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Isolation and potency of Actinomycetes from rhizosphere of nutmeg (*Myristica fragrans* Houtt)

Ferymon Mahulette[1*], Esti Utarti[2], Tri Santi Kurnia[3]
[1]Study Program of Biology Education, Faculty of Teacher Training and Education, Universitas Pattimura
Jl. M. Putuhena, Ambon, Maluku, Indonesia. 97233
[2]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Jember
Jl. Kalimantan Tegalboto No. 37 Jember, East Java, Indonesia. 68121
[3]Study Program of Biology Education, Faculty of Tarbiyah and Teachers Training, Institut Agama Islam Negeri Ambon
Jl. Dr. H. Tarmizi Taher, Sirimau, Ambon, Maluku, Indonesia. 97128
*Email: ferymonm@gmail.com

ABSTRACT. Nutmeg (*Myristica fragrans* Houtt) is commonly cultivated by people in the forests of Moluccas Islands. This plant grows well on relatively infertile soil types. This is presumably due to the presence of symbiotic microbes in the root of nutmeg. The research aimed to isolate, characterize and test the potential of Actinomycetes from rhizosphere of nutmeg. Soil sample were taken from the nutmeg forest in Ambon Island. The Actinomycetes isolation using humic acid vitamin, continued with yeast malt agar (YMA) media. The testing of antibacterial and antifungal activities using YMA media, while cellulolytic activity, phosphate solubilizing, and xylanolytic activity using carboxyl methyl cellulose, Picovskaya agar, and birchwood agar or oat spelt xylan agar. A total of 12 isolates of Actinomycetes were isolated and dominated by *Streptomyces* with various types of aerial mycelia. The substrate mycelium looks brown and cream, while the aerial mycelium looks white and gray. These isolates had the highest inhibitory power against *Escherichia coli* and *Fusarium oxysporum* with indexes of 16.5 mm and 16.0 mm, respectively. The other isolates have the ability of cellulolytic, phosphate solubilizing, and xylanolytic with indexes 3.26, 3.87, and 1.2, respectively. The Actinomycetes isolates that were found can be used as starter to improve the biofertilizer formula for nutmeg.

Keywords: Actinomycetes isolates; antifungal activity; biofertilizer; nutmeg root; *Streptomyces* sp.

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INTRODUCTION

Moluccas has long been known as the Spice Islands in the world. One of the famous spices in this area is nutmeg (*Myristica fragrans* Houtt) (Joseph & George, 2014). Nutmeg grows well on relatively infertile soil types. This is presumably due to the presence of microbes, mainly Actinomycetes associated with the rhizosphere of this plant. Soil in the roots (rhizosphere) of nutmeg that covered by litter is one of the good soils to isolate and test the potential of Actinomycetes, both the potential to produce antimicrobial compounds (Nurkanto & Julistiono, 2014) and produce extracellular enzymes (Baldrian *et al*., 2013). Local plants have their own characteristics in each region. Nutmeg do not grow evenly in Moluccas Islands. The centers for cultivation of this plant are Banda and Ambon Island region (Simanjorang *et al*., 2020). The plants have adapted to the growth environment including microbes that are found around the growing environment (De Zelicourt *et al*., 2013). The adaptation was formed due to the symbiosis of mutualism between plants and various local microbes, including Actinomycetes. These adaptations are often specific to certain types of plants only in certain location (Listiana *et al*., 2018).

Nutmeg can grow on the type of yellowish red soil (podzolic) with low acidity (Ariandi, 2017) The characteristic of this type of soil is the very availability of phosphate limited to plant growth. This type of soil is very good for isolating Actinomycetes which have the ability to dissolve phosphate because these microbes make use of insoluble phosphate for their metabolic and convert it into soluble phosphate for plants (Sineva, 2021). Nutmeg usually grows in the mountainous forests of Ambon Island. The condition of forest soils coated with plant residues (litter) is a good source of xylan

(Nurnawati *et al*., 2014) and cellulose. Litter derived from nutmeg also contains myristicin, a type of phenylpropene which is also allelopathic to plants and microbes. This compound is antimicrobial against various types of bacteria (Nilawati *et al*., 2020). The existence of these allelopathic compounds causes not all microbes to be associated with nutmeg rhizosphere. Microbes that can be associated with nutmeg rhizosphere are microbes that have resistance to stress and have the potential to produce antimicrobial compounds.

Isolation and characterization of Actinomycetes in the nutmeg rhizosphere has never been provided until now. Therefore, this study aimed to isolate and characterize and test the potential of Actinomycetes from rhizosphere of nutmeg. It is necessary to explore by isolating and identifying Actinomycetes in the rhizosphere of the nutmeg. Isolation and identification are important to find specific Actinomycetes strains in soil taken from the rhizosphere of nutmeg and their potential to produce antimicrobial compounds and extracellular enzymes. These strains are expected to be useful to support plant growth and resistance to disease, so that it can reduce the costs of farmers to buy fertilizers and pesticides.

MATERIALS AND METHODS

Samples of soil were taken from nutmeg forests in Lateri Village, Ambon City. Samples were taken at a depth of 10-15 cm and above it there was a litter that falls from a nutmeg tree. Sample preparation and testing were conducted at Laboratory of Microbiology, IPB University.

Isolation of Actinomycetes from rhizosphere of nutmeg. The sample was air dried, mashed and filtered. After the soil was dried, 1 g of the soil sample was weighed and suspended into 90 mL of sterile physiological saline (0.85% NaCl) and dilute serially from 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}. A total of 0.1 mL of samples that have been diluted 10^{-3} and 10^{-4} inoculated into Petri dishes contain humic acid vitamin (HAV) agar (*duplo*) using the spread plate technique with a sterile L-shape glass rod until it was completely absorbed into the media. The composition of HAV media were humic acid (40 ml/l), $CaCO_3$ (0.02 g/l), $FeSO_4$ (0.1 g/l), KCl (1.71 g/l), $MgSO_4$ (5 g/l), Na2 (HPO_4) (0.5 g/l) and bacto agar (18 g/l) and enriched with nalicilic acid (20 mg/l), vitamins (5 ml/l) and cycloheximide (0.05 g/l). Incubate for a week at room temperature (30°C). Characterize of selected Actinomycetes isolates based on the GRAS method (Sukmawaty *et al*., 2020). The observed macroscopic characters of Actinomycetes isolates were the color of substrate and aerial mycelium, while microscopic character was the types of spores formed in the aerial mycelium. The isolates were then purified on Yeast malt agar (YMA) media (1 petri dishes for 8 isolates). The composition of YMA media was 4 g/l yeast extract, 10 g/l malt extract, 4 g/l glucose and 10 g/l agar. The transfers of isolates were using sterile toothpicks than incubate for a week at room temperature.

Analysis of antibacterial activity. A total of 0.1 ml of each target bacterial culture (*Escherichia coli* and *Staphylococcus aureus*) were taken and mix into warm-soft of YMA media, circling Erlenmeyer to stir it and then immediately pour into the dishes filled with sterile YMA media. The composition of the YMA media added cycloheximide (5 mg/l) as an antibacterial. Let it until the media solidifies. After solid, taken 12 of Actinomycetes colonies on a YMA dish using sterile straws and inoculate on media plates that contain target bacteria (1 target bacterial dish for 4 Actinomycetes colonies) (Fig. 1a). Incubate for a week at room temperature (30°C). Observe the clear zone formed around the colonies and measure its diameter. The inhibition zone was determined based on the difference between the diameter of the clear zone and the diameter of the colony which was measured twice horizontally and vertically. The results of the measurement of the clear zone were then compared with the category for response to inhibition of bacterial growth (Surjowardojo *et al*., 2015).

Analysis of antifungal activity. Twelve of purified Actinomycetes colonies were then inoculated by streak on dish containing YMA media using sterile toothpicks. The composition of the YMA media was added by the antibiotic nalidixic acid (5 mg/l) as an antifungal (Sipriyadi *et al*., 2016). Incubate for a week at room temperature (30°C). Each YMA dishes were inoculated with 4 isolates of Actinomycetes. Using a sterile straw, take the *Fusarium oxysporum* (test fungus) colony

and inoculate it on YMA media that has been overgrown with Actinomycetes. *Fusarium* colony was placed in the opposite position to the Actinomycetes isolate and incubate at room temperature for 2-3 days (Fig. 1b). Observe and measure the clear zone formed around colonies. The same procedures were also done for *Penicillium* sp.

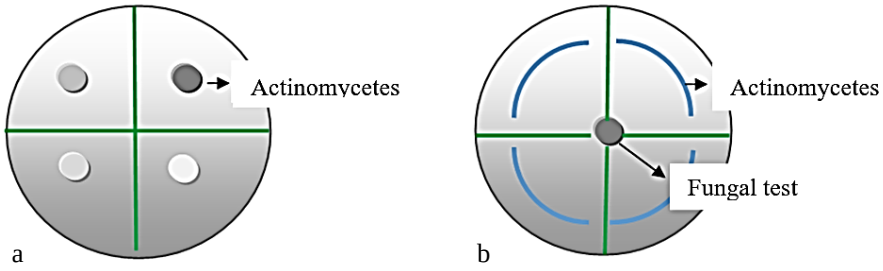

Fig. 1. Scheme placement of Actinomycetes colonies: a. for antagonist test against *E. coli* and *S. aureus*; b. for antagonist test against *F. oxysporum* dan *Penicillium* sp.

Analysis of cellulolytic activity. Twelve of purified Actinomycetes colonies were tested for their cellulolytic activity by inoculating Actinomycetes colonies on 1% carboxy methyl cellulose (CMC) media. The composition of CMC media was CMC composition (0.5 g/100 ml), bacto agar (18 g/l) and yeast extract (1 gr g/100 ml). Incubate at room temperature (30°C) for 3 days. Observe the clear zone formed around colonies. Observation of clear zones add Congo red solution to make it more visible and wash 2 times with 1 M NaCl solution to remove Congo red that was not bound to polysaccharides. The clear zone formed indicates cellulolytic activity (cellulose hydrolysis) which can be determined by cellulolytic index. The cellulose which has not been hydrolyzed appears purple (Pragya *et al*., 2012).

Analysis of phosphate solubilizing activity. A total of 12 purified Actinomycetes colonies were tested for phosphate solubilizing activity by inoculating Actinomycetes colonies on Pikovskaya agar media enriched with 0.1% yeast extract and incubated at room temperature (30°C) for two weeks. The clear zone formed indicates the presence of phosphate solubilizing activity which can be determined by the dissolving index (Wulandari *et al.*, 2019).

Analysis of xylanolytic activity. Twelve of purified Actinomycetes colonies were tested for xylanolytic activity by inoculating Actinomycetes colonies on birchwood xylan (BX) agar (hardwood) and oat spelt xylan (OSX) agar (softwood). The compositions of these two media were BX or OSX (0.5 g/100 ml), bacto agar (18 g/l), yeast extract (1 g/100 ml) and sucrose (10.3 g/100 ml). Incubate at room temperature (30°C) for 3 days. Observe the clear zone formed around colonies. Determination of xylanase activity index was done by adding congo red and 1 M NaCl solution to remove congo red that was not bound to polysaccharides. Clear zone formed indicates xylanolytic activity which can be determined by xylanolytic index (Alvares-Navarrete *et al*., 2015).

Data analysis. Cellulolytic index, phosphate solubilizing index, and xylanolytic index were determined based on the ratio between the diameter of the clear zone formed around colonies against the diameter of the colony. All the index criteria followed the previous studies by Alvares-Navarrete *et al*., 2015, Rudiansyah *et al*., 2017, and Wulandari *et al.*, 2019.

RESULTS AND DISCUSSION

Actinomycetes from rhizosphere of nutmeg. The characterization of Actinomycetes isolates found in the soil of rhizosphere of nutmeg showed that these isolates had various macroscopic and microscopic characteristics. The mycelium substrate looks brown and cream while the aerial mycelium looks white and gray. Reverse and soluble pigments look brown and beige (Fig. 2).

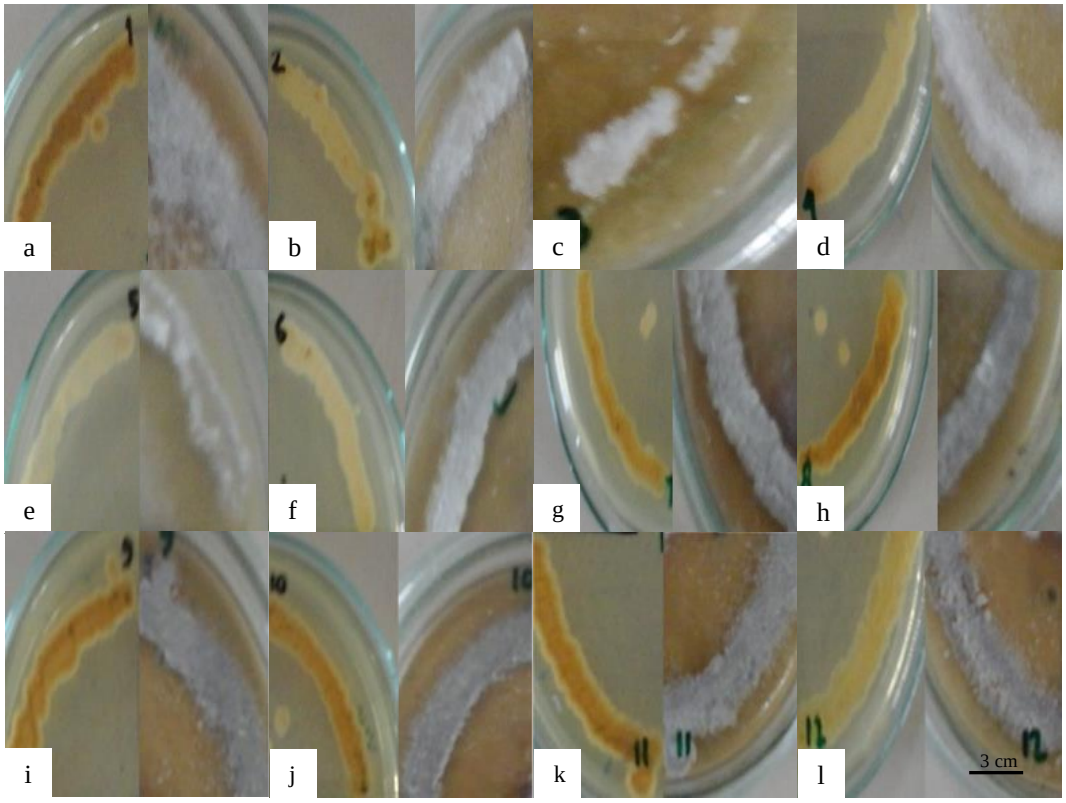

Fig. 2. Macroscopic characteristics of selected Actinomycetes isolates from rhizosphere of nutmeg: a. Isolate AC-01; b. Isolate AC-02; c. Isolate AC-03; d. Isolate AC-04; e. Isolate AC-05; f. Isolate AC-06; g. Isolate AC-07; h. Isolate AC-08; i. Isolate AC-09; j. Isolate AC-10; k. Isolate AC-11; l. Isolate AC-12.

Microscopic observations showed that the isolates had aerial verticilate, rectiflexibiles and spiral spore types. The presence of aerial mycelium of varying types indicates that the possibility of isolates found in rhizosphere soil samples of nutmeg was dominated by the genus *Streptomyces* (Barka *et al*., 2016) (Fig. 3). Actinomycetes were widespread in the soil. One of the best known groups of Actinomycetes was *Streptomyces*. These bacteria have complex interactions with plants and other organisms around them, especially in the rhizosphere. Actinomycetes have an important role in nature. Beside to breaking down organic materials in nature, these bacteria also produce antibiotics and extracellular enzymes. The antibiotics produced by Actinomycetes are streptomycin, irumamycin, and nanaomycin (Takahashi & Nakashima, 2018), while the extracellular enzymes produced include cellulase, chitinase, xylanase, and pectinase (Das *et al*., 2014).

Table 1. Antagonist test of Actinomycetes from rhizosphere of nutmeg against *E. coli* and *S. aureus* on YMA media

Bacterial test	Isolates*	Diameter (mm) Clear zone	Colony of isolates	Inhibition zone	Intensity of inhibition zone	Category
E. coli	AC-01	28.5±0.07	12.0±0.41	16.5	+++++	Strong
	AC-02	17.5±3.53	7.0±2.12	10.5	+++++	Strong
	AC-03	22.5±0.00	10.0±2.12	12.5	++++	Strong
S. aureus	AC-05	25.5±0.14	8.0±0.70	17.5	+	Strong
	AC-06	24.5±0.14	8.5±0.00	16.0	+	Strong
	AC-07	15.0+0.00	7.5±2.12	7.50	+	Medium

Note: * Only display isolates that can form inhibition zone

Actinomycetes as producers of antibacterial compounds. The antagonist test of Actinomycetes from rhizosphere of nutmeg against *E. coli* and *S. aureus* showed that AC-05 isolate more inhibited the growth of *S. aureus* with inhibition zones reaching 17.5 mm (strong category) (Fig. 4a), while inhibition zones against *E. coli* the highest by AC-01 isolate only reached 16.5 mm (strong category) (Table 1). Thus, Actinomycetes inhibits the growth of *S. aureus* than *E. coli*.

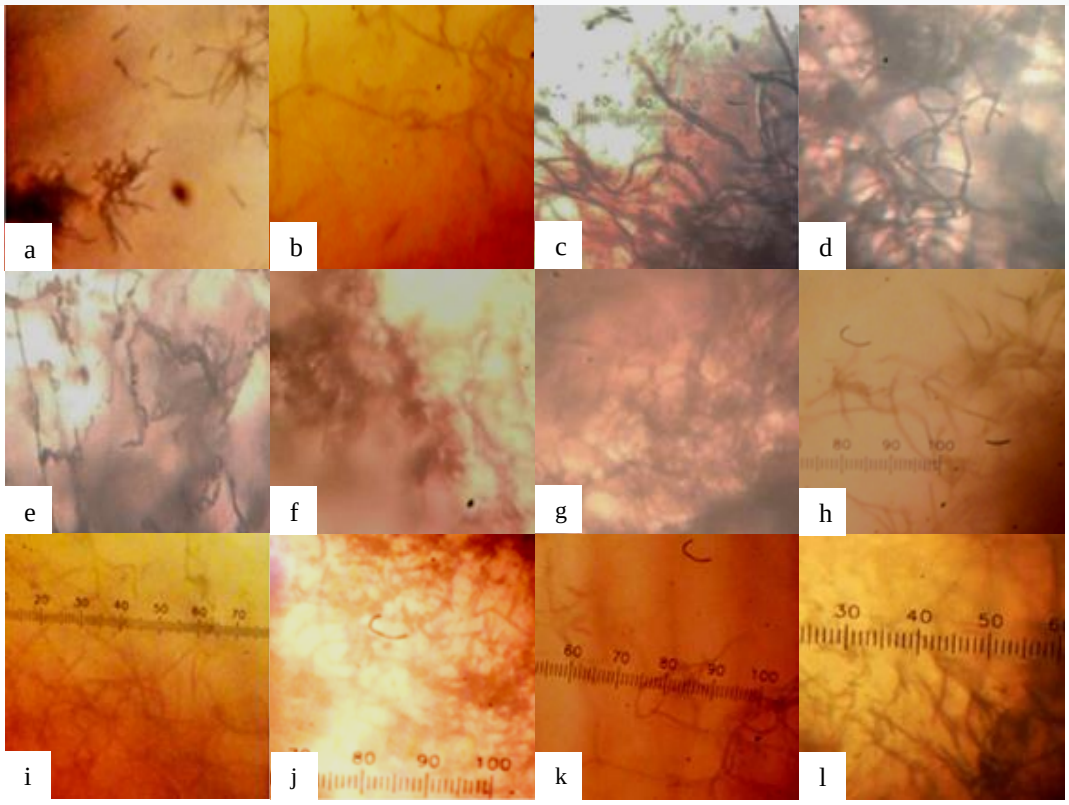

Fig. 3. Microscopic characteristics of selected Actinomycetes isolates from rhizosphere of nutmeg: a. Isolate AC-01; b. Isolate AC-02; c. Isolate AC-03; d. Isolate AC-04; e. Isolate AC-05; f. Isolate AC-06; g. Isolate AC-07; h. Isolate AC-08; i. Isolate AC-09; j. Isolate AC-10; k. Isolate AC-11; l. Isolate AC-12.

Actinomycetes were the producer of antibiotics in nature (van der Meij *et al*., 2017). The results of the antagonist test against *E. coli* and *S. aureus* showed that Actinomycetes more inhibited the growth of *S. aureus* (Gram positive bacteria) than *E. coli* (Gram negative bacteria). The structure of *E. coli* cell with an outer membrane composed of lipopolysaccharides causes the bacterial cell wall was not permeable to the antibiotic compounds produced by Actinomycetes (Sperandeo *et al*., 2017). In contrast, the structure of the *S. aureus* cell wall which was only a peptidoglycan layer on the outside was not an effective barrier to prevent entry antibiotics (Hasyim & Tulak, 2013; Sapkota *et al*., 2020). Inhibition of bacterial activity also comes from the exudate released by nutmeg roots. The exudate was a chemical compound released into the soil to inhibit pathogenic bacteria in the rhizosphere (Baetz & Martinoia, 2014). The root nutmeg contains secondary metabolites had the most antimicrobial effect (Mymand *et al*., 2015). Other specific antibacterial compounds in nutmeg were trimyristin and myristic acid. Both of these compounds have the potential to inhibit Gram positive and negative bacteria (Ali *et al*., 2018).

Actinomycetes as producers of antifungal compounds. The antagonist test of Actinomycetes from rhizosphere of nutmeg against *F. oxysporum* and *Penicillium* sp. show that AC-09 (Fig. 4b) and AC-02 isolates can inhibit the growth of these two pathogenic fungi with inhibition zones reaching 16.0 (strong category) (Table 2). Thus, Actinomycetes inhibits the growth of both types' fungi with the same highest inhibition zone.

Table 2. Antagonist test of Actinomycetes from rhizosphere of nutmeg against *F. oxysporum* and *Penicillium* sp. on YMA media

Fungal test	Isolates*	Diameter (mm)			Intensity of inhibition zone	Category
		Clear zone	Colony of isolates	Inhibition zone		
F. oxysporum	AC-02	14.0±0.70	10.0±0.00	4.0	+++++	Weak
	AC-05	21.0±2.82	10.0±0.00	11.0	+++++	Strong
	AC-06	18.0±4.24	9.0±0.00	9.0	+++++	Medium
	AC-09	25.0±1.41	9.0±0.00	16.0	+++++	Strong
	AC-10	18.0±0.70	10.0±0.00	8.0	+++++	Medium
Penicillium sp.	AC-02	25.0±2.82	9.0±0.00	16.0	+++++	Strong
	AC-06	22.0±4.24	9.0±0.00	13.0	+++++	Strong

Note: * Only display isolates that can form inhibition zone

The Actinomycetes could inhibit the growth of pathogenic fungi. *F. oxysporum* and *Penicillium* sp. were pathogenic fungi. These fungi were often found in nutmeg (Fendiyanto *et al*., 2021). Penicillium was a genus of fungi that was originally known to produce antibiotics. Inhibition of Penicillium growth by Actinomycetes proves that antimicrobial compounds produced by Actinomycetes inhibit the growth of other microbes compared to antimicrobial compounds produced by fungi. Inhibition of Actinomycetes against *F. oxysporum* and *Penicillium* sp. by secreting antifungal secondary metabolites (Tian *et al*., 2022). Besides being produced by Actinomycetes, nutmeg also produce sabinens, terpinenols, and safrol that were antifungal (Ali *et al*., 2018).

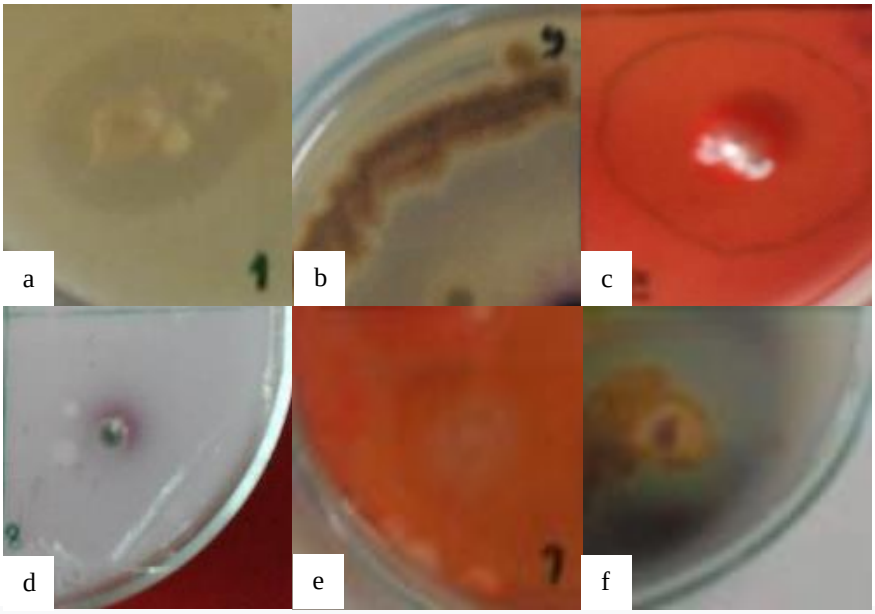

Fig. 4. Potential of selected Actinomycetes isolates from rhizosphere of nutmeg: a. highest antibacterial activity (Isolate AC-05) on YMA media; b. highest antifungal activity (Isolate AC-09) on YMA media; c. highest cellulolytic activity (Isolate AC-05) on CMC media; d. highest phosphate solubilizing activity (Isolate AC-08) on Picovskaya media; e-f. highest xylanolytic activity (Isolate AC-07) BX agar and OSX agar media

Actinomycetes as producers of extracellular enzymes. Beside antimicrobial compounds, Actinomycetes can also produce extracellular enzymes, such as cellulase, phosphate solubilizing, and xylanase (Das *et al*., 2015). The results of the Actinomycetes cellulolytic activity isolated from rhizosphere of nutmeg showed that isolates AC-1, AC-3 and AC-5 (Fig. 4c) have cellulolytic index above 3.00 (high category), whereas phosphate solubilizing activity showed that AC-8 isolates (Fig. 4d) and AC-12 have phosphate dissolving index 1.28 and 0.83 (low category), respectively (Table 3).

Table 3. Activity cellulolytic and phosphate solubilizing test of Actinomycetes from rhizosphere of nutmeg on CMC and Picosvkaya agar media

Activity of microbes	Isolates*	Diameter (mm)		Cellulolytic/ dissolving index	Intensity of inhibition zone	Category
		Clear zone	Colony of isolates			
Cellulolytic	AC-01	2.40±0.07	0.75±0.07	3.26	++	High
	AC-03	1.50±0.00	0.50±0.14	3.00	++	High
	AC-05	1.75±0.07	0.50±0.00	3.50	++	High
	AC-06	2.80±0.00	1.05±0.07	2.66	++	High
Phosphate	AC-08	0.90±0.00	0.70±0.00	1.28	+++++	Low
solubilizing	AC-12	0.75±0.07	0.90±0.84	0.83	++++	Low

Note: * Only display isolates that can form inhibition zone

Another ability of Actinomycetes was produce cellulase enzymes and phosphate solubilizing. The cellulase enzyme can convert cellulose into more simply of sugar. Streptomyces was the largest producer of cellulase in nature (Mohanta, 2014). Cellulolytic activity test showed a clear zone on the agar medium containing 0.5% carboxymethyl cellulose as a carbon source. This indicates the cellulose hydrolysis occur. Thus, Actinomycetes isolates rhizosphere of nutmeg have cellulase enzymes. Characteristics of soil in the rhizosphere of nutmeg that have podzolic type and under the litter allows the found of cellulolytic microbes, including Actinomycetes. Actinomycetes have the ability to decompose complex molecules, specifically lignocellulose (El-Naggar & Abdelwahed, 2012). The ability of Actinomycetes to decompose complex molecules such as litter can be used to reduce agricultural waste.

Phosphorus was one of the important elements for plants. Plants generally contain phosphate ions (PO_4^{3-}) which were usually bound to calcium (Ca^{2+}), aluminum (Al^{3+}), iron (Fe^{3+}) and magnesium (Mg^{2+}) in colloidal soils. The phosphate binding causes these ions cannot be absorbed by the plant optimally. Podzolic soil bind very strongly to phosphate ions. This soil was commonly found in eastern Indonesia, especially in the mountains of Ambon Island. One alternative was increase phosphate ions for plants by using phosphate solubilizing microbes. One group of microbes that have phosphate solubilizing activity was Actinomycetes (Anwar *et al*., 2016). Streptomyces have high phosphate solubilizing activity (Gangwar *et al*., 2014). The low phosphate dissolved index indicates that absorption of phosphate in rhizosphere of nutmeg plat was very limited. The results of the Actinomycetes xylanolytic activity isolated from rhizosphere of nutmeg showed that AC-7 isolates (Fig. 4e-4f) had the highest xylanolytic index of 3.87 and 3.42 (high category) respectively on BX agar and OSX agar (Table 4).

Table 4. Activity xylanolytic test of Actinomycetes from rhizosphere of nutmeg on birchwood xylan agar dan oat spelt xylan agar media

Media	Isolates*	Diameter (mm)		Xylanolytic Index	Intensity of inhibition zone	Category
		Clear zone	Colony of isolates			
BX agar	AC-02	1.40±0.14	0.39±0.01	3.59	+++	High
	AC-07	1.55±0.07	0.40±0.00	3.87	+++	High
	AC-11	1.60±0.14	0.45±0.07	3.55	+++	High
	AC-12	1.85±0.35	0.50±0.14	3.70	+++	High
OSX agar	AC-05	1.90±0.14	1.15±0.07	1.65	++	Medium
	AC-06	3.00±0.28	1.05±0.07	2.85	++	High
	AC-07	1.20±0.28	0.35±0.00	3.42	++	High

Note: *Only display isolates that can form inhibition zone

Xylan was the largest constituent of hemicellulose which was a polymer of β (1-4) D-xylopiranosa (xylose) with a β-1-4-glycoside bond. Xylan can be hydrolyzed enzymatically by xylanase (Nurnawati *et al*, 2014). Xylan was a non-cellulose polysaccharide that abundant in hardwood and annual plants up 30% of the total dry weight of plant biomass (Smith *et al*., 2017). Beside in hardwood plants, xylan was also abundant in soft woody plants (softwood). The xylanolytic activity test showed a clear zone on agar media containing xylan as a carbon source. This indicates

xylan hydrolysis occur. Thus, Actinomycetes isolates from rhizosphere of nutmeg have xylanase enzymes. The xylanase enzyme produced by Actinomycetes in rhizosphere of nutmeg because the soil was located below the litter. Soils that contain a lot of plant residues (litter) were the main source of xylan which can be utilized by microbial xylanase-producing (Nurnawati *et al*., 2014). Nutmeg was an annual woody plant (Karyati, 2014). High xylanolytic activity in birchwood xylan agar because Actinomycetes found in the nutmeg rhizosphere has been adapted to hydrolyze hardwood. Some species of Streptomyces were able to produce more than one type of xylanase when cultivated in a medium containing xylan. The xylanase enzymes produced by Actinomycetes were generally thermostable, so that they were often used in industry, especially the paper processing industry (Selvarajan & Veena, 2017).

This research has successfully isolated various types of Actinomycetes from rhizosphere of nutmeg. The isolates found can be used as starters to design biofertilizer formulas that can be utilized by farmers. Isolates which have high antibacterial ability can also be used as biocontrol agents to control plant pathogens, so that it can reduce the costs of farmers to buy fertilizers and pesticides.

CONCLUSION

The isolation of Actinomycetes from rhizosphere of nutmeg (*Myristica fragrans* Houtt) showed various macroscopic and microscopic characteristics. The presence of aerial mycelia indicates that isolates was dominated by Streptomyces. The Actinomycetes isolates have antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* and antifungal against *Fusarium oxysporum* and *Penicillium* sp. The Actinomycetes isolates also produce extracellular enzymes, i.e., cellulase, xylanase and phosphate solubilizing. The highest cellulolytic, xylanolytic and phosphate solubilizing indices were 3.50, 3.87, and 1.28, respectively. The actinomycetes isolates found can be used as a starter to develop fertilizer formulas and biological control in plant.

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