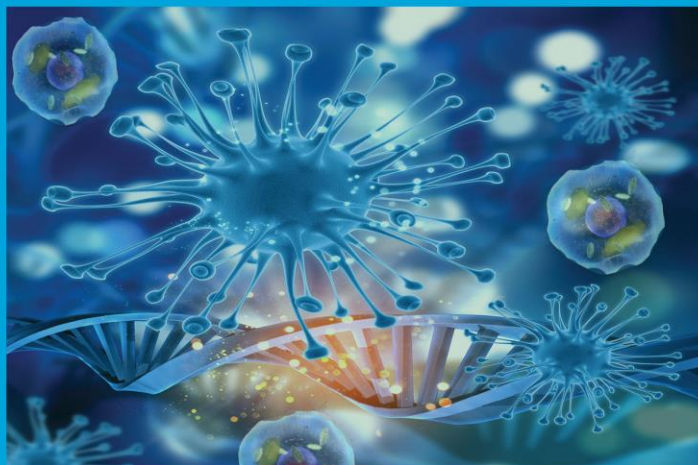


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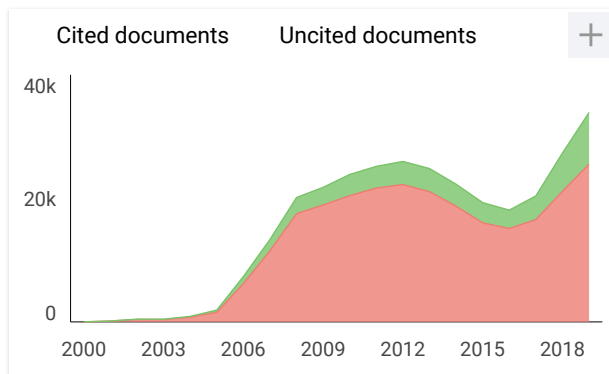
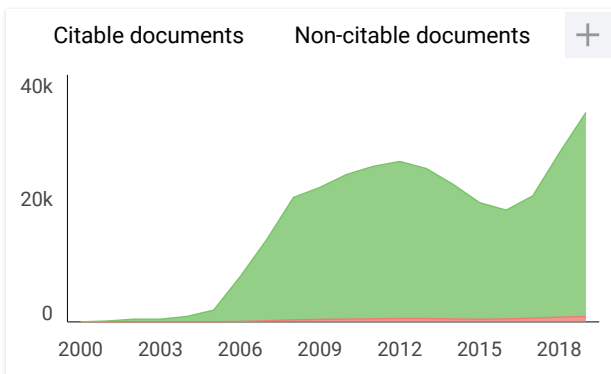
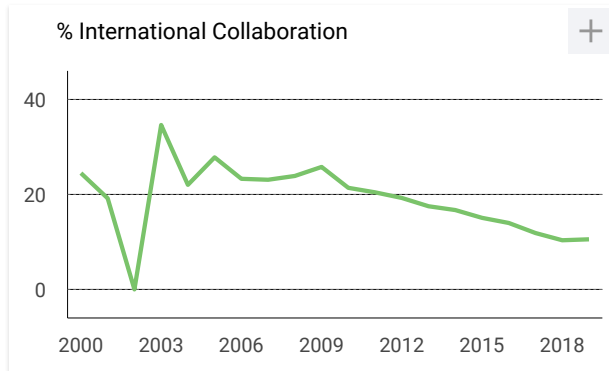
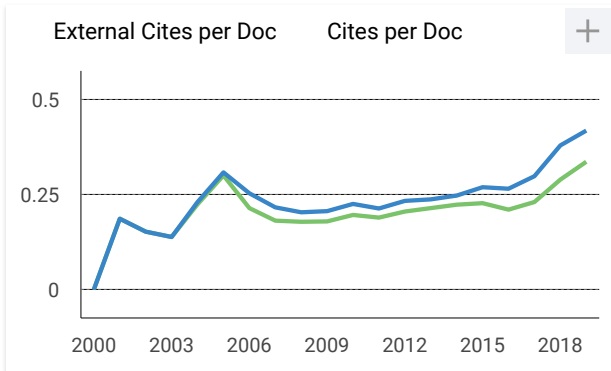
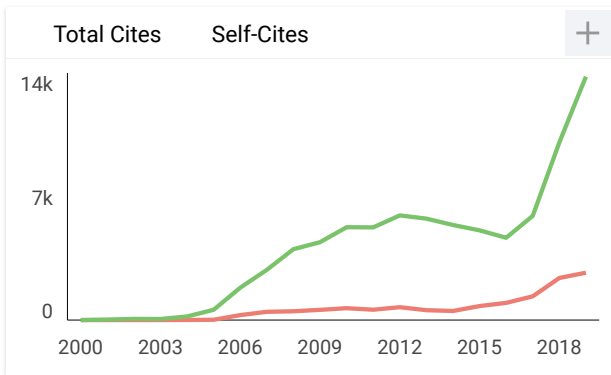
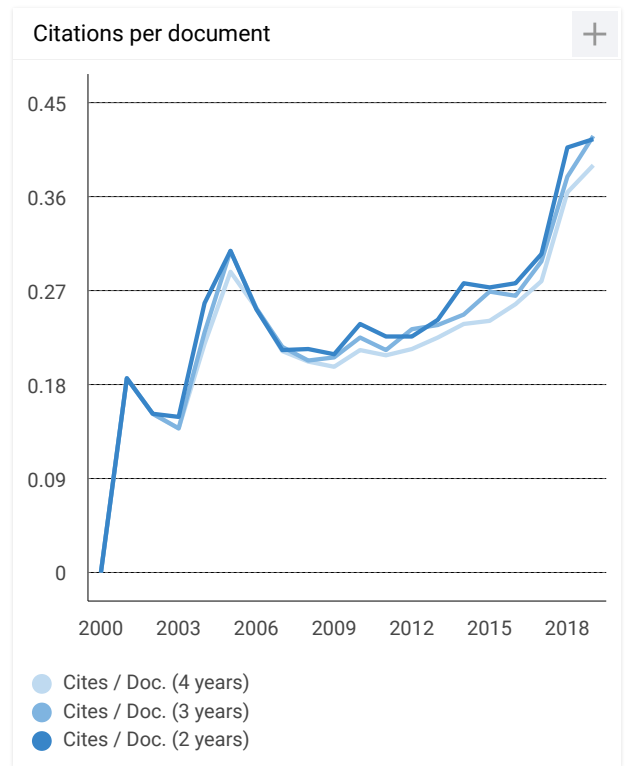
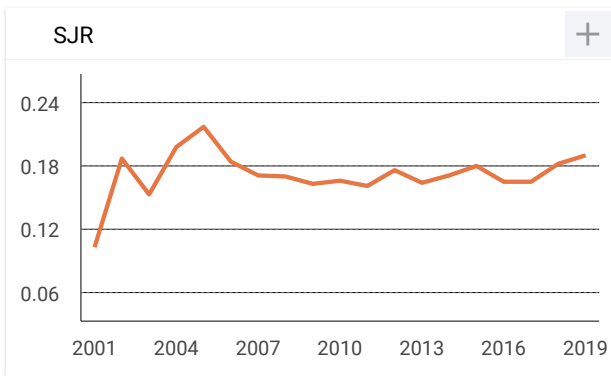
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Isolation and screening of potential proteolytic and amylolytic microbes from Wonorejo Mangrove forest soil, Surabaya, Indonesia

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Ana Mariatul Khiftiyah, Nabela Nur Hanifah, Muhammad Bachruddin, Mar'atus Sholichah, Siti Istiqomah, Selva Rosyta Dewi, Tri Rahayu, Indra Adi Wira Prasetya, Lisa Marjayandari, Nurul 'Aini, Izdihar Tsana, Desi Triwahyuni, Fatimah, and Ni'matuzahroh



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

Ana Mariatul Khiftiyah¹, Nabela Nur Hanifah¹, Muhammad Bachruddin¹, Mar'atus Sholichah¹, Siti Istiqomah¹, Selva Rosyta Dewi¹, Tri Rahayu¹, Indra Adi Wira Prasetya¹, Lisa Marjayandari¹, Nurul 'Aini¹, Izdihar Tsana¹, Desi Triwahyuni¹, Fatimah¹, Ni'matuzahroh^{1,a)}

¹Laboratory of Microbiology, Department of Biology, Faculty of Science and Technology, Universitas Airlangga, Surabaya 60115, East Java, Indonesia

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Abstract. The aims of this research were to isolate and to identify the microbes that have potential amylolytic and proteolytic activities. Microbial isolation was performed on mangrove soil sample from Wonorejo, Surabaya, by plating samples on Nutrient Agar (NA), Potato Dextrose Agar (PDA), Nitrogen free bromothymol blue (Nfb), Carboxymethylcellulose (CMC), and Pikovskaya agar media. Microbial isolates obtained were grown on Bushnell Haas agar enriched with starch or milk to test production of amylase and protease enzymes. The results revealed three kinds of bacteria and six kinds of moulds that were purified and characterised. The two kinds of bacteria had NA1 and CMC1 isolate codes and the two kinds of moulds were PDA20131 and PIKOV1513, which could grow on PDA and Pikovskaya media, and were used to test their amylolytic and proteolytic activities. Results of proteolytic tests revealed 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 growing on the CMC media (CMC1).

Keywords: Amylolytic, growth, microbes, proteolytic.

INTRODUCTION

Mangrove Ecotourism Wonorejo was initiated by Wonorejo subdistrict head and Community Police Communication Forum (FKPM) Rungkut District. The total area of the Ecotourism Mangrove Forest is approximately 700 hectares (648.453 ha).¹ 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.^{2,3} 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.²

Mangrove communities are an ecosystem that provides large quantities of organic matter to the adjacent coastal water in the form of detritus.⁴ Mangrove ecosystems contain active and diverse microbial community that can provides nutrients from dead vegetation through microbial breakdown.⁵

Many reports have been published on bacteria and fungi that degrade cellulose in mangroves of Asia. Tabao and Monsalud (2010)⁶ reported four cellulase producing bacterial strains, such as *Bacillus cereus*, *Bacillus licheniformis*, *Bacillus pumilus*, and *Bacillus* sp., isolated from Philippine mangrove. Research done by Luo et al. showed that 29 fungal isolates from mangrove and marine habitats could produce endoglucanase, xylanase, and laccase.⁷ Four Actinomycetes isolated from mangrove in Kerala, India, could inhibit bacterial and fungal pathogens growth, such as *Staphylococcus aureus* ATCC 25923, *Staphylococcus citreus*, *Bacillus cereus*, *Serratia marcescens*, *Penicillium* sp., *Candida albicans*, *Candida parapsilosis*, and *Cryptococcus neoformans*.⁸ Previous research indicated that there were a number of potential microbes in mangrove soil.

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

EXPERIMENTAL DETAIL

Soil Sampling

Wonorejo mangrove soil samples from each location were taken at depths of 15 and 20 cm,⁹ at three different points. The location of Mangrove Wonorejo is showed in Fig. 1(a). Soil sampling was done using a Belgi drill. Soil samples then put into sterile plastic bags and labeled. Sampling activities are shown in Figure 1(b). All the soil samples were taken to the Laboratory of Microbiology of the Faculty of Science and Technology, Universitas Airlangga. Samples used for isolation purposes were stored in the refrigerator until analysis was performed.



FIGURE 1. (a) Location of Mangrove Wonorejo,¹⁰ (b) Sampling activities.

Microbial Isolation

Isolation of microbes was done by dissolving 1 g of soil from depths of 15 cm and 20 cm. Then the dissolved soil was serially diluted in sterilised distilled water to 10^{-20} . The 10^{-13} , 10^{-14} , and 10^{-15} serial dilutions were carried out on Pikovskaya media according to Ying et al. (2016)¹¹, with modification (in 1 L: 10 g glucose; 5 g $\text{Ca}_3(\text{PO}_4)_2$; 0.2 g NaCl; 0.2 g KCl; 0.1 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.0025 g $\text{MnSO}_4 \cdot 2\text{H}_2\text{O}$; 0.0025 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; 0.5 g yeast extract; 0.5 g $(\text{NH}_4)_2\text{SO}_4$; 15 g agar). While for Carboxymethylcellulose (CMC) and Nitrogen free bromothymol blue (Nfb), media dilutions used were 10^{-13} , 10^{-14} , and 10^{-15} . On the Potato Dextrose Agar (PDA) (Oxoid) and Nutrient Agar (NA) (Oxoid) media dilutions were done up to serials 10^{-17} , 10^{-18} , 10^{-19} , and 10^{-20} . CMC media was made according to Kulkarni et al., with modification (in 1 L: 10 g CMC; 0.75 g KNO_3 ; 0.2 g MgSO_4 ; 0.5 g KH_2PO_4 ; 0.02 g FeSO_4 ; 0.04 g CaCl_2 ; 0.5 g yeast extract).¹² Nfb medium was made according to Andrade et al. (2014)¹³, with modification (in 1 L 5 g malic acid; 4 g KOH; 0.5 g K_2HPO_4 ; 0.05 g FeSO_4 ; 0.01 g MnSO_4 ; 0.1 g MgSO_4 ; 0.2 g NaCl; 0.02 g CaCl_2 ; 3 mL bromothymol blue; 1.75 g agar). Potato Dextrose Agar (PDA) media was supplemented with 2000 ppm chloramphenicol. The plates then were incubated at room temperature for three days. The colonies of microbes present in each media after incubation were observed. Bacteria and mould isolates were randomly selected and then purified and characterised. Characterisation of microbial isolates was done by observing the shape and type of bacteria by Gram staining method, while the characterisation of mould was done by observing the macroscopic characteristics of the colony.¹⁴ Two bacteria and two mould isolates were selected randomly to be used for screening by amylolytic and proteolytic tests.

Screening of Microbial Amylolytic and Proteolytic Activities

The screening test was carried out using Bushnell Haas agar according to Bushnell and Haas, with modification (in 100 mL: 0.1 g K_2HPO_4 ; 0.1 g NH_4NO_3 ; 0.02 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.002 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; 0.005 g FeCl_3 ; 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.¹⁵ Each selected microbial isolate was spotted to the media¹⁶ and

then incubated for 3-6 days at 37°C. Amyolytic activity was determined by evaporating the entire surface to prepare a dish of medium with iodine crystals. Amyolytic activity was observed by the presence of a clear zone around the colonies after exposure to iodine vapor.¹⁷ The observations of proteolytic activity were made by observing the presence of clear zones around colonies that have been grown on Bushnell Haas media enriched with milk. The media would remain opaque if there was no hydrolytic activity.¹⁴

RESULTS AND DISCUSSION

Isolation of potential microbes with proteolytic and amyolytic activities from Wonorejo mangrove soil, Surabaya, was performed. Microbial isolation from depths of 15 and 20 cm of mangrove soil obtained various microbial isolates when grown on PDA, NA, Nfb, Pikovskaya, and CMC media. Based on the results of the isolation, three randomly selected bacterial isolates were purified and further characterised. The three isolates were coded NA1, Nfb1, and CMC1. NA1 and Nfb1 grown on NA and Nfb media, respectively, were derived from a depth of 20 cm, while CMC1 grown on CMC media was derived from a depth of 15 cm (Table 1). The isolates obtained from secondary isolation were further microscopically characterised, i.e. by the shape and bacterial Gram status.

TABLE 1. Microscopic characteristics of bacteria isolated from Wonorejo mangrove soil.

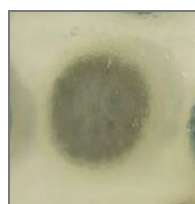
| Isolates | Cell Shape | Gram Type |
|----------|------------|-----------|
| NA1 | Rod | Negative |
| Nfb1 | Coccus | Negative |
| CMC1 | Rod | Negative |

In addition to bacteria isolates, isolation also obtained moulds. Six isolates of moulds grown on PDA and Pikovskaya media were randomly selected for morphological characterisation. Morphological characteristics of each mould are described in Table 2.

TABLE 2. Macroscopic characteristics of molds isolated from Wonorejo mangrove soil.

| Isolates | Morphological Characteristics |
|-----------|--|
| PDA15P4 | Grey, velvety |
| PDA20131 | Yellow, brown edges, velvety |
| PIKOV2015 | Black, granular, cleavage and radial furrow were presence |
| PIKOV1513 | Dark green, velvety, radial furrow was presence |
| PDA20132 | Black, granular, cleavage furrow and radial furrow were presence |
| PDA20145 | Dark green, granular |

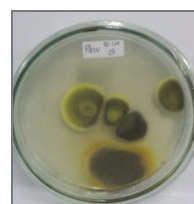
Six isolates of moulds obtained from the isolation were coded PDA15P4, PDA20131, PIKOV2015, PIKOV1513, PDA20132, and PDA20145. PDA15P4 grown on PDA media was derived from soil at a depth of 15 cm. PDA20131, PDA20132, and PDA20145 grown on PDA media were derived from soil at a depth of 20 cm, while PIKOV2015 and PIKOV1513 grown on Pikovskaya medium were derived from soil at depths of 20 cm and 15 cm, respectively. The appearance of each isolate is shown in Fig. 2.



(a)



(b)



(c)

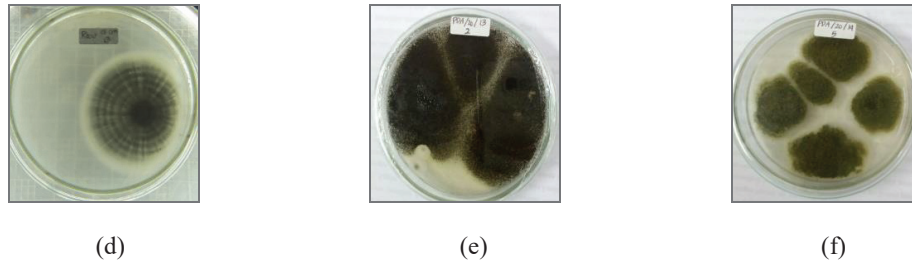


FIGURE 2. Morphology of molds that were obtained from mangrove soil, Wonorejo. (a) PDA15P4; (b) PDA20131; (c) PIKOV2015; (d) PIKOV1513; (e) PDA20132; (f) PDA20145.

Previous research on bacterial isolation from mangrove soils indicated that microbial isolates have various potentials.^{18,19,20,21} Some fungi and bacteria isolated from mangrove soil in Cardoso Island Park, Sao Paulo, Brazil, in summer and winter, could produce amylase, acid phosphatase, and solubilise phosphate.²⁰ Some bacteria isolated from mangrove rhizospheres from Thuwal coastal area, Jeddah, Saudi Arabia had the ability to produce cellulase, protease, lipase, and amylase.¹⁸ *Serratia* sp. isolated by Behera et al. from mangrove soil of the Mahanadi River delta, had potential to solubilise phosphate and produce some organic acids, such as lactic acid, malic acid, and acetic acid.²¹ Other research conducted by Kathiresan and Selvam indicated bacteria that were isolated from rhizospheres of *Rhizophora mucronata* from Pellar estuary in Parangipettai, India had potency to produce indole acetic acid and ammonia, solubilise phosphate, and showed nitrogenase activity.¹⁹

In this research, two isolates of bacteria and two isolates of moulds were selected randomly to be tested for their amyolytic and proteolytic activities. 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 amyolytic activities, while NA1 and PDA20131 isolates showed proteolytic activities (Fig. 2).

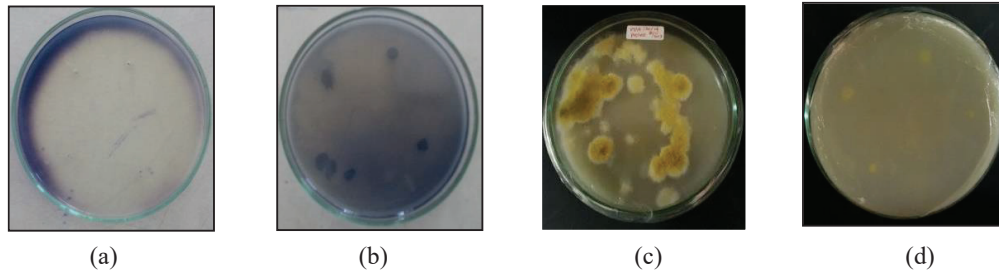


FIGURE 3. Amyolytic and proteolytic screening of selected isolates. (a) and (b) were PIKOV1513 and CMC1 respectively, showed amyolytic activity. (c) and (d) were PDA20131 and NA1 respectively, showed proteolytic activity.

The amyolytic activity of bacteria and moulds was indicated by the formation of clear zones around the isolate colonies when each isolate was grown on Bushnell Haas agar media containing 2% starch. Based on the research, CMC1 and PIKOV1513 isolates were able to form clear zones around the colonies (Fig. 3). The presence of clear zones around the colonies indicated there was starch hydrolysis processes by the microbes.¹⁴ Research conducted by Pupin and Nahas (2013)²⁰ succeeded in isolating fungi and bacteria amylase producers from mangrove soil in Cardoso Island Park, Sao Paulo, Brazil. Other research done by Bibi et al. showed that seven isolates of bacteria, which were EA152, EA154, EA156, EA162, EA164, EA177, and EA179, isolated from rhizospheres of *Salsola imbricata*, *Avicennia germinans*, *Halopeplis perfoliata*, *Halocnemum strobilaceum*, and *Zygophyllum qatarense* were able to produce amylases.¹⁸ Another study done by Ramirez-Eliás et al. showed that there were amylase producing microbes that could be isolated from rhizospheres of mangrove species *Rhizophora mangle* L., *Laguncularia racemosa* (L.) Gaertn.f, *Avicennia germinans* (L.) Stern, and *Conocarpus erectus* L. from Terminos Lagoon, Campeche, Mexico.²²

This study also screened the proteolytic activity of microbes using Bushnell Haas agar media enriched with milk. The ability of bacteria to secrete proteases was indicated by clear zone formation around the microbial isolate.¹⁴ Based on this research, isolates of NA1 bacteria and PDA20131 were shown to produce a clear zone around the microbial colony. Bibi et al. reported that bacterial 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.¹⁸ Other research conducted by

Ramirez-Elias et al. also indicated that there were microbes that had proteolytic activity from rhizospheres of mangrove species *Rhizophora mangle* L., *Laguncularia racemosa* (L.) Gaertn.f, *Avicennia germinans* (L.) Stern, and *Conocarpus erectus* L. from Terminos Lagoon, Campeche, and Mexico.²²

Bacterial isolates, i.e. NA1 and CMC1, and fungal isolates, i.e. PDA20131 and PIKOV1513, had the potential 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 isolates could not be determined yet, they still need further characterisation.

SUMMARY

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

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