *:**
Serretia ITRENQTWNGYKVFGQPVKLTFSFPDYKFSSTNVAGDTGLSKFSAEQQQQAKLSLQSWSD
Rahnella ITRTNQTWNGKGVFDQPVKLTFSFPDYKFSSTNAAGDTGLSKFNVEQQQQAKLSLQSWAD
Yersenia ITRTGQTWNGKNVFDQPAKLTYSFLDSV--SRIPSGDKGFVKFNPAQIETAKLSLQSWAD
Erwinia ITRDNVSWNGANVFGKSANLTFKFLQSA--RSTPDGDTGFVKFNAAQISQAKLALQSWAD
Dickeya ITRDNVSWNGANVFGKSANLTFKFLQSA--RSTPDGDTGFVKFNAAQISQAKLALQSWAD
*** . :*** **.: .:**:.* : **.*: **. * . ***:****:*
Serretia VANITFTEVGAGQKANITFGNYSQDRPGHYDYDTQAYAFLPNTIYQGQNLGGQTWYNVNQ
Rahnella VANITFSEVAATQKANITFGNYSQDQPGHDDYATQAYAFLPGSIYQGKDLSGQTWYNINQ
Yersenia AANIAFTEIASSQKANITFGNYTLSRDGS-PADSQAYAYLPGSG----SPSGSTWYNYNV
Erwinia VANVTFTEVTGNQSANVTFGNYTRDSSGRLDYGSQAYAYLPGSG----SASGTTWYNYNV
Dickeya VANVTFTEVTGNQSANVTFGNYTRDSSGRLDYGSQAYAYLPGSG----SASGTTWYNYNV
.**::*:*: . *.**:*****: . * :****:**.: . .* **** *
Serretia SNVKHPASEDYGRQTFTHEIGHALGLSHPGDYNAGEGNPTYRDASYAEDTREFSLMSYWS
Rahnella YDVKHPASEEYGRQTLTHEIGHALGLSHPGDYNAGQGNPTYQNATYAEDTRQFSLMSYWS
Yersenia DNIRSPDTMEYGRQTLTHEIGHALGLSHPGNYNAGQGYPSYKDVTYAEDTRQFSIMSYWG
Erwinia DNIRSPDTMEYGRQTLTHEIGHALGLNHPGDYNAGEGNPSYSDVTYAEDTRQFSIMSYWS
Dickeya DNIRSPDTMEYGRQTLTHEIGHALGLNHPGDYNAGEGNPSYSDVTYAEDTRQFSIMSYWS
::: * : :*****:**********.***:****:* *:* :.:******:**:****.