The Potential of Nutmeg's Microbes (*Myristica fragrans* Houtt.) as Antagonistic Agents against *Rigidoporus microporus*

Potensi Mikroba Pala (Myristica fragrans Houtt.) sebagai Agens Antagonis terhadap Rigidoporus microporus

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ABSTRAK

Penelitian ini bertujuan untuk mendapatkan isolat khamir dan bakteri asal tanaman pala (*Myristica fragrans* Houtt.) yang berpotensi menghasilkan enzim kitinase serta memiliki kemampuan antagonis terhadap *Rigidoporus microporus*. Isolat khamir dan bakteri diisolasi dari daun dan buah tanaman pala (*M. fragrans*). Hasil isolasi didapatkan sebanyak 35 isolat khamir dan 29 isolat bakteri dengan karakter morfologi yang berbeda. Uji kitinolitik dilakukan secara kualitatif dan parameter yang diamati berupa terbentuknya zona bening di sekitar koloni. Hasil uji kitinolitik menunjukkan sebanyak 4 isolat bakteri yang dapat menghasilkan enzim kitinase (BP 1.2.1, BP 2.1.1, EPBj II.K1, EPBj II. K2) dengan indeks kitinolitik (3,92, 5,38, 2,00, dan 1,66), sedangkan isolat khamir tidak ada yang positif menghasilkan enzim kitinase. Perbedaan nilai indeks menunjukan adanya variasi tingkat aktivitas enzim. Uji antagonis dilakukan dengan metode *dual culture*, dan terdapat 1 isolat khamir dan 14 isolat bakteri yang dapat menghambat pertumbuhan *R. microporus*. Setiap isolat memiliki daya hambat yang berbeda. Berdasarkan presentase nilai hambatan, presentase tertinggi terjadi pada isolat P. K1 (41,1%), P. K2 (50%), dan EPBj II. K6 (42,2%). Indikator uji antagonis adalah terbentuknya zona hambat pada media PDA. Berdasarkan uji hemolisis, isolat khamir dan bakteri (0%) tidak ada yang mampu menguraikan sel darah pada medium. Identifikasi molekuler menunjukkan bahwa isolat P. K1 dan P. K2 tergolong *Bacillus subtillis* dan isolat EPBj II. K6 tergolong *Bacillus aerius* dengan homologi sekuens masing-masing sebesar 100%, dan nilai bootstrap sebesar 99%. Penemuan ini memberikan informasi tentang spesies yang diharapkan dapat mengendalikan jamur akar putih.

Kata kunci: *Bacillus aerius*, *Bacillus subtillis*, enzim kitinase, hemolisis

ABSTRACT

This study aimed to obtain yeast and bacteria from *Myristica fragrans* Houtt., which have the potential to produce chitinase enzymes with antagonistic ability against *Rigidoporus microporus*. Both microorganisms were extracted from the leaves and fruit of nutmeg. A total of 35 yeast and 29 bacterial isolates were obtained, with different morphological characters. The chitinolytic test was carried out qualitatively, and the parameters observed include the clear zones around the colony. A total of 4 bacterial isolates produced chitinase enzymes (BP 1.2.1, BP 2.1.1, EPBj II.K1, and EPBj II. K2) with a chitinolytic index of 3.92, 5.38, 2.00, and 1.66, respectively. Yeast isolates were negative for chitinase enzymes. The difference in index value indicated a variation in enzyme activity. The antagonist test was carried out using a dual culture method. A total of 1 yeast and 14 bacterial isolates inhibited the growth of *R. microporus*, and each has a different inhibitory zone. Based on the percentage of inhibition value, the highest percentage occurred in P.K1 (41.1%), P. K2 (50%), dan EPBj II. K6 (42.2%). The antagonist test indicator includes the formation of inhibitory zones on the medium. Hemolysis test showed that yeast and bacteria are not able to break down blood cells in the medium. The molecular identification showed that P. K1 and P. K2 isolates were classified as *Bacillus subtillis* and EPBj II. K6 were identified as *Bacillus aerius* with 100% sequence homology and 99% bootstrap value respectively. These findings provided information about potential microbes that control white root fungus.

Keywords: *Bacillus aerius*, *Bacillus subtillis*, chitinase enzymes, hemolysis

INTRODUCTION

Nutmeg (*Myristica fragrans* Houtt.) is a native Indonesian plant, which originated from Banda Island and grows effectively in the tropics (Bermawie et al., 2018). It is also known as a spice plant with high economic value and multipurpose, because every part it is used in various industries (Rahardiyan et al., 2020). Nutmeg is known to have antimicrobial activity as showed by Nurhasanah (2014), against pathogenic bacteria, such as *Staphylococcus aureus* and *Escherichia coli*. Tan et al. (2013) studied the content of phenolic compounds in the nutmeg pulp, which has the potential as an antimicrobial substance.

The research on both the antibacterial and anti-fungal compounds in nutmeg (*M. fragrans*) have been widely carried out, however, the presence of microorganisms in these plants has not been detected (Nurhasanah, 2014). *R. microporus* is a pathogenic fungus that causes white root disease in rubber plants (Goh et al., 201). The Central Bureau of Statistics, 2018, stated that Indonesia is the world's largest rubber producer and exporter, therefore, this disease is endemic to the region. It causes the death of rubber in large numbers because, *R. microporus* is easily transmitted from one plant to another.

Pest and plant disease control is generally carried out by using synthetic fungicides. However, its effectiveness has decreased due to an increase in pathogenic resistance, and causing environmental pollution (Nega, 2014). The use of synthetic fungicides also cause adverse effects on human health including acute poisoning, skin and eye irritation, respiratory tract disorders, and stimulating cancer cells growth (Yadav et al., 2017).

One of the effective control measures used in replacing synthetic fungicides includes the environmentally friendly antagonistic agent, such as yeast and bacteria (Talibi, 2014). This is because they easily adapt to various environmental conditions and grow rapidly, therefore, causing both organisms to increase in their natural habitat. An increase in population causes yeast or bacteria to control more

nutrients and living space (Scheuerl et al., 2020).

The research on yeast and bacteria from the nutmeg plant (*M. fragrans*) has not been widely known. Therefore, this study aimed to explore the yeast and bacteria potential obtained from the nutmeg plant (*M. fragrans*), as antagonistic agents against white root fungus (*R. microporus*), as well as their ability to produce chitinase enzymes.

MATERIALS AND METHODS

This research was conducted at the Microbiology Laboratory, Indonesian Center for Agriculture Biotechnology and Genetic Resources Research and Development (ICABIOGRAD), Bogor. The method used were experimental (for antagonistic tests) and descriptive (for chitinolytic and hemolysis tests). Nutmeg plant samples (*M. fragrans*) in the form of leaves and fruit were obtained from the Experimental session of Indonesian Center for Estate Crops Resource and Development, Bogor. Meanwhile, white root fungus (*R. microporus*) is an isolate of Biogen Culture Collection (Biogen CC).

A. Yeast and Bacteria Isolation from Leaves and Nutmeg

Yeast isolation was carried out by using the stratified dilution technique. Leaves and nutmeg samples were subjected to surface sterilization under running water, 70% alcohol, and rinsed using distilled water. Isolation was carried out with 3 treatments, including;
1. DP (The leaves were cut into 1cm long)
2. BT (Old nutmeg was cut into small pieces and seeded)
3. BM (Young nutmeg was cut into small pieces and seeded).

Each sample was weighed as much as 10 g and placed into 90 mL of 0.85% NaCl, and homogenized using a 120 rpm rotator shaker for 15-30 mins, then diluted up to 10-3. Furthermore, 0.1 mL of the dilution result was inserted into Yeast Malt Agar (YMA) medium in a spread plate using a drygalski spatula, and incubation was carried out at room temperature ($\pm$ 28 °C) for 24 hours. The YMA purification was conducted using the quadrant streak method. The incubation was carried out at room temperature ($\pm$ 28 °C) for 24 hours.

Bacterial isolation was performed using direct planting and dilution techniques. There were 5 treatments for bacterial isolation, namely
1. BP (Fruit was cut into 2 parts (part 1 was sterilized with 70% alcohol for 5 mins, part 2 was not sterilized). The sample pieces were attached to the King's B media and silenced for 5 mins, then spread using a drygalsky spatel).
2. P (5 g of nutmeg was cut into small pieces, stored in 45 mL of distilled water in a jam bottle, then shook for 1 hour).
3. PB (Fruit and nutmeg was cut into 4 pieces, placed in 45 mL of distilled water in a jam bottle, and shook for 1 hour).
4. EP (Nutmeg was cut into 4 pieces and the surface was attached to the Kings'B media, the sample was stored in 45 mL distilled water in a jam bottle, then shook 1 hour).
5. EPBj (The surface of the nutmeg was affixed to the Kings'B' media, and stored in 45 mL distilled water in a jam bottle, then shook for 1 hour).

In 2-5 treatments, 1 mL each was collected and diluted up to 10^{-5}, then homogenized. A total of 100 µL from 10^{-3} to 10^{-5} dilutions were inoculated on Kings'B medium by the spread plate method using drygalsky spatels. Incubation was conducted at room temperature ($\pm$ 28 °C) for 24 hours. Bacterial purification was carried out on Kings'B medium by quadrant streak method, and incubated at room temperature ($\pm$ 28 °C) for 24 hours.

B. Chitinolytic Test

Chitinolytic testing was carried out based on Kurniawan et al. (2018) by inoculating yeast and bacterial isolates on a solid chitinase medium. Each of yeast and bacterial colony aged 48 hours was taken

using a sterile inoculation loop, and diluted in 100 µL of sterile distilled water in 1.5 mL microtube, then vortexed until it homogenized. A total of 5 µL of the solution was taken and dropped on the chitin medium surface in a petri dish, each was divided into 2 parts. Samples were incubated at room temperature (± 28 °C) for 7 days. After this, the Petri dishes were dripped with 0.3% Congo Red solution to cover the entire surface of the Petri dishes and left for 2-3 mins, then rinsed with 0.1% NaCl and discarded. Furthermore, observing the presence or absence of clear zones, as well as calculating the chitinolytic index with the following formula were carried out:

$$IK = \frac{(Y2 - Y1)}{Y1}$$

Description:
  IK : Chitinolytic index
  Y2 : clear zone diameter (mm)
  Y1 : colony diameter (mm)

C. Antagonist Test

Antagonist testing was carried out using a dual culture method based on the study of Susilowati and Haryono, (2018) in a PDA medium with chloramphenicol antibiotics. The PDA medium was divided into two parts with a distance of 3 cm, while one side was perforated using a sterile straw measuring ± 6.72 mm. All yeast and bacterial isolates were inoculated into the PDA medium with a 3 cm streak technique using a sterile toothpick.

Yeast and bacteria were incubated for 24 hours at room temperature (± 28 °C). white root fungus (*R. microporus*) was grown on the PDA medium, on the side that had been inoculated with yeast or bacteria. Then, incubation was carried out at room temperature (± 28 °C) for 5 days. The yeast and bacteria growth were observed and the diameter of the white root fungi was measured. In the control experiment, the PDA medium was only inoculated with white root fungus (without yeast or bacteria). Calculation of the yeast and bacterial resistance towards white root fungi was carried out based on the study of Landum et al. (2016), using the following formula:

Presentase penghambatan (%) =

$$\frac{(R1 - R2) \; x \; 100\%}{R1}$$

Description:
  R1 = the diameter of white root fungus control
  R2 = diameter of white root fungus with treatment

D. Hemolysis Test of Yeast Isolates and Potential Bacteria

The hemolysis test on yeast isolates and antagonistic bacteria was carried out to determine the potential of the tested microorganisms as pathogens. This was conducted based on the method of Turista and Puspitasari (2019) using BAP medium (blood agar plate). The yeast and bacteria were inoculated on the blood agar medium by streaking, and incubated for 48 hours at room temperature (± 28 °C). The hemolysis test indicator is a clear zone formation around the isolated breed, indicating that the test isolate decomposed blood cells in the medium. Therefore, it has the potential to become a pathogen. (Adam, 2016).

E. Molecular Identification

The yeast and bacteria isolate that was able to inhibit the growth of *R. microporus* were collected by molecular identification. Bacterial samples were inoculated on Nutrient Broth (NB) media, while the yeast samples were inoculated on Yeast Malt Broth (YMB) media, then homogenized using a rotary shaker for 48 hours at room temperature (± 28 °C).

The DNA isolation procedure follows the steps in the TIANamp Genomic DNA Kit. The measurements of DNA concentration and purity was carried out using a nanodrop 2000 spectrophoto meter. DNA amplification was conducted by Polymerase Chain Reaction (PCR) method

using the Thermal cycler tool. In the yeast sample, ITS rDNA regions were amplified with forward primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and reverse primers ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3'). The PCR was performed on yeast samples for 2 hrs 57 mins with 35 cycles at an initial denaturation procedure of 94 ˚C for 5 mins, then denaturation of 94 ˚C for 1-min, annealing at 55˚C for 1-min, lengthening at 72 ˚C for 2 mins, the final cycle of 72 °C for 10 mins, and hold at 15˚C for ∞. In bacterial samples, 16S rDNA regions were amplified with forward primers16S F (5'-CGCCTGTTT AACAAAAACAT-3 ') and reverse primers 16S R (5'CCGGTCTGA ACCAGATCATGT-3'). The PCR procedure was conducted for 1 hr 58 mins, including 1 initial denaturation cycle of 94 °C for 5 mins, 29 cycles the denaturation of 94 °C for 1 min 30 secs, annealing at 55 °C for 45 secs, lengthening at 72 °C for 1 min the final extending cycle at 72 ˚C for 1 min, and hold at 15 ˚C for ∞. The total volume of PCR was 25 μl consisting of 12.5 μl of GoTaq® Green (Promega), 1 μl (10 pmol) of each primer, 2 μl (about 200 ng) of template DNA, and 8.5 μl of nuclease-free water (NFW).

Visualization of ITS and 16S rDNA regions was performed by electrophoresis, with 1.2 g (1.7%) of agarose, 100 mL of 1x TAE buffer, and 2 μL of red gel. Electrophoresis results were visualized under a UV transilluminator. The PCR products were sent to a sequencing service to obtain the reading. The sequenced data was then edited using the ChromasPro version 2.6.2 application, followed by analysis using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov) to obtain the closest homologous species of the test isolates. The phylogenetic trees were made using the MEGA 7 application with a bootstrap value of 1000, and based on genetic kinship distance using the Neighbor-Joining (NJ) method.

RESULTS

Yeast and Bacteria Isolate from Nutmeg Leaves and Fruits

Yeast and bacterial isolates from samples of nutmeg plants (*M. fragrans*) were obtained from the leaves and fruit. The results showed that a total of 35 yeast isolates were obtained, consisting of (7, 11, and 17 isolates with DP, BT, and BM code) (Table 1). Then a total of 29 bacterial isolates were obtained, consisting of (6, 2, 12, and 9 isolates with BP, P, EP, and EPBj code). The morphological characteristics of the resulting isolates varied. The yeast isolation results consisted of non-pigmented groups (68.57%) including white, and the pigmented isolates (31.42%) including zinc yellow, tangerine, light chrome, light orange, and orange-yellow (Table 1). The results of bacterial isolation showed that the isolates consisted of non-pigmented (17.24%) and the pigmented species (82.75%). The non-pigmented groups include white isolates, while the pigmented include cream, ivory, canary yellow, zinc yellow, tangerine, orange-yellow, and warm gray 1 (Table 2).

Antagonist Test

The antagonist test results obtained 1 yeast (2.85%) and 14 bacterial isolates (48.27%) which have the ability to inhibit the growth of white root fungus *R. microporus* on the PDA incubation medium at room temperature (± 28° C) for 5 days (Figure 1). The parameters observed include the inhibition zone formation and the reduced mycelium of the fungal colonies. Based on the percentage calculation of the resistance value, it was found that each isolate has different antagonistic abilities (Figure 2). Three isolates gave the best results, namely P. K1 (41.1%), P. K2 (50%), and EPBj II. K6 (42.2%) (Figure 3). The control isolates with and without treatments showed different results. The growth of the isolates, either yeast or bacteria, tended to dominate and widened in the test medium (Figure 4).

Table 1. Characteristics of yeast isolates isolated from leaves and nutmeg on Yeast Malt Agar (YMA) medium incubation at room temperature (± 28 °C) for 24 hours

Colony Features		∑ Isolate	Isolate Codes
Color	Texture		
White	Butyrous	15 (42.85%)	DP 1, DP 1.2, DP 2.1, BT 1.1.2, BT 1.3, BT 3.2.2, BM 1.2.1A, BM 1.2.2A, BM 1.3.2B, BM 1.3.3, BM 2.1, BM 2.3.1, BM 2.3.2, BM 3.3.1, BM 3.3.3
White	Mucoid	9 (25.71%)	DP 2.2, BT 1.1.1, BT 2.1.2, BT 3.1, BM 1.1, BM 1.2.1B, BM 1.2.2B, BM 1.3.1A, BM 1.3.2A
Zinc yellow	Butyrous	2 (5.71%)	BM 1.3.1B, BM 2.2
Zinc yellow	Mucoid	4 (11.42%)	DP 2.3, BT 1.2, BT 2.3, BT 3.3
Tangerine	Butyrous	1 (2.85%)	BM 3.3.2
Tangerine	Mucoid	1 (2.85%)	BT 2.1.1
Light chrome	Mucoid	1 (2.85%)	BT 3.2
Light orange	Mucoid	1 (2.85%)	DP 2.4
Orange Yellow	Mucoid	1 (2.85%)	DP 1.3
Total		35 (100%)	

Table 2. Characteristics of bacterial isolates isolated from leaves and nutmeg on Kings'B medium incubation at room temperature (± 28 °C) for 24 hours

Colony Features		∑ Isolate	Isolate Code
Color	Texture		
White	Butyrous	4 (13.79%)	BP 1.1.1, BP.2.2.1, BP 3.1.1, BP 3.2.1
White	Mucoid	1 (3.44%)	P. K2
Cream	Butyrous	1 (3.44%)	P. K1
Cream	Mucoid	8 (27.5%)	EP I. K4, EP III. K2, EP IV. K1, EPBj I. K2, EPBj I. K4, EPBj II. K1, EPBj II. K2, EPBj II. K5
Ivory	Butyrous	1 (3.44%)	EP II. K1
Ivory	Mucoid	4 (13.79%)	EP I. K3, EP II. K2, EPBj I. K3, EPBj II.K6
Canary yellow	Butyrous	1 (3.44%)	EP III. K3
Canary Yellow	Mucoid	1 (3.44%)	EP IV. K2
Zinc Yellow	Mucoid	2 (6.89%)	EP I. K1, EPBj I. K1
Tangerine	Mucoid	3 (10.34%)	EP I. K2, EP III. K1, EPBj II. K4
Orange Yellow	Mucoid	1 (3.44%)	EP II. K3
Warm Grey 1	Butyrous	1 (3.44%)	BP 2.1.1
Warm Grey 1	Mucoid	1 (3.44)	BP 1.2.1
Total		29 (100 %)	

Molecular Identification

The bacteria isolates with the potential to antagonize white root fungi (*R. microporus*), were identified molecularly as P. K1, P. K2, and EPBj II. K6. Those identified were obtained based on the homology value of the sequences in the 16S rDNA area with their closest species, using the BLAST method from NCBI. The sequencing results using 16S rDNA primers in bacteria produced a nucleotide base sequence of P. K1 (803 bp), P. K2 (796 bp), and EPBj II.K6 (1140 bp) isolates.

Hemolysis Test

Hemolysis test was carried out on BAP (Blood Agar Plate) medium at room temperature (± 28 °C) for 2 days. It was observed that none of the yeast and bacteria isolates (0%) indicating positive results was able to break down blood cells in the medium (Figure 5). This showed that both organisms from the nutmeg leaf and fruit (*M. fragrans*) were not pathogenic.

Observations on the BAP medium did not show any clear zone formation around the colony. Based on the search for homology sequences of 16S rDNA area using the BLAST program, it was found that P. K1 and P. K2 isolates were identified as *Bacillus subtilis* with a sequencing of 100% as the closest species (Accession MT605412.1). While EPBj II. K6 isolates were identified as *Bacillus aerius* with a sequence homology of 100% and the closest specie (Accession

MK850542.1) (Table 3). The BLAST results for the three isolates showed an E-value of 0.00, indicating that the sequencing data has a high compatibility rate. The alignment results of P. K1, P. K2, and EPBj II. K6 isolates using the muscle program in MEGA 7 software showed the absence of gaps in the nucleotide base sequence. Based on the phylogenetic tree (Figure 6), P. K1 and P. K2 isolates were in the same clade. While P. K1, P. K2, and EPBj II. K6 isolates have a bootstrap value of 99%. This showed a fairly high level of confidence in the clade formed.

Table 3. The identification results of bacterial isolates from nutmeg based on regional sequence analysis

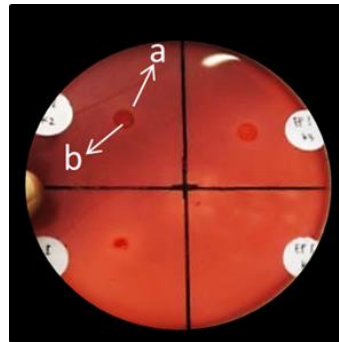| Isolate code	The closest taxon to BLAST results on NCBI	Max score	Query (%)	E-value	Accesion	Similarity (%)
PK1	*Bacillus subtilis*	1483	100	0.0	MT605412.1	100
PK2	*Bacillus subtilis*	1471	100	0.0	MT605412.1	100
EPBj II.K6	*Bacillus aerius*	2621	100	0.0	MK850542.1	100

Figure 1. The chitinolytic test results on solid chitinase medium were incubated at room temperature (± 28 °C) for 7 days. a) clear zone, b) isolate of chitinolytic bacteria
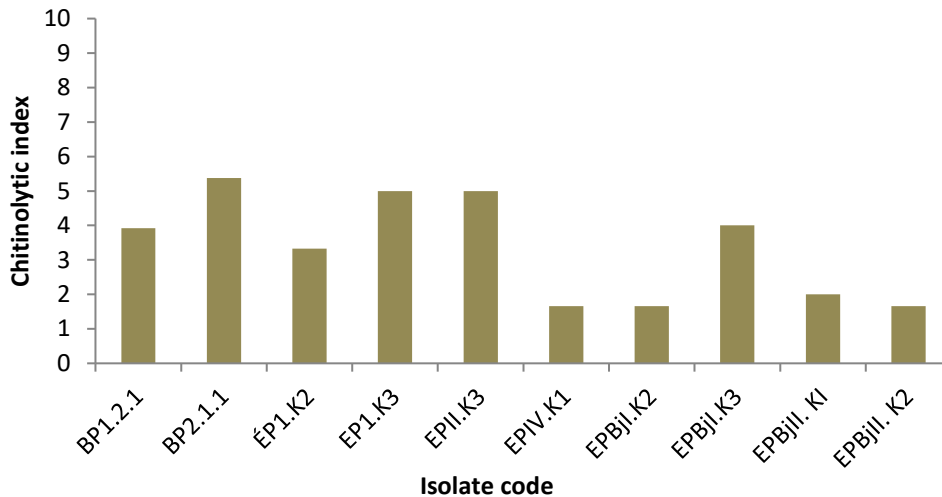

Figure 2. Chitinolytic index graph of bacterial isolates from nutmeg plants
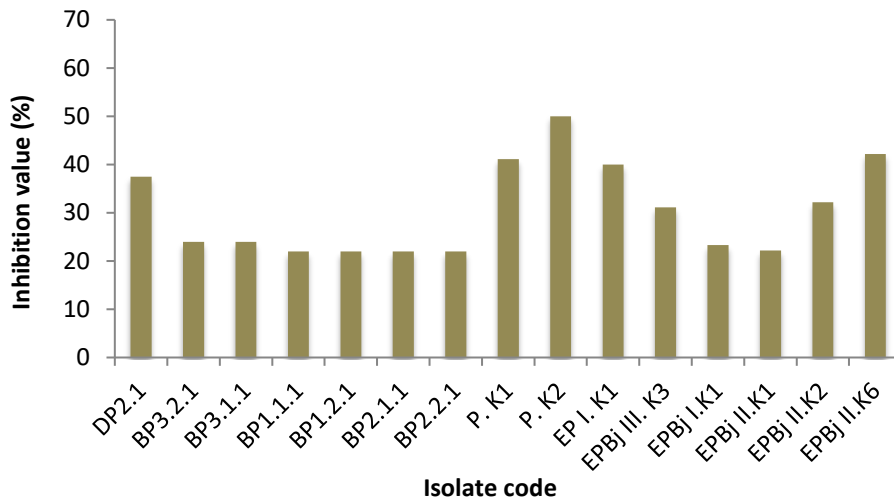
Figure 3. The inhibition value percentage of the antagonist test of yeast isolates and bacteria from nutmeg against white root fungus
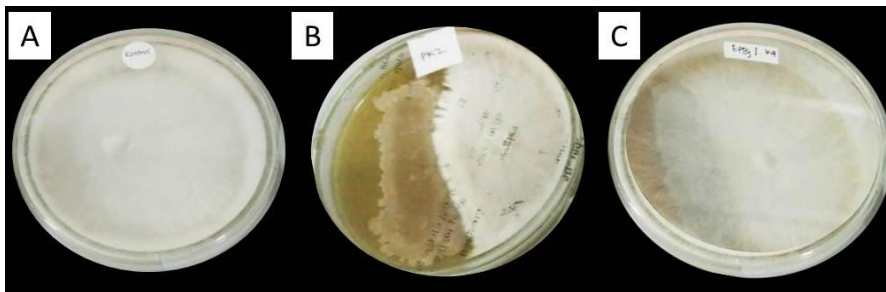

Figure 4. Antagonistic test results against white root fungus. A) Control of isolates, B) Positive results of PK2 isolates antagonists against, C) Negative results of isolates EPBj I K4 antagonists against (*R. microporus*), on PDA medium, incubated for 24 hours at room temperature (± 28 °C)

Figure 5. Hemolysis test of yeast isolates and bacteria from nutmeg On BAP (Blood Agar Plate) medium at room temperature (± 28 °C) for 2 days
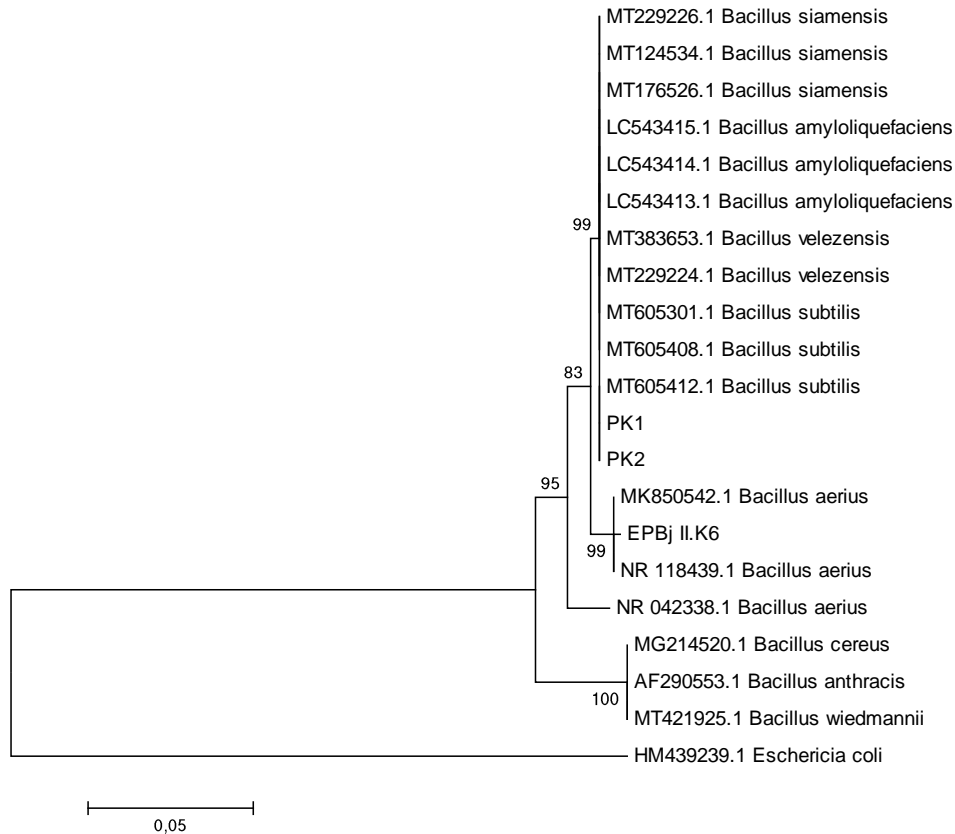
Figure 6. Phylogenetic tree construction of potential antagonistic bacterial isolates (P. K1, P. K2, and EPBj II. K6) from nutmeg based on 16S rDNA area sequence analysis using the Neighbor-Joining 1000 x bootstrap method, MEGA7. *Escherichia coli* as an outgroup

DISCUSSION

Yeast and Bacteria Isolation from Leaves and Nutmeg

The result of yeast and bacteria isolation showed a diversity of isolates. Andreote and eSilva (2017) also stated that their microbial communities are very diverse. The yeast and bacteria diversity showed that the nutmeg plant, especially their leaves and fruit, provides adequate nutrition for various types organisms. Several studies mention the nutritional content of nutmeg plants, including carbohydrates, minerals, protein, Vitamin A, Vitamin B, Vitamin C, folic acid, riboflavin, niacin, thiamine, sodium, potassium, calcium, magnesium, phosphorus, zinc, β-Carotene, and β-Cryptoxanthin (Agbogidi & Azagbaekwe, 2013). Okukpe et al. (2012) stated that the nutmeg plant contains flavonoids, saponins, and alkaloids, which function as anti-fungal substances.

Bacteria have the highest abundance and very varied on diversity (Campbell, 2013). It is influenced by their original environmental conditions (plants), caused by changes in temperature and humidity. Based on Crawford et al. (2012) study, stating that the environmental conditions from where the samples were collected, affect the microorganisms' diversity. Antwis et al. (2017) stated that yeast that lives on plant surfaces is exposed to direct sunlight, drought, and high temperatures. In these extreme conditions, yeast and bacteria generally have pigments and produce mucus. The mucus is an extracellular polysaccharide which functions to help adhere to surfaces, therefore, it is not easily carried away by wind and rain. Extracellular polysaccharides are produced inside the cell and then excreted and accumulated outside the cell. It functions in maintaining the shape, structure, and integrity of the cell wall, and also serves as

a reserve material for the growth and formation of cell components. Extracellular polysaccharides also protect yeast and bacteria from water limitations, help cells to adhere to leaf surfaces, and protect against antimicrobial compounds activity (Crowford et al., 2012).

Chitinolytic Test

The clear zone is formed due to the breaking down of β−1, 4 N-acetylglucosamine homopolymer bonds in chitin by chitinase to become N-acetylglucosamine monomers (Yan & Fong, 2015). The greater the amount of N-acetylglucosamine monomer produced, the greater the clear zone formed around the colony. The clear zone formation is an indicator that the test isolate has a chitinolytic activity after the secretion of the chitinase enzyme produced as its metabolite, therefore providing a clear appearance around the colony (Hardoko et al., 2020). The larger the clear zone formed, the more chitinase enzymes are produced, indicating higher chitinolytic activity. The difference in index value depends on the bacteria's ability to produce chitinase which varies widely. These differences are also caused by diverse gene codes (Haedar et al., 2017). Chitinolytic organisms produce various types of chitinase with varying characteristics and specificity for substrates. This diversity is an attempt to adapt to the various types, and chitin structures available in nature (Haliza & Suhartono, 2012).

Chitin as a substrate induces chitinase enzyme activity, which is regulated through genetic control involving induction of enzyme synthesis at the genetic level. For enzyme synthesis, an inducer is needed, which is in the form of a substrate or a compound, relating to the substrate of the reaction catalyzed by the enzyme. Beier and Bertillson (2013), stated that bacteria produce chitinase for the hydrolysis of chitin to be utilized by microbes as a carbon source. The clear zone's appearance is sharpened by adding congo red to the media.

Congo red binds to various polysaccharides, particularly, acetylglucosamine as a result of chitin degradation by chitinase Bharadwaj et al., 2012).

Antagonist Test

The higher the value of the formed inhibition zone, the greater the ability of the test isolates to inhibit pathogen growth. The difference in antagonistic ability is caused by many conditions, including the speed of spore formation, the number of antibiotic compounds produced, and differences in the specific enzymes yielded. The control isolates with and without treatment showed different results. The growth of the isolates, either yeast or bacteria, tended to dominate and widened in the test medium (Figure 4). This is in line with Sofiana et al. (2020) study, which stated that the control isolates without antagonist treatment showed intact and non-reduced mycelium, high sporulation rates, and larger colony diameter sizes. Isolates treated with antagonists showed reduced mycelium and colony diameter, as well as fewer spores were formed. In limited space and nutrients conditions, pathogenic growth activity is disrupted due to a lack of nutrients. This indicated an antagonistic interaction between the isolates.

Nunes (2012) stated that the inhibited growth of pathogenic colonies was due to the antagonistic properties of the test isolates. Moyano et al. (2016) reported that there was an effect on the growth of mold isolates, in dealing with antagonistic agents (yeasts), such as sporulation and mycelium reduction, as well as the clear zones formation. Antagonistic interaction is a form of protection that includes self-defense, territory, and food (Campbell, 2012). This mechanism occurs due to the competition for space and nutrition, production of anti-fungal compounds, and extracellular enzymes generated by test isolates (Sofiana et al., 2020). Some antagonistic microorganisms have the ability to producevolatile and inhibitory compounds, and hydrolyzing extracellular

enzymes (chitinases, proteases, CMCase), and the ability to form biofilms (Chen et al., 2018; Madigan et al., 2012).

Hemolysis Test

The hemolysis test indicator is a clear zone formation (lysis), which indicated that the isolate lyse red blood cells. The clear zone is formed because the test isolates produced extracellular products that lyse the red blood cells (Jamsari et al., 2018). The complete process of blood lysis is visible from the clear zone. The incomplete hemolysis process showed greenish colored media, due to in significant lysis process caused by no discoloration (Adam, 2016).

Molecular Identification

Identification of P. K1, P. K2, and EPBj II. K6 based on the sequence homology value in the 16S rDNA region with the closest species using the BLAST method from NCBI. 16S rDNA is a useful and powerful marker indicating the presence of bacteria in biological samples, and used as a new technique for bacteria identification at the species level (Srinivasan et al., 2015; Ghosh, 2016). 16S rDNA has areas with a conservative base order (conserved region) and regions with a variable base sequence (variable region) for diversity analysis. The 16S gene is used because it is found in almost all bacteria, with unchanging function, and has a size of 1500 bp which is large enough for bioinformatic purposes (Louca et al., 2019). Several Bacillus species were reclassified based on 16SrDNA, and separated into distinct phylogenetically clusters (Campbell & Kirchman, 2013). The partial 16SrDNA sequences and rRNA gene restriction patterns have been used for the identification or rapid classification of Bacillus species (Cihan et al., 2012).

The sequence comparison is carried out by performing the alignment of all sequences. The alignment aims to determine the homology level of the isolates DNA-base sequence using the control species (Moulia, 2014). Based on the phylogenetic tree, P. K1, P. K2 and EPBj II. K6 isolates had a bootstrap value of 99%, The greater of bootstrap value that appears, the higher of confidence level for the reconstructed tree. According to Faye et al. (2016), a bootstrap value between 70-100% indicates that the branching and phylogenetic tree is unchangeable.

CONCLUSSION

Based on the results, 35 yeast and 29 bacterial isolates were obtained and found to possess different morphological characters. The chitinolytic test showed that 4 bacterial isolates produce chitinase enzymes, namely BP 1.2.1, BP 2.1.1, EPBj II.K1, and EPBj II. K2 with a chitinolytic index of 3.92, 5.38, 2, and 1.66, respectively. While none of the yeast isolates were positive towards chitinase enzymes. Based on the percentage resistance calculation of the antagonistic test, 3 bacterial isolates gave the best results, namely P. K1 (41.1%), P. K2 (50%), and EPBj II. K6 (42.2%). The hemolysis test showed that potential antagonistic isolates were not pathogenic. The molecular identification showed that P. K1 and P. K2 isolates were classified as *Bacillus subtillis*, while that of EPBj II. K6 were categorized as *Bacillus aerius* with each sequence homology of 100%.

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