ANTIMICROBIAL ACTIVITY OF PROTEIN HYDROLYSATES MASH COW (*Pomacea canaliculata)* on *Eschericea coli* and *Staphylococcus aureus* Bacteria

RESEARCH REPORT

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ABSTRACT

Enzymatic protein hydrolysis can be done using the help of natural protease enzymes, one of which is the enzyme bromelain derived from pineapple. Protein hydrolysis by proteases such as bromelain produces hydrolysates containing a mixture of peptides that are bioactive, one of which has antimicrobial activity. One of the mollusks that can be utilized because of its high protein content is the gold snail (*Pomaceae canaliculate)*. This study aims to determine the effect of hydrolysis time and different concentrations of bromelain enzyme in gold snail hydrolysate on antimicrobial activity and to determine the best treatment of hydrolysis time and concentration of bromelain enzyme on antimicrobial activity of gold snail hydrolysate. This research was conducted with the method of Completely Randomized Design (CRD) factorial pattern where factor I is enzyme concentration (1%, 5%, 10%) and factor II is the length of hydrolysis time (6 hours, 12 hours, 18 hours). Based on the results of the analysis of antimicrobial activity of snail protein hydrolysate, it showed inhibitory activity only on *Eschericea coli* bacteria with the highest Diameter of Inhibition Area (DDH) which was in the treatment of 18 hours hydrolysis time with 10% enzyme concentration of 8.8 mm. Determination of the *Minimum Inhibitory Concentration* (MIC) and *Minimum Bactericidal Concentration* (MBC) values of *Eschericea coli* bacteria is at 50% concentration of gold snail protein hydrolysate, while in *Staphylococcus aureus bacteria* at 100% hydrolysate concentration.

Keywords: Antimicrobial Activity, *Eschericea coli*, Hydrolysis, Goldfish, *Staphylococcus aureus*

FOREWORD

Praise be to God Almighty for His blessings and grace, so that the author can complete the research proposal on the **ANTIMICROBA ACTIVITY of KEONG MAS PROTEIN HYDOLYSAT (*POMACEAE CANALICULATE)*** well. The purpose and objective of the research proposal is to complete one of the curriculum requirements that must be followed to obtain a Bachelor's degree in the Food Technology Study Program, Faculty of Engineering, UPN "Veteran" East Java.

The ease and smoothness in the preparation of this research proposal cannot be separated from the help and support of various parties. For this reason, on this occasion with respect the author expresses his gratitude for his help and guidance to:

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The author fully realizes that this research proposal is far from perfection. Therefore, the author really hopes for constructive criticism and suggestions for future writing to be better.

Surabaya

Author

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CHAPTER I
INTRODUCTION

A. Background

Gold *snail* or *golden apple snail* (GAS) is a type of freshwater mollusk originating from the rainforest along the Paraguay and Parana Rivers that cut through Paraguay, Brazil, Bolivia and Argentina (Joshi, 2005). Gold snail (*Pomacea canaliculata)* is a class of *mollusca* that has been known as a pest that is quite disturbing for the growth of rice plants, and a threat to farmers (Dewi, 2014). Based on the results of proximate testing of gold snail meat, the protein content ranges from 16-50% and almost 40% of its body weight consists of protein, so gold snail is one of the animal food ingredients that can be developed and studied more deeply because it has high potential (Fitsari *et al.,* 2015).

The protein content of snails can be considered as a valuable resource. One of the utilization is that it can be used as a protein hydrolysate. Hydrolysate is a mixture of protein hydrolysis results consisting of peptides and free amino acids. Protein hydrolysate is obtained from the hydrolysis process. According to Jaziri *et al.* (2017) protein hydrolysis is the process of breaking down proteins into simpler compounds (amino acids) either by enzymes, bases or acids. The most efficient method of protein hydrolysis is using enzymes, because hydrolysis with acids / bases can damage some amino acids and also produce toxic compounds, while enzymatic hydrolysis can reduce peptide size, change characteristics and improve the quality of the protein (Jaziri *et al.,* 2017).

Enzymatic protein hydrolysis can be done with the help of natural protease enzymes, one of which is bromelain enzyme from pineapple. Bromelin enzyme is an endopeptidase enzyme that has a sulfhydryl group (-SH) at the active site. Basically, this enzyme is obtained from pineapple plant tissues (Supartono 2004). The advantage of bromelain compared to other plant proteases is that bromelain is easily obtained and available from the beginning of fruit development until the fruit ripens although there are fluctuations in its proteolytic activity (Maurer, 2001). Bromelain has a C-terminal cutting specificity that is broad enough to the amino acid residues that make up its substrate including arginine, lysine, tyrosine, and phenylalanine so that it can produce a high degree of hydrolysis (Whitaker, 2003).

Enzyme activity is also strongly influenced by the length of hydrolysis and the concentration of enzyme added. According to Anggraini *et al.* (2015), the

longer the incubation time will cause the enzyme to hydrolyze the protein longer, so that more peptide bonds will be hydrolyzed. Meanwhile, the reaction rate will be directly proportional to the enzyme concentration, where the higher the enzyme concentration, the higher the reaction rate with a certain concentration limit.

Hydrolysis of proteins by proteases such as bromelain produces hydrolysates containing a mixture of peptides that are bioactive. According to Najafian and Babji (2011) protein hydrolysates by protease enzymes have bioactive properties including antioxidant and antibacterial properties. Research from Kusumaningtyas *et al.,* (2015) stated that, the results of milk casein hydrolysis using the protease enzyme bromelain showed that peptides before and after fractionation were able to inhibit the growth of *E.coli, S. Typhimurium and L. monocytogenes,* but antimicrobial peptides from milk casein hydrolysis with protease *Lactobacillus acidophilus* DPC6026 did not show any antibacterial activity on the three bacteria.

Peptides do not show any activity when inside the protein sequence but show more activity when released through enzymatic hydrolysis such as antimicrobial activity. Bioactive peptides such as antimicrobial peptides have potential as natural antimicrobials on pathogenic bacteria, for example against *Staphylococcus aureus* and *Escherichia coli* bacteria. This is based on previous research from Ulagesan *et al* (2018), on the hydrolysate of the snail species *Cryptozona bistrialis* through an enzymatic hydrolysis process using three types of enzymes, namely papain, trypsin and pepsin, showing that the three protein hydrolysates with concentrations of 50 µg/ml, 25 µg/ml and 15 µg/ml were analyzed for their antibacterial activity against *S. aureus and P. aeruginosa*. Only the papain-digested protein hydrolysate from *C. bistrialis* had activity against the tested pathogens.

Peptides from protein hydrolysates can kill microorganisms in a very short time (pathogen-elimination mechanism) (Zoysa *et al.* 2009). The antimicrobial mechanism is largely determined by the presence of bioactive peptide compounds composed of cationic amino acids (arginine, histidine and lysine). Cationic amino acids are not easily denatured in the form of short peptides (20 amino acids) (Sathyan *et al.* 2012; Zoysa *et al.* 2009). Cationic amino acids in the form of short peptides can interact with negatively charged microbial cell membranes so that they can inhibit or kill microbes because these interactions can affect the

permeability of microbial cell membranes (antimicrobial peptides can work like detergents) or these peptides directly enter the cytoplasm of microbial cells and inhibit the work of DNA, RNA synthesis processes and enzymatic reactions (Jenssen *et al.* 2006; Mihajlovic and Lazaridis 2010).

Several studies on testing the antimicrobial activity of gold snail using protease enzymes are still very limited. Therefore, this study used the potential of peptides that act as antimicrobials with the treatment of hydrolysis time and different concentrations of goldfish bromelain enzyme in goldfish hydrolysate. Antimicrobials from goldfish snail protein hydrolysate are expected to be one of the renewable food preservatives, besides being able to become conventional antibiotics.

B. Research Objectives

1. Knowing the effect of hydrolysis time and different concentrations of bromelain enzyme in gold snail hydrolysate on antimicrobial activity on *Staphylococcus aureus* and *Escherichia coli* bacteria.
2. Knowing the best treatment of hydrolysis time and concentration of bromelain enzyme on the antimicrobial activity of *Staphylococcus* aureus and *Escherichia coli* on goldfish protein hydrolysate.

C. Benefits of Research

1. Provide information to the public about the benefits of gold snail hydrolysate as an antimicrobial.

CHAPTER II
LITERATURE REVIEW

A. Goldfish (*Pomaceae canaliculate*)

The *golden apple snail* (GAS) is a freshwater mollusk native to the rainforests along the Paraguay and Parana Rivers that cut through Paraguay, Brazil, Bolivia and Argentina. In Asia, the conch was first recognized in Taiwan in 1979 and is now widely distributed throughout the Asian continent. Along with its spread, the carp has now become one of the most dangerous rice pests in rice-producing countries in Asia, such as the Philippines, Vietnam, Thailand and Indonesia (Joshi, 2005). The damage to rice plants by one carp snail can damage young rice plants by 7-24 stems per day (Salleh *et al*., 2012).

The gold snail is a mollusk that is designated as a plant disrupting organism (pest) or major pest of rice (Oryza sativa) in rice fields. This organism has the potential to become a major pest because rice fields are a suitable habitat for its development, so that the carp snail can reproduce very quickly and is able to damage rice plants in a short time (Hendarsih and Kurniawati, 2009).

Carp snails are one of the major problems in rice production. Carp snails have the same morphology as rice snails. The shell is puckered round, golden yellow in color, 1.2-1.9 cm in diameter, 2.2-3.6 cm in height and weighs 4.2-15.8 g. Carp snails reproduce oviparously and produce eggs. A female gold snail can lay 500 eggs in a week with a breeding period of 3-4 years. Gold snails lay eggs in the morning and evening, the eggs will hatch within 7-14 days and by the 60th day the snails have become adults and can reproduce (Ruslan and Harianto, 2009). The food of the gold snail is generally young and soft plants, such as rice seedlings, vegetables, and water hyacinth (Budiyono, 2006).

According to Ardhi (2008), the gold snail has special characteristics that distinguish it from other types of snails in the same habitat. Gold snails have a brown shell and creamy white to reddish-gold or orange flesh. Their body size varies greatly depending on the availability of food. The diameter of the shell can reach up to 4 cm and weighs 10-20 grams.

Conch Mas (Ramdani, 2018)

1. Chemical Content of Goldfish (*Pomaceae canaliculate)*

Gold snails have a high nutritional content. From the results of the proximate test, the protein content in gold snails ranges from 16-50% and almost 40% of its body weight consists of protein which is a building block for living things. In every 100 g of gold snail meat, it contains 83 calories of food energy, 12.2 g of protein, 0.4 g of fat, 6.6 g of carbohydrates, 3.2 g of ash, 61 mg of phosphorus, 40 mg of sodium, 17 mg of potassium, 12 mg of riboflavin, 1.8 mg of niacin and other food nutrients such as vitamin C, Zn, Cu, Mn, and Iodine. In addition to containing many of the nutrients above, animals from the mollusk family are also rich in calcium (Fitsari *et al.,* 2015).

Table 1. Nutrient Content of Goldfish

NO.	Nutrient Content	Total	
		Meat	
1.	Crude Protein (%)	52,7 [a]	51,8[b]
2.	Crude Fat (%)	3,2[a]	13,62[b]
3.	Crude Fiber (%)	5,59[a]	6,09[b]
4.	Ash Content (%)	15,3[a]	24%[b]
5.	Metabolic energy (kcal/kg)	-	2094[b]

Source: [a]Sulistiono (2007);[b] Julfereina (2008)

The results of research by Kamil *et al.* (1998) showed that gold snail flour has a moisture content of 8.03-8.73%, protein content of 65.50-70.67%, fat content of 1.27-1.43%, ash content of 9.13-10.47%, crude fiber content of 8.19-9.59%, and salt content of 0.56-1.69%. Research on gold snail hydrolysate stored for 2 years at room temperature, which shows that gold snail consists of 16 essential and non-essential amino acids, where glutamic acid is the amino acid component with the highest level and has the potential to become a *flavor enhancer* (Ramdani, 2018).

B. Protein Hydrolysate

Protein hydrolysis is the process of breaking down proteins into simpler compounds (amino acids) either by enzymes, bases or acids. Hydrolysis using acids/bases can damage some amino acids and also produce toxic substances (Jaziri *et al.,* 2017). All proteins will produce amino acids when hydrolyzed, but there are some proteins that besides producing amino acids also produce protein molecules that are still bound (West, 2014).

Theoretically, the most efficient method of protein hydrolysis is using enzymes, because enzymes produce peptides that are less complex and easily broken down. In addition, enzyme hydrolysis can produce hydrolyzate products that avoid changes and damage to non-hydrolytic products (Johnson and Peterson, 1974). Protein hydrolysis is influenced by the concentration of hydrolyzing ingredients, temperature and hydrolysis time and air pressure. An increase in enzyme concentration will increase the volume of fish protein hydrolysate which is insoluble into soluble nitrogen compounds. The speed of enzyme catalysis increases at higher enzyme concentrations, but when the enzyme concentration is excessive, the process is inefficient. To increase the hydrolysis activity, commercial proteolytic enzymes can be used (Syahrizal, 1991).

According to Rasco and Kristiansson (2000), enzymatic protein hydrolysis is an efficient way of protein hydrolysis to dissolve fish protein. Proteins that are hydrolyzed using enzymes are highly dependent on the type of enzyme, substrate, and hydrolysis conditions which include pH, temperature, incubation time and hydrolysis enzyme concentration.

Enzymatic hydrolysis can reduce the size of peptides, change the characteristics and improve protein quality (Jazari *et al*., 2017). Enzymatic protein hydrolysis has been widely used as a food supplement, functional food, *flavour enhancer*, cosmetic ingredient and fortification of fruit extracts and soft drinks (*Zheng et al*., 2006). Various enzymatic protein hydrolysis studies have been conducted, such as milk protein, mackerel and fish waste (Liu and Chiang, 2008).

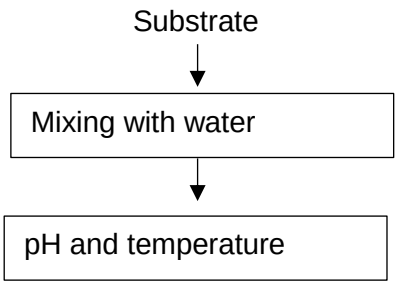
Enzymatic protein hydrolysis has advantages over protein hydrolysis with acids and alkalis because the resulting peptide products have specific amino acid composition and sequence according to the type of protease used. In addition, enzymatic protein hydrolysis takes place under *milder* conditions (not under

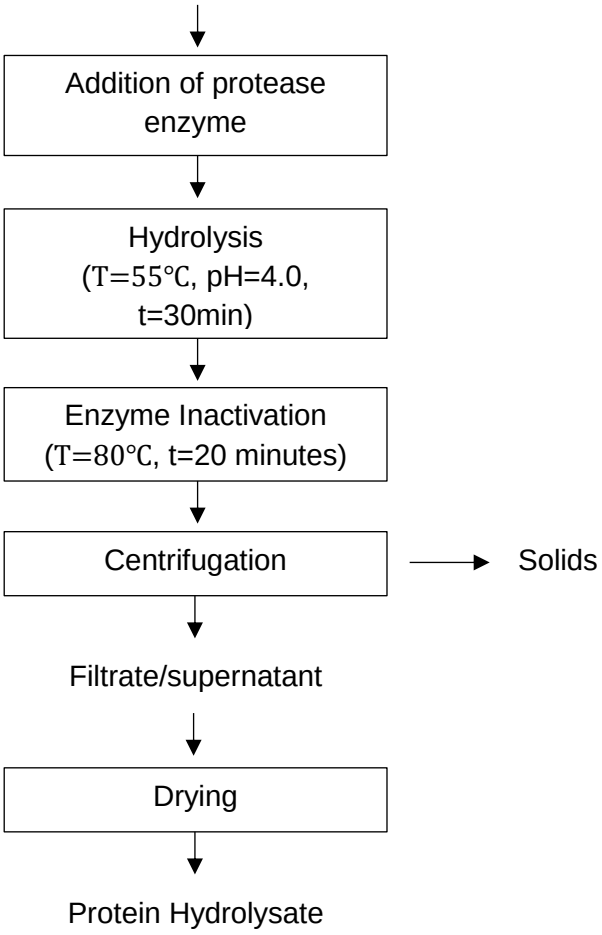
extreme conditions) than using acids or bases so that it does not damage the amino acids produced. Hydrolysis using NaOH tends to damage essential amino acids (tryptophan, cysteine or serine) and change the conformation of the L-amino acid structure into D-amino acids, this form cannot be consumed by humans (Whitaker, 2003).

Compared to chemical hydrolysis, enzymatic hydrolysis has more advantages, such as mild reaction conditions, low unwanted products, and high product quality and yield. Peptides do not show any activity when inside the protein sequence but show more activity when released by enzymatic hydrolysis such as antimicrobial, antioxidant, antidiabetic, anti-inflammatory, and anti-hypertensive capabilities. Hydrophobic amino acids are important for the antioxidant and antimicrobial properties of protein hydrolysates, they can enhance their interaction with lipid targets or enter into target organs through hydrophobic association (Ulagesan *et al.,* 2018). Protein hydrolysates containing cationic amino acids in their composition will exhibit higher antimicrobial activity (Najafian and Babji, 2012).

The conditions during the hydrolysis process can be controlled through the degree of hydrolysis (DH) produced. The degree of hydrolysis is the percentage (%) of free amino groups released during the hydrolysis process to the total nitrogen contained in the substrate. The percentage of peptide bond breakage was calculated using DH, during hydrolysis proteolysis monitoring parameters were determined at various time intervals. A higher percentage of DH indicates that a greater amount of peptides are present in the solution (Ulagesan *et al.,* 2018). The degree of hydrolysis (DH) of proteins is largely determined by several factors including the type of protease used, enzyme concentration, temperature, pH and hydrolysis time (Haslaniza *et al.,* 2010). Therefore, it is very important to optimize some of these factors to obtain the optimal DH.

The following is the process of making protein hydrolysate according to Widadi (2011):





Flowchart of Protein Hydrolysate Preparation (Widadi, 2011)

C. Enzymes

According to Yasid and Nursanti (2005) enzymes are a class of proteins that are most widely found in living cells. To date, approximately more than 2000 enzymes have been identified, each of which functions as a catalyst for chemical reactions in living systems. Enzyme synthesis occurs in the cell and most enzymes can be obtained from extraction from tissues without damaging their function.

Enzymes have tremendous catalytic power, which is usually much greater than synthetic catalysts. Enzymes accelerate chemical reactions without the formation of side products. The catalytic activity of enzymes depends on the integrity of their structure as proteins. For example, if the enzyme is reacted with strong acids or incubated with trypsin, treatments that will cut the polypeptide chain resulting in a conformational structure that can cause the catalytic activity to be

lost. Furthermore, heat treatment and pH treatment that is far from its normal state will also eliminate its catalytic activity (Lehninger, 1993).

Enzymes that act as catalysts in protein hydrolysis reactions are called proteolytic enzymes or proteases. Because what is broken is the bond in the peptide chain, it is also called peptidase. There are two kinds of peptidase, namely endopeptidase and exopeptidase (Naiola and Widyastuti, 2007).

Generally, the application of proteolytic enzymes in the preparation of protein hydrolysates can use endopeptidase to produce protein hydrolysates with a high content of amino acids or short-chain peptides. However, it requires a long incubation time (Febrianto, 2013). The combination of endopeptidase and exopeptidase begins by using endopeptidase to break down proteins into peptides and continued with exopeptidase to break down peptides into amino acids or chained peptides (Rawlings *et al*., 2007).

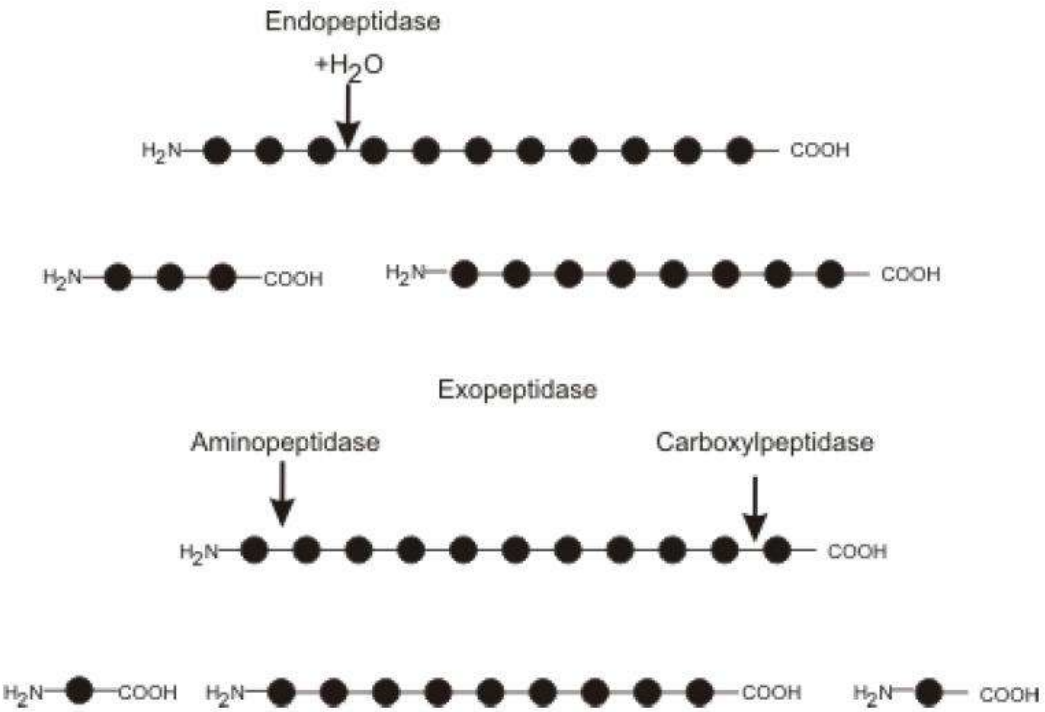


Figure 4. Endopeptidase and exopeptidase mechanisms (Wikimedia, 2015)

The mechanism of action and activity of enzymes are influenced by several factors, namely:

a) Temperature

Temperature can accelerate the reaction process, but at a certain temperature point the speed of the reaction catalyzed by the enzyme will begin to decrease or even no longer appear its activity. The temperature condition where the enzyme can produce the highest activity is called the optimum temperature. Since enzymes are protein-structured, and proteins can be damaged by heat, at certain high temperatures enzyme activity begins to decrease and even disappear. This is very possible due to denaturation or damage to the enzyme structure that can cause damage to the enzyme in whole or in part, especially on the active side (Wang *et al.,* 2009).

b) Hydrogen Potential (pH)

pH is one of the important factors that must be considered when working with enzymes. This is because enzymes can only work at certain pH conditions. A pH condition where the enzyme can work with the highest activity is called the optimum pH. Conversely, at certain pH conditions, the enzyme is completely inactive or even damaged. This is because enzymes are protein molecules whose stability can be affected by the acidity of the environment and in extreme acidity conditions can be damaged (Wang *et al.,* 2009).

c) Substrate

Biochemical reactions catalyzed by enzymes are also affected by the amount of substrate. When testing the substrate concentration from low to high on the enzymatic reaction speed, at first a comparable relationship will be obtained stating that the reaction speed will increase as the substrate concentration increases, but then results will be obtained stating that at a certain highest substrate concentration, the reaction speed no longer increases. In this condition, the substrate concentration becomes saturated and the reaction speed becomes maximum or called maximum speed (Wang *et al.,* 2009).

d) Enzyme Concentration

The reaction speed of a material that uses enzyme assistance depends on the concentration of the enzyme. At a certain substrate concentration, the reaction speed increases with increasing enzyme concentration (Wang *et al.,* 2009).

D. Bromelin Enzyme

Bromelin is one of the proteolytic enzymes or proteases, which is an enzyme that catalyzes the breakdown of proteins into amino acids by building blocks through hydrolysis reactions. Hydrolysis (hydro = water; lysis = loosen or disrupt) is the breaking down of large molecules into smaller units by the combination of water. In protein digestion the peptide bonds are broken with the insertion of water components, -H and -OH, at the chain end (William *et al*, 2002).

Bromelin enzyme is an endopeptidase enzyme that has a sulfhydryl group (-SH) at the active site. Basically, this enzyme is obtained from pineapple plant tissues (Supartono, 2004). Bromelin can be obtained from pineapple plants from the stalk, skin, leaves, fruit, and stem in different amounts. The enzyme content is more in the flesh of the fruit, this is indicated by its higher activity compared to the activity of the stem (Supartono, 2004).

Bromelin enzyme is a type of protease enzyme that is able to hydrolyze peptide bonds in proteins into smaller molecules, namely amino acids, so that they are easily digested by the body. Bromelin enzyme is present in all tissues of the pineapple plant. About half of the protein in pineapple contains bromelain protease. Among various types of fruit, pineapple is a source of protease with high concentrations in ripe fruit (Purwaningsih, 2017).

Bromelin enzyme from pineapple plant tissues has the same potential as papain found in papaya which can digest proteins by 1000 times its weight. Bromelin can be obtained from pineapple plants from the stalk, skin, leaves, fruit, and stem in different amounts. The enzyme content is more in the flesh of the fruit, this is indicated by its higher activity compared to the activity of the stem (Supartono, 2004). Meanwhile, according to Herdiyastuti (2006), the content of bromelin enzyme is more in the stem which has been underutilized. The distribution of bromelain in pineapple stems is uneven and depends on the age of the plant. The content of bromelain in tissues that are not old, especially those that are gummy, is very little and sometimes even absent (Herdyastuti, 2006). The optimum pH for bromelain enzyme is usually in the range of 5.5 - 8.0 and can break down glycyl, analyl and leucyl peptide bonds. Bromelin enzyme can be activated at pasteurization temperature and if thermal denaturation occurs, it cannot be reactivated (irreversible) (Novaes *et al*., 2015).

Bromelin belongs to the sufrihydyl group that contains proteolytic enzymes. It also contains peroxides, phosphoric acid, some protease inhibitors, and calcium-binding organics. Bromelin enzyme hydrolyzes proteins containing peptide bonds into simpler amino acids. In this case cysteine endopeptidase specifically cleaves peptide bonds at carbonyl groups such as those found in arginine or aromatic amino acids i.e. phenylalanine or tyrosine (Gautam *et al.*, 2010).

Bromelain is a group of endoproteases that are commonly used in protein hydrolysis. In addition, bromelain has a fairly broad cutting specificity towards amino acid residues that make up its substrate including arginine, lysine, tyrosine, and phenylalanine so that it is able to produce a high degree of hydrolysis (Whitaker, 2003). Bromelain has been widely applied to various processes in the food industry such as making protein hydrolysates, stabilizers in the beer industry, tenderizers in the meat and fish processing industry, the baking industry, the leather and textile tanning industry (Haslaniza *et al.,* 2010).

Bromelin activity is influenced by several things, namely the part of the pineapple plant as the source of the enzyme, the type of substrate, inhibitors, and the type of precipitant used for bromelin purification (Esih, 2006). Bromelin enzyme isolated from the flesh of ripe pineapple fruit has higher activity than bromelain enzyme isolated from leaves and unripe pineapple fruit. The optimum condition for the enzymatic reaction of bromelain from the flesh of ripe pineapple fruit is achieved at pH 6.5 at a temperature of 50 ℃for 20 minutes, (Priya *et al*., 2012).

Bromelin enzyme belongs to the glycoprotein class, which is a protein that contains one oligosaccharide part in each molecule, which is covalently bound to the enzyme's polypeptide chain. The amino acid sequence around the active site: -Cys - Gly - Ala - Cys - Trp-Asn - Gly - Asp - Pro - Cys - Gly - Ala - Cys - Cys - Trp. Cysteine (Cys) indicates the active site (Gautam *et al.*, 2010).

E. Antimicrobial Peptides

Protein is an important substance in the body. Amino acids are the main components of proteins that have metabolic functions in the body and are divided into two groups, namely essential and non-essential amino acids (Mandila and Hidajati, 2013). Essential amino acids are amino acids that cannot be made by the body and must be obtained from food sources of protein. Non-essential amino

acids are amino acids that can be made by the human body. Protein quality is judged by the ratio of amino acids contained in the protein (Winarno, 2008).

Antimicrobial peptides are components that haveevolved and permanently present in the innate immune system and are found across all classes of life. The fundamental difference is in prokaryotic cells and eukaryotic cells, which are the targets of antimicrobial peptides. These peptides constitute a spectrum of antibiotics antibiotic spectrum. Antimicrobial peptides are proven to kill gram-positive bacteria and gram-negative bacteria, including strains that are resistant to conventional antibiotics, mycobacteria, capsule-encased viruses, fungiand even cancer cells. Unlike most conventional antibiotics, antimicrobial peptides can boost immunity by functioning as an immunomodulator.

Peptides are a collection of 2-20 amino acids bound to each other through peptide bonds. According to Korhonen and Pihlanto (2006), bioactive peptides are specific protein fragments that have a positive impact on functional and body conditions. Bioactive peptides generally have a low molecular weight and are hydrophobic. Bioactive peptides generally consist of 2-20 amino acids and many bioactive peptides have more than one functional property. These amino acids can be derived from plants or animals such as milk, cheese, yogurt, fish, meat, nuts and kefir (Korhonen and Pihlanto, 2006).

Proteins in intact form have low bioactivity while proteins that have been hydrolyzed with enzymes will increase their bioactivity because the protein has been released from the long bonds of its fragments. Bioactive peptides have potential as antihypertensive, antioxidant, opioid antagonist, antibacterial, antithrombotic, and immunomodulatory compounds (Murray & Fitzgerald, 2007).

Antimicrobial peptides (AMPs) are usually composed of 12-50 amino acids and have an amphipathic structure (Lai & Gallo 2009). Antimicrobial peptides are usually positively charged and hydrophobic which allows them to interact with negative charges on the surface of the bacterial cell membrane. Subsequently, the peptide inserts and forms a pore that results in membrane damage and bacterial cell death (Bechinger & Gorr 2017).

The factors that affect the activity of antimicrobial peptides include:

1. Peptide Payload

The majority of antimicrobial peptides characterized to date carry a net positive charge, ranging from +1 to as much as +9 and can contain highly

pronounced cationic domains. The initial interaction with the membrane is primarily electrostatic and there is a strong correlation between cationic charge and biological activity with an increase in charge correlating with an increase in activity and also with an increase in the concentration of peptide bound to the membrane interface beyond (Lee *et al.,* 2016).

Membrane charge density also plays an important role in mediating the binding and selectivity of cationic AMP (*Antimicrobial Peptide)*. The partitioning of cationic AMP into zwitterionic lipids is generally weak with a high dissociation constant. The presence of negatively charged lipids such as phosphatidylglycerol (PG) and cardiolipin in microbial membranes mediates electrostatic interactions with cationic peptides with greatly reduced dissociation rates. However, while the contribution of electrostatic interactions forms the basis of selective peptide binding to bacterial membranes, this alone cannot be used to explain selectivity towards bacteria on host cells. As described above, enhanced electrostatic interactions are accompanied by non-linear concentration-dependent cell toxicity, once the charge threshold is exceeded (Lee *et al.,* 2016).

2. Secondary Structure

Various structures have been characterized for AMPs which can be generally classified into four main structural groups: helical peptides, β-strand/sheet peptides, mixed helical/sheet peptides and extended non helical/sheet peptides. Most AMPs undergo a conformational transition from flexible unstructured in solution to a specific structured or more rigid conformation upon interaction with membranes. In the case of helical peptides, amino acid substitutions that significantly disrupt the helical structure can lead to a dramatic decrease in activity (Lee *et al.,* 2016).

3. Hydrophobicity

Hydrophobicity is critical for interaction with membranes as it controls the extent to which the peptide can partition into the membrane layer. AMPs typically contain around 50% hydrophobic residues (Lee *et al.,* 2016). According to Chen *et al* (2007) that an optimal percentage of hydrophobicity is able to increase activity against microbial cell membranes. In addition, increasing hydrophobicity beyond the optimal point will cause loss of antimicrobial activity and increased toxicity to mammalian cells.

Antimicrobial peptides from marine molluscs function as antimicrobials with a broad spectrum, namely having an antimicrobial ability mechanism that can act to inhibit or even kill gram-positive bacteria, gram-negative bacteria, fungi, yeasts, viruses, and protozoa (Li *et al*. 2011; Zoysa *et al.* 2009; Nam *et al.* 2015).

Marine life from the gastropod class generally has antimicrobial activity in the form of antimicrobial peptides composed of amino acids arginine, lysine, leucine, serine, glycine, alanine, proline and cysteine (Sathyan *et al*. 2012; Zoysa *et al.* 2009; Li et *al.* 2011). Amino acids responsible for antimicrobial peptides are positively charged (cationic) amino acids and hydrophobic amino acids. Bioactive peptide compounds in gastropods with activity as antioxidant peptides and antimicrobial peptides are basically determined by positively charged (cationic) amino acids and hydrophobic amino acids (Sathyan *et al*. 2012; Nam *et al.* 2015; Sila and Ali 2016).

Table 2: Antimicrobial Activity of Animal Protein Hydrolysates

Source	Peptide Sequence	Target Microbes	Fractionation	Enzymes
Sea Snails (Dolashka *et al.,* 2015)	EPPYQLLAKFIKAGNGR	*E.coli, S.epidermidis, E.faecium, S.aureus*	2 and 10 kDa by HPLC	-
Oyster (*Crassostrea gigas)* (Liu *et al.,* 2008)	ATSGGACVILTPLHA	*E.coli, Pseudomonas aeruginosa, Bacillus subtilis, S.aureus*	5-10 kDa with DEAE Sephadex A-25 column, Sephadex G-25 and HPLC	Alcalase and bromelain
Leatherjacket (*Meuchenia sp*.) (Salampessy *et al.,* 2010)	-	*E. coli, B. cereus, S. aureus and C. albicans*	Fraction 12 by RP-HPLC	Bromelin
Goat milk (Kusumaningtyas, 2015)	-	*E.coli, S. Typhimurium and L. monocytogenes*	Fraction <10 kDa	Bromelin

F. Mechanism of Action of Antimicrobial Peptides

According to Waluyo (2004), antimicrobials are chemical substances obtained or formed and produced by microorganisms, these substances have the power to inhibit the activities of other mycororganisms even in small amounts. Definition of antimicrobials according to Rostinawati (2009), antimicrobials are chemical substances produced by a microbe that has antimicrobial properties.

According to Pelczar & Reid (1979) Antibacterial compounds are defined as biological or chemical compounds that can inhibit the growth and activity of bacteria. The ability of an antibacterial or antimicrobial substance to inhibit growth is influenced by several factors, among others:

- Concentration of antimicrobial agent
- Storage time
- Ambient temperature
- Properties of bacteria that include type, concentration, age and microbial state
- Physical and chemical properties of food including moisture content, pH, type and amount of compounds in it

The specialty of the mechanism of action of antimicrobial peptides from mollusks is that one of them can kill microorganisms in a very short time (pathogen-elimination mechanism) because it can interact with nucleic acids (binding to microbial DNA) from microbial cells, so that microbes are more quickly damaged and experience death. The nature of killing or inhibiting microbes by damaging their nucleic acids makes antimicrobial peptides from molluscs difficult to experience resistance to microbes (Zoysa *et al*. 2009).

The mechanism of action of AMP is by disrupting metabolism and destroying microbial membranes. The amount of AMP will increase during infection, inflammation, or epidermal differentiation. The interaction between AMP and microbes is electrostatic, which is the interaction between AMP which is cationic with anionic components of bacteria, fungi, or viruses. The composition of cationically charged amino acids makes it easy for AMP to attach and enter the cell through the gap that has formed on the membrane. It can also enter the cell directly to bind intracellular molecules that are important for living cells (Gunawan and Efendi, 2018).

The mechanism of action of AMP as shown in the figure shows that (a) AMP will attach to the outer part of the microbial membrane. (b) Integration of AMP

on the membrane causes the outer surface of the membrane to thin, then stretching occurs in the bilayer layer (c) Then a temporary gap is formed in the transition phase (d) Lipids and AMP move towards the inside of the membrane (e) AMP diffusion to the intracellular target (f) The target cell membrane will be damaged to form fragments (Gunawan and Efendi, 2018).

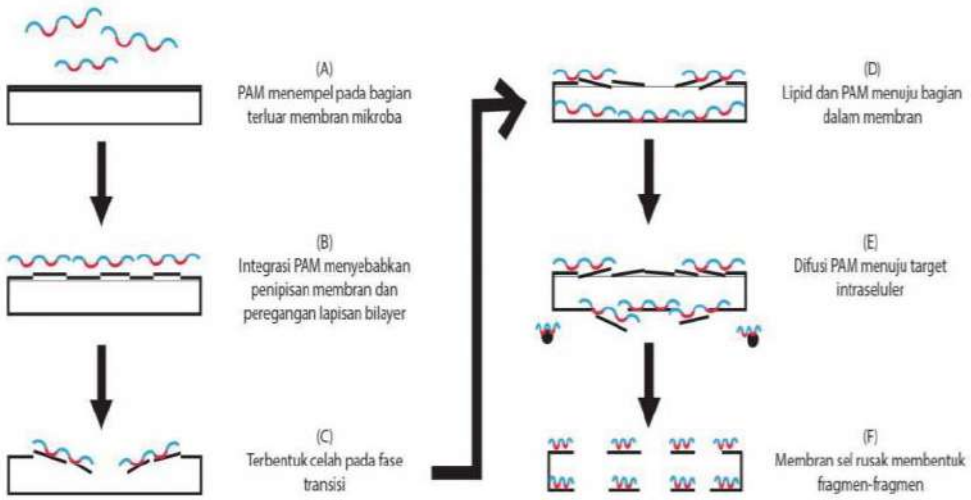
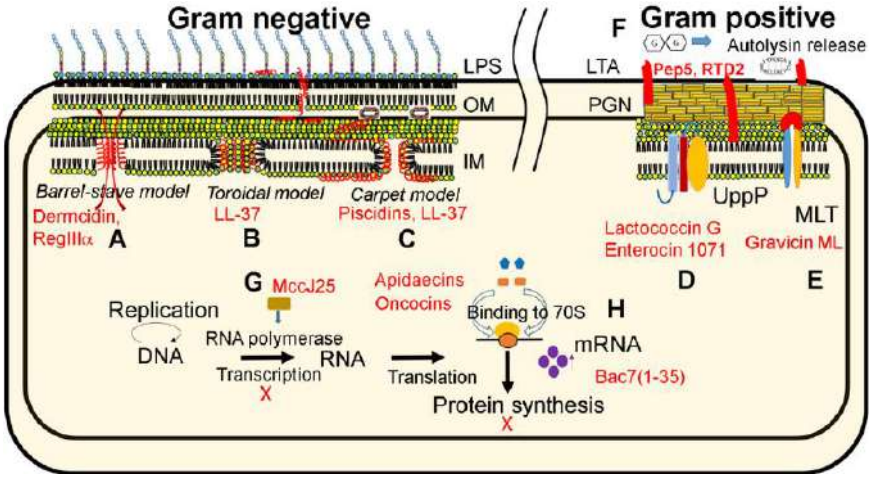


Figure 5. Mechanism of Action of Antimicrobial Peptides (Gunawan and Efendi, 2018)

There are three electrostatic models that explain AMP activity on bacterial membranes: (a) the stave model, in which the peptide would be inserted perpendicularly in the bilayer, together generating a pore; (b) the carpet model, which suggests that the peptide is absorbed in parallel in the bilayer and, upon reaching an adequate covering age, produces a detergent effect and destroys the membrane; and (c) the toroidal pore model, which suggests that the peptide is inserted perpendicularly in the lipid bilayer and generates regional membrane curvature, in which the pore is formed by the peptide and the phospholipid head group (Meri *et al.,* 2019).

A large number of AMPs (*Antimicobial Peptidas) are* thought to kill bacteria through mechanisms that damage the membrane and intracellular targets. Adsorption of AMP on the bacterial surface will be electrostatically driven between the cationic peptide and negatively charged cell wall components such as *Lipoteichoic Acid* from Gram-positive bacteria and lipopolysaccharides from Gram-

negative bacteria (Malanovic and Lohner, 2016). Overall, the mechanism of action of antimicrobial peptides on gram-positive and gram-negative bacteria is the same.



Mechanism of Action of Antimicrobial Peptides on Gram-Positive and Gram-Negative Bacteria (Wang *et al.,* 2015)

G. Decision Analysis

A decision is the conclusion of a process to choose the best course of action from a number of existing alternatives (Siagian, 2007). Decision making is the process of choosing an alternative way of acting with an efficient method according to the situation. This process is used to find and solve existing problems that are carried out based on existing knowledge and information. Decisions can be made from existing decision alternatives. There are three aspects that play a role in decision analysis, namely intelligence, perception and philosophy (Hariwan *et al.*, 2016).

H. Theoretical Foundations

Antimicrobials are very useful for the health and food fields. This is because the use of antimicrobials can be used as food preservatives to help inhibit the growth of bacteria that cause spoilage in food. The ability of antimicrobial activity due to the presence of bioactive compound components contained in a food ingredient.

The antimicrobial activity of gold snail protein hydrolysate using natural protease enzymes in the form of bromelain enzymes on *E.coli* and *S.aureus* bacteria has not yet been studied, so this has the potential to be used as research in order to produce renewable natural antibacterials and benefit the community. This study uses the test bacteria *S.aureus* as gram-positive bacteria and *E.coli* as

gram-negative bacteria. *Escherichia coli* and *Staphylococcus aureus* bacteria are bacteria that exist in the human body, but their presence in food is a contamination (Swarbrick, 2004).

Escherichia coli is a gram-negative bacterium that can cause harm to the body if the *Escherichia coli* entering the body is around 106 cells/ml. Similarly, food poisoning can be caused by enterotoxin contamination from *Staphylococcus aureus* which is a gram-positive bacterium. The amount of toxin that can cause poisoning is 1.0 µg/gr of food (Efendi *et al.,* 2014).

Some bioactive compounds that can act as antimicrobial agents are bioactive peptides. Bioactive peptides can be produced from animal proteins, one of which is goldfish snail, by breaking the peptide bond of the protein so that a shorter structure with a certain amino acid composition and sequence is produced. One method that can be used is by protein hydrolysis. Protein hydrolysis is the process of breaking down proteins into simpler compounds (amino acids) either by enzymes, bases or acids (Jaziri *et al.,* 2017).

Protein hydrolysate is a result of the process of breaking down proteins into simple peptides and amino acids through hydrolysis (Kristinsson, 2007). Theoretically, the most efficient method of protein hydrolysis is using enzymes, because enzymes produce peptides that are less complex and easily broken down. The advantages of enzymatic hydrolysis are that the resulting peptide products have a specific amino acid composition and sequence according to the type of protease used.

Based on previous research from Ulagesan *et al.,* (2018), on the hydrolysate of the snail species *Cryptozona bistrialis* through an enzymatic hydrolysis process using three types of enzymes, namely papain, trypsin and pepsin, it shows that the three protein hydrolysates with concentrations of 50µg/ml, 25 µg/ml and 15 µg/ml were analyzed for their antibacterial activity against S. aureus and P. aeruginosa. Only the papain-digested protein hydrolysate from C. bistrialis had activity against the tested pathogens.

In the study of Satyan *et al.,* (2012) Peptides having antimicrobial sequence motifs were identified from histone-H2A derived from mollusks namely *C. madrasensis*, *S. cucullata*, *M. casta*, *F. gracilis,* and *B. vittata* and named Moluskin. The high similarity of Molluskin in terms of physicochemical properties

and molecular structure with other AMP histone-H2A derivatives with proven antimicrobial activity strongly favors it to be an antimicrobial peptide.

The success of obtaining bioactive peptide pieces is largely determined by the protein source and enzyme specificity, so enzyme selection is an important stage to obtain bioactive peptides and will determine the bioactivity of the resulting peptides (Kumar *et al.,* 2013). In this study, one of the natural protease enzymes is bromelain obtained from young pineapple fruit which is widely available in Indonesia and needs to be optimized. The advantage of bromelain compared to other plant proteases is that bromelain is easily obtained and is available from the beginning of fruit development to ripe fruit although there are fluctuations in its proteolytic activity (Maurer, 2001). In addition, bromelain is also active both in the form of pure enzymes and still in the form of pineapple fruit juice so that the utilization of bromelain to produce bioactive peptides from goldfish protein hydrolysates has never existed before.

Bromelin is a cysteine protease that can be isolated from pineapple fruit. Bromelin is also an endoprotease that breaks down proteins into peptides (Arshad *et al*., 2014). The cutting specificity of bromelin is quite broad against the amino acid residues that make up its substrate including arginine, lysine, tyrosine, and phenylalanine so that it can produce a high degree of hydrolysis (Whitaker, 2003). Bromelin can be used to produce bioactive peptides such as antimicrobial peptides from *Meuschenia sp.* fish protein that can inhibit *Staphylococcus aureus* and *Bacillus cereus.*

In addition, enzyme activity is also strongly influenced by the length of hydrolysis and the concentration of enzyme added. According to Anggraini *et al.* (2015), the longer the incubation time will cause the enzyme to hydrolyze the protein longer, so that more peptide bonds will be hydrolyzed. Meanwhile, the reaction rate will be directly proportional to the enzyme concentration, where the higher the enzyme concentration, the higher the reaction rate with a certain concentration limit.

Based on the previous study of Dolashka *et al.,* (2011) they have isolated and analyzed several bioactive compounds, peptides, glycopeptides, hemocyanins, from sea snails. Also biochemically and pharmacologically active peptides in hemolymph of garden snail Helix lucorum and sea snail R. venosa were analyzed. Some of them, rich in Cys, Pro, Ser or Gly residues showed high

antimicrobial activity against S. aureus and low activity against Klebsiella pneumoniae because they were found to penetrate the bacterial cell membrane and accumulate in the cytoplasm.

The antimicrobial activity of antimicrobial peptides is usually positively charged and hydrophobic which allows them to interact with negative charges on the surface of the bacterial cell membrane. Furthermore, the peptide inserts and forms a pore that results in membrane damage and bacterial cell death (Bechinger & Gorr 2017). According to Malanovic and Lohner (2016), a large number of AMPs (*Antimicobial Peptidas)* are thought to kill bacteria through mechanisms that damage membranes and intracellular targets. AMP adsorption on the bacterial surface will be electrostatically driven between the cationic peptide and negatively charged cell wall components such as *Lipoteichoic Acid* from Gram-positive bacteria and lipopolysaccharides from Gram-negative bacteria.

Based on the study of Dolashka *et al* (2011), they identified several novel proline-rich antimicrobial peptides with molecular masses between 3000 and 9500 Da from the hemolymph of *R. venosa* conch and garden snail *H. lucorum* showing strong structural subunits antimicrobial activity against Gram-positive (Gram +) and Gram-negative (Gram -). Especially, *Staphylococcus* species are becoming increasingly resistant to many commonly used antibiotics including penicillin, tetracycline, etc., thus making the study of new natural antimicrobial peptides very important.

I. Hypothesis

The hypothesis of this study is that it is suspected that the length of hydrolysis and the concentration of bromelain enzyme affect the antimicrobial activity of goldfish protein hydrolysate.

CHAPTER III
RESEARCH METHODOLOGY

A. Time and Place of Research

The research was conducted in the Food Analysis Laboratory and Microbiology Laboratory, Food Technology Study Program, Faculty of Engineering, UPN "Veteran" East Java from December 2020 - February 2021.

B. Materials Used

The raw materials used in this study were gold snail (*Pomacea canaliculata)* obtained from rice fields around the Semambung Village area, Semambung District, Sidoarjo Regency, and natural protease enzyme from young pineapple fruit (bromelain enzyme). Chemicals used were: distilled water, bovine serum albumin (BSA), NaOH, $CuSO_4$, sodium carbonate, Na.K tartrate, *Follin Ciocalteu* Reagent*,* Nutrient Agar, Nutrient Broth and Plate Count Agar.

C. Tools Used

The tools used in the analysis of antimicrobial activity of goldfish protein hydrolysate include centrifugation, spectrophotometer, *magnetic stirrer*, incubator, colony counter, micropipette, bunsen, *cotton swab*, *paper disk,* petri disk, Erlenmeyer, test tube and measuring cup.

D. Research Methods

1. Research Design

Testing the antimicrobial activity of gold snail hydrolysate using an experimental design in the form of a complete randomized design (CRD) factorial pattern with 2 factors each repeated 2 times. The data obtained from the analysis were processed using *Analysis of Variance* (ANOVA) so that the interaction and significant differences between each treatment were known. If there is a significant difference, further tests are carried out using the DMRT (*Duncan't Multiple Range Test)* method at 5%. Data analysis using the help of the SPSS Statistics 17.0 for Windows program.

The mathematical model that applies to the Randomized Complete Factorial Design design (Kusriningrum, 2008) is as follows:

$$Y =_{ijk} \mu + \alpha_i + \beta_j + (\alpha) + \beta_{ij}\varepsilon_{ijk}$$

Where,

Y_{ijk} = observation result on i-th treatment j-th replication

μ = common mean value

α_i = the effect of the i-th level of factor A

β_j = the effect of the jth level of factor B

$(\alpha)\beta_{ij}$ = interaction effect of the i-th level of factor A and the j-th level of factor A.

B factor

ε_{ij} = residual effect (experimental error) of the i-th level of factor A and the level of

of factor B in the kth replication

2. Research Variables

a. Changeable Variables

Factor I. Hydrolysis Time of Goldfish Protein

T1 = Hydrolysis time 6 hours

T2 = 12 hours hydrolysis time

T3 = 18 hours hydrolysis time

Factor II. Bromelin Enzyme Concentration

S1 =Concentration 1% (v/b)

S2 = 5% concentration (v/b)

S3 = Concentration 10% (v/b)

From the combination of the two factors, nine treatments were obtained as follows:

T \ S	S1	S2	S3
T1	T1S1	T1S2	T1S3
T2	T2S1	T2S2	T2S3
T3	T3S1	T3S2	T3S3

Description:

T1S1 = 6 hours hydrolysis time with 1% bromelain enzyme concentration

T1S2 = 6 hours hydrolysis time with 5% bromelain enzyme concentration

T1S3 = 6 hours hydrolysis time with 10% bromelain enzyme concentration

T2S1 = 12 hours hydrolysis time with 1% bromelain enzyme concentration

T2S2 = 12 hours hydrolysis time with 5% bromelain enzyme concentration

T2S3 = 12 hours hydrolysis time with 10% bromelain enzyme concentration

T3S1 = 18 hours hydrolysis time with 1% bromelain enzyme concentration

T3S2 = 18 hours hydrolysis time with 5% bromelain enzyme concentration

T3S3 = 18 hours hydrolysis time with 10% bromelain enzyme concentration

b. Fixed Variable

- The ratio of snail meat and distilled water = 1: 2 (w/b)
- Hydrolysis Temperature = 54 C^o
- Enzyme inactivation heating temperature = 90 C^o
- Enzyme inactivation heating time = 10 minutes
- Centrifugation/separation time = 30 minutes
- Centrifugation/separation speed = 3000 rpm

E. Observed Parameters

- **Initial Raw Materials**
  a. Water Content (AOAC 2012 925.10)
  b. Ash Content (AOAC 2012 923.03)
  c. Dissolved Protein Content Lowry Method (Sudarmadji et al., 1997)
- **Bromelin Enzyme Extract Coarse**
  a. Yield
  b. Enzyme Activity Lowry Method (Sudarmadji et al., 1997)

c. Enzyme Specificity

- **Hydrolysate**
  b. Degree of Hydrolysis of TCA method (Silvestre *et al.,* 2013)
  c. Dissolved Protein *Content/Lowry* (Sudarmadji *et al.,* 1997)
  d. Total Peptide Concentration (Yang *et al.,* 2020)
  e. Antimicrobial activity testing by Disc Diffusion method (Banjara *et al.,* 2012)
  f. Testing the *Minimum Inhibitory Cocentration* (MIC) value (Heryudi *et al., 2015)*
  g. Testing the *Minimum Bactericidal Concentration* (MBC) Value (Heryudi *et al., 2015)*

F. Research Procedure

1. Preparation of Crude Bromelin Enzyme from Pineapple

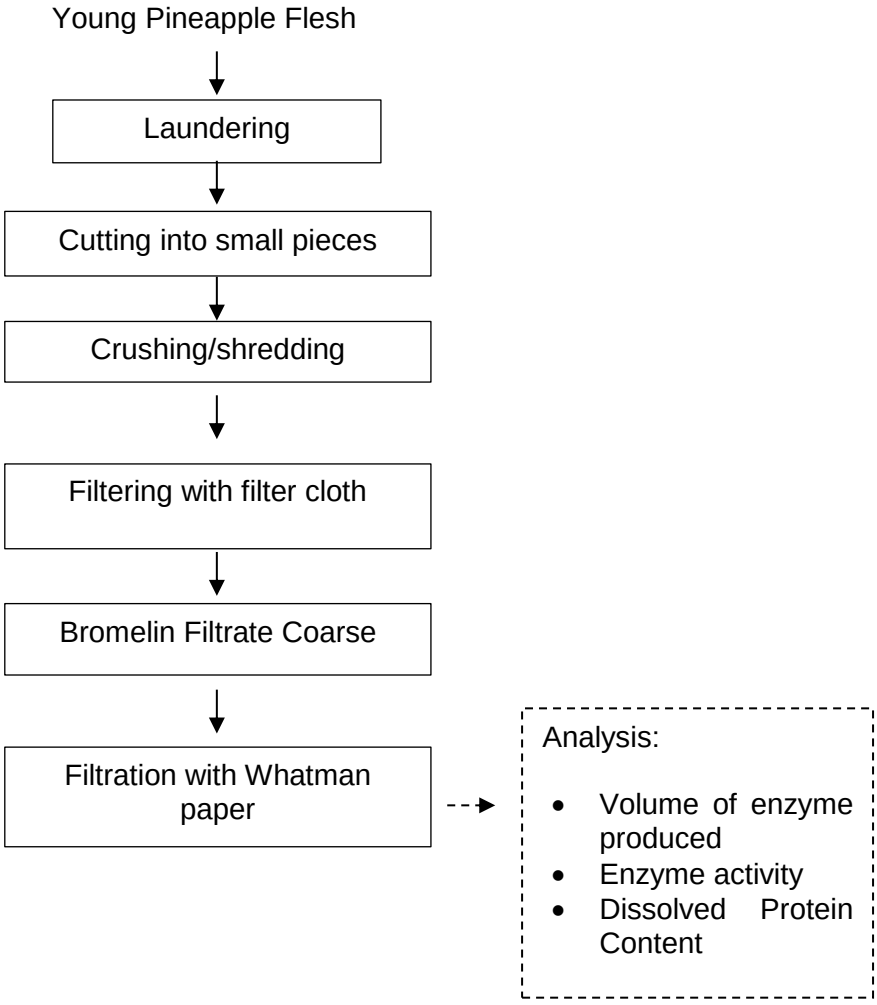
a. The flesh of young pineapple fruit that has been peeled, washed using running water until clean and cut into small pieces then weighed.
b. The flesh of the pineapple is then pureed using a blender without using water.
c. The crushed pineapple is then wrapped in a cloth and squeezed to get the filtrate.
d. The filter results were filtered again using Whatman paper to obtain crude bromelain enzyme extract.

The flowchart of the crude bromelain enzyme extract preparation procedure can be seen in **Figure 5**.

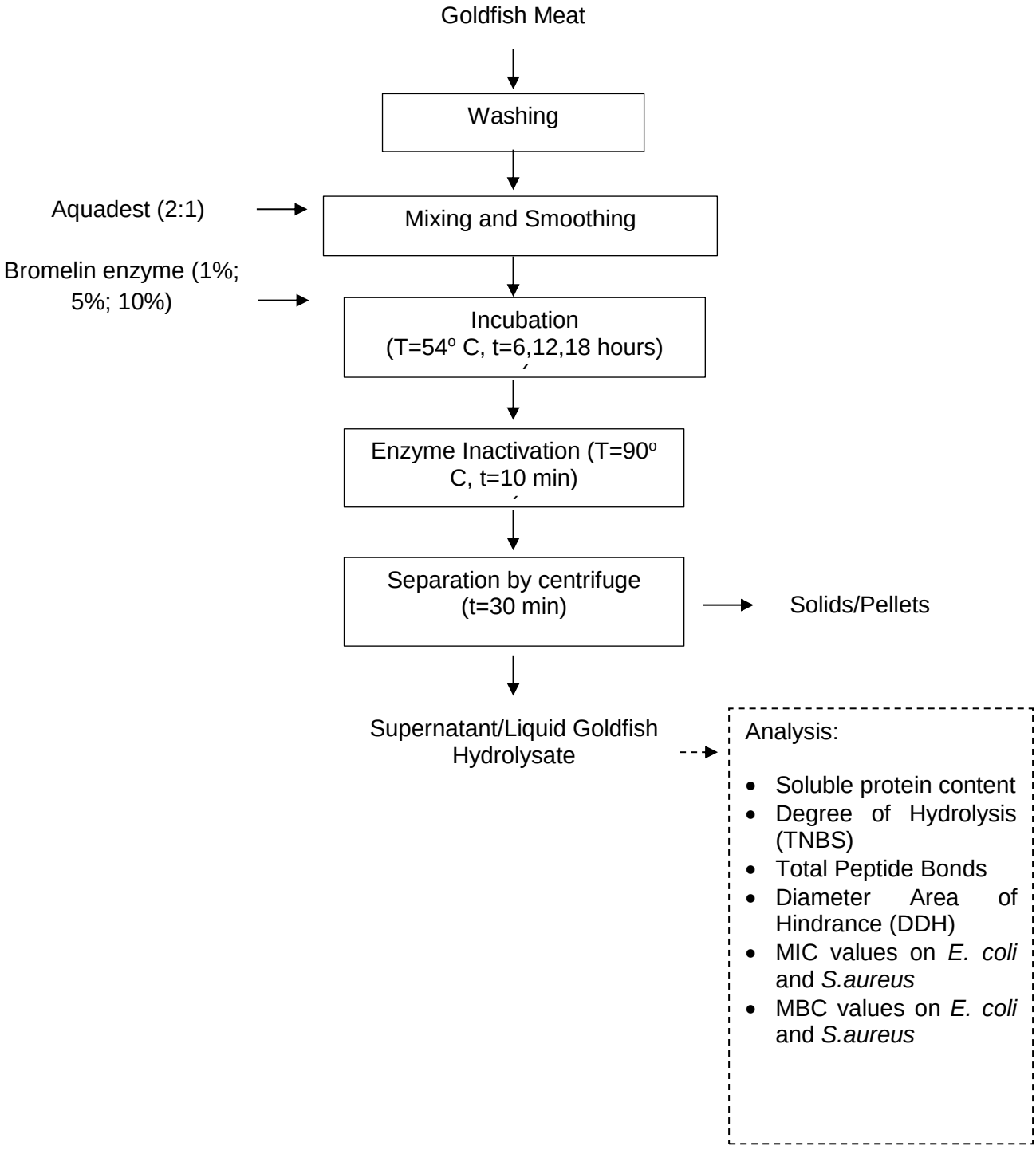
2. Preparation of Goldfish Hydrolysate

a. Carp snail meat is washed under running water to remove any dirt.
b. Aquadest was added to the gold snail meat in a ratio of (2:1) and pulverized using a blender.
c. *Bromelin* enzyme concentrations of 1%, 5% and 10% were added to the mixture and hydrolyzed/incubated at 54^{o} C for 6,12,18 hours
d. The enzyme was activated at 90^{o} C for 10 minutes.
e. The liquid and supernatant were separated using a *centrifuge* at 3000 rpm for 30 minutes.
f. The supernatant was removed and used as hydrolysate.

The flowchart of the procedure for making the goldfish bromelain enzyme can be seen in **Figure 6.**



Flowchart of the process of making crude bromelain enzyme extract (Erpiana, 2018)


Goldfish Meat
Washing
Aquadest (2:1)
Mixing and Smoothing
Bromelin enzyme (1%; 5%; 10%)
Incubation (T=54° C, t=6,12,18 hours)
Enzyme Inactivation (T=90° C, t=10 min)
Separation by centrifuge (t=30 min)
Solids/Pellets
Supernatant/Liquid Goldfish Hydrolysate
Analysis:
Soluble protein content
Degree of Hydrolysis (TNBS)
Total Peptide Bonds
Diameter Area of Hindrance (DDH)
MIC values on E. coli and S.aureus
MBC values on E. coli and S.aureus

Flowchart of the process of making goldfish hydrolysate
(Modified by Widadi, 2011)

CHAPTER VI
RESULTS AND DISCUSSION

The analysis conducted in this study included analysis of raw materials: goldfish meat and crude bromelain enzyme extract from pineapple pulp. The analysis continued with testing on the resulting goldfish meat hydrolysate including analysis of soluble protein content, Degree of Hydrolysis, Total peptide bonds, Diameter of *Inhibition* Area (DDH), *Minimum Inhibitory Concentration (*MIC) on *E. coli* and *S.aureus, Minimum Bactericidal Concentration* (MBC) on *E. coli* and *S.aureus.*

B. Raw Material Analysis Results

Analysis of raw materials carried out is analysis of water content, ash content and total protein content. The results of raw material analysis can be seen in table 3 as follows:

Table 3. Raw material analysis results of conch meat

Component	Analysis Result	Literature	
		a	b
Water Content (%)	86,22 ± 0,304	83,85	76,97
Ash Content (%)	2,07 ± 0,251	1,54	2,15
Total Protein Content (%)	15,09 ± 0,176	14,79	14,01

Source: [a] Hamid *et al.* (2015);[b] Ilhandzahina (2020)

The results of the analysis of goldfish meat raw materials in the manufacture of goldfish protein hydrolysate were found to have a moisture content of 86.22%, ash content of 2.07% and crude protein content of 15.09%. The results of research by Hamid *et al.* (2015) found that the nutritional content of goldfish meat is 83.85% moisture content, 1.54% ash content, 14.79% crude protein content, while the results of research by Ilhandzahina (2020) showed that the nutritional content of goldfish meat is 76.97% moisture content, 2.15% ash content and 14.01% crude protein content. Based on these results, there are differences in results from the literature with this study. This is thought to be due to several factors that influence it.

The factors that are thought to influence are the amount and food available, the process of storing meat in a frozen state for too long causes some of the content to change, the initial boiling process to separate the meat and shell causes the water content to increase. This is supported by Winarno (1997) that the boiling

process, when the water medium becomes hot, this heat will be transferred to food ingredients which causes tissue changes in food ingredients. This is what causes the high water content in the treatment of cooking methods by boiling.

C. Analysis Result of Coarse Bromelin Enzyme Extract

Analysis of crude bromelain enzyme extract included: Analysis of yield, enzyme activity, enzyme specificity and enzyme soluble protein content using the lowry method. The results of crude bromelain enzyme extract analysis can be seen in Table 4 as follows:

Table 4. Analysis Result of Enzyme Coarse Extract

Component	Analysis Result	Literature
Yield (%)	70,89 ± 0,0587	71,17 [a]
Enzyme Activity (U/ml)	2,849 ± 0,5038	5,373[a]
Enzyme Specificity (U/mg)	0,251 ± 0,5051	0,521[a]
Soluble Protein Content (mg/ml)	13,333 ± 0,1167	10,299[a]

Source: [a]Wuryanti (2014)

The isolation process is carried out to obtain crude extract of bromelin enzyme. In this study, crude bromelain enzyme was isolated from the flesh of young pineapple fruit. Bromelin enzyme is a class of sulfhydryl protease enzymes that can hydrolyze proteins to produce simpler amino acids (Gautam *et al.,* 2010). If the crude extract of bromelain enzyme has been obtained, the protein can be tested quantitatively using a spectrophotometer.

Analysis of crude bromelain enzyme extract used in the preparation of goldfish protein hydrolysate showed that the crude bromelain enzyme extract had a volume yield of 70.89%, enzyme activity of 2.849 U/ml, enzyme specificity of 0.251 U/mg and soluble protein content of 13.333 mg/ml. According to Wuryanti (2004), crude bromelain enzyme activity with pH 7.5 was 5.373 U/ml, enzyme specificity 0.521 U/mg and soluble protein content 10.299 mg/ml. There are differences in the results of this study with the literature, this is thought to be due to differences in the maturity of the pineapple fruit flesh used in addition to storage conditions. This is due to the nature of enzymes that are easily denatured due to several factors such as temperature, pH and storage time (Sarkar *et al.,* 2017). Bromelin enzyme can be activated at pasteurization temperature and if thermal denaturation occurs, it cannot become active again (irreversible) (Novaes *et al.,* 2015).

D. Analysis Result of Goldfish Proteate Hydrolysate

1. Dissolved Protein Content

Based on the results of the analysis of variance (Appendix 2), it can be seen that there is a significant interaction ($p \leq 0.05$) between the treatment of hydrolysis duration and the concentration of bromelain enzyme on the soluble protein content of the goldfish protein hydrolysate produced. The soluble protein content value obtained from the hydrolysate sample test shows how much protein is successfully broken down from the total protein of the starting material and dissolved in the hydrolysate. The higher the soluble protein content value, the more optimal the hydrolysis process is. The average value of soluble protein content of the goldfish protein hydrolysate can be seen in Table 5 as follows:

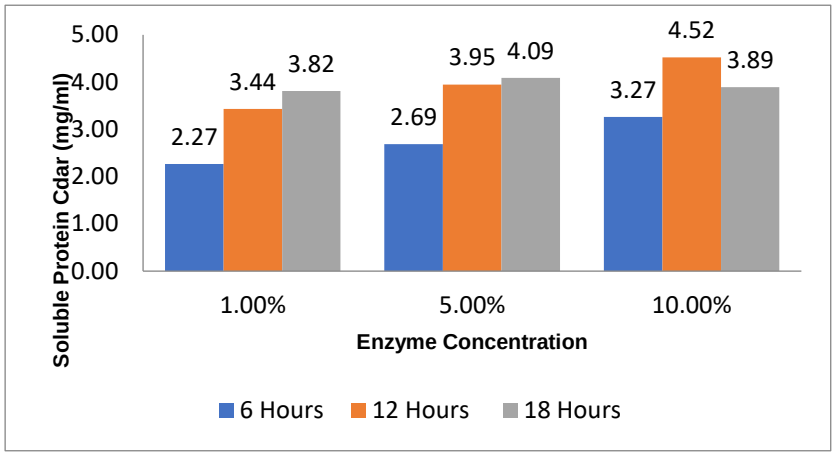
Table 5. Mean value of soluble protein content of goldfish protein hydrolysate with enzyme concentration treatment and hydrolysis duration

Treatment		Average Soluble Protein Content (%)	DMRT 5%	Notation
Enzyme Concentration	Hydrolysis Duration			
1%	6 Hours	2,265 ± 0,106	-	a
1%	12 Hours	3,435 ± 0,502	0,438	c
1%	18 Hours	3,815 ± 0,007	0,4097	b
5%	6 Hours	2,685 ± 0,106	0,4276	c
5%	12 Hours	3,95 ± 0,028	0,4444	c
5%	18 Hours	4,09 ± 0,056	0,4485	c
10%	6 Hours	3,265 ± 0,077	0,4512	c
10%	12 Hours	4,52 ± 0,028	0,4539	c
10%	18 Hours	3,89 ± 0,098	0,4529	c

Description: mean values followed by different letters state significantly different ($p \leq 0.05$)

Table 5 shows that the average soluble protein content of protein hydrolysate from gold snail ranged from 2.265% - 4.52%. The lowest protein content results were obtained from the 6-hour hydrolysis treatment with 1% enzyme concentration, while the highest results were obtained from the 12-hour hydrolysis treatment with 10% enzyme concentration. The results of this study showed lower results than Ilhandzahina (2020) on goldfish protein hydrolysate using bromelain enzyme with a hydrolysis time of 3 hours, which amounted to 8.28%. However, these results have higher results than Putra *et al.* (2018) on goldfish protein hydrolysate using commercial papain enzymes, which amounted to 1.81%.

The relationship between enzyme concentration and hydrolysis duration on the soluble protein content of goldfish protein hydrolysate can be seen in **Figure 8.**



The relationship between hydrolysis time and bromelain enzyme concentration on the soluble protein content of goldfish protein hydrolysate**.**

Figure 8 shows that the more enzyme concentration added and the longer the hydrolysis time, the soluble protein content in the resulting goldfish protein hydrolysate tends to increase. This is because the increase in the concentration of enzyme added causes an increase in the soluble nitrogen content in the protein hydrolysate so that the soluble protein content of the goldfish protein hydrolysate increases. In addition, the longer the hydrolysis time causes the more soluble protein in the hydrolysate to increase because the bromelain enzyme is able to cut peptide bonds in the inner or middle part so as to produce more short-chain peptide bonds. However, in the treatment of 10% enzyme concentration with 18 hours of hydrolysis, the resulting soluble protein content decreased. This is because the addition of excess enzymes will cause constant protein levels because the addition of bromelain enzyme in the hydrolysis process has decreased activity.

This is supported by Agustina (2008) that protein content increases as the concentration of enzyme added increases. This shows that as the enzyme concentration increases, the speed of the hydrolysis reaction increases, however, at a certain limit, excessive enzyme addition will result in a constant amount of hydrolysate because the enzyme addition is no longer active. The longer the hydrolysis time, the more peptide bonds are produced. Nafi *et al.* (2014) stated that during hydrolysis the interaction between the enzyme and the substrate which is

getting longer causes an increase in the breaking of peptide bonds into simpler ones so that protein solubility will also increase.

2. Degree of Hydrolysis (DH)

The degree of hydrolysis is a parameter that can be used as monitoring of the hydrolysis process because it states the percentage of peptide bonds that are released due to the hydrolysis process (Silvestre *et al.,* 2013). Based on the results of analysis of variance (Appendix 4), it can be seen that there is a significant interaction ($p \leq 0.05$) between the concentration of bromelain enzyme and the duration of hydrolysis on the Degree of Hydrolysis (DH) of goldfish protein hydrolysate. The average value of Hydrolysis Degree (DH) of goldfish protein hydrolysate can be seen in Table 6 as follows:

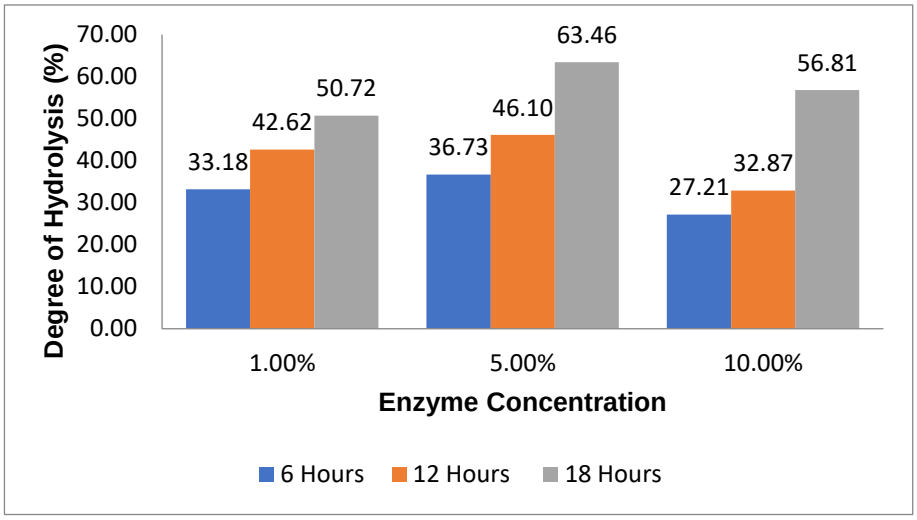
Average value of Hydrolysis Degree (DH) of gold snail protein hydrolysate with enzyme concentration treatment and hydrolysis duration**.**

Treatment		Average Degree of Hydrolysis (%)	DMRT 5%	Notation
Enzyme Concentration	Hydrolysis Duration			
1%	6 Hours	33,175 ± 0,106	-	a
1%	12 Hours	42,615 ± 0,615	1,4399	c
1%	18 Hours	50,715 ± 0,756	1,3469	b
5%	6 Hours	36,725 ± 0,190	1,4058	b
5%	12 Hours	32,87 ± 0,014	1,461	d
5%	18 Hours	63,46 ± 0,834	1,4744	e
10%	6 Hours	27,205 ± 0,784	1,4833	f
10%	12 Hours	46,1 ± 0,933	1,4921	h
10%	18 Hours	56,805 ± 0,091	1,4887	g

Description: mean values followed by different letters state significantly different ($p \leq 0.05$)

The average degree of hydrolysis of the goldfish protein hydrolysate in **Table 6** ranged from 27.205% to 63.46%. The treatment of 10% enzyme concentration with a hydrolysis time of 6 hours produced the lowest degree of hydrolysis, namely 27.205%, while the treatment of 5% enzyme concentration with a hydrolysis time of 18 hours produced the highest degree of hydrolysis, namely 63.46%. The results of this study are not much different from the results of Ilhandzahina's research (2020) on gold snail hydrolysate using bromelain enzyme, which amounted to 69.44%, but higher than the *Cryptozona bistrialis* snail protein hydrolysate using papain enzyme in Ulagesan *et al.'s* research (2018) of 27-29.5%.

The relationship between the treatment of enzyme concentration and the duration of hydrolysis on the results of the degree of hydrolysis of goldfish protein hydrolysate can be seen in **Figure 9.**



The relationship between hydrolysis time and bromelain enzyme concentration on the Degree of Hydrolysis (DH) of gold snail protein hydrolysate**.**

Figure 9 shows that the higher the enzyme concentration and the longer the hydrolysis time, the higher the degree of hydrolysis. This is because the availability of a large amount of enzyme can increase the speed of hydrolysis of a fixed amount of substrate. The higher the concentration of enzyme added, the more peptide bonds that can be hydrolyzed. If the degree of hydrolysis produced tends to be lower, it can be known that it is because the concentration of enzyme added is less but the availability of substrate is greater than the amount of enzyme available, so the speed in cutting peptide bonds in the substrate is lower. In addition, the low degree of hydrolysis (DH) at the highest enzyme concentration of 10% is very likely due to the presence of inhibitors that are still present in the goldfish protein hydrolysate, thus blocking the contact of protein and enzyme (aqueous phase) which causes the affinity of the substrate to the catalytic side of the enzyme to be low. Figure 9, shows that the hydrolysis speed increased very fast at the beginning of hydrolysis, from 6 hours to 12 hours. However, the hydrolysis speed showed a decrease at the hydrolysis time of 18 hours. This is because the peptide bond cutting reached the maximum speed and then the peptide bond cutting activity by the bromelain enzyme began to decrease because the available substrate will decrease so that it will cause inhibition of the active side

of the enzyme and the peptide bond cutting activity by the enzyme will be disrupted which will indirectly cause the hydrolysis rate to reach the stationary phase.

Haslaniza *et al.* (2010) stated that the degree of protein hydrolysis is determined by several factors including the type of protease used, enzyme concentration, temperature, pH and hydrolysis time. This is also supported by Restiani (2016) who stated that the low DH at low enzyme concentration levels can be caused by the availability of many substrates, in addition to the availability of substrates that are more than the number of enzymes causing *irreversible* inhibition of the catalytic side of the enzyme so that the enzyme is unable to carry out the activity of cutting peptide bonds and indirectly causing the rate to reach the stationary phase.

3. Total Peptide Concentration

Based on the results of analysis of variance (Appendix 6), it can be seen that there is a significant interaction ($p \leq 0.05$) between the treatment of enzyme concentration and hydrolysis duration on the concentration of total peptides of goldfish protein hydrolysate produced. The average value of total peptide concentration of goldfish protein hydrolysate can be seen in Table 7 as follows:

Table 7: Average value of total peptide concentration of goldfish protein hydrolysate by enzyme concentration treatment with hydrolysis duration.

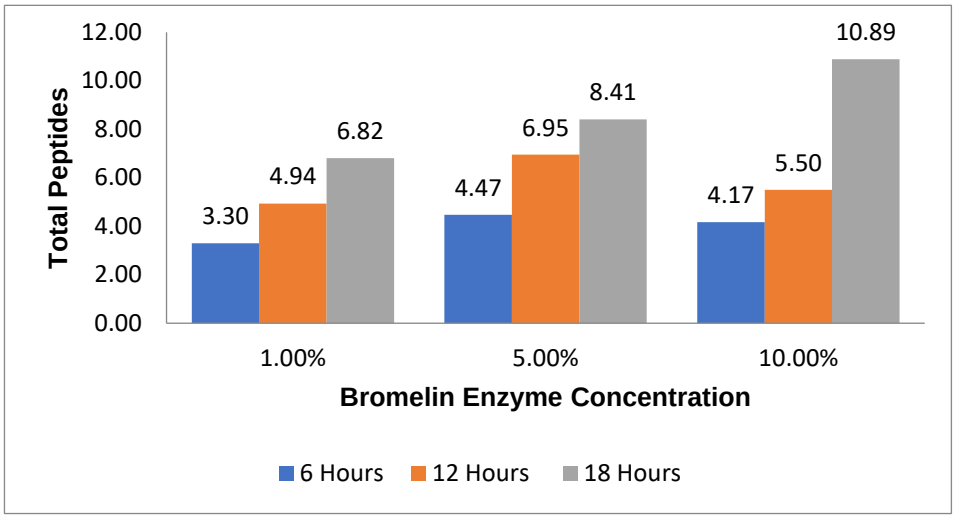
Treatment		Total Peptide Concentration (mg/g)	DMRT 5%	Notation
Enzyme Concentration	Hydrolysis Duration			
1%	6 Hours	3,3 ± 0,098	-	a
1%	12 Hours	4,935 ± 0,205	0,3267	c
1%	18 Hours	6,815 ± 0,007	0,3056	b
5%	6 Hours	4,47 ± 0,254	0,319	b
5%	12 Hours	6,95 ± 0,028	0,3315	d
5%	18 Hours	8,41 ± 0,127	0,3346	e
10%	6 Hours	4,17 ± 0,141	0,3366	e
10%	12 Hours	5,5 ± 0,028	0,3386	g
10%	18 Hours	10,89 ± 0,098	0,3378	f

Description: mean values followed by different letters state significantly different ($p \leq 0.05$)

Table 7 shows that the average concentration of total peptides in goldfish protein hydrolysate ranged from 3.3 - 10.89 mg/g. The lowest results were obtained from the treatment of 1% enzyme concentration with a hydrolysis time of 6 hours, while the highest results were obtained from the treatment of 10% enzyme concentration with a hydrolysis time of 18 hours. These results are better than the

research of Seniman *et al.* (2014) which produced total peptide concentration values between 0.35 - 6.90 mg/g in catfish protein hydrolysate using papain enzyme. The concentration of peptides is influenced by the length of time of hydrolysis of goldfish protein, the longer the hydrolysis time, the higher the concentration of total peptides in the hydrolysate product.

The relationship between the treatment of enzyme concentration and hydrolysis duration on the concentration of total peptides of goldfish protein hydrolysate can be seen in **Figure 10.**



The relationship between hydrolysis time and bromelain enzyme concentration on total peptide concentration of goldfish protein hydrolysate**.**

Figure 10 shows that the longer the hydrolysis time used and the more enzyme concentration added, the higher the total peptide concentration of the goldfish protein hydrolysate produced. This is because the longer the hydrolysis time, the more peptides will be released as a result of bromelain enzyme activity. Meanwhile, the more enzyme concentration added, the more peptide chain breaks so that the more peptides produced. In the treatment of 10% enzyme concentration at a hydrolysis time of 12 hours to 18 hours, there was a significant increase. This is because the substrate that binds to the enzyme at a hydrolysis time of 18 hours is more, so the speed of enzyme activity will increase significantly and the resulting peptide bond will also increase.

This is supported by Seniman *et al.* (2014) that the total peptide concentration is closely related to the hydrolysis time of the hydrolysate used, the peptide content increases with increasing hydrolysis time because more peptides

are released as a result of proteolytic enzyme activity. In addition, differences in the rate and pattern of hydrolysis can be due to differences in the nature of the enzyme cutting sites as well as the accessibility of peptide bonds to each protease.

E. Antimicrobial Activity Analysis Results

1. Diameter of Area of Inhibition (DDH)

In the antibacterial activity test, the method used is the disc diffusion method. The results of the antibacterial test are based on the measurement of the Diameter of Inhibition Area (DDH) of bacterial growth formed around the disc paper. The test bacteria used were *Streptococcus aureus* as gram-positive bacteria and *Eschericea coli* gram-negative. Based on the results of analysis of variance (Appendix 8), it can be seen that there is a significant interaction ($p \leq 0.05$) between the treatment of enzyme concentration and hydrolysis duration on the DDH of goldfish protein hydrolysate produced. The average value of DDH of goldfish protein hydrolysate can be seen in Table 8 as follows:

Table 8. Average Value of Diameter of Inhibition Area (DDH) of Goldfish Protein Hydrolysate by Treatment of Enzyme Concentration with Hydrolysis Duration

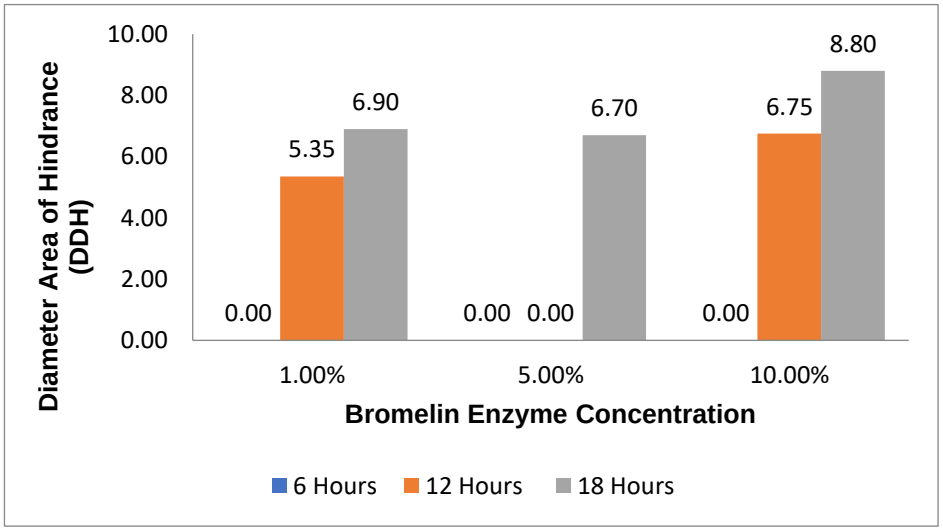
Test Bacteria	Treatment	Diamter of Inhibition Area (mm)	DMRT 5%	Notation

Eschericea coli	A1B1	-	-	a
	A1B2	5,35 ± 0,494	0,6594	a
	A1B3	6,9 ± 0,141	0,6719	d
	A2B1	-	0,6079	a
	A2B2	-	0,6499	b
	A2B3	6,7 ± 0,424	0,6655	c
	A3B1	-	0,6345	a
	A3B2	6,75 ± 0,353	0,6695	c
	A3B3	8,8 ± 0,282	0,6735	e
Staphylococcus aureus	A1B1	-		
	A1B2	-		
	A1B3	-		
	A2B1	-		
	A2B2	-		
	A2B3	-		
	A3B1	-		
	A3B2	-		
	A3B3	-		

Description: mean values followed by different letters state significantly different (p≤ 0.05); the sign (-) indicates ineffective in inhibiting bacterial growth.

Table 8 shows that the gold snail protein hydrolysate only showed inhibition on *E.coli* bacteria with an average diameter of 5.3 - 8.8 mm. The lowest result was obtained from the treatment of 1% enzyme concentration with 12 hours of hydrolysis, while the highest result was obtained from the treatment of 3% enzyme concentration with 18 hours of hydrolysis. The DDH results of the goldfish snail hydrolysate produced have differences with the results of research by Dolashka *et al.* (2011) on sea snail protein hydrolysate using enzymatic hydrolysis can inhibit the activity of *S.aureus* bacteria *(*gram positive) and *E.coli* bacteria (gram negative). In addition, in the research of Aissaoui *et al.* (2016) on *scorpionfish* protein hydrolysate was able to inhibit the growth of *S.aureus* bacteria with a DDH of 31 mm while in *E.coli* bacteria the resulting DDH was 19 mm*.* This is because several factors that affect DDH are the sensitivity of the organism, incubation conditions, agar diffusion speed, and protein content in each raw material to be hydrolyzed. In addition, according to Beswika (2009) in his research, the difference in the size of the inhibition zone can be caused by large molecules of secondary metabolite compounds that have difficulty diffusing in the agar medium.

The relationship between the treatment of enzyme concentration and hydrolysis duration on the Diameter of Inhibition Area (DDH) of goldfish protein hydrolysate on *E.coli* bacteria can be seen in **Figure 11.**



The relationship between hydrolysis time and concentration of bromelain enzyme on the DDH value of *E.coli* bacteria of goldfish protein hydrolysate

Figure 11 shows that the more enzyme concentration added and the longer the hydrolysis time, the greater the DDH produced in inhibiting the growth of *E.coli* (gram negative) bacteria. This is because if the more enzyme concentration is added, the more protein cutting into peptides that have bioactive properties, one of which is the antimicrobial properties produced. In addition, if the longer the hydrolysis time, it will provide an opportunity for enzymes to cut peptide bonds for longer so that they can produce more bioactive peptides.

The antimicrobial DDH value of the goldfish protein hydrolysate showed that the test sample was more active in inhibiting *E.coli* (gram negative) than *S.aureus* (gram positive). The mechanism of antimicrobial peptides in inhibiting the activity of gram-positive and gram-negative bacteria is not known for certain. Likewise, its effectiveness whether it is easier to inhibit the activity of gram positive or gram negative bacteria, when viewed from the structure of the bacterial cell wall, gram negative bacteria have a more complex structure than gram positive bacteria. The existence of antimicrobial peptides that are dominated by positive charges in goldfish hydrolysate causes antimicrobial peptides to more easily inhibit the growth of gram negative bacteria than gram positive (Rachmawati, 2015). This can be seen from previous research which shows that the presence of antimicrobial

peptides such as $rBPI_{21}$ which are dominated by positive charges more effectively inhibit the activity of gram negative bacteria than gram positive (Domingues *et al.,* 2014).

According to Domingues *et al.,* (2014) on the negativity of *E.coli* (gram negative) and *S.aureus* (gram positive) bacteria showed that the cell membrane of *E.coli is* more negative than *S.aurues*, thus affecting electrostatic interactions in the initial phase of cationic attachment of antimicrobial peptides to the surface of the bacterial anionic membrane, so this is what causes the antimicrobial protein $rBPI_{21}$ to more effectively inhibit the growth of gram negative than gram positive. Almost 90% of the negative charge of the gram negative cell wall is contributed by lipopolysaccharides which are able to overcome the electonegativity of the thick peptidoglycan in the gram positive cell wall, so that positively charged peptides penetrate the gram negative bacterial cell wall more quickly.

The enzymatic hydrolysis process will cause the formation of bioactive peptides, one of which has the ability as an antimicrobial. According to Ulagesan *et al.,* (2018) amino acids that have antimicrobial abilities are amino acids that are hydrophobic, so that later these amino acids are able to interact with lipid targets or enter target organs through hydrophobic associations. Based on research by Ilhandzahina (2020), it is stated that 20.21% of amino acid hydrolysate of goldfish protein consists of hydrophilic amino acids, while for hydrophobic amino acids it is 9.54%. These results indicate that the more dominant amino acid component in goldfish protein hydrolysate is hydrophilic amino acids.

2. Minimum Inhibitory Concentration (MIC)

Testing the antimicrobial activity of goldfish protein hydrolysate using the MIC method. The MIC test uses a dilution / dilution method, namely with a concentration of 100%, 50%, 25%, 12.5% and 6.25% goldfish protein hydrolysate and then added test bacteria, namely *Eschericea coli* and *Staphylococcus aureus and* then observed turbidity or turbidimetry of the solution with the help of a spectrophotometer. The MIC test is defined as the lowest value of antimicrobial

concentration that will inhibit the growth of microorganisms after 24 hours incubation. Inhibition of bacterial growth is characterized by clear media then later determined as MIC. MIC values seen from the absorbance value of each tube can be seen in table 9:

Minimum Inhibitory Concentration (MIC) Value of Goldfish Protein Hydrolysate with Bromelin Enzyme Concentration Treatment with Hydrolysis Duration on *Eschericea coli* bacteria

Treatment	Hydrolysate concentration									
	100%		**50%**		25,00%		12,50%		6,25%	
	Before	After	**Before**	**After**	Before	After	Before	After	Before	After
A1B1	0,986	0,83	**0,872**	**1,275**	0,423	1,375	0,347	1,4	0,343	1,41
A2B1	1,027	0,89	**0,898**	**0,93**	0,468	1,41	0,418	1,43	0,404	1,47
A3B1	1,128	0,93	**0,93**	**0,98**	0,679	1,4	0,542	1,44	0,401	1,325
A1B2	1,008	0,87	**0,82**	**0,87**	0,754	1,134	0,653	1,203	0,619	1,346
A2B2	1,163	0,9	**0,91**	**0,835**	0,616	1,087	0,814	1,148	0,568	1,192
A3B2	1,267	0,945	**0,954**	**0,652**	0,617	1,255	0,453	1,38	0,529	1,38
A1B3	1,507	0,88	**0,87**	**0,512**	0,455	0,976	0,376	1,251	0,323	1,31
A2B3	1,149	0,93	**0,94**	**0,492**	0,53	1,08	0,467	1,233	0,41	1,45
A3B3	1,268	0,97	**0,965**	**0,54**	0,575	1,002	0,397	1,114	0,35	1,34
Flat	1,167	0,905	**0,906556**	**0,787333**	0,568556	1,191	0,496333	1,288778	0,438556	1,358111
Description	Down		**Down**		Up		Up		Up	

Description: "Increase" indicates absorbance value after incubation > absorbance value before incubation, which means that there is bacterial growth; while "Fixed" or "Decrease" indicates absorbance value after incubation ≤ absorbance value before incubation, which means that bacterial growth is inhibited.

Table 10: *Minimum Inhibitory Concentration* (MIC) Value of Goldfish Protein Hydrolysate with Bromelin Enzyme Concentration Treatment with Hydrolysis Duration on *Staphylococcus aureus* bacteria.

Treatment	Hydrolysate concentration									
	100%		50%		25,00%		12,50%		6,25%	
	Before	**After**	Before	After	Before	After	Before	After	Before	After
A1B1	**0,73**	**0,825**	0,546	1,235	0,529	1,265	0,511	1,305	0,491	1,45
A2B1	**0,806**	**0,981**	0,627	1,335	0,598	1,365	0,568	1,41	0,362	1,45
A3B1	**0,686**	**0,513**	0,49	0,46	0,436	1,225	0,364	1,38	0,358	1,46
A1B2	**0,786**	**1,034**	0,572	1,29	0,392	1,33	0,374	1,355	0,437	1,444
A2B2	**1,05**	**1,01**	0,981	1,245	0,508	1,295	0,449	1,335	0,464	1,4
A3B2	**0,774**	**0,899**	0,597	1,245	0,461	1,3	0,394	1,355	0,378	1,45
A1B3	**0,786**	**0,672**	0,796	0,77	0,673	0,987	0,51	1,312	0,33	1,43
A2B3	**0,891**	**0,7**	0,67	0,976	0,531	1,18	0,44	1,45	0,41	1,567
A3B3	**0,912**	**0,69**	0,874	0,817	0,65	1,12	0,476	1,241	0,352	1,289
Flat	**0,824556**	**0,813778**	0,683667	1,041444	0,530889	1,229667	0,454	1,349222	0,398	1,437778
Description	**Down**		Up		Up		Up		Up	

Description: "Increase" indicates absorbance value after incubation > absorbance value before incubation, which means that there is bacterial growth; while "Fixed" or "Decrease" indicates absorbance value after incubation ≤ absorbance value before incubation, which means that bacterial growth is inhibited.

Table 9 shows that the concentration of 100% hydrolysate in all treatments has decreased the absorbance value which means that bacterial growth is inhibited and at a concentration of 25%, 12.5% and 6.25% has increased absorbance value which means there is bacterial growth. At a concentration of 50%, it can be seen that the absorbance value before incubation and after incubation decreased, so this concentration was determined as the MIC value of goldfish protein hydrolysate against *E.coli* growth using the UV-Vis Spectrophotometer method. Meanwhile, **Table 10** shows that the 100% concentration shows that the absorbance value before incubation and after incubation decreases, while at concentrations of 50%, 25%, 12.5% and 6.25% the absorbance value increases, which means there is bacterial growth. So that the concentration of 100% is determined as the MIC value of goldfish protein hydrolysate against the growth of *S.aureus* bacteria. This is slightly different from the results of research from Lima *et al.,* (2014) which states the MIC value of collagen hydrolysate from *Penicillum aurantiogriseum* against gram-positive bacteria (*S.aureus)* at a concentration of 55% and on gram-negative bacteria (*E.coli* and *B.subtilis*) showing MIC values at concentrations of 6.25% and 50%.

The minimum inhibition of *E.coli* bacteria growth by turbidimetric test occurred at a concentration of 50% because it began to look clearer than the small concentration tubes below, namely 25% concentration tubes up to 6.25% tubes. The higher the concentration of hydrolysate added, the less bacterial activity can be reduced, while the minimum inhibition of *S.aureus* bacterial growth occurred at a concentration of 100% because the solution looked clearer than before incubation. This is due to the greater content of antibacterial compounds such as antimicrobial peptides in the hydrolysate. Determination of MIC through turbidimetric testing is done by looking at the turbidity of the test sample in the tube instead of looking at the density of the color of the solution in the tube. According to Dewi (2010), the higher the concentration of the color of an extract solution, the greater the level of inhibitory activity against bacterial growth seen in the clearer solution. while the increase in absorbance value at a concentration of 25% (E*.coli* bacteria) and 50% (*S.aureus* bacteria*)* is not entirely due to bacterial growth, but can also be caused by a higher concentration, so that it can affect the absorption of light by dead bacterial cells in the solution (Watson, 2005).

3. Minimum Bactericidal Concentration (MBC)

Based on the results of the MIC value test, it is known that at concentrations of 100% and 50% of the goldfish protein hydrolysate shows inhibition of bacterial growth of *S.aureus* and *E.coli* by showing changes in the turbidity of the suspense solution after incubation for 24 hours. Furthermore, to determine the lowest concentration of protein hydrolysate as an antibacterial agent needed to kill bacteria *S.aureus* and *E.coli* can be done with MBC (*Minimum Bactericidal Concentration*) testing. The MBC value is seen from the number of bacterial colonies that can be seen in Table 11:

Minimum Bactericidal Concentration (MBC) Value of Goldfish Protein Hydrolysate with Enzyme Concentration Treatment with Hydrolysis Duration on *Eschericea coli* bacteria

Treatment	Hydrolysate Concentration				
	100%	50%	25%	12,50%	6,25%
	Colony Count (CFU/ml)				
A1B1	0	0	41	55	169
A2B1	0	0	23	56	77
A3B1	0	0	0	0	9
A1B2	0	0	0	54	67
A2B2	0	0	0	17	33
A3B2	0	0	0	0	27
A1B3	0	0	24	67	74
A2B3	0	0	0	25	57
A3B3	0	0	0	0	0
Flat	0	0	29,3	45,67	64,125
Description		**MBC**			

Description: "0" indicates no growth of *Eschericea coli* bacteria.

Table 11 shows that at the highest concentration of goldfish protein hydrolysate tested, there was no bacterial growth. To calculate the number of bacteria in each concentration, the number of bacteria was calculated using the *total plate count* (*TPC*) technique. The TPC test results showed that in all treatments with 50% hydrolysate concentration there was no growth of *E.coli* bacteria, it can be stated that at that concentration the goldfish protein hydrolysate is able to kill bacteria so that the MBC value in goldfish protein hydrolysate with enzyme concentration treatment and hydrolysis duration is at 50% hydrolysate concentration.

Table 12. *Minimum Bactericidal Concentration* (MBC) Value of Goldfish Protein Hydrolysate with Enzyme Concentration Treatment with Hydrolysis Duration on *Staphylococcus aureus* bacteria

Treatment	Hydrolysate Concentration

	100%	50%	25%	12,50%	6,25%
	Colony Count (CFU/ml)				
A1B1	0	41	61	78	TBUD
A2B1	0	21	41	57	98
A3B1	0	0	35	66	95
A1B2	0	0	21	55	76
A2B2	0	0	19	52	68
A3B2	0	0	8	25	49
A1B3	0	0	13	36	53
A2B3	0	0	0	30	46
A3B3	0	0	0	14	33
Flat	0	31	28,3	45,8	57,5
Description	**MBC**				

Description: "0" indicates no growth of *Staphylococcus aureus* bacteria.

Table 12 shows that at a concentration of 100% hydrolysate there was no growth of *S.aureus* bacteria, it can be stated that this concentration can be stated as the MBC value. So it can be seen that at a concentration of 50% protein hydrolysate of gold snail is not only able to inhibit the activity of *E.coli* bacteria, but also able to kill these bacteria. This is different from the test on *S.aureus* bacteria which showed its minimum killing ability at a concentration of 100%.

CHAPTER V
CONCLUSIONS AND SUGGESTIONS

A. Conclusion

1. The results of the analysis of the goldfish protein hydrolysate obtained, it can be seen that there is a significant interaction ($p \leq 0.05$) between the treatment of enzyme concentration and hydrolysis duration on the soluble protein content, degree of hydrolysis and total peptide concentration.
2. Antimicrobial activity testing showed inhibitory activity only on *Eschericea coli* bacteria with the highest Diameter of Inhibition Area (DDH) in the treatment of 18 hours hydrolysis time with 10% enzyme concentration of 8.8 mm. Determination of the *Minimum Inhibitory Concentration* (MIC) and *Minimum Bactericidal Concentration* (MBC) values for *Eschericea coli* bacteria is at 50% concentration of goldfish protein hydrolysate, while for *Staphylococcus aureus* bacteria at 100% hydrolysate concentration.
3. Antimicrobial activity testing on gold snail protein hydrolysate with enzyme concentration treatment and hydrolysis duration showed that the most effective inhibition on gram negative bacteria, namely *Escherichia coli*.

B. Advice

1. To obtain high antimicrobial activity, it is necessary to purify the hydrolysate using SDS-PAGE so that only the antimicrobial peptide components contained in the goldfish protein hydrolysate.
2. Further studies need to be carried out regarding the length of time the antimicrobial ability of the gold snail hydrolysate can last.

LITERATURE

Agustina, F. 2008. Formulation and Isothermic Study of Water Sorption of Instant Corn Porridge. Thesis. Postgraduate Program. Bogor Agricultural University. Bogor

[AOAC] *Association of Official Analytical Chemistry. 2012. Official Method of Analysis* 923.03, *Chapter* 32.1.05. Gaithersburg (US): AOAC.

[AOAC] *Association of Official Analytical Chemistry. 2012. Official Method of Analysis* 925.10, *Chapter* 32.1.03. Gaithersburg (US): AOAC.

Banjara RA, Jadhav SK, Bhoite SA. 2012. Antibacterial activity of di-2- ethylaniline phosphate screened by paper disc diffusion method. Journal of Applied Pharmaceutical Science. 2012:02(7):230- 3.

Bechinger B, Gorr SU. 2017. *Antimicrobial peptidas: Mechanism of action and resistance*. J Dent Res. 96:254-260.

Bloom William, Don W. Fawcett. 2002. Textbook of histology. 12th edition. Translation of Jan Tambayong. Jakarta: EGC.

Budiyono, S. 2006. Techniques for Controlling Carp Snails in Rice Plants. Journal of Agricultural Sciences, 2(2): 128-133.

Cazzaniga, N.J. 2002. Old species and new concepts in the taxonomy of pomacea (gastropoda: Ampullariidae). Biocell 26 (1):71-81.

Dolashka *et al.,* 2015. Bioactive Compounds Isolated From Garden Snails. J. BioSci. Biotechnol. 147-155

Domingues, M.M., Silva, P.M., Franquelim, H.G., Carvalho, F.A., Castanho, M.A.R.B., and Santos, N.C. 2014. Antimicrobial Protein rBPI $_{21}$ - Induces Surfec Changes On Gram Negative And Gram Positive Bacteria. *Nanomedicine: Nanotechnology, Biology, And Medicine*. Vol 10:543-551.

Effendi F, Anna P. Roswiem, Ernie Stefani. 2014. Antibacterial Activity Test of Probiotic Kombucha Tea Against *Escherichia coli* and *Staphylococcus aureus* Bacteria. Pharmacy Study Program, Bogor College of Industrial Technology and Pharmacy.

Febrianto, N.A. 2013. Protein Hydrolysate from Cocoa Beans and Coffee Beans. Jember: Warta of Coffee and Cocoa Research Center. pp. 20-23.

Gautam, S.S., Mishra, S., Dash, V., Amit, K. and Rath, G. 2010. Cooperative study or extraction, purification and estimation of bromelain from stem and fruit of pineapple plant. Thai J. Pharm., Sci. 34, 67-76.

Gunawan H & Effendi R.M.R.A. 2018. *Antimicrobial Peptides*. Department of Skin and Gender Health Sciences. Vol. 45 No. 1. 52-58

Hamid, S.A., Halim, N.R.A., and Sarbon, N.M. 2015. Optimization of Enzymatic Hydrolysis Conditions of Golden Apple Snail (*Pomaceae canaliculate*) Protein by Alcalase. International Food Research Journal 22 (4)

Haryawan, P., Muhammad Kholil, and Ade A.D.N. 2016. Analysis of decision making on determining the best hand antiseptic liquid with the *analytical hierarchy process* (AHP) method. PASTI Journal. 9(2).

Haslaniza, H., Maskat, M.Y., Wan Aida, W.M. and Mamout, S. 2010. The effects of enzyme concentration, temperature and incubation time on nitrogen content and degree of hydrolysis of protein precipitate from cockle (Anadara granosa) meat wash water. International Food Research Journal, 17: 147-152.

Hendarsih-Suharto and N. Kurniawati. 2009. "Toxicity of Rerak and Saponins to the Goldfish (Pomaceae Canaliculata Lamarck)". Agrikultura, 16(2).

Herdyastuti, N. (2006). Isolation and Characterization of Bromelin Enzyme Coarse Extract from Pineapple Stem (Ananas comusus L. merr. Berk. Research. Biology vol. 12, 75-77.

Holo H, Nilssen, Ness IF. 1991. Lactococcin A, a new bacteriocin from Lactococcus lactis subsp. cremoris: isolation and characterization of the protein and its gene. Journal of Bacteriology.173(12): 3879-3887.

Ilfandzahina, Maghfiroh Oktafiani. 2020. "Identification of *Umami* Component and Preparation of *Flavor Enhancer* from Goldfish (*Pomacea canaliculate*)". Thesis. Faculty of Engineering, Food Technology, UPN "Veteran" East Java.

Johnson AH, Peterson MS. 1974. *Encyclopedia of Food Technology*. Wesport Connecticut: The AVI Publ. Co. Inc. Vol. II.

Joshi. 2005. Misfolding of glycoproteins is a prerequisite for peptide: N-glycanase mediated deglycosylation. FEBS Lett 579(3):823-6

Kirk R E, Othmer J B. 1953. *Encyclopedia of Chemical Technology*. Volume IX. New York: The Interscience Encyclopedia Inc.

Koesoemawardani D, Nurainy D, Hidayati S. 2008. The process of making protein hydrolysate of raw fish. *Journal of Natu Indonesia* 13(3): 256-261.

Kristiansson, and G. A. Rasco, Fish Protein Hydrolysates: Production, Biochemical, and Functional Properties. Crit. Rev. Food Sci. Nutr., 40, 43. 2000.

Kumar S, Chouhan S, Sanghi A, Teotis UVS. 2013. Antioxidative Effect Of Yak Milk Caseinate Hydrolyzed With Three Different Proteases. Vet World 6: 799-802.

Lai Y, Gallo RL. 2009. AMPed up immunity: How antimicrobial peptidas have multiple roles in immune defense. Trends Immunol. 30:131-141.

Lehninger AL. 1993. *Basic Biochemistry I*. Maggy Thenawidjaja, translator. Jakarta: Erlangga Publishers. Translation of : *Principles of Biochemistry*.

Li H, Parisi MG, Parrinello N, Cammarata M, Roch P. 2011. *Molluscan Antimicrobial Peptidas, A Review from Activity-based Evidences to Computer-assisted Sequences. Journal International Scholarly Research.* 8: 85-97.

Luo HY, Wang B, Li ZR, Chi CF, Zhang QH, He GY. 2012. Preparation and evaluation of antioxidant peptides from papain hydrolysate of Sphyrna lewini muscle protein. Food Science Technology. 51: 281- 288.

Malanovic, Nermina and Karl Lohner. 2016. Antimicrobial Peptides Targeting Gram-Positive Bacteria. Institute of molecular biosciences, biophysics division, University of Graz. 9:59

Mandila, S.P. and N. Hidajati. 2013. Identification of amino acids in silk worms (Tubifex sp.) extracted with acetic and lactic acid solvents. UNESA J. of Chemistry, 2(1):103- 109.

Maulida, R and Guntarti, A. 2015. Effect of Black Rice (Oryza Sativa L.) Particle Size on Extract Yield and Total Anthocyanin Content. Fharmaciana. Vol. 5 No. 1: 9-16.

Meri Amerikova, Ivanka Pencheva El-Tibi, Vania Maslarska, Stanislav Bozhanov & Konstantin Tachkov. 2019. Antimicrobial activity, mechanism of action, and methods for stabilization of defensins as new therapeutic agents, Biotechnology & Biotechnological Equipment, 33:1, 671-682.

Murray, R. K., Granner, D. K. & Rodwell, V. W. 2009. Harper's Biochemistry (27th ed.). Jakarta: EGC Medical Books.

Nagasawa T, Yonekura T, Nishizawa N, Kitts DD. 2001. In Vitro and in vivo inhibition of muscle lipid and protein oxidation by carnosine. Moll Cell Biochem, 225.29-34.

Naiola E & N Widhyastuti. 2007. Semi Purification and Characterization of Protease Enzyme of Bacillus sp. Berk. Biological Research (13): 51-56

Najafian L and Babji AS. 2012. A review of fishderived antioxidant and antimicrobial peptidas: their production, assessment, and applications. Peptidas. 33: 178-185.

Nam BH, Seo JK, Lee MJ, Kim YO, Kim DG, An CM, Park NG. 2015*. Functional Analysis of Pacific oyster (Crassostrea gigas) β-thymosin: Focus on antimicrobial activity. Journal of Fish and Shellfish Immunology.* 45: 167-174.

Novaes, L.C.d.L. et al., 2015. Stability, purification, and applications of bromelain: a review. Biotechnology Progress, pp.1-34.

Prakash, A. 2001. Antioxidant Activity Medallion Laboratories-Analytical Progress. Volume 19. Number 2. pp 1-4.

Priya, S. P., Jayakumar, Mathai, V., Chintu & Babu, S. 2012. Immobilization and Kinetic Studies of Bromelain: A Plant Cysteine Bromelin From Pineapple (Ananas comosus) Plant Parts. Int J Med Health Sci., 1 (3): 10-16.

Purwaningsih, Indah. 2017. Potential of Bromelin Enzyme of Pineapple Fruit Juice (Ananas comosus I.) in Increasing Tofu Protein Content. Poltekkes Kemenkes Pontianak. Vol 6 No 1.

Poedjiadi, A and Supriyanti, T.2009. Fundamentals of Biochemistry Revised Edition Jakarta: UI-Press.

Rachmawati, Andhy I.B. 2015. "Effectiveness Test of Protein Hydrolysate of Melinjo Seed (*Gnetum gnemom L.)* as Antibacterial Agent". Thesis. Faculty of Pharmacy, University of Jember.

Rajapakse N, Mendis E, Jung WK, Je JY, Kim SK. 2005. *Purification of a radical scavenging peptide from fermented mussel sauce and its antioxidant properties*. Food Res Int, 38.175-82.

Rawlings N.D., Morton F.R., & Barret A.J. 2007. *An Introduction to Peptidases and the MEROPS Database.* In: Polaina J, MacCabe AP, editors. Industrial Enzymes: Structure, Function and Application. Netherlands: Springer. pp. 161-179.

Restiani, Ratih. 2016. Enzymatic Hydrolysis of Nyamplung (*Calophylum Inophyllum)* Seed Cake Protein Using Bromelain. Biology Study Program, Faculty of Biotechnology, Universitas Kristen Dua Wacana Yogyakarta.

Rostinawati, Tina. 2009. Antibacterial Activity of Ethanol Extract of Rosella Flower (Hibiscus sabdariffa L) against Escherichia coli, Salmonella typhi and Staphylococcus aureus by Agar Diffusion Method. Padjajaran University: Jatinangor.

Ruslan and Harianto. *2009*. Management of Goldfish Pests.Yogyakarta: PT Citra Aji Parama.

Saito K, Jin DH, Ogawa T, Muramoto K, Hatakeyama E, Yasuhara T. 2003. *Antioxidative properties of tripeptide libraries prepared by the combinatorial chemistry.* J Agric Food Chem, 51.3668-74.

Sarkar, S., Ahmed, M., Mozumder, N.H.M.R. & Saeid, A., 2017. Isolation and characterization of bromelain enzyme from pineapple and its utilization as anti-browning agent. *Process Engineering Journal*, 1, pp.52-58.

Sathyan N, Philip R, Chaithanya ER, Kumar PRA. 2012. *Identification and molecular characterization of molluskin, a histone- H2A-derived antimicrobial peptide from molluscs. Review Article International Scholarly Research Network.* 2012:1-6.

Seniman, M.S.M., Yusop, S.M., and Babji, A.S. 2014. Production of Enzymatic Protein Hydrolysates From Freshwater Catfish (*Clarias batrachus*). AIP Conference Proceefdings 1614,323.

Setiabudi, R. 2007. Introduction to Antimicrobials, in Pharmacology and Therapy 5th Edition. Jakarta: FKUI Publishing House.

Siagian, Sondang. 2007. Managerial functions revised edition. Jakarta. Bumi Aksara.

Soeparno. 2011. *Nutrition* Science *and Meat Nutrition*. Gajah Mada University Press. Yogyakarta.

Sudarmadji, S., B. Haryono, & Suhardi. 1997. Analytical Procedures for Food and Agricultural Materials Fourth Edition. Yogyakarta: Liberty Publishers.

Sulistiono. 2007. Management of Goldfish (Pomacea canaliculata). Repository. Department of Aquatic Resources Management. Bogor Agricultural University.

Supartono. (2004). Characterization of Neutral Protease Enzyme from Fresh Pineapple Fruit. Jurnal MIPA Universitas Negeri Semarang 27 (2), 134-142.

Syahrizal FSNA. 1991. Microbiology of fish sauce made by enzymatic hydrolysis. [Thesis]. Bogor: Faculty of Agricultural Technology, Bogor Agricultural University.

Ulagesan S, Kim JH. Antibacterial and antifungal activities of proteins extracted from seven different snails. Appl Sci, 2018; 8(8), 1362.

Virtanen, T., Pihlanto, A., Akkanen, S., Korhonen, H. 2006. Development of Antioxidant Activity in Milk Whey During Fermentation with Lactic Acid

Bacteria. Biotechnology and Food Research, MTT Agrifood Research Finland, Jokiomen, Finland.

Waluyo, Lud. 2004. General Microbiology. UMM PRESS, Malang.

Wang WY and De Mejia EG. 2005. A new frontier in soy bioactive peptidas that may prevent age-related chronic diseases. Compr Rev Food Sci Food Saf. 4.63-78.

Whitaker, JR. 1994. *Principles of Enzymology for the food Sciences*. Marcel Dekker Inc. New York.

Widadi, I.R. 2011. Preparation and Characterization of Protein Hydrolysate from Dumbo Catfish (Clarias gariepinus) Using Papain Enzyme [Thesis]. Faculty of Fisheries and Marine Science, IPB.

Winarno, F. G. (*2008*). Food Science and Nutrition. Jakarta: Gramedia Pustaka Utama

Winarsi H, 2007. Natural antioxidants and free radicals potential and application in health. Yogyakarta. Kanisius.

Wu Hui-chun, Chen HM, Shiau CY. 2003. Free amino acids and peptides as related to antioxidant properties in protein hydrolysates of mackerel (Scomber austriasicus). Food Research. 36: 949-957.

Wuryanti. 2004. Isolation and Determination of Specific Activity of Bromelin Enzyme from Pineapple Fruit (*Ananas comosus L*.). J.Kim. Science & Appl. Vol. 7 (3)

Yazid, E and Nursanti, L. 2006. Biochemistry Practicum Manual for Analysis Students. Yogyakarta: Andi Yogyakarta.

Zoysa MD, Nikapitiya C, Whang I, Lee JS, Lee J. 2009. *Abhisin: A potential antimicrobial peptide derived from histone H2A of disk abalone (Haliotis discus discus)*. Journal of Fish and Shellfish Immunology. 27 : 639- 646.

Appendix 1. Analysis Procedure

1. Analysis Procedure

a. Moisture Content (AOAC 2012 925.10)

The dishes were oven dried at $130℃$ for 15 minutes, then cooled in a desiccator for 10 minutes. The dried cup was weighed before use. About 2 g of sample was weighed into the cup, then dried in an oven at 130°C for 1 hour, cooled in a desiccator and weighed until the weight was constant. Moisture content was calculated using the following formula:

$$\text{Moisture content (\%bk)} = a\text{-}(b\text{-}c)a \times 100\ \%$$

Description:

a: initial sample weight (g)

b: weight of sample and cup after drying (g)

c: weight of empty cup (g)

b. Ash Content (AOAC 2012 923.03)

The porcelain cup was dried in a $105℃$ oven for 15 minutes and cooled in a desiccator. The dried porcelain cup was weighed and the weight recorded before use. A total of 3.0-5.0 g of sample was weighed in the porcelain cup and placed in an electric furnace at $550℃C$ until complete ashing. After the ashing is complete, the sample cup is cooled in a desiccator, then weighed. Weighing is repeated until a fixed weight is obtained. Calculation of ash content is done by using the following formula:

$$\text{Ash content (\%)=C-AB-A } x \text{ 100\%}$$

Description:

a: weight of empty cup (g)

b: weight of cup + initial sample (g)

c: weight of cup + dry sample (g)

c. Degree of Hydrolysis method *soluble protein content in* TCA (Silvestre *et al*, 2013)

A total of 1 mL of sample was added with 1 mL of 10% TCA, then incubated for 30 minutes after which it was centrifuged at 3000 rpm for 15 minutes. The supernatant obtained was then analyzed for soluble protein content using the Lowry method. The percentage of hydrolysis degree (DH) was determined by the following equation:

$$DH\ (\%) = \frac{Protein\ terlarut\ dalam\ 10\ \%\ TCA\ (mg)}{Total\ Protein\ (mg)}\ x\ 100\%$$

d. Soluble protein content of the Lowry method (Sudarmadji et al., 1997).

A total of 0.05 grams of sample was dissolved to 50 ml. The solution was taken as much as 1 ml and then added up to 4 ml. Adding 5.5 ml of reagent (3) into each test tube, mixed evenly using a vortex and incubated for 15 minutes. Add 0.5 ml of reagent (4) into each test tube, mixed evenly using a vortex and incubated for 30 minutes. Measurement of absorbance at a wavelength of 650 nm using a spectrophotometer. Blanko is made in the same way but without the next sample dissolved protein can be calculated by the formula obtained from the standard curve with the formula y = ax + b obtained from the standard curve.

e. Total Peptide Bond Concentration (Yang *et al.,* 2020)

The protein hydrolysate was mixed with 15% TCA solution (2:1) at 25℃ for 1 hour. The mixture was then centrifuged for 10 min. The supernatant was then treated with the Follin-phenol method and the absorbance was measured with a spectrophotometer at a wavelength of 680 nm.

f. Antibacterial Activity Testing by Disc Diffusion Method

The test bacterial suspension was inoculated on NA media as much as 0.1 mL, then leveled with a hockey stick and allowed to dry. Paper discs that have

been soaked in gold snail protein hydrolysate in each treatment for 15 minutes are then placed on the surface of the media aseptically. The clear zone around the disc paper was observed.

g. Antibacterial Activity Testing with MIC Method

Bacterial preparations of *Staphylococus aureus* and *Eschericea coli* stored on agar media were taken with a sterile ose needle, then implanted on an inclined agar medium by scratching. Bacteria that have been scratched on agar media are incubated in an incubator at 37 ℃ for 1 x 24 hours. Bacteria that have been incubated are taken colonies from the inclined agar media using a sterile ose needle and then put into NB until the turbidity is the same as the McFarland standard 1. Then as many as 7 sterile test tubes were prepared. Each test tube was labeled 1-7, then tube 6 was labeled K(+) which is a positive control, which is a tube containing bacterial suspension equivalent to McFarland 1 turbidity standard. Tube 7 was labeled K(-) which is a negative control, which is a tube containing NB medium. Tube 1 was filled with 4 ml of 100% concentration of gold snail protein hydrolysate. Tubes 2-5 were filled with 2 ml of NB liquid medium. Then take 2 ml of solution from tube 1, put into tube 2, mixed until homogeneous so as to obtain a concentration of 50%. The same thing was done until tube 5 until all extract concentrations were obtained in a ratio of 1:2 (w/v). To test turbidity, 1 ml of bacterial suspension media that has been equalized with McFarland 1 turbidity standard was taken and put into test tubes labeled 1-5. Then all tubes were incubated at 37℃ for 1 x 24 hours with 2 repetitions of incubation. After each incubation, the turbidity was observed using the turbidimetric method or turbidity observation using a UV-Vis *Spectrophotometer* with a wavelength of 600 nm. If the turbidity of the tube is still equal to or more turbid than the K(+) tube containing the McFarland 1 bacterial suspension, it means that the bacteria can still grow well, but when the solution in the tube looks clearer than the K(+) tube, it means that bacterial growth is starting to be inhibited, this is what shows the Minimum Inhibitory Concentration (MIC). After turbidimetric observation at the 2nd incubation, the TPC test was then carried out to determine the bacteriostatic and bacteriocidal properties.

h. *Total Plate Count* (TPC) Test

The TPC test was carried out on PCA (Plate Count Agar) media containing the concentration of goldfish protein hydrolysate from the petri dish that looked the clearest, made 3 treatments or 3 petri dishes. Then each Petri dish was incubated at 37℃ for 1x24 hours. Colonies were counted using a *colony counter*.

Data and Analysis of Variance of Soluble Protein Content

Dissolved Protein Content Analysis Table

Treatment	Test I	Test II	Total	Average	STDEV
A1B1	2,34	2,19	4,53	2,265	0,10606602
A1B2	3,08	3,79	6,87	3,435	0,50204581
A1B3	3,81	3,82	7,63	3,815	0,00707107
A2B1	2,76	2,61	5,37	2,685	0,10606602
A2B2	3,93	3,97	7,9	3,95	0,02828427
A2B3	4,13	4,05	8,18	4,09	0,05656854
A3B1	3,21	3,32	6,53	3,265	0,07778175
A3B2	4,50	4,54	9,04	4,52	0,02828427
A3B3	3,96	3,82	7,78	3,89	0,09899495
Total	31,72	32,11	**63,83**		
Average	3,5244444	3,567778			

Two-way Table

A (enzyme)	B (Hydrolysis time)			AMOUNT	AVERAGE
	B1	B2	B3		
A1	4,53	6,87	7,63	19,03	6,34333333
A2	5,37	7,9	8,18	21,45	7,15
A3	6,53	9,04	7,78	23,35	7,78333333
AMOUNT	16,43	23,81	23,59	63,83	
AVERAGE	5,4766667	7,936667	7,863333		

Analysis of Variance (ANOVA) Table

SK	db	JK	KT	Fhit	Ftab (0.05%)
Treatment	8	8,14	1,02	31,03*	3,23
A	2	1,56	0,78	23,82*	4,26
B	2	5,88	2,94	89,57*	4,26
AB	4	0,70	0,18	5,37*	3,63
Error	9	0,30	0,03		
Total	17	8,44			

Notes: *) significant effect or interaction (F. count> F. table)

Appendix 3. 5% DMRT Test Table of Dissolved Protein Content

Sample Code	Average	A1B1	A1B3	A2B1	A1B2	A2B2	A2B3	A3B1	A3B3	A3B2	P	SSR	LSR
		2,265	2,685	3,265	3,435	3,815	3,89	3,95	4,09	4,52			
A1B1	2,265												
A1B3	2,685	0,42									2	3,199	0,4097
A2B1	3,265	1	0,58								3	3,339	0,4276
A1B2	3,435	1,17	0,75	0,17							4	3,42	0,4380
A2B2	3,815	1,55	1,13	0,55	0,38						5	3,47	0,4444
A2B3	3,89	1,625	1,205	0,625	0,455	0,075					6	3,502	0,4485
A3B1	3,95	1,685	1,265	0,685	0,515	0,135	0,06				7	3,523	0,4512
A3B3	4,09	1,825	1,405	0,825	0,655	0,275	0,2	0,14			8	3,536	0,4529
A3B2	4,52	2,255	1,835	1,255	1,085	0,705	0,63	0,57	0,43		9	3,544	0,4539
NOTATION		**a**	**b**	**c**	**c**	**c**	**c**	**c**	**c**	**c**	**s.e**		**0,12807333**

Data and Analysis of Degree of Hydrolysis (DH)

Treatment	Test I	Test II	Total	Average	STDEV
A1B1	33,10	33,25	66,35	33,175	0,10606602
A1B2	42,18	43,05	85,23	42,615	0,6151829
A1B3	50,18	51,25	101,43	50,715	0,75660426
A2B1	36,86	36,59	73,45	36,725	0,19091883
A2B2	32,86	32,88	92,2	46,10	0,01414214
A2B3	62,87	64,05	126,92	63,46	0,834386
A3B1	27,76	26,65	54,41	27,205	0,78488853
A3B2	45,44	46,76	65,74	32,87	0,93338095
A3B3	56,74	56,87	113,61	56,805	0,09192388
Total	387,99	391,35	779,34		
Average	43,11	43,48333			

Two-way Table

A (enzyme)	B (Hydrolysis time)			AMOUNT	AVERAGE
	B1	B2	B3		
A1	66,35	85,23	101,43	253,01	84,3366667
A2	73,45	92,2	126,92	292,57	97,5233333
A3	54,41	65,74	113,61	233,76	77,92
AMOUNT	194,21	243,17	341,96	779,34	
AVERAGE	64,736667	81,05667	113,9867		

Analysis of Variance (ANOVA) Table

SK	db	JK	KT	Fhit	Ftab (0.05%)
Treatment	8	2331,36	291,42	822,01*	3,23
A	2	299,68	149,84	422,65*	4,26
B	2	1888,14	944,07	2662,94*	4,26
AB	4	143,54	35,89	101,22*	3,63
Error	9	3,19	0,35		
Total	17	2334,56			

Notes: *) significant effect or interaction (F. count> F. table)

Appendix 5. 5% DMRT Test Table of Degree of Hydrolysis

Sample Code	Average	A1B1	A1B3	A2B1	A1B2	A2B2	A2B3	A3B1	A3B3	A3B2	P	SSR	LSR
		27,205	32,87	33,175	36,725	42,615	46,1	50,715	56,805	63,46			
A1B1	27,205												
A1B3	32,87	5,665									2	3,199	1,3469
A2B1	33,175	5,97	0,305								3	3,339	1,4058
A1B2	36,725	9,52	3,855	3,55							4	3,42	1,4399
A2B2	42,615	15,41	9,745	9,44	5,89						5	3,47	1,4610
A2B3	46,1	18,895	13,23	12,925	9,375	3,485					6	3,502	1,4744
A3B1	50,715	23,51	17,845	17,54	13,99	8,1	4,615				7	3,523	1,4833
A3B3	56,805	29,6	23,935	23,63	20,08	14,19	10,705	6,09			8	3,536	1,4887
A3B2	63,46	36,255	30,59	30,285	26,735	20,845	17,36	12,745	6,655		9	3,544	1,4921
NOTATION		**a**	**b**	**b**	**c**	**d**	**e**	**f**	**g**	**h**	**s.e**		**0,421023884**

Data and Analysis of Total Peptides

Treatment	Test I	Test II	Total	Average	STDEV
A1B1	3,23	3,37	6,6	3,3	0,09899495
A1B2	5,08	4,79	9,87	4,935	0,20506097
A1B3	6,81	6,82	13,63	6,815	0,00707107
A2B1	4,29	4,65	8,94	4,47	0,25455844
A2B2	6,93	6,97	13,9	6,95	0,02828427
A2B3	8,50	8,32	16,82	8,41	0,12727922
A3B1	4,07	4,27	8,34	4,17	0,14142136
A3B2	5,52	5,48	11	5,5	0,02828427
A3B3	10,96	10,82	21,78	10,89	0,09899495
Total	55,39	55,49	110,88		
Average	6,1544444	6,165556			

Two-way Table

A (enzyme)	B (Hydrolysis time)			AMOUNT	AVERAGE
	B1	B2	B3		
A1	66,35	85,23	101,43	253,01	84,3366667
A2	73,45	92,2	126,92	292,57	97,5233333
A3	54,41	65,74	113,61	233,76	77,92
AMOUNT	194,21	243,17	341,96	779,34	
AVERAGE	64,736667	81,05667	113,9867		

Analysis of Variance (ANOVA) Table

SK	db	JK	KT	Fhit	Ftab (0.05%)
Treatment	8	2331,36	291,42	822,01*	3,23
A	2	299,68	149,84	422,65*	4,26
B	2	1888,14	944,07	2662,94*	4,26
AB	4	143,54	35,89	101,22*	3,63
Error	9	3,19	0,35		
Total	17	2334,56			

Notes: *) significant effect or interaction (F. count> F. table)

Appendix 7. 5% DMRT Test Table of Total Peptide Concentration

Sample Code	Average	A1B1	A1B3	A2B1	A1B2	A2B2	A2B3	A3B1	A3B3	A3B2	P	SSR	LSR
		27,205	**32,87**	**33,175**	**36,725**	**42,615**	**46,1**	**50,715**	**56,805**	**63,46**			
A1B1	27,205												
A1B3	32,87	5,665									2	3,199	1,3469
A2B1	33,175	5,97	0,305								3	3,339	1,4058
A1B2	36,725	9,52	3,855	3,55							4	3,42	1,4399
A2B2	42,615	15,41	9,745	9,44	5,89						5	3,47	1,4610
A2B3	46,1	18,895	13,23	12,925	9,375	3,485					6	3,502	1,4744
A3B1	50,715	23,51	17,845	17,54	13,99	8,1	4,615				7	3,523	1,4833
A3B3	56,805	29,6	23,935	23,63	20,08	14,19	10,705	6,09			8	3,536	1,4887
A3B2	63,46	36,255	30,59	30,285	26,735	20,845	17,36	12,745	6,655		9	3,544	1,4921
NOTATION		**a**	**b**	**b**	**c**	**d**	**e**	**f**	**g**	**h**	**s.e**		**0,421023884**

Data and Analysis of Diameter of Area of Hindrance (DDH)

Treatment	Test I	Test II	Total	Average	STDEV
A1B1	0,00	0,00	0	0	0
A1B2	5,00	5,70	10,7	5,35	0,49497475
A1B3	7,00	6,80	13,8	6,9	0,14142136
A2B1	0,00	0,00	0	0	0
A2B2	0,00	0,00	0	0	0
A2B3	7,00	6,40	13,4	6,7	0,42426407
A3B1	0,00	0,00	0	0	0
A3B2	7,00	6,50	13,5	6,75	0,35355339
A3B3	9,00	8,60	17,6	8,8	0,28284271
Total	35	34	69		
Average	3,8888889	3,777778			

Two-way Table

	B (Hydrolysis time)				
A (enzyme)	**B1**	**B2**	**B3**	**AMOUNT**	**AVERAGE**
A1	0	10,7	13,8	24,5	8,16666667
A2	0	0	13,4	13,4	4,46666667
A3	0	13,5	17,6	31,1	10,3666667
AMOUNT	0	24,2	44,8	69	
AVERAGE	0	8,066667	14,93333		

Analysis of Variance (ANOVA) Table

SK	db	JK	KT	Fhit	Ftab (0.05%)
Treatment	8	223,75	27,97	387,26*	3,23
A	2	26,67	13,34	184,64*	4,26
B	2	167,61	83,81	1160,40*	4,26
AB	4	29,47	7,37	102,00*	3,63
Error	9	0,65	0,07		
Total	17	224,40			

Notes: *) significant effect or interaction (F. count> F. table)

Appendix 9. 5% DMRT Test Table Diameter Area of Inhibition (DDH)

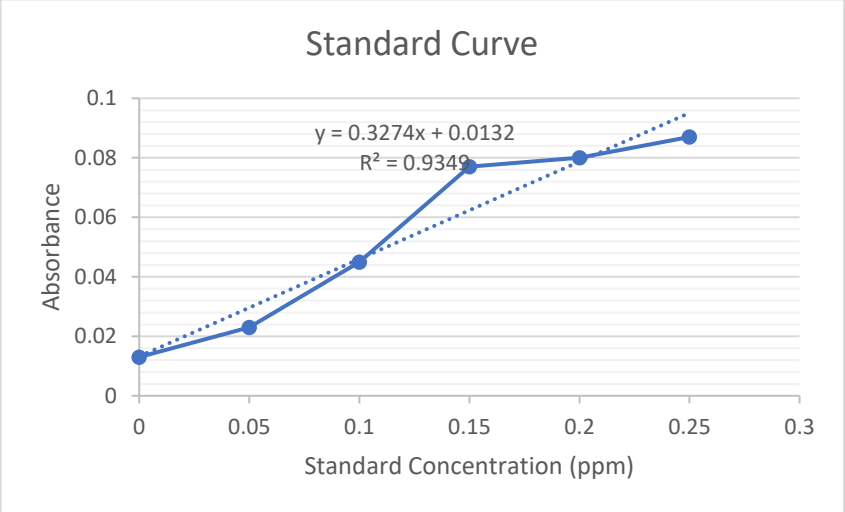
Sample Code	Average	A1B1	A2B1	A3B1	A2B2	A1B2	A2B3	A3B2	A1B3	A3B3	P	SSR	LSR
		0	0	0	0	5,35	6,7	6,75	6,9	8,8			
A1B1	0												
A2B1	0	0									2	3,199	0,6079
A3B1	0	0	0								3	3,339	0,6345
A2B2	0	0	0	0							4	3,42	0,6499
A1B2	5,35	5,35	5,35	5,35	5,35						5	3,47	0,6594
A2B3	6,7	6,7	6,7	6,7	6,7	1,35					6	3,502	0,6655
A3B2	6,75	6,75	6,75	6,75	6,75	1,4	0,05				7	3,523	0,6695
A1B3	6,9	6,9	6,9	6,9	6,9	1,55	0,2	0,15			8	3,536	0,6719
A3B3	8,8	8,8	8,8	8,8	8,8	3,45	2,1	2,05	1,9		9	3,544	0,6735
NOTATION		**a**	**a**	**a**	**b**	**c**	**c**	**c**	**d**	**f**	**s.e**		**0,190029238**

Appendix 10. Lowry's Dissolved Protein Standard Curve

- **BSA Standard Solution Table**

Concentration (ppm)	Absorbance
0	0,013
0,05	0,023
0,1	0,045
0,15	0,077
0,2	0,08
0,25	0,087

- **Standard Curve**

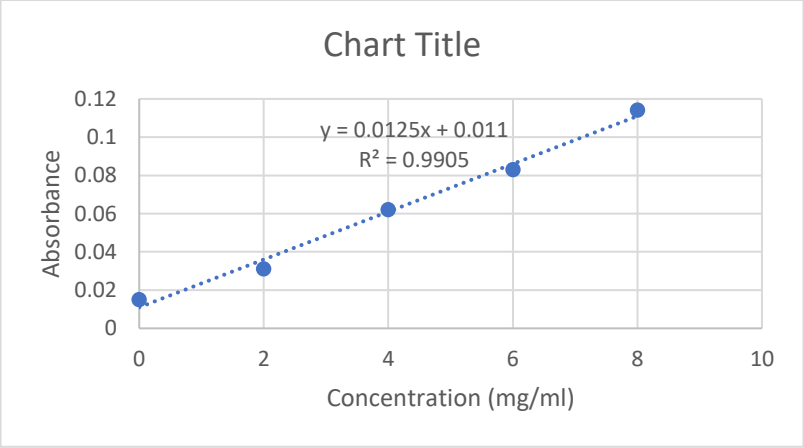


Standard Curve for Total Peptide Concentration

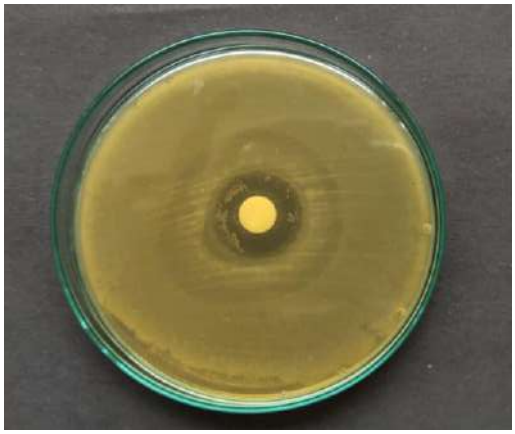
- **Standard Solution Table**

Concentration	absorbance
0	0,015
2	0,031
4	0,062
6	0,083
8	0,114

- **Standard Curve**



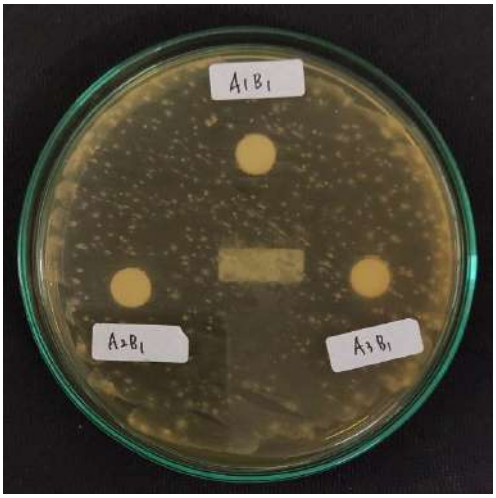
Documentation of Diameter of Inhibition Area (DDH) Testing



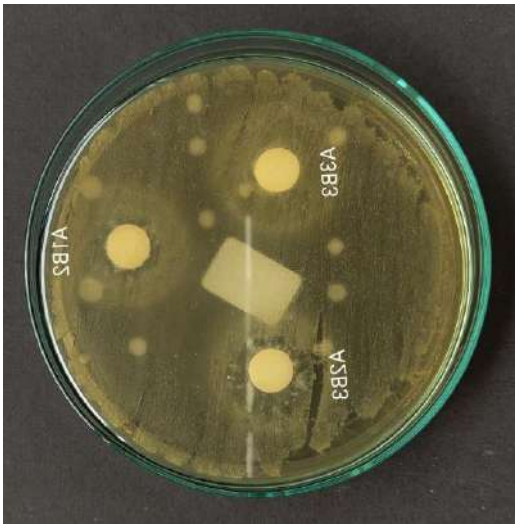
Positive Control *E.coli*

Positive Control *S.aureus*

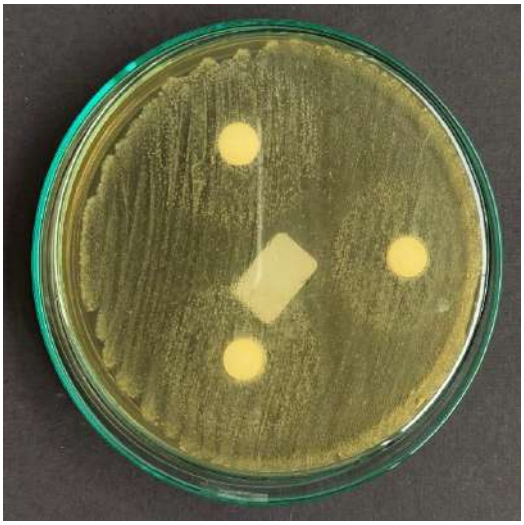
Hydrolysis Time 6 Hours
(E.coli)



Hydrolysis Time 6 Hours
(*S.aureus)*

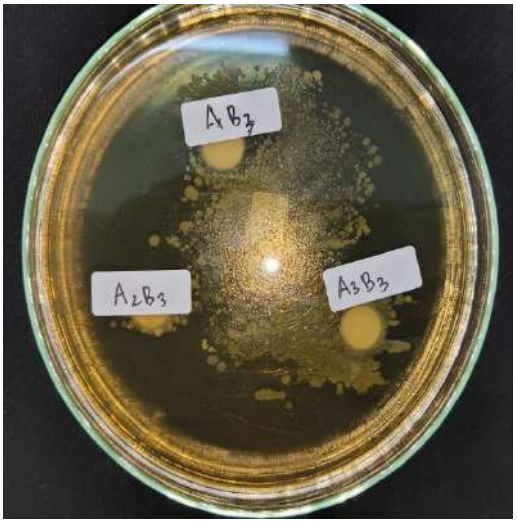


12 Hours Hydrolysis Time
(E.coli)



12 Hours Hydrolysis Time
(*S.aureus)*

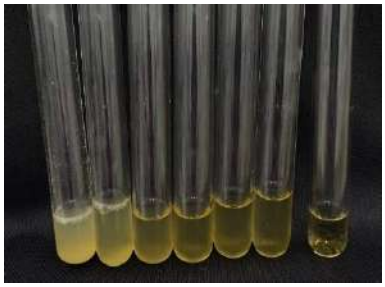
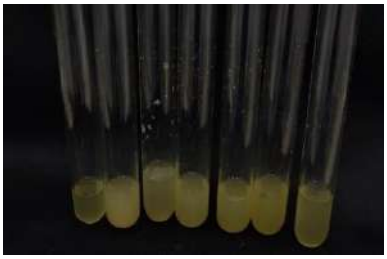
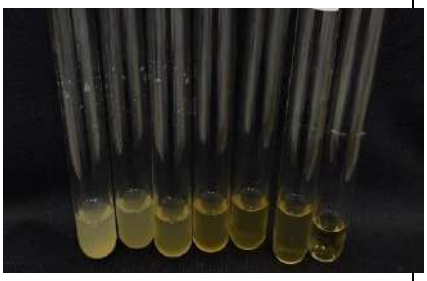
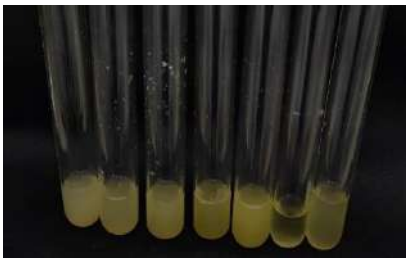
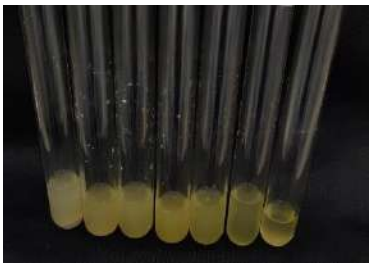
18 Hours Hydrolysis Time
(E.coli)



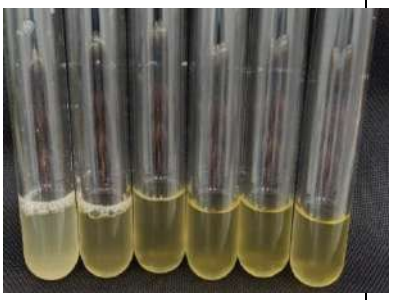
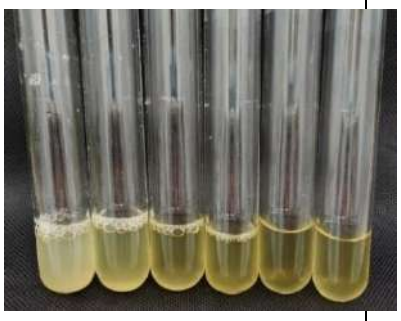
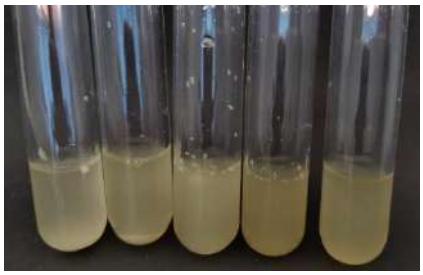
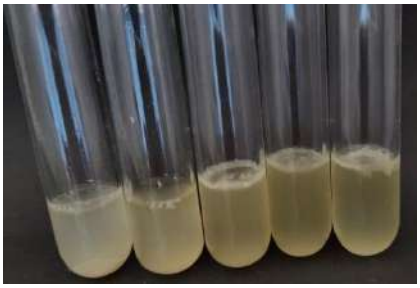
18 Hours Hydrolysis Time
(*S.aureus)*

Appendix 13. MIC Testing Documentation

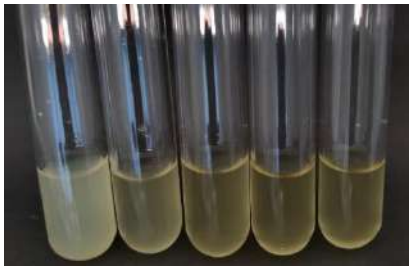
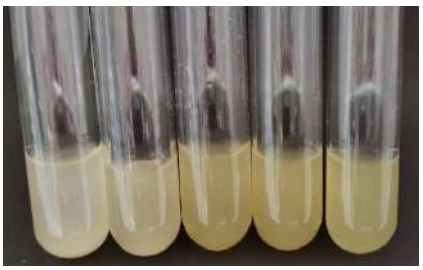
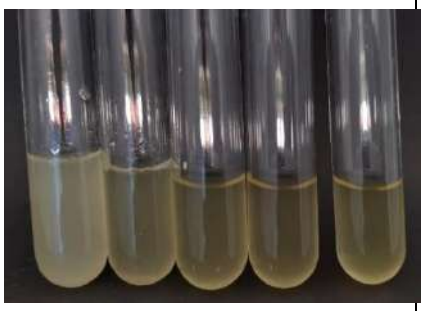
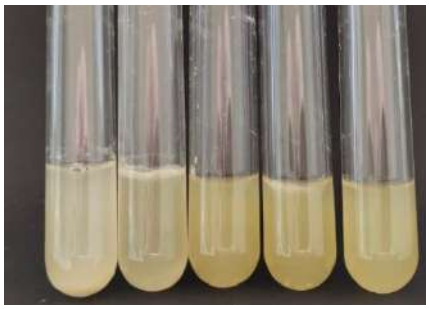
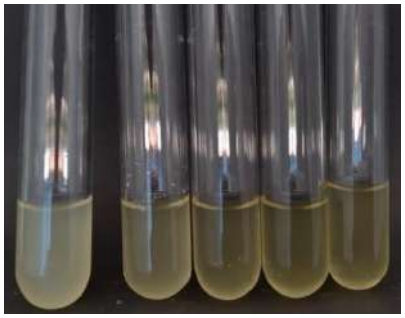
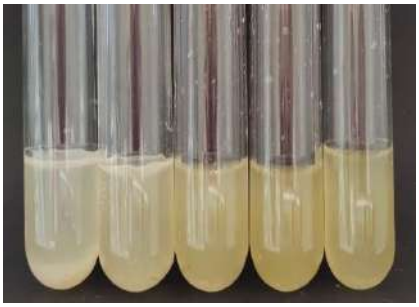
- **Hydrolysis Time 6 Hours *E.coli***

Enzyme Concentration	Before Incubation	After incubation
1%		
5%		
10%		

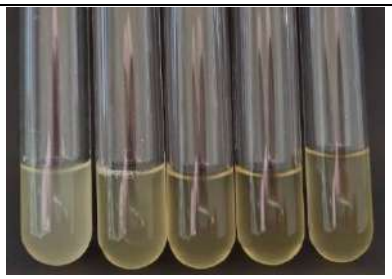
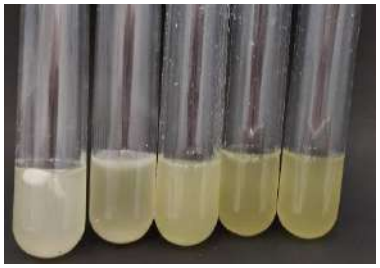
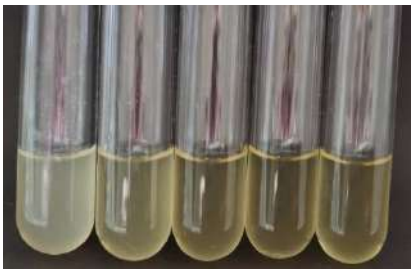
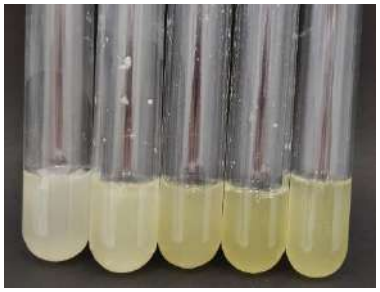
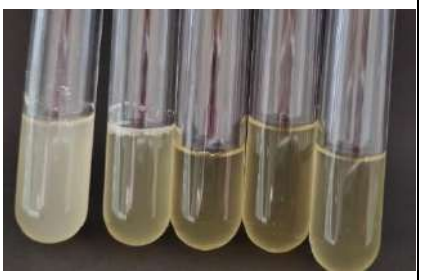
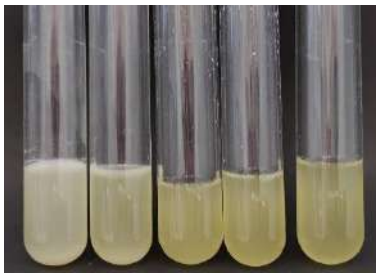
- **Hydrolysis Time 6 hours *S.aureus***

Enzyme Concentration	Before Incubation	After Incubation
1%		
5%		
10%		

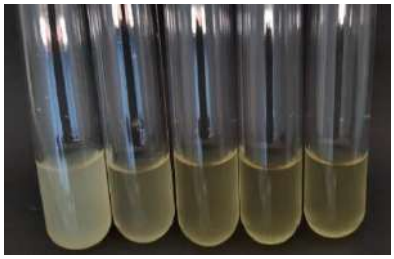
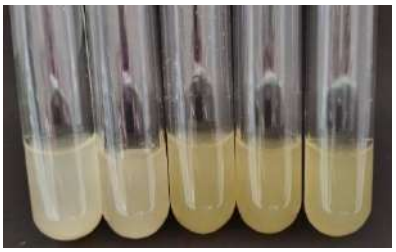
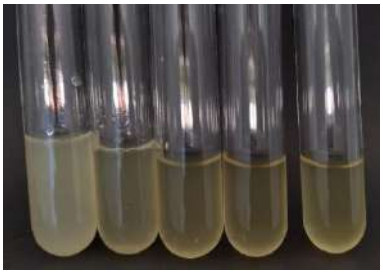
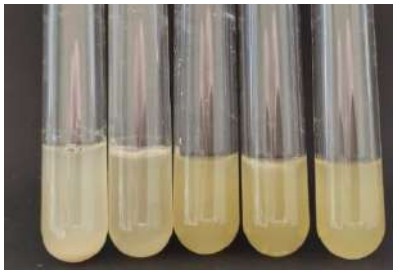
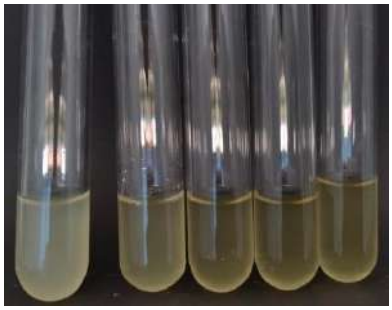
- **Hydrolysis Time 12 Hours *E.coli***

Enzyme Concentration	Before Incubation	After Incubation
1%		
5%		
10%		

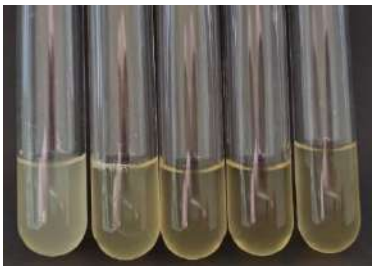
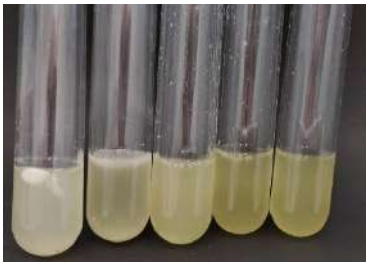
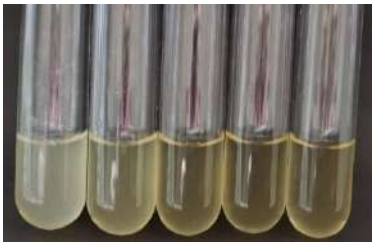
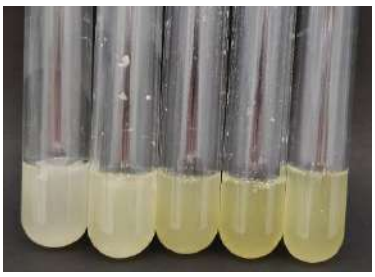
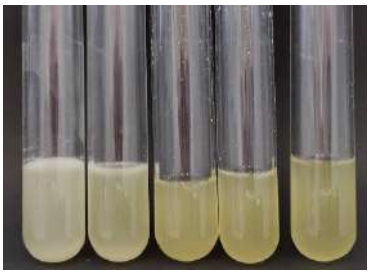
- **Hydrolysis Time 12 Hours *S.aureus***

Enzyme Concentration	Before Incubation	After Incubation
1%		
5%		
10%		

- **18 Hours Hydrolysis Time *E.coli***

Enzyme Concentration	Before Incubation	After Incubation
1%		
5%		
10%		

- **18 Hours Hydrolysis Time *S.aureus***

Enzyme Concentration	Before Incubation	After Incubation
1%		
5%		
10%		

Appendix 14. MBC testing