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CONDITIONAL ACCEPTANCE LETTER

April 20, 2018

Dear Author(s),

We would like to congratulate you on the *acceptance* of your manuscript ***"Isolation and Screening Proteolytic and Amylolytic Potential Microbes from Wonorejo Mangrove Forest Soil, Surabaya, Indonesia"*** in ICGRC 2018 Proceeding. However, some correction may be done by the author. Therefore, you are requested to submit the revised manuscript in ICGRC 2018 email for further process and pay publication fee (2.700.000 IDR) to this following account.

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048 Screening Result ICGRC 2018

2 messages

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Isolation and Screening of Potential Proteolytic and Amylolytic ~~Potential~~ Microbes from Wonorejo Mangrove Forest Soil, Surabaya, Indonesia

Abstract. The aims of thise research were to isolate and to ~~find out~~identify the microbes that have ~~a~~ potentialey ~~in~~ amylolytic and proteolytic activities. Microbial isolation was performed on mangrove soil sample ~~of~~ from Wonorejo, Surabaya, by plating samples on NA, PDA, Nfb, CMC, and Pikovskaya ~~A~~agar mediaum. Microbial isolates obtained were grown on Bushnell Haas agar ~~which~~ enriched with starch orand milk to test production of amylases and proteases enzymes ~~production~~. The results revealeds three kinds of bacteria and six kinds of moulds that were~~which had been~~ purified and characteriszed. The two kinds of bacteria had~~with~~ NA1 and CMC1 isolate codes and the two kinds of moulds were~~,~~ PDA20131 and PIKOV1513, which could grow on PDA and Pikovskaya mediaum, and were used to test their amylolytic and proteolytic activities. Results of proteolytic tests revealeds that there were two microbial isolates that could produce proteases, i.e. PDA20131 ~~-~~and a Gram-~~-~~negative rod bacteria (NA1). The isolates that produced amylases were PIKOV1513 and a Gram-~~-~~negative rod bacteria capable of~~to~~ growing on the CMC mediaum (CMC1).

INTRODUCTION

Mangrove Ecotourism Wonorejo ~~is~~ was initiated by Wonorejo sub~~-~~district head and Community Police Communication Forum (FKPM) Rungkut District. The total area of the Ecotourism Mangrove Forest is~~of~~ approximately 700 hectares (648.453 ha) (Idajati et al., 2016). The mangrove soil depends on the sediment carried by river water, which is generally rich in organic matter and has a high nitrogen value. The deposition of sediments in mangrove forests is controlled by spatial geomorphology, hydrodynamics, and vegetation (Adame et al., 2010; Furukawa and Wolanski, 1996). A general pattern within these systems is the even distribution of sediments across the intertidal zone of the riverine mangrove forests, with suspended sediments more accumulated within the mangrove forest fringe zone (Adame et al., 2010).

Mangrove communities are ~~highly~~ an ecosystem that~~which~~ provides large quantities of organic matter to the adjacent coastal water in the form of detritus (Behera et al., 2017). Hence, it is rich in energy and contains a large active microbial population, both attached and living free (Bano et al., 1997). Mangrove leaves contain components of water-soluble organic materials such as tannins and sugars, and consist of a polymeric component called lignocellulo~~selytic~~ (Behera~~,~~ et al., 2017)~~.~~). Cellulose degradation by bacteria and fungi occurs exocellularly, because cellulose is not water soluble. Cellulose degradation ~~results~~ products are used by ~~the~~ microbes as the main energy source in their~~its~~ metabolism ~~.~~(Behera et al., 2017).

Many reports have been published on bacteria and fungi that degrade cellulose in mangroves of Asia ~~are also well documented~~. Tabao and Monsalud (2010) reported ~~cellulase producing~~ five cellulase producing bacterial strains, such as *Bacillus cereus*, *Bacillus licheniformis*, *Bacillus pumilus*, and *Bacillus* sp., isolated from Philippine mangrove. Luo et al. (2005) screened ~~twenty nine~~29 fungal isolates from mangroves (and other marine sources) in Thailand, Hong Kong, and Vietnam~~,~~ for extracellular enzyme activity and found that most of these fungal isolates produced endoglucanases and were mostly Ascomycetes, except for a b~~B~~asidiomycete, *Calathella mangrovei*, and a mitosporic fungus, *Cirrenalia tropicalis*. ~~The~~ Previous research~~es that have been done before~~ indicated that there were a number~~ny~~ of potential microbes in mangrove soil.

Surface colour of mangroves shows the community of microbes ~~that~~ living there. In the subsurface zone, orange and brown colourations indicates that there are ~~any~~ partially oxidiszed conditions due to the release of O_2

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by living mangrove roots, while the rest of the layers are gray and black, indicatinges that there areis any reduce conditions due to the microbial sulfate reduction (Castro-Rodriguez et al., 2018). Therefore, in this study take samples of soil were taken from the surface close to mangrove roots.

Mangrove Wonorejo is located in an estuary area that gets streams from various rivers, one of which is Kali Wonorejo (River). So that the community of mangrove microbes can be influenced from by organic and inorganic materials from Kali Wonorejo. The aim of this study was to explore potential microbes of mangrove soil Mangrove, located in the Mangrove Ecotourism Wonorejo, Surabaya, Indonesia, as amylolytic and proteolytic microbes.

METHODS

Soil Sampling

Wonorejo mangrove soil samples from each location were taken at depths of 15 and 20 cm (Imchen et al., 2017) at three different points. The lLocation of Mangrove Wonorejo is showed inby Figure 1a. Soil sampling was done using a Belgi drill. Soil samples then put into a sterile plastic bags and labeled. Sampling activities are shownis figured in Figure 1b. All the soil samples were taken to the Laboratory of Microbiology of the Faculty of Science and Technology, Airlangga University. S, samples used for isolation purposes were stored in the refrigerator until analysis was performed.

Microbial Isolation

Isolation of microbes was done by dissolving 1 g of soil from depths of 15 cm and 20 cm. T, and then the dissolved soil was serially dilluted in steriliszed distilled water tountil 10^{-20}. The 10^{-13}, 10^{-14}, and 10^{-15} serialesdilutions were was carried out on Pikovskaya mediaum according to Ying et al. (2016), with modification (in 1 L: glucose 10 g glucose; 5 g $Ca_3(PO_4)_2$ 5 g; NaCl 0.2 g NaCl; KCl 0.2 g KCl; 0.1 g $MgSO_4.7H_2O$ 0.1 g; 0.0025 g $MnSO_4.2H_2O$ 0.0025 g; 0.0025 g $FeSO_4.7H_2O$ 0.0025 g; 0.5 g yeast extract 0.5 g; 0.5 g $(NH_4)_2SO_4$ 0.5 g; 15 g agar 15 g). W, while for CMC and Nfb, mediaum used dilutions used were 10^{-13}, 10^{-14}, and 10^{-15}., Oon the medium of PDA (Oxoid) and NA (Oxoid) media dilutions were was done up to serials 10^{-17}, 10^{-18}, 10^{-19}, and 10^{-20}. CMC mediaum was made according to Kulkarni et al. (2018), with modification (in 1 L: CMC 10 g CMC; KNO_3 0.75 g KNO_3: $MgSO_4$ 0.2 g $MgSO_4$; KH_2PO_4 0.5 g KH_2PO_4; $FeSO_4$ 0.02 g $FeSO_4$; $CaCl_2$ 0.04 g $CaCl_2$; 0.5 g yYeast Eextract 0.5 g). Nfb medium was made according to Andrade et al. (2014), with modification (in 1 L; 5 g malic acid 5 g; KOH 4 g KOH: K_2HPO_4 0.5 g K_2HPO_4; $FeSO_4$ 0.05 g $FeSO_4$; $MnSO_4$ 0.01 g $MnSO_4$; $MgSO_4$ 0.1 g $MgSO_4$; NaCl 0.2 g NaCl; $CaCl_2$ 0.02 g $CaCl_2$; 3 mL bromothymol blue 3 mL; 1.75 g agar 1.75 g;). PDA mediaum was added supplemented with chloramphenicol 2000 ppm chloramphenicol. The plates then were incubated at room temperature for three days. The colonies of microbes present in each media after incubated incubation were observed. Bacteria and mould isolates were randomly selected and then purified and characteriszed. Characteriszation of microbial isolates was done by observing the shape and type of bacteria by Gram staining method (Cappuccino and Sherman, 2014), while the characterizsation of mould was done by observing at the macroscopic characteristics of the colony. Two bacteria and two mould isolates were selected randomly to be used for screening byof amylolytic and proteolytic tests.

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Screening of Microbial Amylolytic and Proteolytic Activitiesy

The screening test was carried out using Bushnell Haas Agar agar according to Bushnell and Haas (1941), composition with modification (in 100 mL: K_2HPO_4 0.1 g K_2HPO_4; KH_2PO_4 0.1 g; NH_4NO_3 0.1 g NH_4NO_3; 0.02 g $MgSO_4.7H_2O$ 0.02 g; 0.002 g $CaCl_2.2H_2O$ 0.002 g; $FeCl_3$ 0.005 g $FeCl_3$; agar 2 g agar), supplemented with milk (2 g/100 mL) for screening proteolytic activity, and supplemented with starch (2 g/100 mL) for amylolytic activity. Each selected microbial isolate was spotted to the medium media (Hasan, 2015) and then incubated for 3-6 days at 37 °C. Amyolytic activity was determined by evaporating the entire surface to prepare a dish with iodine crystals. Amylolytic activity was observed in by the presence of a clear zone around the colonies after exposure to iodine vapor (Kasavi et al., 2016). The observations of proteolytic activity were made by observing the presence

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of clear zones around colonies that have been grown on Bushnell Haas ~~medium~~ media enriched with milk. The ~~medium~~ media would remain opaque if there was no~~t any~~ hydrolytic activity (Cappuccino and Sherman, 2014).

RESULTS AND DISCUSSION

Isolation of potential microbes ~~in~~ with proteolytic and amylolytic activities from Wonorejo mangrove soil, Surabaya, was performed~~has been done~~. Microbial isolation from depths of 15 and 20 cm of mangrove soil obtained various microbial isolates when grow~~i~~ng on PDA, NA, Nfb, Pikovskaya, and CMC medi~~a~~um. Based on the results of the isolation, three randomly selected bacterial isolates were purified and further characteri~~s~~zed. The three isolates were coded NA1, Nfb1, and CMC1. NA1 and Nfb 1 grow~~i~~ng on NA and Nfb medi~~a~~um, respectively, were derived from a depth of 20 cm, while CMC1 grow~~i~~ng on CMC medi~~a~~um was derived from a depth of 15 cm (Table 1). The isolates obtained ~~ing~~ from secondary isolation were~~as~~ further microscopically characteri~~s~~zed, i.e. by the shape and bacterial Gram status.

In addition to bacteria isolates, isolation also obtained moulds. Six isolates of moulds grow~~i~~ng on PDA and Pikovskaya medi~~a~~um were randomly selected for morphological characteri~~s~~zation. Morphological characteristics of each mould are~~is~~ described in Table 2.

PIKOV1513, PDA20132, and PDA20145. PDA15P4 grow~~i~~ng on PDA medi~~a~~um was derived from soil at a depth of 15 cm. PDA20131, PDA20132, and PDA20145 grow~~i~~ng on PDA medi~~a~~um were derived from soil at a depth of 20 cm, while PIKOV2015 and PIKOV1513 grow~~i~~ng on Pikovskaya medium were derived from soil at ~~a~~ depth~~s~~ of 20 cm and 15 cm, respectively. The appearance of each isolate is shown in Figure 2.

In this research, two isolates of bacteria and two isolates of moulds were selected randomly to be tested for their amylolytic and proteolytic activities~~y~~. The two bacterial isolates used were isolates CMC1 and NA1, while mould isolates used were PDA20131 and PIKOV1513. Based on the results of screening, it was found that PIKOV1513 and CMC1 isolates showed amylolytic activities, while NA1 and PDA20131 isolates showed proteolytic activities (Figure 2).

The amylolytic activity of bacteria and moulds was indicated by the formation of clear zones around the isolate colonies when each isolate was grown on ~~the~~ Bushnell Haas a~~A~~gar medi~~a~~um containing 2% starch. Based on the research, CMC1 and PIKOV1513 isolates were able~~known~~ to form clear zones around the colonies. The presence of clear zones around the colonies indicated there was ~~any~~ starch hydrolysis processes by the microbes (Cappuccino and Sherman, 2014). Research ~~that had been~~ conducted by Pupin and Nahas (2013) ~~had~~ succeeded in isolating fungi and bacteria amylase producers from mangrove soil in Cardoso Island Park, Sao Paulo, Brazil. O~~A~~nother research ~~that had been~~ done by Bibi et al. (2017) showed that seven isolates of bacteria, which were EA152, EA154, EA156, EA162, EA164, EA177, and EA179, isolated~~ing~~ from rhizospheres of *Salsola imbricata*, *Avicennia germinans*, *Halopeplis perfoliata*, *Halocnemum strobilaceum*, and *Zygophyllum qatarense* were kno~~a~~ble~~wn~~ to produce amylases. Another study~~research that had~~ done by Ramírez-Elías et al. (2014) showed that there were ~~any~~ amylase producing microbes that could be isolated from rhizospheres of mangrove species~~,~~ *Rhizophora mangle* L., *Laguncularia racemosa* (L.) Gaertn., *Avicennia germinans* (L.) Stern, and *Conocarpus erectus* L.~~,~~ from Terminos Lagoon, Campeche, Mexico.

This study also screened the proteolytic activity of microbes using Bushnell Haas a~~A~~gar medi~~a~~um ~~which was~~ enriched with milk. The ability of bacteria to secrete proteases was indicated by clear zone formation around the microbial isolate (Cappuccino and Sherman, 2014). Based on this~~e~~ research, isolates of NA1 bacteria and PDA20131 were sh~~kn~~own to produce a~~have~~ clear zone around the microbial colony. Bibi et al. (2017) reported that b~~a~~ecterial isolates, EA152, EA154, EA165, EA170, EA177, and EA179, that were isolated from rhizospheres of *Salsola imbricata*, *Avicennia germinans*, *Halocnemum strobilaceum*, and *Zygophyllum qatarense* had the ability to produce proteases. O~~A~~nother research conducted by Ramirez-Elias et al. (2014) also indicated that there were microbes that had proteolytic activity from rhizospheres of mangrove species~~,~~ *Rhizophora mangle* L., *Laguncularia racemosa* (L.) Gaertn., *Avicennia germinans* (L.) Stern, and *Conocarpus erectus* L.~~,~~ from Terminos Lagoon, Campeche, Mexico

Bacterial isolates, i.e. NA1 and CMC1, and fungal isolates, i.e. PDA20131 and PIKOV1513, had the potential~~cy~~ to produce proteolytic and amylolytic enzymes. Therefore, it was necessary to identify the isolates to determine the species. In this study, the names of bacteria and mould~~s~~ isolates could not be determined yet, they~~it~~ still need further characteri~~s~~zation.

CONCLUSION

Microbial isolation from mangrove soil in Wonorejo, Surabaya obtained three bacterial isolates and six isolates of moulds. Bacterial isolates –CMC1 (rod, Ggram-–negative) and PIKOV1513 were foundknown to produce amylolytic enzymes, whereas bacterial isolates NA1 (rod, Ggram-–negative) and PDA20131 were foundknown to produce proteolytic enzymes.

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