SCREENING OF ENZYME-PRODUCING MARINE ACTINOMYCETES DERIVED FROM MANGROVE LITTERS OF OESAPA BEACH, KUPANG

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ABSTRACT

Marine Actinomycetes derived from mangrove litters have been successfully isolated from the Mangrove Forest area of Oesapa beach in Kupang, East Nusa Tenggara. Isolation was conducted by placing mangrove leave litters on Sabouraud Dextrose Agar (SDA) and then purified by using streak plate technique. Isolation brought out 5 purified marine actinomyetes isolates namely: Sr-1, Sr-2a1, Sr-2a2, Sr-2b and Sr-3. Screening for enzyme production was carried out for four hydrolytic enzymes: Protease, Amylase, Lipase and Cellulase. Four isolates tested positive for protease catalytic activity, three isolates showed both amylase and cellulase activities while lipase activity shown in all isolates. Actinomycetes colonies was characterized by having filamentous growth, gram-positive staining, earth-like smell, powdery texture colonies and occasional pigment production. These isolate colonies showed a good perspective for alternative sources of enzyme production derived from local natural resources. Moreover, these hydrolytic enzymes when yielded as crude extract, could be implemented as feed additive formulated in animal feeds. By doing so, it could help increase livestock productivity through enhancing nutrient availability and absorption while reducing animal excretion or waste at the same time.

Keywords *: hydrolytic enzymes, mangrove litters, marine actinomycetes, morphological characterization.*

1. INTRODUCTION

As human population tends to rise in time, a basic need for food sustainability become increasingly important. This would certainly lead to increase activities in agriculture for fulfilling such demands ranging from plant crops, fishery to animal farming. One type of agriculture that is advancing and industrialized is Animal Farming. Over the decade, requirements for animal protein has risen which subsequently resulted in livestock production intensification (Harvey et al., 2017). However, producing high-quality standards of animal products remain a challenge in certain parts of the country, and East Nusa Tenggara (NTT) is no exceptions. In order for an animal farming to thrive and advance, a wide variety of factors should be taken into account. One major aspect necessary for ensuring good quality of animal productivity is feedstuff (FAO, 2021). A feedstuff should be specifically formulated to ensure all the nutrients an animal need that could speed up its growth (*Animal Feed*, 2021). It is also a common practice that feedstuff is added with feed additives to further boost the wellbeing of livestock as well as assisting in nutritional absorption ("Livestock Farmers Can Improve Quality with Feed Additives," 2017).

A kind of feed additive that is widely used in feed additive manufacturing is enzyme. Several benefits expected by using such additives is that it could act to counter the anti-nutritional factors contained in animal feed, enhancing nutritional accessibility, and supplement the lack of enzymes inside the animal body (Duranthon, 2012). Enzymes applied in animal feed could come from many sources including microorganisms. One advantage is that microorganism is easily cultured and could bring out more enzyme yield (Imran et al., 2016). Enzymes it produces for instance: Amylase, Lipase,

Protease, Phytase, Xylanase, Xylanase are just to name a few. Many studies have found the significance of Actinomycetes microorganism as alternative source of enzymes that could be applied in many aspects including animal agricultural (Mukhtar et al., 2017). It is also found in a wide range of habitat including in decomposed litter (Thong, 2003).

Research on Actinomycetes isolation was conducted at the Mangrove Forest of Oesapa Beach, Kupang, East Nusa Tenggara with the main goal to screen its ability on producing 4 hydrolytic enzymes that could be applied as animal feed additive which were: Cellulase, Amylase, Protease and Lipase. The result of this research would hopefully open more possibilities of using microorganism derived from local resources especially Actinomycetes, that would in turn decrease the dependence on imported animal feed products. It is also in line with target of the Governor of East Nusa Tenggara to establish an Animal Feed factory in the province and also to support the agenda of the Ministry of Agriculture to promote livestock production commodity for the next 5 years (Lewokeda, 2020).

2. METHOD

Isolation and Purification

The source for Marine Actinomycetes isolation was taken from leaf litters of Mangrove Forest in the Oesapa beach area of Kupang, East Nusa Tenggara province. Mangrove litters were taken aseptically and put inside plastic sealed bags before taken into the laboratory. The litters were then placed on Sabouraud Dextrose Agar (SDA) with duplo repetition. After incubation at room temperature for a week, the colonies were selected for Actinomycetes isolates to further underwent purification process (Aghamirian & Ghiasian, 2009; Rudiansyah et al., 2019). Such colonies would be differed by having a distinctive earth-like scent, crusty colony appearances, filamentous growth and could often secreted pigments (Joseph et al., 2021; Ortenberg & Telsch, 2003). Purification applied selective media Yeast-Malt (YM) Agar to enhance isolate growth (El Karkouri et al., 2019).

Screening for Hydrolytic Enzymes

Purified isolates of Marine Actinomycetes were screened for its ability to produce hydrolytic enzymes: Cellulase, Amylase, Lipase and Protease. Cellulase test was conducted by inoculation on YM agar added with 1% of Carboxy Methyl Cellulose (CMC). Media grown with the isolates were submerged with 0,1% of Congo-Red (CR) solution and rinsed thrice with NaCl 1 M solution each for approximately 15 minutes. Enzyme produced would be indicated by clear zone around the colonies (Sazci et al., 2008).

YM Agar with 1% tapioca starch was used for Amylase test. After flooding the media with Lugol's iodine solution, enzyme production would be indicated by clear zone surrounding the colonies (Putri et al., 2021). For Lipase test, the isolates were streaked on YM Agar with 1% olive oil and 0,1% Rhodamine-B dye. Enzyme production would be observed by the colour changing of the colony to reddish in appearance as it absorbs the dye (Pramiadi & Yulianti, 2014). Protease production was analysed by using YM Agar with 1% of skim milk that would display clear zones around the colony

after incubation. Isolate(s) with at least one enzyme production would be gram-stained and morphologically characterized its colony that was grown on YM Agar (Alnahdi, 2012). Gram-staining applied 4 steps in which each colony was 1) smeared and heat-fixed with crystal violet in an object glass for 60 seconds, rinsed, followed by 2) lugol's iodine addition as mordant, rinsed again, 3) flooded with ethanol 95% as decolorizing agent and at last, 4) stained with a counterstain which was safranin. Gram-positive cells would show violet in colour under microscopic observation whereas Gram-negative would display reddish colour (Smith & Hussey, 2005; Tripathi & Sapra, 2021). Morphological characteristics observed including: form, elevation, margin, texture, opacity and colour on growth media (Tankeshwar, 2021).

3. RESULTS AND DISCUSSION

Isolation process brought out 5 Marine Actinomycetes isolated from leaf litters of the Mangrove Forest in Oesapa beach area. Typical colony appearances were able to be differed after incubation by having crusty colony texture, distinctive soil smell, filament-type growth and for some isolates were also observed to produce pigments (Joseph et al., 2021; Ortenberg & Telsch, 2003). Those isolates were later labelled as: Sr-1, Sr-2a1, Sr-2a2, Sr-2b and Sr-3.

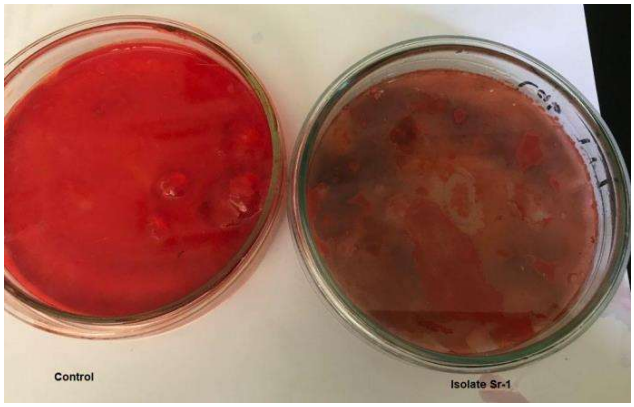

Figure 1. Positive Result of Cellulose Test on Isolate Sr-1.

Cellulase activity was shown in 3 isolates, Sr-1, Sr-2a2, and Sr-3 indicated by clear zones formed around the colonies. CR dye would be strongly attached to cellulose polymer creating a dark reddish colour on the growth media. If cellulase enzyme is present, cellulose would be degraded that disrupted this attachment. Consequently, if rinsed with NaCl solution, the dye would be easily removed producing cellulolytic zone marked with clear areas around the colony (McDonald et al., 2012). An example of a positive Cellulase test is shown on Figure 1. above. Amylase activity was shown in 3 isolates which were, Sr-1, Sr-2a2, and Sr-2b. Positive results for Amylase enzyme was shown by amylolytic zone formed around the colonies. This zone would have a clear area because of amylum decomposition by the enzyme. In the presence of amylum, when added with Lugol's iodine, charge exchange reaction would occur that reflect blue colour when various wavelength is absorbed (Saenger, 1984). However, if the substrate was degraded by Amylase, such charge reaction could not proceed

leaving a clear area in the surrounding colonies (Goedecke, 2016). An example of a positive Amylase test is shown on Figure 2. below.

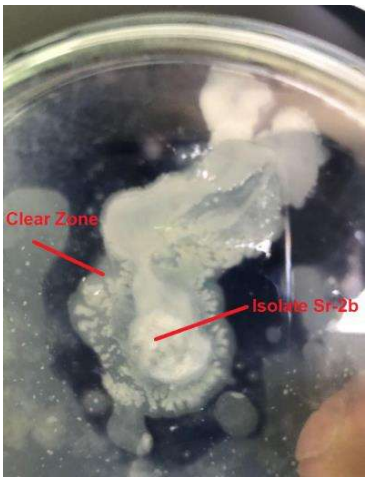

Figure 2. Positive Result of Amylose Test on Isolate Sr-2b.

For Lipase screening, positive result was indicated by the ability of the colonies to absorb the Rhodamine-B dye (Jette & Ziomek, 1994). Under lipolytic activity, the lipid substrate decomposed by Lipase would result in fatty acids released in the media that formed bonds with the dye producing slight reddish colour inside the colonies (Pramiadi & Yulianti, 2014). Based on the analysis, all isolates were capable of secreting this enzyme. One reason that could influence such result is that the microenvironment where the samples where taken was from a much polluted area with high lipid content possibly coming from domestic wastes. An example of a positive Lipase test is shown on Figure 3. below. Protease analysis showed 4 isolates able to produce the enzyme: Sr-1, Sr-2a2, Sr-2b, and Sr-3. When added with skim milk, the growth media would produce cloudy texture that when the protein substrate was decomposed into its monomers (amino acids), the surrounding colonies would have clear zones (Perwendha et al., 2020). An example of a positive Protease test is also shown on Figure 4. below.

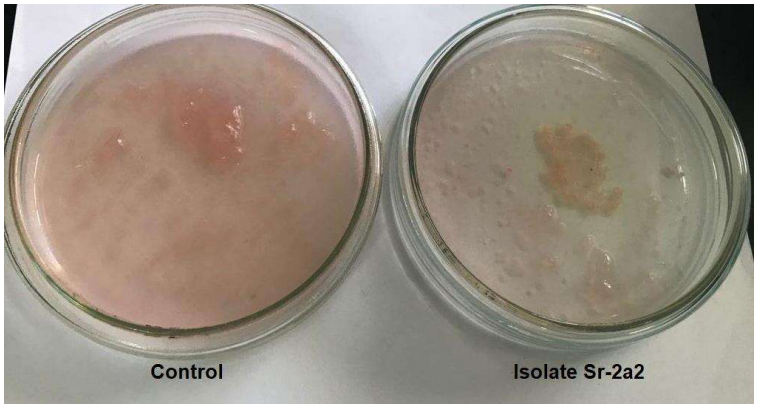

Figure 3. Positive Result of Lipase Test on Isolate Sr-2a2.

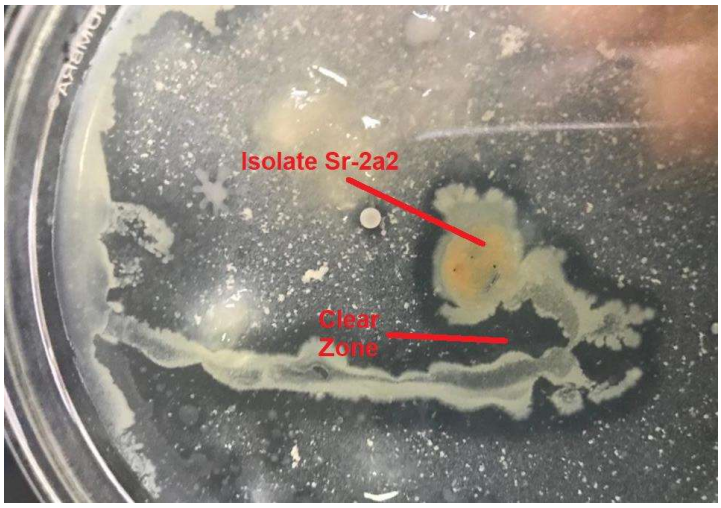

Figure 4. Positive Result of Protease Test on Isolate Sr-2a2.

Gram-staining procedure was also conducted at the colonies. Because Actinomycetes are Gram-positive, staining would make the cells become violet under microscopic observation. Based on the procedure, all isolates displayed violet. This group of bacteria would have a thicker peptidoglycan layer. When stained with Crystal violet, the primary dye will bind to the cell wall of either Gram-negative or -positive. Addition with Lugol's iodine at the second step would act as fixative to further strengthen the bond of the iodine-peptidoglycan complex. 95% Ethanol at the third step would then function as decolourizer that dissolve the lipid. However, as Gram-positive has low-lipid content, this stage would not give any significant impact that when added with the counter-stain at the last step, Safranin, the dye would not react with the peptidoglycan, leaving the cell wall violet in colour from the crystal violet at the first stage (Bruckner, 2017). Examples of a positive Gram-staining is shown on Figure 5. below.

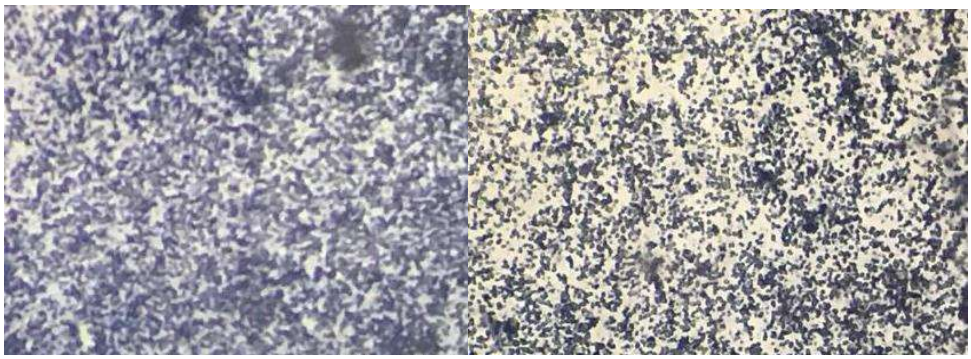

Figure 5. Positive Result of Gram-Staining Test on Isolate Sr-2a1 (left) and Sr-2b (right).

The isolates were also observed for its morphological characteristics grown on agar media (YM Agar). All colonies displayed opaque and crusty texture, with most of it possessing filamentous growth. Even though some isolates did not exhibit filamentous growth on YM agar, however, it did show filamentous growth on other media such as YM + CMC 1% and YM + skim milk. Another

characteristic that was also sensed in all colony isolates was the earth-like smell it produced. (Sivonen, 1982) isolated a chemical substance called Geosmin that was shown to be responsible for such odour. The characteristics of morphological traits observed from the 5 Marine Actinomycetes colony isolates in detail is shown on Table 1. below.

Table 1. Morphological Characteristics on YM Agar

Characteristics	Sr-1	Sr-2a1	Sr-2a2	Sr-2b	Sr-3
Form	Irregular	Filamentous	Irregular	Filamentous	Irregular
Elevation	Crateriform	Crateriform	Crateriform	Crateriform	Crateriform
Margin	Undulate	Filiform	Lobate	Filiform	Undulate
Texture	Crusty	Crusty	Crusty	Crusty	Crusty
Opacity	Opaque	Opaque	Opaque	Opaque	Opaque
Colour on YM Agar	Light brown	Light brown	Light brown	Light brown	Light brown
Colony Appearance					

Based on enzyme screening results, isolate Sr-1 and Sr-2a2 showed to possess all the hydrolytic enzymes tested. However, Sr-2a2 isolate showed preferably higher catalytic activities when compared to Sr-1 especially regarding Amylase and Protease enzymes. Moreover, this could be further analysed quantitively by using spectrophotometry to determine enzyme concentration of each isolate (Roskoski, 2007). A more detail on hydrolytic enzymes each isolate has could be seen on Table 2. below.

Table 2. Hydrolytic Enzyme(s) Produced from Each Isolates

Enzyme	Sr-1	Sr-2a1	Sr-2a2	Sr-2b	Sr-3
Cellulase	+	-	+	-	+
Amylase	+	-	++	+	-
Lipase	+	+	+	+	+
Protease	+	-	+	+	+

Enzyme screening and morphological characteristics from the result above could indicate that the species were most probably from *Streptomyces* spp. However, further analysis at molecular level related to genetic sequencing is still imperative in order to identify the exact species of the colony isolates. Application as animal feed additives could also be conducted at the earliest stage by extracting crude enzymes to be formulated in animal feed and then given to animals to see their physiological responses in terms of growth and development (*in vivo*). Other alternative is by combining crude enzyme with raw materials of animal feed which will then be analysed its catalytic activity in degrading animal feed substrates.

4. CONCLUSION

Based on the results explained above, several things could be deduced, as follows:

1. Marine Actinomycetes isolated from mangrove litters have been conducted successfully with 5 isolates was resulted from this research.
2. Leaf litters from Mangrove Forest of Oesapa beach has proven to become a source for Actinomycetes-derived enzyme explorations from local natural resources.
3. From the screening for enzyme production, the isolate showed potentials in producing 4 kinds of hydrolytic enzymes: Cellulase, Amylase, Lipase and Protease with isolate Sr-1 and Sr-2a2 exhibited catalytic activities for all enzymes.
4. Further studies should be conducted at the molecular level to identify the exact species of each isolate as well as biochemical characteristics to observe their ability to metabolize certain substrates.
5. More sorts of enzyme could also be tested especially for the ones also applied as feed additives such as: Chitinase, Xylanase, Phytase, and Ligninase.

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