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Isolation and Characterization of Amylolytic Bacteria from Peatlands Area in Central Kalimantan

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Abstract: *Peatlands in Central Kalimantan, Indonesia, are ecologically significant due to their acidic, organic-rich conditions, yet their microbial communities remain underexplored, particularly regarding their enzymatic potential. This study aimed to isolate and characterize amylolytic bacteria from peat soil collected at a depth of 143–150 cm in the LAHG CIMTROP area of Central Kalimantan. A total of 44 bacterial isolates were obtained using serial dilution and the gridding method. Screening for amylase activity identified isolate ENS31 as having the highest activity, shown by a clear halo on starch agar. ENS31 was further subjected to biochemical characterization and enzyme activity assays. The amylase enzyme exhibited optimal activity at pH 7 and 50°C, with a specific activity of 3.5 U/mg, and retained activity up to pH 12 (2 U/mg). These results indicate that ENS31 produces an enzyme stable in alkaline conditions despite originating from an acidic environment, demonstrating potential for industrial applications requiring pH and temperature-tolerant biocatalysts. This study contributes to understanding the functional diversity of peatland microbes and highlights tropical peat ecosystems as promising sources of novel enzymes.*

Keywords: *Amylolytic Bacteria, Amylase Activity, Peatland, ENS31*

INTRODUCTION

Indonesia has approximately 21 million hectares of peatlands spread across various regions, including Sumatra Island (35%), Kalimantan (32%), Papua (30%), and other islands (3%) (Susandi et al., 2015). Central Kalimantan alone accounts for around 4.6 million hectares of peatland, according to the Ministry of Environment and Forestry (Kementerian Lingkungan Hidup dan Kehutanan–KLHK). These peatlands are ecologically important due to their large carbon storage capacity and unique soil characteristics, such as high-water content, naturally acidic pH (3.0–4.5), and a porous structure that supports water retention (Mustamo et al., 2016). Their dark coloration reflects the high concentration of organic matter, particularly humus (Leifeld et al., 2020).

Peat soils in Indonesia are formed under different environmental conditions, resulting in distinct physical, chemical, and biological characteristics between regions. For instance, peatlands in Sumatra are generally shallower, more frequently disturbed due to intensive agricultural conversion, and exhibit higher decomposition levels, while Papua's peatlands are relatively undisturbed, deeper, and more pristine due to limited human activity (Maftu'ah et al., 2021; Wahyunto et al., 2012). In contrast, Central Kalimantan's peatlands are characterized by intermediate peat depth, chronic exposure to past land-use changes (e.g., logging, Mega Rice Project), seasonal water fluctuations, and recurring peat fires (Osaki et al., 2016; Wahyunto et al., 2012). These historical and environmental factors contribute to a unique peat profile like moderately decomposed, nutrient-poor, highly acidic, and affected by periodic drought and rewetting cycles.

Despite their ecological importance, tropical peatlands are highly vulnerable to degradation from drainage, fire, and land-use change (Kitson & Bell, 2020; Turetsky et al., 2015). Such disturbances accelerate the decomposition of organic material and release significant amounts of greenhouse gases (Pulunggono et al., 2022). In this context, microbial activity, particularly bacteria and fungi, plays a crucial role in both maintaining and potentially altering the ecological balance of peat ecosystems. Microorganisms in peat soils drive essential processes in the carbon and nutrient cycles, particularly through enzymatic activities that decompose complex organic compounds (Kitson & Bell, 2020).

Microorganisms are essential in these ecosystems, especially in nutrient cycling, organic matter decomposition, and the carbon cycle. One key microbial function is the enzymatic hydrolysis of carbohydrates by amylolytic bacteria, which produce amylase enzymes to break down starch into simpler sugars (Erfianti et al., 2023; Fauzi Akbar et al., 2013). While the presence and industrial potential of amylase-producing microbes have been widely studied in various environments, there remains limited understanding of their occurrence, diversity, and ecological function in tropical peat soils, especially in Central Kalimantan. Given the distinct environmental conditions of Central Kalimantan's peat, such as its long-term waterlogging, high acidity, and organic richness, the microbial communities and their enzymatic adaptations may differ significantly from those in peatlands in Sumatra or Papua.

Furthermore, with the growing interest in biotechnological applications of microbial enzymes for industries such as bioenergy, food processing, and agriculture, understanding the amylolytic potential of indigenous peat microbes may offer novel biocatalysts adapted to extreme conditions. However, exploring such microbial resources in Central Kalimantan peat is still underrepresented in scientific literature. Therefore, this study aims to isolate and characterize amylolytic bacteria from Central Kalimantan peat soil, conduct biochemical identification, and optimize conditions for amylase activity. This research will contribute to the ecological understanding of microbial functions in tropical peat ecosystems and the potential discovery of novel amylase-producing strains for industrial applications, particularly those adapted to acidic and anaerobic conditions.

RESEARCH METHODS

Materials and Tools

The materials used in this study include peat soil samples, 0.9% w/v NaCl solution, Luria-Bertani (LB)-Agar medium (1% w/v NaCl; 0.5% w/v yeast extract; 1% w/v tryptone; and 2% w/v agar), Lugol's solution, DNS solution (1% w/v DNS; 0.4 M NaOH; and 30% w/v K–Na-tartrat), 1% soluble starch solution, pH buffer solutions (citrate buffer, phosphate buffer, carbonate buffer), Congo Red, distilled water, Luria-Bertani Broth (LB-broth), cotton, filter paper, aluminum foil, ethanol, and other supporting materials.

Instruments used in this research consist of Oscillator Orbital Shaker Spring KANGJIAN KJ-201BD, centrifugal machine 8001-1, SAFAS Uvmc1 spectrophotometer, analog stirring hot plate AHS-12A Model, TOMY SEIKO High-Pressure Steam Sterilizer LBS-325, IKEME Micropipette Single Channel Adjustable Pipette 100-1000µL.

Methods

Peat *Sampling*

Peat soil sampling was conducted around the Peat Forest Nature Laboratory (Laboratorium Alam Hutan Gambut – LAHG), managed by CIMTROP (Centre for

International Cooperation in Sustainable Management of Tropical Peatland), Universitas Palangka Raya. This site is located within the Sebangau peat swamp forest in Central Kalimantan, an area recognized for its ecological integrity and extensively used in previous research as a representative tropical peat ecosystem (MIRMANTO, 2009; Ritzema et al., 2014). It is undisturbed or minimally disturbed peat swamp forest conditions, making it a suitable reference area for microbial ecological studies. The selected sampling location was situated near tree root zones to target the rhizosphere region, where microbial density and activity, especially of enzyme-producing bacteria, are typically higher due to root exudates and organic matter input (Ling et al., 2022). Soil samples were collected using a sterile core sampler at a depth of 143–150 cm below the ground surface, targeting a deeper peat layer under anaerobic and acidic conditions. The peat soil at this depth was blackish brown, slightly watery, and rich in organic matter, consistent with typical mature peat characteristics. The collected soil was immediately placed in sterile plastic bags and stored in a cool box containing ice packs to maintain a temperature of approximately ±4°C, following the method adapted from Silvianingsih et al. (2021), to preserve microbial viability prior to laboratory analysis (Silvianingsih et al., 2021).

Bacteria Isolation

The bacterial isolation process was carried out using the serial dilution method, namely by dissolving 1 gram of peat soil in 0.9% NaCl 10 mL, then diluting to 10-7. Then every 100 µL of the serial dilution solution was taken and then spread on each petri dish containing LB-Agar media and incubated for 72 hours. After that, the bacterial colonies were separated based on their morphology and inoculated by gridding method.

Screening of Amylolytic Bacteria

To find out which isolates can produce amylolytic activity, an activity test was carried out with LBA media which had been added with 1% soluble patu. This process is done by transferring the selected bacterial colonies into a Petri dish containing media with the streak plate method, then incubated for 24 hours at room temperature. After incubation, the bacterial isolates were then flushed with lugol solution (KI 10%; I2 5%), allowed to stand for 5 minutes, then rinsed with distilled water and observed for the formation of halo areas (Sarian et al., 2017).

Amylase Production

Bacterial isolates with the clearest halo region were selected for amylase enzyme production. Enzyme production was carried out by taking bacterial isolates and transferring them into 20 mL of LB-broth solution, then agitated using a shaker at 160 rpm for 18 hours. Then, 1% of the isolate solution was mixed into 200 mL LB-broth and incubated for 24 hours to obtain a high-density bacterial suspension. Furthermore, the suspension was centrifuged at 3500 rpm for 15 minutes. From the centrifugation, the supernatant was obtained in the form of crude extract of amylase enzyme which was then used for amylase activity tests.

Enzyme Assay

In this study, the optimization process of hydrolysis conditions by the amylase enzyme was carried out with a focus on two main parameters, namely pH and temperature. pH optimization was carried out at pH 3, 4, 7, 9, 11, and 12 using citrate buffer, phosphate buffer,

and NaOH-glycine buffer. Temperature optimization was performed at 40, 50, 60, 70, and 80 ºC.

Amylase activity was measured by the DNS method (Miller, 1959). At first, 500 µL of 1% soluble starch was mixed with 500 µL of crude extract of amylase enzyme, then incubated at 50 ºC for 30 minutes. Then, 1 mL of DNS solution (1% 3,5-dinitrosalicylic acid; 2N NaOH; 30% Na-K-tartrate) was added. The reaction was stopped by heating the mixture at 100 ºC for 10 minutes. Then the mixture was diluted 10 times using distilled water. The amylase enzyme activity assay was performed in duplo and using a control. Furthermore, the solution was measured for absorbance using UV-Vis spectroscopy instrumentation at a wavelength of 500 nm. The Bradford method was used to determine the protein content produced to obtain the specific activity (equation 2).

$$\text{Unit Activity } \left(\frac{\text{Unit}}{\text{mL}}\right) = \frac{\text{Glucose concentration (mM)}}{\text{Enzyme volume (mL) x incubation time (minute)}} \quad (1)$$

$$\text{Specific Activity } \left(\frac{\text{U}}{\text{mg}}\right) = \frac{\text{Unit activity } \left(\frac{\text{Unit}}{\text{mL}}\right)}{\text{Protein concentration } \left(\frac{\text{mg}}{\text{mL}}\right)} \quad (2)$$

Biochemical Test

Bacterial biochemical tests carried out in this experiment include indole, methyl red, Voges-Proskauer, citrate utilization, motility, and gas test and H2S test. For enzymatic activity tests, catalase, oxidase, gelatinase, protease, urease and amylolytic tests were also performed. The sugar fermentation test was conducted using TSIA (Triple Sugar Ion Agar) media. Then the decarboxylation and lysine deamination tests were carried out using LIA (Lysine Iron Agar).

RESULTS AND DISCUSSION

The isolation of microorganisms from peat soil in the LAHG CIMTROP area of Central Kalimantan at a depth of 143–150 cm (coordinates: 2°19'23"S 113°53'17"E – Figure 1) was based on the ecological significance and unique properties of this site. Central Kalimantan peatlands are known for their intermediate peat depth, acidic pH, high organic content, and seasonal hydrological fluctuations, which shape unique microbial habitats (Osaki et al., 2016; Wahyunto et al., 2012). Compared to the more decomposed peat in Sumatra or the pristine, deep peat in Papua, Central Kalimantan peat soils provide a distinct ecological niche for microbial communities, especially in deeper layers influenced by root exudates and anaerobic conditions (Maftu'ah et al., 2021). Recent research has revealed that microorganisms found in the root zone are higher in diversity and number. These microorganisms play a role in nutrient cycling and plant protection, as well as influencing the roots' development (Malook et al., 2021).

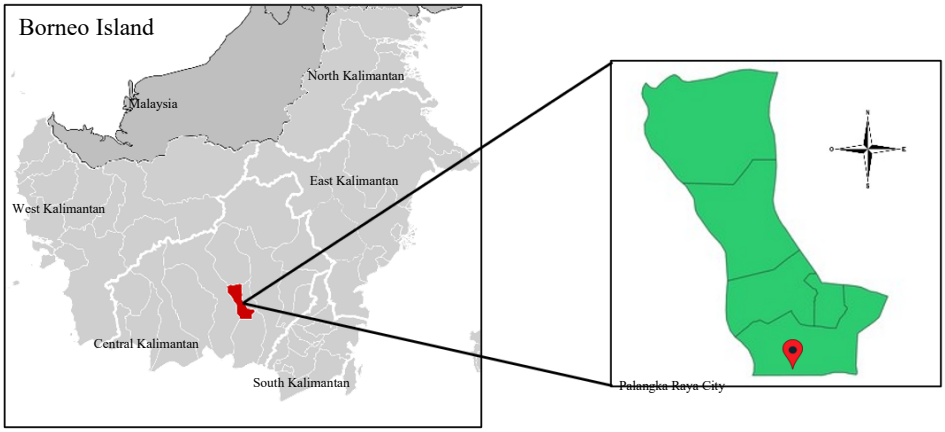

Figure 1. Sampling Site

The isolation process began with dilution of peat soil samples to the 10^{-7} level using physiological solution, which aims to reduce the possibility of cross-contamination between colonies. Inoculation of the diluted samples was carried out on LB-Agar media and incubation for 72 hours resulted in colonies with varying morphologies. The colonies had cream, white, pink, and pale-yellow colors, as well as different surfaces. This diversity of colony morphology reflects the diversity of microorganism species successfully isolated, which is very important for the next step in identifying and understanding the characteristics of each microorganism.

The gridding method was employed to systematically separate bacterial colonies on solid media, enabling clear identification and easier analysis. By placing colonies within clearly marked grid boxes with adequate spacing, this approach ensures that each developed colony originates from a single microbial cell, minimizing the possibility of overlapping growth or contamination (Figure 2). From this study, a total of 44 distinct bacterial colonies were successfully isolated from peat soil. Two different layout strategies were used: the first involved dividing the plate into sectors, while the second used a square grid system. Each colony was assigned a number corresponding to its isolate identity. For example, colony number 1 is labeled as ENS1, where "ENS" represents the isolate code used in this research. This numbering system allows for accurate tracking during biochemical characterization, enzymatic activity screening, and subsequent analyses. Previous studies have demonstrated that the gridding technique is effective for obtaining reliable and pure microbial isolates, particularly when preparing for further assessments such as metabolic profiling or enzyme production (Bentley et al., 1992).

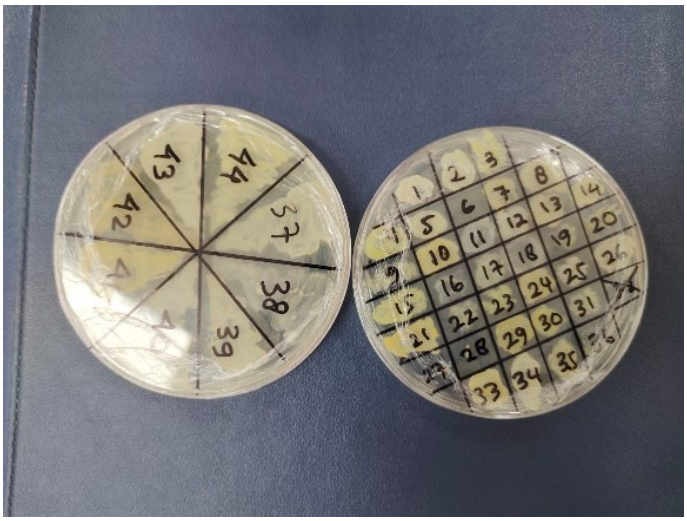

Figure 2. Bacteria Isolated from peatland Gridding method applied for the isolation and identification of bacterial colonies from peat soil. (Left) Petri dish divided into sectors (numbered 37–44) to facilitate colony grouping and preliminary localization. (Right) Petri dish using the gridding technique, where each colony is placed within a clearly marked square grid (numbered 1–36) to ensure proper separation and traceability

Bacteria that had amylolytic activity were selected by observing the formation of clear areas around colonies that had been inoculated on LB-Agar media containing 1% soluble starch. The clear area around the colony would only be seen after the bacteria growth media was stained with iodine solution. The larger the clear zone, the greater the amylase activity formed. Forty-four isolates were selected for amylolytic activity in this study, but only two isolates showed a clear zone, namely isolates ENS31 and ENS42.

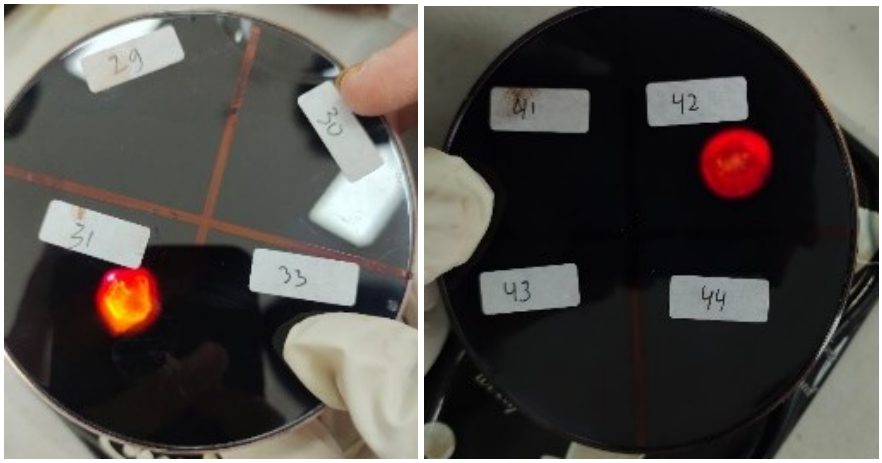

Figure 3. Screening Amilolitic Bacteria

The clear zone in the selection of amylolytic bacteria was formed because the amylase enzyme excreted by bacteria can hydrolyze soluble starch in LB-Agar media into oligosaccharides. Oligosaccharides could not bond with the iodine solution, so a dark blue colorless part was formed around the colony. On the other hand, iodine solution could bind

with carbohydrates such as soluble starch, so a dark blue color was formed on LB-Agar media that had been added with 1% soluble starch (Puspasari et al., 2011).

Table 1. Biochemical Test

Biochemical Test	Result
IMVIC Test	
Indol	-
Metil red	+
VP	-
Citrate utilization	-
Enzyme Activity Test	
Catalase	+
Oxidase	-
Gelatinase	-
Protease	+
Starch Hydrolisis	+
Urease	-
Addition Test	
Gas	-
H_2S	-
Motility	-
Sugar Fermentor	
Glucose	-
Lactose	+
Sucrose	+
Amino Acid Utilization	
Lysin decarboxilation	+
Lysin deamination	+

Furthermore, ENS31 showed the most prominent amylolytic activity, as indicated by the largest clear zone on starch-containing LB-Agar medium after iodine staining. ENS31 was further analyzed to be characterized using several biochemical tests (Table 1). Then, amylase enzyme production was carried out from ENS31 to obtain enzyme crude extract so that the resulting amylase activity test could be carried out.

The IMVIC tests consisted of an indole, methyl red, VP, and citrate tests. Indole test is done to detect the ability of bacteria to produce indole as a metabolic product that utilizes tryptophan. The test showed that ENS31 isolate did not produce indole. Methyl red test was conducted to detect the ability of bacteria to produce stable mixed acid as the final product of glucose metabolism. Isolas ENS31 showed a change in color to red, indicating that the methyl red test results were positive.

VP test was performed to detect the ability of bacteria to metabolize pyruvate into a neutral intermediate product called “acetoin”. The citrate test was performed to detect the ability of bacteria to utilize citrate as the sole source of energy (Hall, 2013; Tille, 2014). Isolate ENS31 showed negative results in the VP and citrate utilization test.

Enzymatic activity tests include catalase, oxidase, gelatinase, protease, urease, and starch hydrolysis tests. The catalase test is carried out to detect the ability of bacteria to utilize H_2O_2 into H_2O and O_2. Oxidase test is performed to detect the ability of bacteria to use oxygen as an electron acceptor and donor during the oxidative phosphorylation process. The gelatinase test is performed to detect the ability of bacteria to produce gelatinase. The appearance of clear areas around the colonies characterizes the results of gelatinase, protease, and hydrolysis tests. The urease test is characterized by a change in the color of the media to pink due to the presence of pH indicators in the media and changes in pH that occur. Isolate

ENS31 showed positive results in starch hydrolysis, protease, and catalase tests. This suggests the isolate may play multiple roles in organic matter decomposition in peat ecosystems, contributing to nutrient cycling.

Additional tests were conducted, including gas, H2S, and motility tests. Cracks in the media characterize the gas test. H2S is characterized by the media turning black. The motility test detects whether the bacteria can move with flagella. Isolate ENS31 showed negative results in gas, H2S, and motility tests.

The sugar fermentation test uses TSIA media, which shows bacteria's ability to ferment three sugar types: glucose, lactose, and sucrose. Isolate ENS31 showed negative results in the fermentation test of the three types of sugar. Finally, the amino acid utilization test consists of lysine decarboxylation and lysine deamination tests that show the ability of bacteria to use lysine as a carbon source. The decarboxylation and lysine deamination test results of ENS31 isolate showed positive results.

Furthermore, the crude extract of the enzyme produced by ENS31 was produced, and its amylolytic activity was carried out using the DNS method. The principle of the DNS method is based on the interaction between the DNS solution and the product produced. The enzyme activity unit was measured by calculating the reducing sugar content resulting from the enzymatic reaction through DNS-reducing sugar interaction and dividing by the volume and incubation time according to equation 1. Previously, the DNS method used a glucose standard curve to determine reducing sugar concentration. PH and temperature optimization were conducted to determine the highest amylolytic activity.

The protein content produced by ENS31 isola was measured using the Bradford method, and absorbance was measured at a wavelength of 550 nm. Previously, a BSA standard curve was made using the Bradford method (Kielkopf et al., 2020). Protein content is needed to determine the specific activity of the enzyme (U/mg) through the division of enzyme specific activity and protein content according to equation 2.

The specific activity of the enzyme increased as the pH increased, starting from pH three until it peaked at pH 7, reaching 3.5 U/mg (Figure 4). However, the specific activity showed a slight increase at pH 12, although not as significant as the specific activity at pH 7. A decrease in activity at extreme pH (too acidic or basic) indicates that the enzyme structure becomes unstable at these conditions, causing denaturation or loss of catalytic function (Gupta et al., 2003). This finding is consistent with previous reports that microbial enzymes isolated from acidic environments do not always exhibit acid-stable activity (Lynd et al., 2002). Enzymatic adaptation may reflect the internal cellular conditions or micro-niche variability within the peat matrix, allowing enzyme secretion to be optimized for specific substrates rather than ambient pH. However, although its optimum pH is at neutral pH (pH 7), the amylolytic bacterium ENS31 has the advantage of still showing activity at pH 12. This ability indicates that the bacteria can survive in extreme alkaline environments, so it has the potential to be used in various industrial applications that require stable enzymes under alkaline conditions.

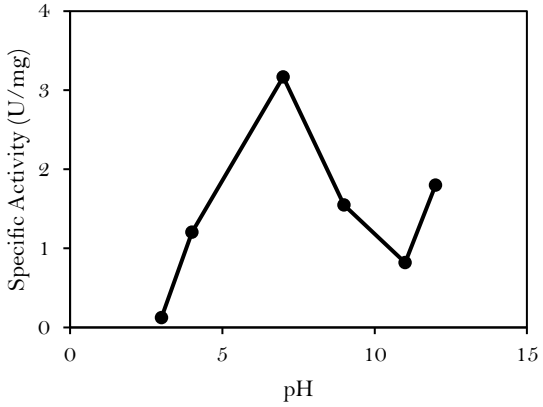

Figure 4. Specific activity of amylase from ENS31 towards pH

The graph of incubation temperature against the specific activity of amylase enzyme from ENS31 isolate shows an increase in the specific activity of the enzyme along with the increase in temperature up to 50°C, reaching 1.8 U/mg (Figure 5). The specific activity decreased drastically when the temperature was raised again until almost no activity at 80°C. The optimum temperature at 50°C indicates that the enzyme works most efficiently. The sharp decline after the optimum temperature indicates that the enzyme undergoes thermal denaturation, in which the protein's three-dimensional structure is damaged by high temperature (Frima et al., 2020).

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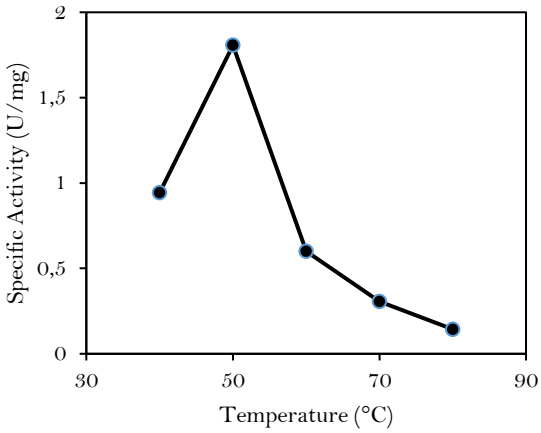

Figure 5. Specific activity of amylase from ENS31 towards temperature

The uniqueness of the amylolytic activity of ENS31 is its ability to maintain enzyme activity at highly alkaline pH (up to pH 12), although the optimum pH is at neutral conditions. This ability shows that this bacterium can be used in industries or biotechnology requiring resistant enzymes at extreme pH. The optimum temperature of 50°C also makes this enzyme suitable for processes that require enzyme stability at moderate temperatures.

CONCLUSIONS

This study successfully isolated the amylolytic bacterium, ENS31, from peat soil in the CIMTROP area, Central Kalimantan, as an underexplored acidic and organic-rich environment. ENS31 produced amylase with optimal activity at pH 7 and 50°C, reaching a specific activity of 3.5 U/mg, and retained 2 U/mg activity even at pH 12. These characteristics highlight its potential as a robust biocatalyst for industrial applications requiring enzyme stability under extreme conditions. The findings address the knowledge gap on functional microbial diversity in Central Kalimantan peatlands and suggest that such ecosystems can be valuable sources of enzymes with unique properties suitable for biotechnology.

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