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ISOLATION AND SCREENING OF AMYLASE PRODUCING BACTERIA FROM PALM OIL LIQUID WASTE

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ABSTRACT

Amylase is the most studied enzyme in biotechnology and also accounts for 25% of the total enzyme market worldwide. In industry, amylase has potential applications in the bakery, textile, detergent, syrup, pharmaceutical, and medical industries. One of the potential sources of amylase is microbes, because it is easy to isolate, culture, and also genetically engineered. Liquid waste is one of the most potential and abundant sources of microbes. In this study, the authors used palm oil waste as a source of amylase-producing microbes. The purpose of this study was to isolate and screen bacteria that showed amylase activity. Bacteria were isolated from palm oil liquid waste samples using the serial dilution method and the number of CFUs was calculated. The isolates were then characterized morphologically including Gram staining, elevation, color, edge, cell shape and also catalase reaction. Characterization results obtained 7 Gram positive isolates and 8 Gram negative isolates, the shape of cocci (12 isolates) and bacilli (3 isolates) and convex elevation and white isolates. Amylase activity was detected using nutrient agar media supplemented with 1% starch and the amylolytic index was measured. Amylolytic activity was indicated by the presence of a clear zone around the isolate. Of the 15 isolates, only 1 isolate showed amylase activity, namely LCF 4 with an amylolytic index of 0.57. Further research is recommended to identify and determine enzyme activity.

Introduction

The use of enzymes in the industrial world is increasingly widespread due to the rapid development of enzyme application technology, fermentation technology and genetic engineering. One of these enzymes is amylase. Amylase is classified as a saccharidase, which is an enzyme that cleaves polysaccharides. The main function of amylase is to break down starch in food so that it can be used by the body. Amylase is also synthesized in plant fruit during ripening, causing the fruit to be sweeter (Ariandi, 2016). The enzyme α-amylase (1,4-α-D-Glucan glucano hydrolase, E.C.3.2.1.1) is a key enzyme in the metabolism of living organisms that use starch as a carbon source and energy source. α-amylase is an endo-enzyme that can hydrolyze α-1,4-glycoside bonds in straight-chain or branched starch polymer units to

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produce glucose (Purnawan et al., 2015). Amylase is the most studied enzyme in biotechnology and accounts for 25% of the total enzyme market worldwide. In industry, amylase has applications in the bakery, textile, detergent, syrup, pharmaceutical, and medical industries (Fentahun and Kumari, 2017). Amylase can be derived from various sources, including plants, animals and microorganisms. Amylase from microorganisms generally meets industrial needs, this is because the sources are abundant and easy and fast to culture, and the enzymes produced are also more stable (Albejo et al., 2017).

Research on the isolation and identification of amylase-producing bacteria has been carried out quite a lot. In a study conducted by Luang-In et al (2019) it was reported that amylase-producing bacteria were isolated from soil taken from forests in Mahasarakham province, Thailand. The results showed that 13 bacterial isolates that were successfully isolated showed amylase activity in 1% starch media. A total of 12 Gram positive bacteria, rod-shaped and identified as Bacillus spp. 1 bacterial isolate with Gram negative and rod-shaped identified as *Enterobacter cloacae* (Luang-In et al., 2019). In another study it was reported that amylase-producing bacteria were isolated from hot springs in Singgahan Tuban, East Java. Of 103 isolates, 51 isolates showed amylase activity with the formation of a clear zone around the colony on solid media containing 1% starch (Novitasari and Herdyastuti, 2014).

Industrial waste mostly produces liquid or solid waste that still contains organic which has undergone decomposition. There are still many industries that dispose of their waste into open waters so that in a relatively short time it will cause a foul odor as a result of the fermentation of waste. The content of the waste produced is dominated by organic compounds, although inorganic materials are also found. This is indicated by the high concentration of BOD in the waste. Palm oil liquid waste (LCKS) is one of the potential sources of enzymes that produce microbes. In several studies, microbes from LCKS were isolated to be tested for their lipolytic activity (Chairunnisa et al., 2020). Until now, the number of studies reporting on amylase-producing microbes from palm oil wastewater is still very limited. In this study, researchers isolated and screened amylase-producing microbes from LCKS. To the best of the author's knowledge, isolation and screening of amylase-producing bacteria from palm oil wastewater in oil palm plantations in Aceh Tamiang has never been done before.

Materials And Methods

Sampling

LCKS sampling was carried out at palm oil mill in Aceh Tamiang. Samples were collected using sterile plastic tubes. At the time of sampling the temperature and pH were measured. pH and temperature measurements were carried out to determine the optimum conditions for microbial growth on a laboratory scale The LCKS collected consisted of 2 types, namely waste from the anaerobic pond (LCKSA) and from the cooling pond (LCKSP). The sample was stored in a cooler box to inhibit or stop the microbial growth and taken to the laboratory for further analysis (Nedwell, 1999).

Isolation and Enumeration

Approximately 1% of the sample was diluted successively in sterile distilled water, and the aliquots were then aseptically incubated in Nutrient Broth growth medium at 37°C for 24 h. The aliquots in the last dilution were spread onto NA media and incubated for 48 hours at 37°C. Growing colonies were counted in Colony Forming Units using a colony counter. The microbial isolates obtained were then purified for further analysis (Elijah et al., 2013).

Bacteria Characterization

The bacterial isolates obtained were characterized morphologically using the Gram staining method. Ose needles are sterilized and allowed to cool for 30 seconds. Using a clean slide, place 1 loopful of

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bacterial colonies in the center of the slide. 1 drop of water was placed on the isolate to make a suspension. The slide was heated by passing it over a Bunsen flame 2-3 times to fix it. After that, the crystal violet was dripped on the preparation evenly, and waited for 1 minute. The preparations were rinsed with running water and then the iodine solution was dripped on the preparations and waited for 1 minute. The preparations were then rinsed again with running water. Decolorization was carried out by dripping decolorization liquid and rinsed again with running water. Then add safranin and wait for 1 minute. The preparations were then rinsed with running water and dried. Observations were made using a microscope with a magnification of 100x. Bacteria that show blue or purple under a microscope are Gram-positive bacteria, while bacteria that are red in color indicate Gram-negative bacteria (Claus, 1992).

Amylase Activity Screening

The purified bacterial isolates were then spot onto selective amylolytic media in a petri dish and incubated at 37°C for 24 hours. After incubation, the media was then flooded with 1% (w/v) Lugol's iodine solution and isolates showing a clear zone around the isolates indicated that the isolates produced amylase enzymes. The amylolytic index was then measured by the formula $\frac{colony\ diamater\ (mm)}{clear\ zone\ diameter\ (mm)}$ (Kiran et al., 2018).

Results and Discussion

Sampling process

Sampling of palm oil liquid waste (LCKS) was carried out at palm oil mill in Aceh Tamiang. Samples were collected using sterile plastic tubes and at the time of sampling the temperature and pH were measured. Each sample was collected in the amount of ± 50 mL. The samples were stored in a cooler box and further analyzed in the laboratory on the same day.

The results of pH and temperature measurements show that LCKSP has a pH of 4.3 and a temperature of 60°C while LCKSA has a pH of 6 and a temperature of 43.8°C. Palm oil liquid waste is a by-product of the processing of palm oil into palm oil or Crude Palm Oil (CPO). The LCKS processing process aims to be used safely in accordance with the Decree of the State Minister of the Environment No.28 of 2003 concerning Technical Guidelines for Utilization of Wastewater from the Palm Oil Mill Industry. The pH condition of LCKSP was at pH 4.3, this condition could suppress the growth of fungi and bacteria. This situation was possible because in the cooling pond there has been an increase in volatile fatty acids including acetic acid, butyric acid and propionic acid. The growth of fungi and bacteria will increase by 19.04% and 14.81%, respectively, with an increase in the pH of the LCKS to 6. This occurs due to the neutralization process due to the formation of a buffer that neutralizes the pH (Nursanti, 2013).

Bacterial Isolation and Enumeration

Of the 2 samples isolated, only bacteria from LCKSA samples were successfully isolated, with a total CFU of 1.3 x 10^8 CFU/mL. The results of bacterial isolation from LCKSA are shown in the following figure.

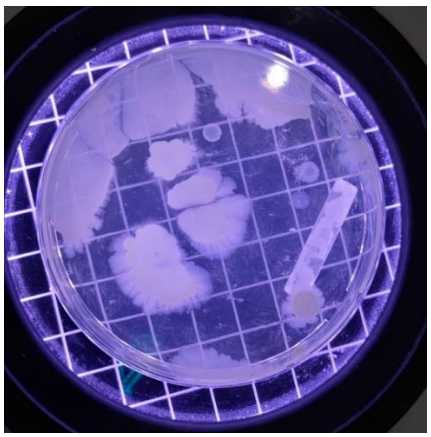

Figure 1. Bacteria isolated from LCKSA

In the LCKSP sample, no bacteria were found, this was due to the influence of the low pH of the sample. One of the important factors in bacterial growth is the pH value. Bacteria need an optimum pH for optimum growth, which is in the pH range of 6.5-7.5. The

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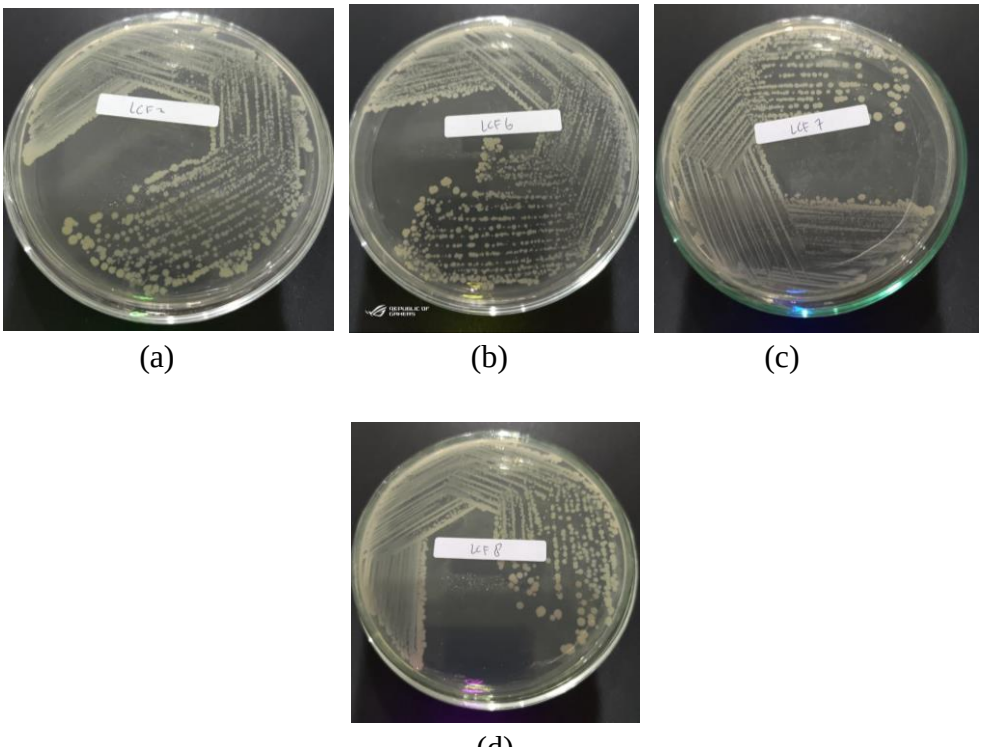
maximum pH value for bacterial growth of most bacterial species is 4 to 9. The effect of pH on bacterial growth is related to enzyme activity. This enzyme is needed by some bacteria to catalyze reactions associated with bacterial growth. If the pH in a medium or environment is not optimal, it will interfere with the work of these enzymes and ultimately interfere with the growth of the bacteria itself (Suriani et al., 2013). A decrease in pH can change the physiology of cells. Cytoplasmic acidification causes inhibition of enzyme activity, resulting in reduced catabolic flux through glycolysis so that the rate of biochemical energy synthesis decreases. The decrease in energy production along with the increase in energy use to overcome cytoplasmic acidification causes limited energy for biomass synthesis. Under these conditions, the specific growth rate decreases progressively and growth eventually stops (Subagiyo et al., 2015). From the isolation results, 15 isolates were selected for further analysis, and coded LCF 1, LCF 2, LCF 3 to LCF 15.

Isolate Characterization

Prior to characterization, the 15 isolates were purified by streaking 4 quadrants onto a new growth medium. The purified isolate is shown in the following figure and The results of the characterization of each isolate are shown in the following table.



(a) (b) (c)

(d)

Figure 2. Results of purification of several isolates by streak 4 quadrant method

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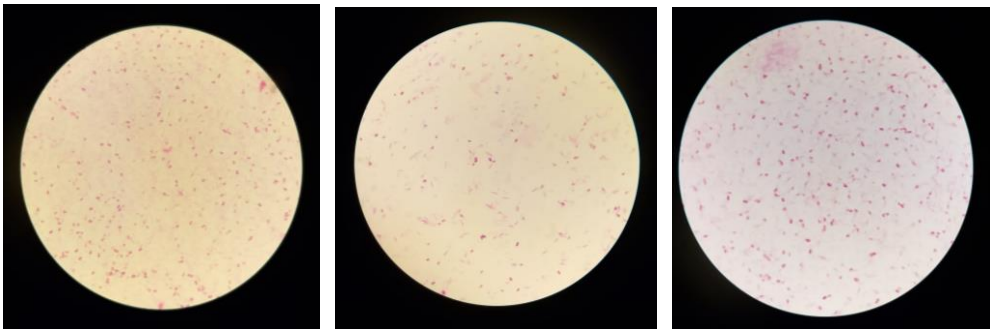
Table 1. Characterization of isolates

Isolates	Gram staining	elevation	colour	edge	morphology	Catalase test
LCF 1	+	Convex	White	entire	Streptococcus	-
LCF 2	-	Convex	White	entire	Diplobacil	-
LCF 3	-	Convex	White	entire	Monococcus	-
LCF 4	+	Convex	White	entire	Monococcus	-
LCF 5	-	Convex	White	entire	Diplococcus	-
LCF 6	-	Convex	White	entire	Streptococcus	-
LCF 7	+	Convex	White	entire	Monococcus	-
LCF 8	-	Convex	White	entire	Monococcus	-
LCF 9	+	Convex	White	entire	Streptococcus	-
LCF 10	-	Convex	White	entire	Monococcus	-
LCF 11	+	Convex	Cream	entire	Monococcus	-
LCF 12	-	Convex	Cream	entire	Streptobacil	-
LCF 13	-	Convex	White	entire	Diplobacil	-
LCF 14	+	Convex	White	entire	Streptococcus	-
LCF 15	+	Convex	White	entire	Streptococcus	-



(a) (b) (c)

Figure 3. Gram staining of isolates (a) LCF 5; (b) LCF 7; (c) LCF 9

The results of microscopic photos of Gram staining of several isolates are shown in the following figures. The morphological characterization of each bacterium is highly variable and is the first step in identifying the bacteria. From the results of morphological characterization, it was found that all isolates did not show catalase activity, which means that all isolates did not produce catalase enzyme to hydrolyze hydrogen peroxide. 7 isolates showed Gram positive, all isolates had convex colonies and entire edges. In a study reported by Chairunnisa et al (2019), bacterial isolates isolated from palm oil wastewater showed morphological characteristics including irregular, round, or circular shapes, entire and undulate edges, white, cream and yellow colony colors and flat elevation, convex, and raised (Chairunnisa et al., 2019).

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Amylase activity

Of the 15 isolates tested, only 1 isolate showed amylase activity, namely LFC 4 with an amylolytic index of 0.57. The amylolytic activity of LCF 4 is shown in the following figure.

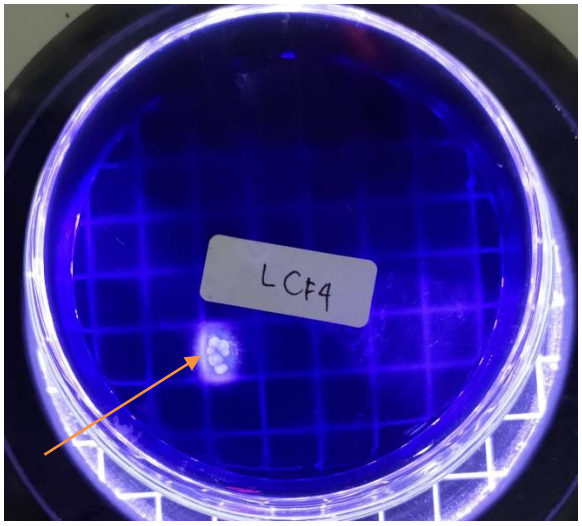

Figure 4. Amylase activity of LCF 4. The clear zone due to starch hydrolysis in the media was indicated by arrows

The formation of a clear zone around the bacteria is an indication of amylase activity, this is because the starch contained in the media is hydrolyzed by the bacterial extracellular amylase enzyme, while the dark blue color is formed through the reaction between starch and iodine (Fachrial et al., 2019). The isolation of amylase-producing bacteria from palm oil effluent is still very little reported, most of these amylase-producing bacteria are isolated from the soil. In a study reported by Gebreselema (2015), amylase-producing bacteria and actinomycetes were isolated from soil samples around the University of Gondar, Ethiopia. Of the 18 isolates that were isolated, 5 isolates showed the highest amylolytic activity and based on the morphological and biochemical characterization of the bacteria identified as *Bacillus* and *Streptomyces* (Gebreselema, 2015). Research on bacteria isolated from palm oil effluent mostly studies lipase activity. In another study, it was reported as many as 2 bacterial isolates isolated from palm oil wastewater in the Malimping-Banten area, showing lipase activity and identified as *Bacillus velezensis* and *Chryseobacterium gleum.* POME is reported to contain substantial concentrations of carbohydrates, proteins, lipids, nutrients and minerals favored by microbial growth. In POME, there are 2 types of carbohydrates, namely soluble and insoluble carbohydrates. The amount of soluble carbohydrates is very small compared to insoluble carbohydrates, such as hemicellulose, cellulose and starch. The presence of carbohydrate substrates in POME causes amylase activity in the microbes contained within (Nurul-Adela et al., 2016).

Conclusions

A total of 15 bacterial isolates were isolated from palm oil wastewater, there was 1 isolate that showed amylase activity with an amylolytic index of 0.57. For future research, it is recommended that amylase-producing bacteria be identified molecularly and their enzyme activity measured quantitatively.

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