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pH and Temperature Optimization of Several Bacterial Isolates from Mangrove Waters in the Mandeh Area to Produce Cellulase Enzyme

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Abstract: Enzymes serve as vital biological catalysts, playing a crucial role in numerous industrial processes to accelerate specific biochemical reactions. In this study, we aimed to optimize cellulase enzyme production by investigating the influence of temperature and pH on four bacterial isolates: PUA-18, PUA-21, PUA-28, and PUA-38. The experiment was carried out in triplicates using a one-factor-at-a-time design, with pH and temperature as the chosen factors and statistically analyzed using one-way ANOVA followed by Tukey's Honest Significant Difference (HSD). The results showed that the optimum pH for PUA-18, PUA-28, and PUA-38 isolates was pH 6, while PUA-21 showed optimum activity at pH 9. The optimum temperature for PUA-18 was 35°C, PUA-21 was 27°C and PUA-28 and PUA-38 were 31°C. After optimization, the cellulase enzyme production increased by 145% for PUA-18 (0.0598-0.1463 U/mL), 35% for PUA-21, 45% for PUA-28 and 39% for PUA-38. These findings add to the limited literature and address the broader implications of using cellulolytic bacteria from mangrove environments for industrial applications.

Keywords: Optimum Conditions, Enzyme Activity, Bacterial Cellulase, Cellulolytic Isolates

Introduction

Cellulases, which are essential components of industrial enzymatic processes, have been commercially available for more than three decades (Kuhad *et al*., 2016). Their widespread use emphasizes their critical role as catalysts, significantly improving the efficiency and advancement of various industrial processes. Cellulases are widely recognized as the most important hydrolytic enzymes in modern biotechnology, demonstrating versatility in industries such as food, feed, textiles, and pulp (Kuhad *et al*., 2011; Marlida and Zain, 2007). Ongoing research in the bioconversion of cellulosic materials reflects a collaborative effort to advance large-scale conversion processes, which hold the promise of widespread benefits to humanity.

The natural world, particularly ecosystems such as mangroves, provides a fertile environment for microorganisms to produce cellulase enzymes, providing a valuable resource with numerous applications. Within the mangrove ecosystem, the litter produced by the vegetation serves as a primary source of organic material, feeding a variety of aquatic organisms. Notably, this leaf litter contains cellulose, a carbon source that can be used by cellulolytic microorganisms to grow while also producing cellulases. This intrinsic relationship highlights the remarkable potential of harnessing cellulase from microorganisms in mangrove environments, making it a valuable resource for applications in biotechnology, industry, and beyond (Irfan *et al*., 2012).

Previous research on cellulolytic bacteria associated with mangrove habitats in Indonesia yielded promising results. Dewiyanti *et al*. (2021) investigated mangrove aquatic ecosystems in Aceh Province, identifying 21 isolates capable of producing cellulase enzymes among 49 successfully purified isolates. Kurniawan *et al*. (2018) conducted a study in Sungai Liat and Tukak Sadai, Bangka Belitung and identified five types of isolates-*Bacillus pumilus*, *Pseudomonas aeruginosa*, *Bacillus amyloliquefacien*, *Bacillus alvei* and *Bacillus coagulans* with *Pseudomonas aeruginosa* being the most efficient cellulase producer.

Despite the ecological importance of mangroves, there is a noticeable lack of research on optimizing cellulase production from mangrove aquatic bacteria, particularly in the Indonesian context. This study aims to close this

research gap by investigating the cellulase production potential of mangrove bacteria in Indonesia, with the goal of providing valuable insights for both environmental and industrial applications.

Materials and Methods

Preparation of Bacterial Isolates

The isolates used were those available in the Biotechnology Laboratory of Andalas University, identified with the respective codes: PUA-18, PUA-38, PUA-28, and PUA-21. Before proceeding to the next stages, these four isolates were subcultured. Subculturing of the isolates was carried out by inoculating a loopful of each isolate onto NA agar slants and incubating them for 24 h at room temperature.

Bacterial Growth and Enzyme Production

To initiate the culture, a starter medium was created using a sterile 250 ml Erlenmeyer flask containing 100 ml of CMC broth (CMC 10, $MgSO_4.H_2O$ 0.2, KNO_3 0.75, K_2HPO_4 0.5, $FeSO_4.H_2O$ 0.02, $CaCl_2$ 0.04 and bacto-pepton 2 g/L) as per Astuti *et al*. (2022). The medium was then inoculated with 1-2 loopfuls of bacterial isolates and incubated at room temperature with agitation set at 150 rpm for 24 h. Following this, 5 ml of the inoculum was transferred into a production medium consisting of 95 mL of CMC broth in another 250 mL Erlenmeyer flask.

Observation of bacterial cellular growth and enzyme activity in the production media was conducted at intervals of 4 h. Growth was determined by measuring the culture absorbance at 600 nm using a UV-Vis Spectrophotometer. Enzyme activity was done by extraction of crude enzyme and subsequent cellulase assay using the modified DNSA method of McKee (2017). The growth and enzyme activity measurements are halted upon the observation of a declining trend. The bacterial growth profile and enzyme activity profile are compared and the time of the highest enzyme activity is identified for determining the optimum harvest time. All experiments were performed in triplicate and mean values were reported.

Crude Enzyme Extraction

Cellulase enzyme is harvested by transferring 1 mL of bacterial culture into a sterile 1.5 mL Eppendorf tube. Afterward, the bacterial culture underwent a 10 min centrifugation at 10,000 rpm to separate the supernatant, which was then used as the crude enzyme extract.

Determination of Cellulase Activity

Cellulase activity was assayed by a modified DNSA method of McKee (2017). 250 µL of crude enzyme extract with 250 µL of 1% w/v CMC in phosphate buffer solution. This mixture was incubated for 30 min at 30°C. Following that, 500 µL of DNSA (3,5-dinitrosalicylic acid) reagent was added to stop the enzymatic reaction. The solution was then heated for 5 min in a boiling water bath before being immediately cooled in an ice water bath. The cellulase activity was calculated using the formula from Irawati (2016), which involves converting absorbance values obtained from a standard glucose concentration:

$$EA = \frac{C}{weight\ of\ glucose \times T} \times \frac{H}{E}$$

where:
EA = Enzyme activity (Unit/mL)
C = Glucose concentration (ppm)
weight of glucose is 180 g/moL
T = Reaction time (30 min)
H = Total enzyme-substrate volume (mL)
E = Enzyme volume (mL)

pH Influence on Cellulase Production

Each isolate was treated with varying media pH ranging from 5-9 to determine the effect of pH. Incubation is carried out at the optimum harvest time for each isolate, with agitation set at 150 rpm and at room temperature. The cellulase activity of the isolates is then tested to determine the optimal pH for cellulase production. If the highest activity is found exactly at the pH range limit, further testing at higher or lower pH levels is performed.

Temperature Influence on Cellulase Production

To determine the effect of temperature, each isolate was treated with a different incubation temperature of 23, 27, 31, 35, and 39°C. Incubation takes place at the optimum harvest time and media pH for each isolate, with agitation set to 150 rpm. The isolate's cellulase activity is then tested to determine the optimal temperature for cellulase production. If the highest activity is found exactly at the temperature range limit, further testing at higher or lower temperature levels is carried out.

Data Analysis

Data were statistically analyzed using one-way ANOVA followed by Tukey's Honest Significant Difference (HSD) test with SPSS version 27 and a significance level of $p<0.05$ was applied to determine the statistical significance.

Results and Discussion

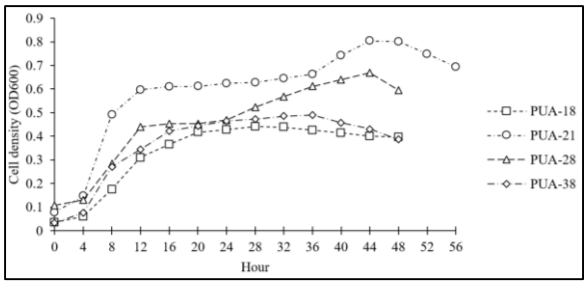
Growth Curves and Cellulase Activity

The bacterial isolates display varying growth profiles (Fig. 1). Isolates PUA-18 and PUA-38 typically follow a

growth pattern with four common growth phases. Isolates PUA-21 and PUA-28 show diauxic growth, a phenomenon where microbial populations undergo two stages of exponential growth when provided with two different carbon sources. This behavior highlights the dynamic nature of microbial responses (Salvy and Hatzimanikatis, 2021). An example of diauxic growth was observed in a study conducted by Buendia-Kandia *et al.* (2018) on *Clostridium acetobutylicum* ATCC 824, a cellulolytic bacterium. In this study, diauxic growth occurred when the bacterium ceased growth after the depletion of glucose and then resumed growth as it began to utilize cellobiose in the subsequent growth stage. This demonstrates how microorganisms have highly dynamic responses to changes in available carbon sources and can undergo two phases of exponential growth to maximize resource utilization.

Differences in the growth curves of PUA-21 and PUA-28 are evident, with PUA-21 exhibiting a stationary phase following diauxie, while PUA-28 directly enters a death phase. According to Liu (2020), bacterial cell growth typically follows four main phases: The lag phase, the exponential phase, the stationary phase, and the death phase. However, the observation of these phases can vary and may not always be distinct. Changes in the composition of the growth medium can lead to variations, such as an exponential phase with a gradual growth rate, often caused by a lack of essential nutrients or the accumulation of metabolites, which can eventually serve as a secondary nutrient source (Stanbury *et al*. 2013). These findings emphasize the dynamic and adaptable nature of microbial growth in response to different environmental conditions.

The patterns of enzyme synthesis in many bacterial isolates were comparable, except for PUA-38, which shows a significant increase with a peak at 36 h (0.2471 U/mL) and a subsequent slight drop (Fig. 2). These fluctuations in enzyme synthesis can be linked to the dynamics of microbial growth. During the exponential phase of growth, bacterial cells divide rapidly and actively synthesize proteins, including enzymes. The length of the incubation period is a crucial factor in this process. A longer incubation period provides more time for bacterial populations to proliferate, potentially enhancing enzyme production. However, an excessively prolonged incubation period can lead to nutrient depletion, the accumulation of metabolic byproducts, and changes in bacterial physiology, all of which can impact enzyme synthesis. Therefore, choosing the right incubation period is essential for optimizing enzyme yields (Allison and Vitousek, 2005). Research conducted by Vimal *et al*. (2016) demonstrated that the optimal incubation period for cellulolytic bacterial isolates varied, with different isolates showing optimal incubation periods at 48, 72, and 96 h.



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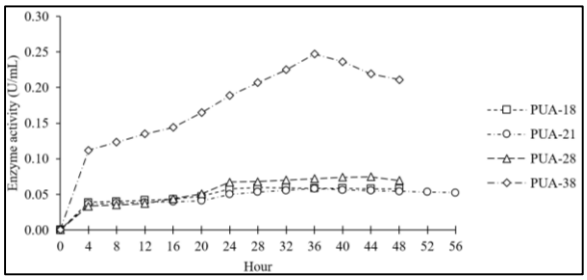

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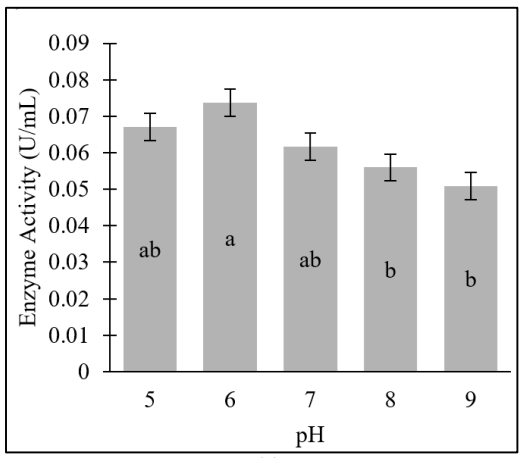
Fig. 1: Curves of; (a) Growth of bacterial isolates; (b) Resulting cellulase activity



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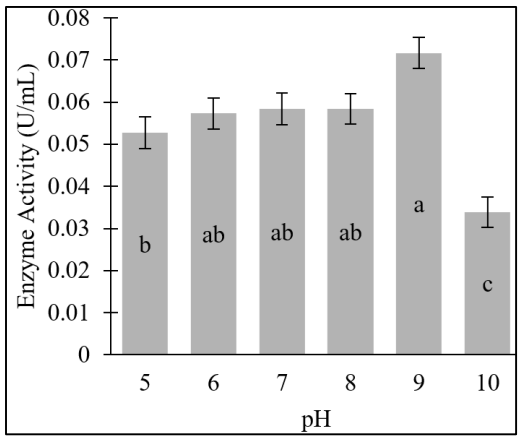

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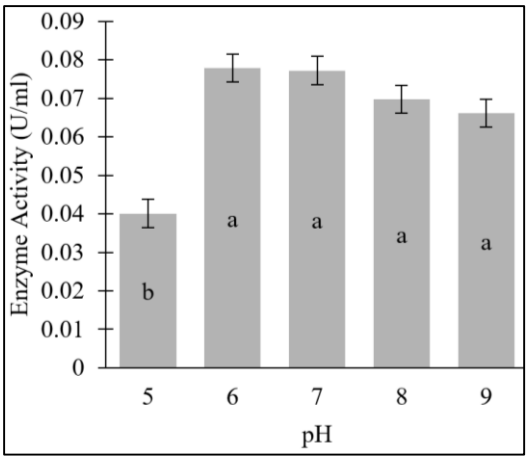

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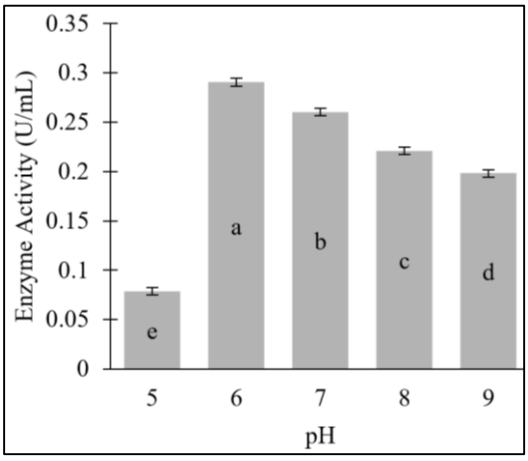
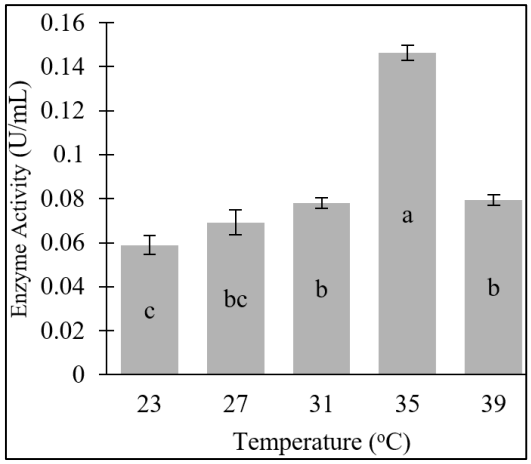


(d)

Fig. 2: pH effects on enzyme activity of; (a) PUA-18; (b) PUA-21; (c) PUA-28; (d) PUA-38 (Means with different superscript letters are statistically significantly different at (p 0.05)

Effects of pH

The effect of pH on cellulase activity is presented in Fig. 3. The cellulase activity responses to changes in media production pH were investigated. Cellulase activity in PUA-18 varied across pH levels, peaking at pH 6 and decreasing at pH 8 and 9, indicating an optimal pH of 6. PUA-21 showed varying cellulase activity in response to pH changes, with the highest activity observed at pH 9, indicating that optimal cellulase activity occurs at higher pH levels, particularly near pH 9. In the case of PUA-28, cellulase activity varied, with the highest mean activity found at pH 6, indicating an optimal pH of around 6. PUA-38 cellulase activity varied significantly across pH levels, with the highest mean activity observed at pH 6, indicating optimal cellulase activity around pH 6. Overall, cellulase activity responses to pH changes are isolate-specific. pH affects bacterial cellulase production in several ways. First, pH influences the regulation of cellulase gene transcription. Bacteria have regulatory mechanisms that respond to pH changes by activating or inhibiting cellulase gene expression. This ensures cellulase production at the optimal pH level for efficient cellulose degradation (Behera *et al*., 2017). PUA-18, PUA-21, and PUA-28 exhibit a stable response, indicating their ability to maintain gene regulation in the face of pH variations. Furthermore, pH affects the stability and activity of cellulase. Enzymes function best within a specific pH range. Deviations from this pH range can lead to decreased enzyme activity or denaturation. Additionally, according to Al Azkawi *et al*. (2018), pH can also affect the composition of bacterial populations. Bacteria have different pH tolerances and capabilities for cellulase production. pH changes can support the growth of specific cellulolytic bacterial species, leading to changes in cellulase production.



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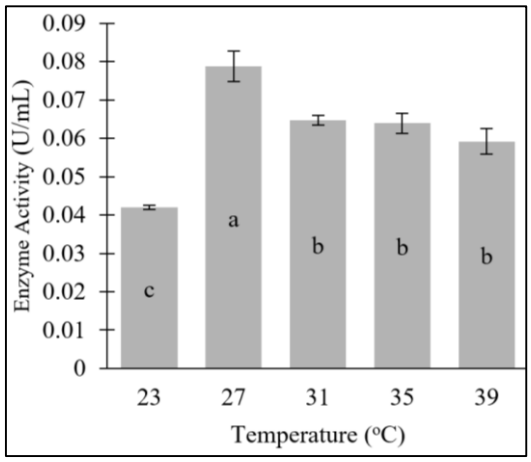

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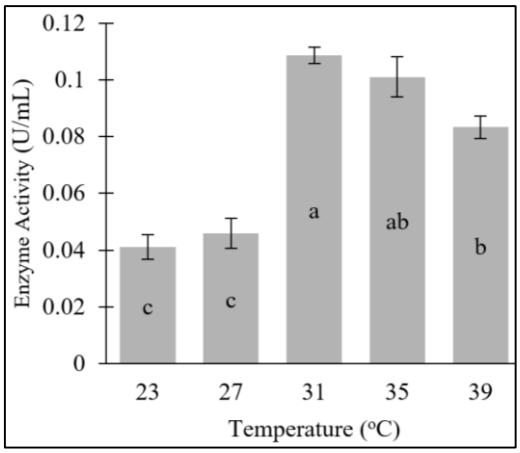

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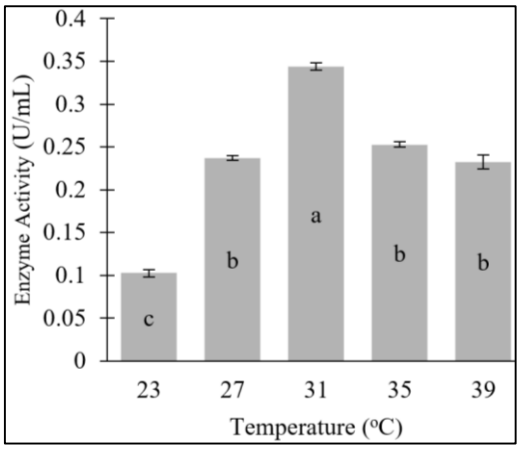

(d)

Fig. 3: Temperature effects on enzyme activity of; (a) PUA-18; (b) PUA-21; (c) PUA-28; (d) PUA-38 (Means with different superscript letters are statistically significantly different at P 0.05)

Effect of Temperature

Figure 3 illustrates how temperature affects cellulase activity. Cellulase enzyme activity responses to temperature changes in four isolates (PUA-18, PUA-21, PUA-28, and PUA-38) revealed distinct trends. Isolate PUA-18 showed an increase in temperature from 23-35°C, peaking at 35°C. PUA-21 demonstrated peak activity at 27°C, with a decrease at 31°C. Isolate PUA-28 increased significantly at 31°C, maintained high activity at 35°C and decreased at 39°C, indicating an optimal temperature range of 31-35°C. PUA-38 activity increased significantly at 27°C, increased again at 31°C and then decreased at 35°C, with a further decline at 39°C, indicating that the optimal cellulase activity temperature is around 31°C. These findings highlight the unique responses of each isolate to temperature variations. Consistent with previous studies, such as Shaikh *et al*. (2013). They reported that isolates the CDB27 and CDB30 exhibited optimal temperature for cellulase enzyme production at 40 and 50°C, respectively.

Table 1: Cellulase production before and after optimization

	Cellulase Activity (U/mL)		
Isolate	Before	After	Increase of cellulase activity (%)
PUA-18	0.0598	0.1463	145
PUA-21	0.0583	0.0787	35
PUA-28	0.0747	0.1086	45
PUA-38	0.2471	0.3438	39

Temperature plays a crucial role in bacterial enzyme production as bacteria can regulate enzyme synthesis in response to temperature changes. Each bacterial species has an optimal temperature range for enzyme production, where specific enzyme production can be induced or suppressed at certain temperatures. Temperature also affects the gene expression involved in enzyme synthesis through transcriptional regulation (Ruff *et al*. 2015). Furthermore, temperature also influences protein folding and stability, which directly impacts enzyme activity. High temperatures can cause enzyme denaturation, while low temperatures can reduce enzyme production (Berg *et al*., 2002).

Cellulase Activity Before and After Optimization

Optimal pH and temperature boost the isolates cellulase activity. PUA-18, PUA-21, PUA-28, and PUA-38 all had significant increases in cellulase activity after optimization, with PUA-18 showing the greatest increase at 145% (Table 1). This underscores the effectiveness of optimization techniques in enhancing cellulase production across various bacterial isolates. Our study aligns with another research study done by Abou-Taleb *et al*. (2015) focused on cellulase-producing strains, *Bacillus alcalophilus* S39 and *Bacillus amyloliquefaciens* C23. It was found that an initial pH of 7 and incubation temperatures of 30 and 45°C resulted in the highest cellulase activity for *Bacillus alcalophilus* S39 and *Bacillus amyloliquefaciens* C23, respectively.

Conclusion

This study successfully identified the pH and temperature conditions that maximize cellulase enzyme production in bacterial isolates. A systematic optimization process resulted in significant increases in enzyme production across all four bacterial isolates. These findings have practical implications, emphasizing the importance of pH and temperature optimization in increasing cellulase enzyme activity in bacterial isolates derived from the Mandeh mangroves. This study's findings contribute to a better understanding of cellulase production processes, which could have applications in a variety of industries. A key recommendation for future studies is to investigate and optimize the production medium. Investigating the composition of nutrients,

carbon sources and other growth-promoting factors within the medium may provide valuable insights into enhancing cellulase production.

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Author's Contributions

Feskaharny Alamsjah: Conceived the original idea for the study, contributed to the design and planning of the experiment, provided guidance and supervision throughout the research, reviewed and provided critical feedback on the manuscript.

Dwiky Rahmadi Aswan: Designed the experiment approach and methodology, conducted the experiment and collected data, drafted the initial manuscript.

Anthoni Agustien: Reviewed and revised the manuscript for clarity and coherence, provided necessary resources and equipment for the study.

Ethics

No ethical issues arose during the course of this research. The authors declare no conflicts of interest in this study.

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