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Optimizing Carbon and Nitrogen for Protease Production in Endophytic Bacteria from Mandeh, Pesisir Selatan

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Abstract-The protease enzyme possesses the ability to hydrolyze proteins into peptide bonds and amino acids, serving as a biocatalyst that accelerates reactions, thus holding potential across various industries. The aim of this research is to determine the influence of carbon and nitrogen sources, as well as their concentrations, on the optimum protease activity of a collection of bacterial isolates from *Sonneratia* sp. in the Mandeh area, Pesisir Selatan. The research employed an experimental methodology, and protease activity was assessed using the Takami method. The results of this study indicated that bacterial isolates EUA-131 and EUA-135 exhibited optimum activity with the addition of 1% glucose and 1% $NaNO_3$, while isolate EUA-136 showed optimum activity with the addition of 1.5% maltose and 1% KNO_3.

Keyword-*Sonneratia* SP., Bacterials, Takami Method, Protease

I. INTRODUCTION

The cells of every living organism consist of chemicals that have the ability to catalyze or accelerate biochemical reactions and function as biocatalysts, known as enzymes. Enzymes exhibit superior catalytic efficiency, adjustable activity, and high specificity compared to catalysts from chemical or synthetic sources. These advantages have expanded the application of enzymes across various industries, such as chemistry, food, and pharmaceuticals [1]. Alkaline proteases constitute 60–65% of the global industrial market [2]. The protease market is estimated to grow from USD 3.32 billion in 2023 to USD 4.45 billion in 2028, with a compound annual growth rate (CAGR) of around 6% during the projected period (2023–2028)[3].

Protease is an enzyme with the ability to break down complex protein molecules into simpler forms. Microorganisms are known to play a crucial role in enzyme production technology, both intracellular and extracellular, on an industrial scale[4,5]. Most reactions within living cells require enzymes as catalysts, and these enzymes play a vital role in life [6,7,8]. Enzymes from microorganisms have advantages over those from animals or plants. These advantages stem from the high chemical diversity of microorganisms, ease of mass cultivation, and genetic manipulation capabilities [9].

The advantage of microbial enzyme production also lies in its capacity for large-scale production using established fermentation techniques. The success of a fermentation process requires favorable environmental and nutritional conditions for microorganisms [10]. Bacterial protease production can be influenced by various factors, including physical, chemical, and nutritional aspects. Physical factors involve variables such as inoculum concentration, aeration, temperature, and time. Meanwhile, incubation and pH are also physical factors, while nutritional aspects involve carbon sources, nitrogen sources, and metal ions [11]. Protease production is highly susceptible to various environmental stimuli, especially in the context of nutrient limitations such as the

availability of nitrogen and carbon sources [12,13,14]. It has been reported that the expression of protease genes can be inhibited in the presence of preferred nitrogen sources, and this process is controlled by the nitrogen catabolite repression (NCR) pathway.The biotechnology laboratory at Universita Andalas has available collections of 4 bacterial isolates from the species Sonneratia sp. from the Mandeh area, Pesisir Selatan, where 3 isolates were obtained, namely EUA131, EUA-135, and EUA136, which are producers of protease enzymes. To produce enzymes that have high activity so that they can be used, optimization of microbes must be carried out, namely through engineering the composition of the medium with the addition of carbon and nitrogen source concentrations.

II. RESEARCH METHODS

2.1 Rejuvenation of Protease Producing Bacteria

The isolate rejuvenation process bacteria was carried out on three isolates by streaking each pure culture with 1 ose of culture pure on a petri dish containing NA media, then incubated for 24 hours at 30^0C, then inoculated on upright and inclined media and stored in an incubator.

2.2 Growth Curve and Protease Activity Assay

This growth curve can be determined by providing 100 mL of protease production medium in a sterile 250 mL Erlenmeyer. Then I inoculated 1-2 loops of the oblique culture of each isolate on the protease production medium. Incubated at room temperature (inoculum) with agitation of 150 rpm for 24 hours. After that, pipetted 5 mL of inoculum into 95 mL of protease production medium in a 250-mL Erlenmeyer.Sampling 3 mL of bacterial culture every 2 hours, the calculation of bacterial biomass was carried out by determining the dry weight of the bacterial cells.Add 1 mL of bacteria taken from the treatment medium and then centrifuge at 6000 rpm for 5 minutes. Centrifuged isolates will produce supernatant and bacterial cell deposits. The microtubes containing the precipitated bacterial cells were baked in the oven at 100°C for 30 minutes, then cooled to room temperature, and the dry weight was weighed. The dry weight obtained is reduced by the weight of the empty microtubes to obtain the bacterial biomass. The sample was stopped after a decrease in the growth of bacterial isolates, and the growth profile of each bacteria was obtained.

2.3 Protease Enzyme Activity Testing

Protease activity testing was carried out using the Takami method (1989), in which casein was used as a substrate. Starting with making 1% casein substrate, the substrate solution was pipetted into a test tube as much as 0.5 mL, then added the available enzyme solution and pipetted as much as 0.5 mL, added 0.25 TrisHCl buffer 50 mM pH 8.0, and incubated at 30^0C for 15 minutes. After that, 0.5 mL of TCA was added and incubated at room temperature for 20 minutes. Then the test tube was vortexed and centrifuged at 6000 rpm for 20 minutes. After that, 0.375 mL of the supernatant was taken and transferred to a new test tube, after which 1.25 mL of $NaCO_3$ and 0.25 mL of 1N Folin Ciocelteu were added. The incubation results were measured with a spectrophotometer at 578 nm. One unit of protease activity is defined as the amount of enzyme that can produce 1 mol of tyrosine product per minute under measurement conditions.

2.4 The Influence Of Carbon Source Concentration And Its Concentration In The Production Medium On Protease Activity

The influence of carbon sources on enzyme production medium on enzyme activity was conducted using 250-mL Erlenmeyer flasks containing 50 mL of production medium, where the basal carbon source was supplemented with maltose, glucose, lactose, and fructose at a concentration of 1% each. Samples were taken at the optimum time, and protease activity was tested. After obtaining the highest activity from the carbon source, the concentration of that carbon source was then varied (0.5%, 1%, 1.5%, 2%). Subsequently, the production medium was centrifuged at 6000 rpm at a temperature of 30°C for 20 minutes to obtain the enzyme solution, and the enzyme activity was determined.

2.5 The influence t of Nitrogen Source Concentration and Its Concentration in Production Medium on Protease Activity

The effect of nitrogen sources on enzyme production medium on enzyme activity was carried out by providing a 250 mL Erlemeyer containing 50 mL of enzyme production medium that had been added with a carbon source that had optimum activity and inorganic nitrogen (KNO_3, $NaNO_3$, NH_4Cl, and $(NH_4)_2SO_4$) with a concentration of 1% each. After obtaining the highest activity from the nitrogen source, the concentration of the nitrogen source was varied (0.5%, 1%, 1.5%, 2%). Furthermore, the

production medium was centrifuged at 6000 rpm at 30°C for 20 minutes to obtain an enzyme solution and determine the enzyme activity.

III. RESULTS AND DISCUSSION

3.1 Growth Curve and Protease Activity Assay

Growth and activity test from some isolate bacteria protease producers seen in the following figure this.

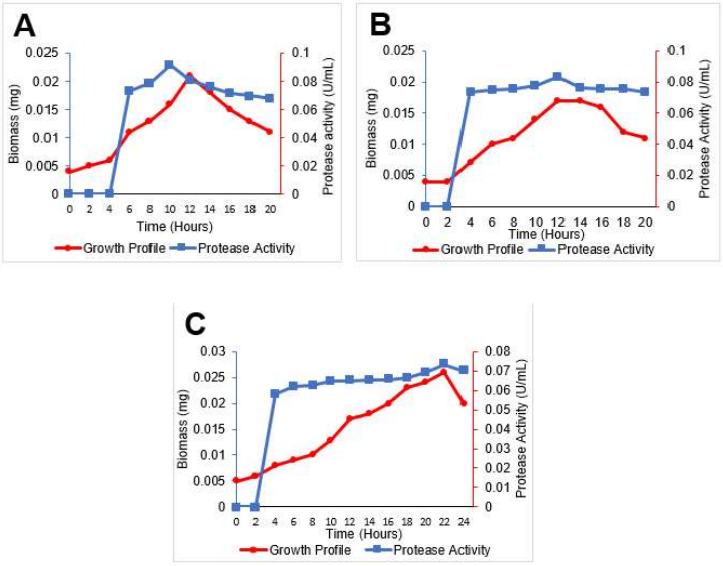

Figure1. Growth curve and protease activity of (A) EUA-131 Isolate, (B) EUA-135 Isolate and (C) EUA-136 Isolate

Figure 1 shows that the EUA-131 (A) isolate experienced an increase in activity from the first 4 hours of incubation until it reached its optimal production time of 10 hours. The EUA-135 isolate (B) reached its optimal production time of 10 hours. The EUA136 isolate (C) reached the optimal time for enzyme production at 22 hours. After reaching the optimal production period, the three isolates experienced a decrease in enzyme activity. Each microorganism has an optimum time for the production of protease enzymes, which varies depending on the type of microorganism. The difference in microbial types can influence the enzyme activity produced by each isolate. Furthermore, the enzyme activity of each microbe is influenced by the quantity and sequence of amino acids formed. There is a possibility that the enzyme activity of the isolate is also affected by the quantity of enzymes and amino acids produced by different isolates (Agustien, 2010).

3.2 Effect of Carbon Source and Its Concentration on Protease Activity

Effect of Carbon Source and Its Concentration on Protease Activity isolates is shown in Figure 2 below this

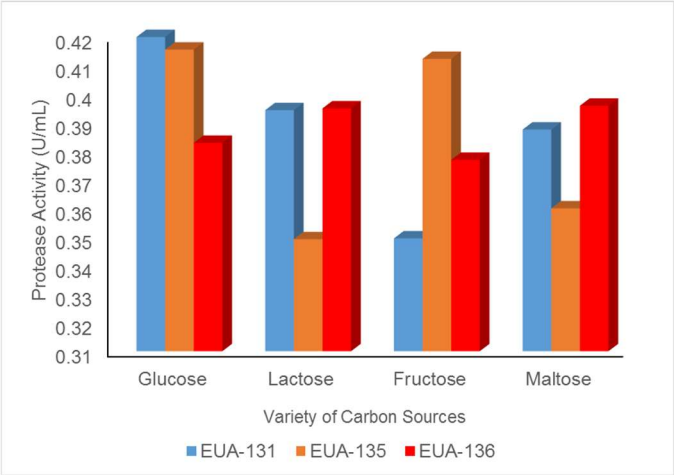

Figure 2. Variations in carbon sources for 3 isolates (EUA-131, EUA-135, and EUA-136)

The result of the research indicate that protease production from isolates is differently influenced by various carbon sources. Figure 2 show the EUA-135 and EUA-136 isolate highest activity with the addition of glucose, while isolate EUA-136 exhibits high protease activity with the addition of maltose. Protease production reaches its peak when glucose is consumed. As a well-known fact, some hydrolase enzymes only form when the carbon source in a medium is almost entirely utilized by microbes. With the likelihood that, by employing shake flask culture methods, glucose can be utilized by organisms in higher concentrations in a relatively short period, resulting in a significant increase in protease production [15].

Isolate EUA-136 has the highest protease activity compared to isolates EUA-131 and EUA-135. This is because the high activity of proteinase detected with maltose can be explained by the role of maltose in maintaining the morphological integrity of bacterial cells. When maltose undergoes metabolism, the β-glucose-1-phosphate released from maltose can serve as a precursor for cell wall synthesis. Additionally, maltose acts as an effective protective agent for the cell membrane and proteins during the microbial dehydration process. Therefore, the observed high protease activity with maltose can be attributed to maltose's role in preserving the morphological integrity of bacterial cells. This ensures a close relationship between protease and cells during growth in maltose, reducing the possibility of the autoproteolytic release of protease-related proteins into the culture medium [16].

Carbohydrates are essential nutrients for microbial growth. Bacteria can absorb and utilize various carbon sources as building blocks for their synthesis. Additionally, carbon sources also serve as the primary energy source in bacterial metabolism [17].

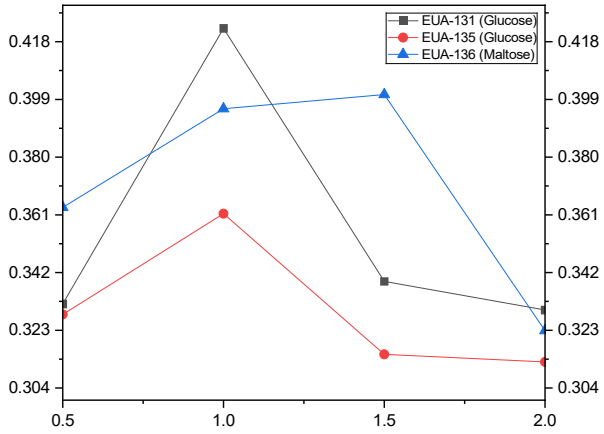

Figure 3. Variations in carbon source concentration for 3 isolates (EUA-131, EUA-135, and EUA-136)

Figure 3 shows that he optimal protease activity in isolate EUA-131 occurs at a concentration of 1%, reaching 0.4224 U/mL. Isolate EUA-135 shows the highest activity at 0.3614 U/mL, but when the glucose concentration is increased by 1%, the protease activity of both isolates decreases. Meanwhile, in isolate EUA-136, the peak activity occurs at a concentration of 1.5%, which is 0.4006 U/mL, but when the concentration is increased to 2%, its protease activity becomes low. The findings of this study indicate that the higher the concentration of the carbon source, the higher the protease activity produced. However, there is a certain limit where protease activity begins to be inhibited. The presence of carbohydrates in the medium can enhance the growth of microorganisms, but if the amount is too high, it can negatively impact protease production. This possibility is due to the catabolic repression mechanism, indicating that in the absence of glucose, protease functions to provide peptides or amino acids as a carbon or energy source, besides serving as a nitrogen source. Therefore, protease synthesis can be repressed when the cell energy status is high in the presence of glucose. It is now known that the catabolite control protein (CcpA) is responsible for the regulation mechanism of glucose catabolism and acts as a signal for repression in protease synthesis [16]. In addition, the main factor of catabolite repression is related to the cAMP receptor in its genetic regulation [18].

3.3 Effect of the Nitrogen Source and Its Concentration on Protease Activity

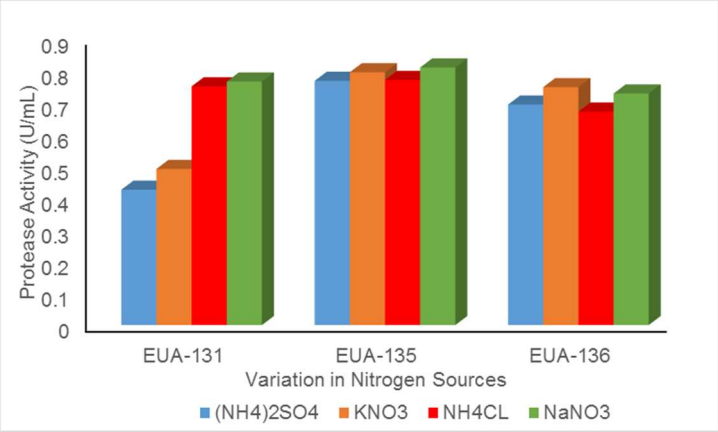

Figure 4. Variations in Nitrogen sources for 3 isolates (EUA-131, EUA-135, and EUA-136)

Isolate EUA-131 and EUA-135 exhibited the highest activity at $NaNO_3$ concentrations of 0.7655 U/mL and 0.8095 U/mL, respectively. Meanwhile, isolate EUA-136 showed the highest activity with the addition of KNO_3 at 0.7478 U/mL.

Figure 4 demonstrate that the addition of sodium nitrate can enhance protease activity. This is because sodium nitrate provides nitrate (NO_3) as a nitrogen source. Nitrate is an oxidized form of nitrogen that bacteria can utilize for growth and protein synthesis. In Figure 4, the protease activity of isolate EUA-136 increased with the addition of potassium nitrate, in line with another study by Jindal et al[19], indicating that the addition of potassium nitrate to the culture medium of Bacillus amyloliquefaciens could enhance the production of protease and amylase enzymes. A study concluded that nitrate stress increases protease activity, leading to increased protein degradation [16,17].

The addition of potassium nitrate and sodium nitrate can increase protease activity by providing nitrate (NO_3) as a nitrogen source. Nitrate is an oxidized form of nitrogen that bacteria can utilize for growth and protein synthesis. Some bacteria have nitrate metabolism pathways that allow them to take up nitrate from the environment and use it as a nitrogen source. In this metabolic pathway, nitrate is converted into ammonium (NH^{4+}) through nitrate reduction, involving enzymes such as nitrate reductase. Ammonium can then be used for protein synthesis, including the synthesis of protease enzymes. Potassium nitrate contains nitrate that is easily accessible to bacteria. When potassium nitrate is added to the growth medium, bacteria with nitrate metabolism pathways will use the nitrate from potassium nitrate for protein synthesis, thereby increasing protease activity [20,21].

The regulation of potassium and sodium pumps in fermentation media plays a crucial role in maintaining the ion balance within bacterial cells, influencing the activity of protease enzymes and overall protease enzyme production. The potassium pump regulates the concentration of K^+ ions inside bacterial cells, while the sodium pump regulates the concentration of Na^+ ions. Achieving the right ion balance, including K^+ and Na^+ concentrations, is essential in the production of protease enzymes. However, the regulation of potassium and sodium pumps in fermentation media must be adjusted to the specific needs and

characteristics of the process [22]. The concentration of K^+ and Na^+ ions inside bacterial cells can influence the activity of protease enzymes and overall protease enzyme production. For example, a study found that the addition of sodium nitrate increased the activity of protease in microorganisms. However, more research is needed to determine the specific effect of K+ and Na+ ion concentrations on protease activity in fermentation media [17].

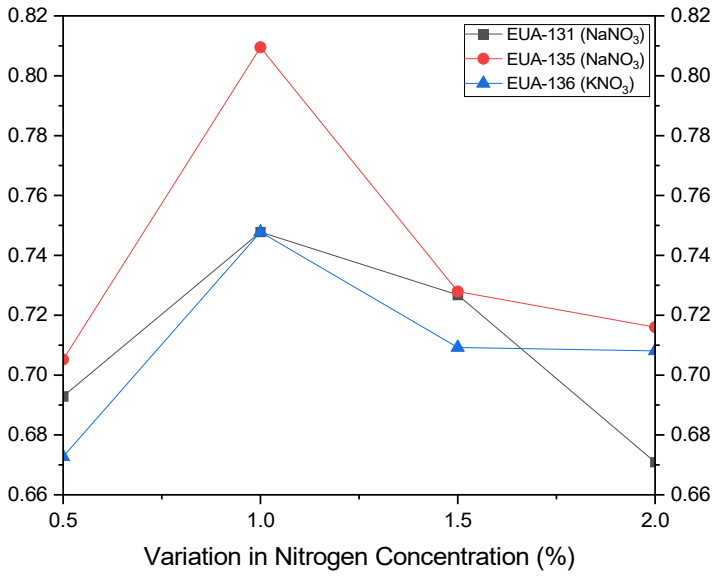

Figure 5. Variations in nitrogen source concentration for 3 isolates (EUA-131, EUA-135, and EUA-136)

Figures 5 indicate that all isolates are optimal with the addition of a 1% nitrogen source. The higher the nitrogen concentration, especially in the form of certain nitrogen compounds, can have adverse effects on protease activity. Several reasons why protease activity may decrease with an increase in nitrogen concentration include negative inhibition, where high nitrogen concentrations can have a negative inhibitory effect on protease enzyme activity. Some nitrogen compounds can directly interact with the enzyme, altering its structure or active function and thereby inhibiting protease activity [23]. Increased nitrogen levels can alter the characteristics of the reaction environment around the protease enzyme. Changes in pH or ion balance in the environment can affect the enzyme's conformation [22]. Some microorganisms have genetic regulation mechanisms that respond to nitrogen levels in the environment. Increased nitrogen levels can trigger regulatory responses that reduce the expression of protease genes, decreasing enzyme production [24]. The presence of specific nitrogen compounds, especially in high amounts, can compete with the natural substrate of the protease enzyme. This competition can inhibit the enzyme's access to its substrate and reduce its catalytic activity.

IV. CONCLUSION

Based on the research conducted, it can be concluded that Isolate EUA131 and EUA-135 show optimum protease activity at 1% glucose, while Isolate EUA-136 performs optimally at 1% maltose. Isolates EUA-131 and EUA-135 exhibit optimum performance at 1% NaN03, and EUA-136 performs optimally at 1% KNO3 after fermentation in the protease production medium at a temperature of 30°C, agitation of 150 rpm for 20-24 hours. Optimizing carbon and nitrogen sources in protease production is essential for cost efficiency, quality control, and industrial applications. This research provides a foundation for optimal production conditions, enhancing efficiency and sustainability in the biotechnology industry.

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