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Optimization Of Temperature And Ph For The Protease Production By Endophytic Fungi From Mangrove Sonneratia Alba

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Abstract — The research about optimization of temperature and pH for the protease production from mangrove Sonneratia alba endophytic fungi in Mandeh Area, Pesisir Selatan District was conducted from March to June 2023 at Biotechnology Laboratory, Andalas University, Padang. This research aims to determine the optimum temperature and pH of endophytic fungal isolates of mangrove plants Sonneratia alba from the Mandeh area, Pesisir Selatan District in producing protease and to determine the enzyme production after optimization. The research method used in this study was experimental and descriptive. Protease activity was determined using the Takami method. The results of the study showed that the optimum temperature for isolates EUA-124 and EUA-126 in producing protease is 34°C the optimum pH for isolate EUA-124 in producing protease is 7 and the optimum pH for isolate EUA-126 in producing protease is 6. Protease production by isolates EUA-124 increased by 8.621% and protease production by isolate EUA-126 increased by 3.334%.

Keywords — Endophytic fungi; Mangrove; Optimization; pH; Protease; Temperature

I. INTRODUCTION

Enzymes are biological compounds that serve as catalysts, speeding up biochemical and biological reactions (Panneerselvam, 2015). Without the use of a catalyst, an enzyme can accelerate a reaction 108 to 1011 times faster (Poedjiadi, 2006). Protease enzymes are one class of enzymes that is crucial to the expansion of the industry. Around 75% of enzyme applications sold globally for industrial use are hydrolytic enzymes, and roughly 60% of those are proteolytic enzymes (Ningthoujam& Kshetri, 2010; Rai *et al*., 2010).

Proteases are enzymes that catalyze the breaking of peptide bonds within proteins and polypeptides, producing oligopeptides or free amino acids as a result. Proteinases, which catalyze the degradation of protein molecules into more manageable fragments, and peptidases, which hydrolyze polypeptide fragments into amino acids, are two types of protease enzymes. Every live cell produces proteases for intracellular use or to be released into the environment for nutritional and defensive purposes. Aside from being physically necessary, they have also found various crucial functions to play in the health, food, textile, and medicine industries over the years (Correa *et al*., 2014).

Proteases, also known as proteolytic enzymes, are commercially important because they are used in bioremediation and waste treatment, detergents, cosmetics, and leather manufacture, silk degumming, animal cell culture, contact lens cleaning, therapy and diagnosis, and the pharmaceutical, photographic, and food industries (Rao *et al.*, 1998). Accordingly, many proteases

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are being studied by the pharmaceutical industry as possible therapeutic targets or as diagnostic and prognostic agent biomarkers (Turk, 2006). Proteases also serve important functions in plants, contributing to the processing, maturation, or destruction of certain protein sets in response to developmental cues or changes in environmental conditions. Furthermore, proteases are thought to be insecticides because they are required for the complete digestion of complex insect cuticles (Anwar & Saleemuddin, 1998; Gupta *et al.*, 2002; Harrison & Bonning, 2010; Hasan *et al.*, 2013; Kumar & Takagi, 1999; Murthy & Naidu, 2010; Nielsen & Oxenboll, 1998).

Protease activity, like that of most enzymes, is controlled by a variety of variables. Temperature, pH, and enzyme activators are among the most influential parameters (Leboffe, 2012). The majority of processes industry requires enzymes that can withstand extreme conditions. So it is very important for enzymes used to have optimal conditions in the range a wide range of temperatures and pH (Bizuye, 2014). The appropriate incubation period will result in maximum protease production with the resulting high enzyme activity (Yuniati, 2015).

Plants, animals, and microbes all can create proteases. Microorganisms are a source of very beneficial enzymes because these microorganisms have faster growth than animals and plants (Yuliana & Nuniek, 2014). Due to their lower production costs and greater stability, microbial enzymes are among the most significant of them. Since microbes can manufacture such a wide range of enzymes, their ability to produce proteases has increased recently. According to Tavano (2013), they produce nearly two-thirds of the proteases produced worldwide. Since fungi produce a wider variety of enzymes than bacteria do, and mycelia are easier to separate from the culture medium, fungi have increased their production of proteases in recent years (Veloorvalappil et al., 2013; Haddar et al., 2010). Plants can act as a reservoir for a variety of microorganisms known as endophytes (Bacon *et al.*, 2000).

Endophytes are microorganisms that live inside their hosts' tissues and carry out ecological functions without endangering the host. They are the source of numerous novel biomolecules, such as enzymes, and have been found all over nature (Pinheiro *et al.*, 2012). Endophytic fungi are intracellular live organisms in healthy plant tissues, which induce the host to produce secondary metabolites. This induction can be caused by genetic recombination or coevolution (Sugijanto *et al.*, 2004; Sia *et al.*, 2013). Huang in White *et al* (2014) stated that there is a correlation between the presence of endophytic fungi and the host plant's ability to produce secondary metabolites. The ability of endophytic fungi to synthesize secondary metabolites is an opportunity for large-scale production in a short time without causing ecological damage. The fungi found in mangrove endophytes have a significant potential to create secondary metabolites. The leaves of the mangrove plants serve as a habitat for many kinds of microfungi. Endophytic fungi play a crucial part in the process of plant nutrient uptake (Chanway, 1996).

Biotechnology Laboratory Andalas University has collections of endophytic fungal isolates from *sonneratia alba* namely EUA-120, EUA-121, EUA-122, EUA-123, EUA-124, EUA- 125, EUA-126, and EUA-127, and two isolates positive protease-producing with the codes EUA-124 and EUA-126. Based on the enormous ability of endophytic fungi and the biodiversity that exists in Indonesia, the prospect of research on endophytic fungi from plants in Indonesia is very large. Mangrove areas are the main ecosystems that support important life in coastal and marine areas. Many benefits can be taken from mangrove plants (Saprudin & Halidah, 2012). Considering its potential as a mangrove plant that lives in the tropics is very large, it is important to conduct research on endophytic fungi of mangrove plants as a producer of protease enzymes. This study aims to analyze and explore the optimum condition of protease production by endophytic fungi of mangrove plants.

II. METHOD OF RESEARCH

A. Growth Profile of Fungi and Protease Activity Assay

The growth profile of fungi and protease activity assay is the first step that is carried out. The fungal growth profile was carried out by measuring the biomass value of the fungal isolate for seven days. 5 ml of inoculum was pipetted into 95 ml of protease production medium in a 250 ml Erlenmeyer. Empty Eppendorf is weighed using analytical scales then 1,5 ml of fungal isolate inoculum is extracted, then centrifuged for 15 minutes at 6000 rpm. After centrifugation, supernatant was formed and the supernatant was discarded. Distilled water is added 2 times into the Eppendorf and then centrifuged again, after which it is put into an 80˚C oven and then weighed until the weight is stable. If the weight is stable, the biomass value of the fungi is calculated by using the following formula:

$$biomass\ value = constant\ weight - eppendorf\ weight$$

Protease activity in the crude enzyme extract was determined according to the method of Takami (1989) using casein as a substrate. 2% casein substrate was made, then the substrate solution was pipetted into a 14 ml Eppendorf for 0.5 ml. Then 0.5 ml of enzyme solution was pipetted into the tube and 0.25 Tris-HCL buffer 50 mM pH 8.0 and incubated at room temperature for 15 minutes. 0.5 ml of TCA was added and incubated at room temperature for 20 minutes. Then centrifuged at 6000 rpm for 20 minutes. After centrifugation 0.375 ml of the supernatant was taken and transferred into a new test tube. After that, the addition of Na_2CO_3 as much as 1.25 ml and 1N Folin Ciocalteu as much as 0.25 ml. The results of the incubation were then measured with a spectrophotometer with a wavelength of 578 nm. All the experiments were done in duplicate and mean values are presented. The enzyme activity was calculated by following the formula:

$$\text{PA} = \frac{Asp-A}{Ast-Abl} \text{ X } \frac{1}{T}$$

PA: Protease Activity Unit (Units/mL)

Asp: Sample Absorbance

Abl: Blank Absorbance

Ast: Standard Absorbance

T: Incubation Time (minute)

B. Determine the Optimum Temperature for Protease Enzyme Production

After determining the growth profile of fungi and protease activity assay, optimization of temperature was conducted. To determine the effective temperature for protease production by the fungal species, fermentation was carried out at 2°C intervals in the range of 28, 30, 32, 34, and 36°C. Incubated for its optimum period of protease production on the second day.

C. Determine the Optimum pH of Protease Enzyme Production

Optimization of pH is carried out after optimization of temperature. The temperature used in pH optimization fermentation is 34^0C obtained from temperature optimization. To determine optimal pH, fungus cultures were cultivated in a 250 mL flask containing a 100 mL optimized medium with different pH ranges 4.0; 5.0; 6.0; 7.0; and 8.0. The pH of the medium was adjusted by using 1 N HCl or 1 N NaOH. The flasks were incubated for their harvesting period of protease production on the second day.

III. RESULT AND DISCUSSION

A. Growth Profile and Protease Activity Assay Curve

Observations of the growth curve and protease activity assay curve of two mangrove endophytic fungal isolates (EUA-124 & EUA-126) are presented in Figure 1. and Figure 2.

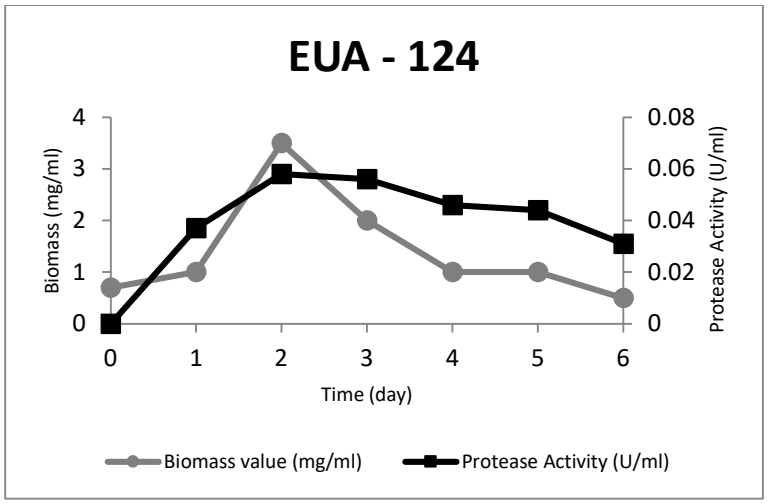

Figure 1. Growth profile and protease activity of isolate EUA-124 after being fermented on protease production media at 33°C, 100 rpm agitation for 6 days

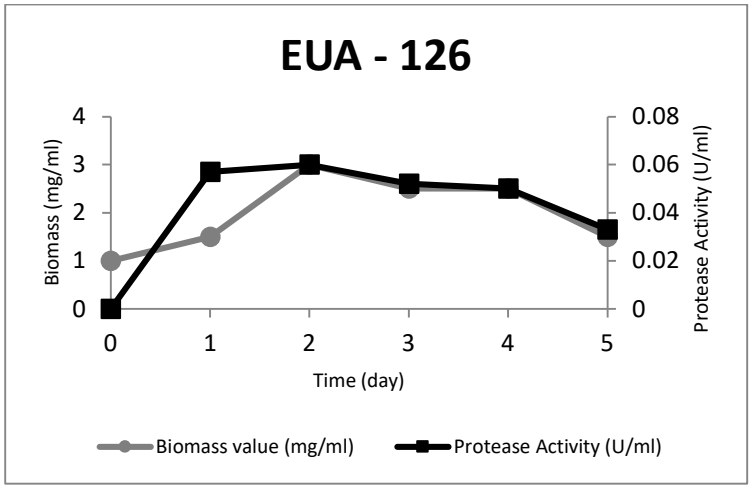

Figure 2. Growth profile and protease activity of isolate EUA-126 after being fermented on protease production media at 33°C, 100 rpm agitation for 5 days

In Figure 1, the growth curve of isolate EUA-124 shows that the lag phase occurred for a day. The lag phase is a phase in which cells adapt to the new environment and the formation of enzymes to break down substrates takes longer (MooreLandecker, 1996). In this phase, cells are biochemically active but not dividing (Patil *et al*., 2008). In isolate EUA-126 (Figure 2) the lag phase was not detected. This is due to the media used for inoculum being the same as the medium used for production, therefore the fungi do not need to adapt for a long period. If the growth medium and environment are the same as the previous medium, no adaptation time may be required (Suprihatin, 2010).

The exponential phase of isolate EUA-124 occurred from day 1 to day 2. The availability of sufficient nutrients allows cells to divide so that cell numbers increase rapidly (Patil *et al.*, 2008). While the exponential phase in isolating EUA-126 significantly occurred until day 2. The exponential phase is the most suitable phase for starters because metabolic activity takes place actively and optimally and can synthesize materials quickly and in constant quantities (Purkan, 2014). In this phase growth rate is strongly influenced by the medium in which it grows such as pH and nutrient content, as well as environmental conditions including temperature (Suprihatin, 2010).

The stationary phase of isolates EUA-124 and EUA-126 started on day 2. Stationary phase duration in both isolates was very short, this is due to in that phase the metabolism of both isolates being very high. Many compound's secondary metabolites

can be harvested in the stationary phase (Gandjar, 2006). According to Simanjuntak *et al*. (2002), the fungus produces secondary metabolites with the highest intensity at the end of the exponential phase or the beginning of the stationary phase.

The death phase of EUA-124 and EUA-126 occurred after day 3 where there is a visible decrease in the amount of biomass. The death phase occurs when the medium runs out of nutrients, presence of toxic substances, and depletion of energy reserves in the cell, then the fungal population will decrease number. The death rate accelerates exponentially; depending on the species, all cells die within a few days (Brock *et al.,* 2003).

Based on the growth profile, the best time to harvest the fungus is on day 2 for both isolates, when the fungus is in the logarithmic phase. The activity of the fungus and growth rate increase during this phase (Fardiaz, 1988).

Figure 1 also shows the highest protease activity of isolate EUA-124 with an enzyme activity value of 0.058 U/ml and isolate EUA-126 (Figure 2) with an enzyme activity value of 0.06 U/ml on day 2. This is the optimum time to harvest the enzyme. The increase in protease activity value is thought to be caused by the high metabolic activity of microbial cells in cell division and enzyme synthesis. As the number of cells increases, the secretion of enzymes increases so that protease activity will also increase. This is to the statement of Das (2013), where enzyme secretion depends on the number of cells and the growth phase of specific microorganisms. According to Choliq (2008), the ability of fungi to produce high protease enzymes is influenced by differences in the variation of protease encoding genes for each type and strain of fungi. Variances in protease activity can also be caused by differences in substrates used for fermentation as extracellular enzyme production can be affected by protein substrates and medium composition (Vermelho *et al*. 1996).

Extracellular enzymes are synthesized by microorganisms during the exponential phase until the stationary phase following the pattern of their growth curve. The highest enzyme activity is attained as the exponential phase ends in the growth curve (Ire *et al.,* 2011). This is also supported by Qadar *et al.* (2009) and Kumaraswamy *et al*. (2012), which state that optimal protease enzyme production is attained at the end of the exponential phase or early stationary phase.

Protease activity in both isolates decreased on day 3. The decrease in protease activity can be caused by insufficient nutrients and substrates in the production medium (Srividya & Mala, 2009). Lack of nutrients causes stressful and unfavorable conditions for microorganisms, decreasing enzyme activity (Khusro, 2016). Nutrients and substrates that are depleted result in the amount of enzymes secreted also decreasing. Decreased protease activity can also occur along with cell autolysis due to the accumulation of various enzymes in the medium (Olajuyigbe, 2013). In addition, crude enzymes if left too long in the production medium will become unstable (Rabelo *et al*., 2011).

B. Temperature Optimization for Protease Production

The results of temperature optimization for protease production by EUA-124 and EUA-126 with 2 days incubation time are presented in the following figure.

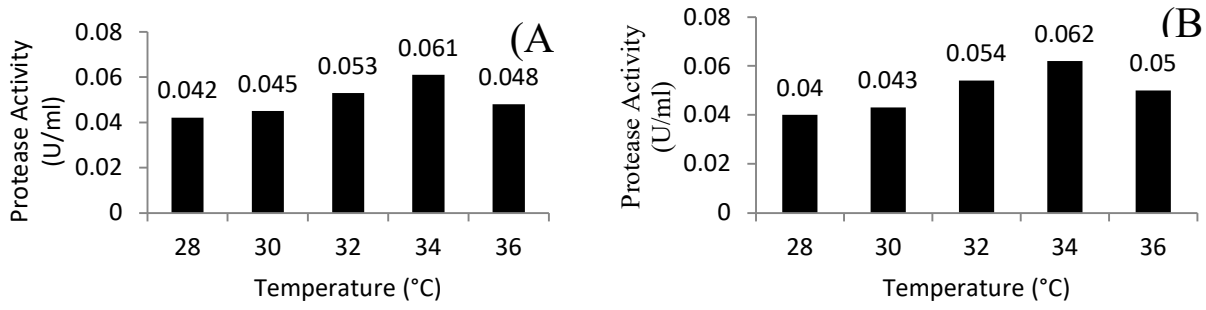

Figure 3. Histogram of optimum temperature for protease production of isolates EUA-124 (A) and EUA-126 (B) with temperature variations, 100 rpm agitation for 2 days.

Isolates EUA-124 and EUA-126 had the highest protease activity values at 34ºC with enzyme activity values of 0.061 U/ml and 0.062 U/ml. In this study, protease activity at low temperatures tended to be low. This is as stated by Garret (1999) that at low temperatures, enzymatic reactions take place slowly, an increase in temperature will accelerate the reaction by increasing

the average energy of the reactant molecules, causing the activation energy of the reaction to be lower, thus increasing the reaction rate until the optimum temperature is reached and the enzymatic reaction reaches its maximum.

Enzyme activity increases as temperature increases until the optimal temperature is reached. Each enzyme has an optimal temperature to produce the highest enzyme (Sayem *et al*., 2006). An increase in temperature causes enzyme activity to increase due to an increase in kinetic energy. This can increase the intensity between the enzyme and the substrate. Increased collision intensity will make it easier to form enzyme complexes with substrates so that more products will be formed (Noviyanti *et al.*, 2013). But at 36°C protease activity in both isolates decreased. This is due to the denatured proteins in the enzyme causing a decrease in enzyme activity to return to the minimum phase. Increased environmental temperature around the enzyme will cause the breakage of hydrogen bonds, ionic bonds, or hydrophobic interactions so that the tertiary structure of the enzyme changes, which causes the enzyme fold structure to open on the surface so that the active side of the enzyme changes resulting in a decrease in enzyme activity (Whitaker, 1994).

C. pH Optimization for Protease Production

pH affects the speed of enzyme activity in catalyzing a reaction. This is because the concentration of hydrogen ions affects the dimensional structure of the enzyme and its activity. Each enzyme has an optimum pH at which its three-dimensional structure is most conducive to binding the substrate. When the hydrogen ion concentration changes from the optimum concentration, the enzyme activity is progressively lost until eventually the enzyme becomes non-functional (Lehninger, 1997).

The results of pH optimization for protease production by EUA-124 and EUA-126 with 2 days incubation time are presented in the following figure.

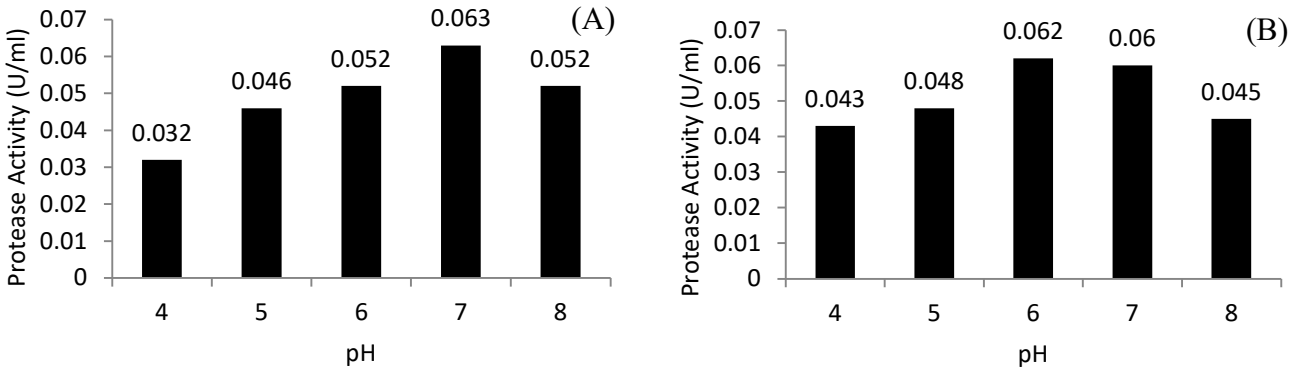

Figure 4. Histogram of optimum pH for protease production of isolates EUA-124 (A) and EUA-126 (B) at 34°C with pH variation, 100 rpm agitation for 2 days.

Figure 4 shows the optimum pH for EUA-124 with the highest protease activity at pH 7 with a protease activity value of 0.063 U/ml. The highest activity of EUA-126 with an enzyme activity value of 0.062 U/ml was at pH 6. This may be related to the habitat of the endophytic fungus itself, by the literature that says the optimum temperature and pH conditions of protease production are likely to reflect the climatic conditions found in environments inhabited by the original host plant (Zaferanloo *et al.* 2013). In this study, endophytic microbial samples were taken in the manner mangrove area with an average pH ranging from 6 to 7. This is also supported by Suparnorampius *et al*., (2020) who stated that the optimum pH for the growth of a proteolytic microbe ranges from 5-7 which is related to enzyme activity in the process of catalyzing growth reactions. If it is not optimal, the resulting enzyme work reaction can affect the growth rate of the microbe.

The highest protease enzyme activity is at the appropriate ionization level to bind to the substrate. It has a very stable enzyme conformation so the effectiveness of enzyme-substrate binding is high (Malle *et al*., 2015). Enzyme activity that decreases due to changes in pH is caused by changes in the ionic state of the substrate and enzyme. These changes can occur in amino acid residues that function to maintain the tertiary and quaternary structures of active enzymes (Palmer, 1981).

Each strain of microorganisms has a different optimum pH to be able to produce protease. Previous research conducted by Devi *et al.* (2012), in their research, found that A. niger was able to produce the highest protease enzyme activity on day 7 with

a pH range between neutral to alkaline (7-9). While the results of research by Coral *et al.* (2013), found that alkaline protease produced by *A. niger* Z1 produced an enzyme activity value of 11.95 U/mg at pH 8.5, incubation time of 5 days, and temperature of 25°C. This occurs because the isolate of Z1 has alkalophilic properties, so it has optimum growth at pH 7-12.

D. Protease Activity Before and After Optimization

The observation results of protease activity conditions before and after optimization are presented in the following table.

Table 1. Protease activity conditions before and after optimization

Isolate	Protease Activity		Protease activity increase (%)
	Before	After	
EUA-124	0.058	0.063	8.621
EUA-126	0.06	0.062	3.334

Table 1 shows that optimization affects the protease activity of the isolate. The fungal isolate EUA-124 showed an increase in protease activity of 8.621% of the initial activity of the enzyme, while the fungal isolate EUA-126 showed an increase in protease activity by 3.334% after optimization to the protease production of fungal isolate. This proves that optimization with variations in temperature and pH can affect fungi in enzyme production by the statement of Beg (2003) that protease production is greatly influenced by factors such as pH, temperature, and incubation period.

IV. CONCLUSIONS

Based on the research result, it can be concluded that:

1. Optimum temperature for isolates EUA-124 and EUA-126 in producing protease is 34° C. The optimum pH for isolate EUA-124 in producing protease is 7 and the optimum pH for isolate EUA-126 in producing protease is 6.
2. Protease production by isolate EUA-124 increased by 8.621% and protease production by isolate EUA-126 increased by 3.334%.

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